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ORIGINAL ARTICLE

Suppression of Human Hepatoma Growth in vivo by a Monoclonal Antibody Against a Mr 45,000 Protein

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ABSTRACT

The monoclonal antibody T2-2 was originally raised against the colorectal carcinoma cell line LS174T and was found to bind to several other human carcinomas, including hepatoma and ovarian cancer. The goal of this study was to investigate the antitumor activity of mAb T2-2 in human tumor models and further characterize the antigen. mAb T2-2 inhibited the growth of human hepatocellular cell line SMMC 7721 in vivo and in vitro. Western blot analysis revealed that this mAb recognizes an unique Mr 45,000 band from tissue extracts of human hepatocellular carcinoma (HCC), which localizes to the cell periphery. In vitro cell assays indicate that T2-2 decreases cell adhesion to laminin, implying the functional role of T2-2 antigen in cell-matrix interaction and cell migration.

INTRODUCTION

Human hepatocellular carcinoma (HCC) is a common, fatal malignancy that is present worldwide (1, 2). Unfortunately, no great progress in its prevention and treatment has been made. Most cases of hepatoma are incurable, as extensive resection is not possible, and the disease is rarely identified at an early stage. Furthermore, at the present time, systemic chemotherapy is of limited value due to lack of effective chemotherapeutic regimens (3). Therefore, HCC has become the leading cause of death in cancer patients (1). Prevention and treatment of this neoplasm are now major concerns.

As potent candidates for cancer therapy, therapeutic antibodies have been widely applied in cancer prognosis and treatment due to their specificity and efficacy (4). Promising antibodies are under investigation for both HCC prevention and treatment. Effective antibodies include ^{131}I conjugated anti-CD147 antibody fragment HAb18 F(ab′)$_2$ for HCC diagnosis and therapy that will soon reach market (5) and ^{131}I-labeled Hepama-I specific for the SMMC 7721 cell line which is now in clinical trial (6). Antibodies and antibody conjugates targeting HBV or HCV surface antigens (7, 8) have been applied for HCC prevention and therapy (9, 10).

In the present report, we describe the characterization of mAb T2-2, which exhibits highly effective anti-HCC potential both in vitro and in vivo. The target antigen recognized by mAb T2-2 was identified as a Mr. 45, 000 tumor-assoicated antigen which was expressed both on colorectal carcinoma and hepatoma, but not present on normal colon and liver. Our results demonstrate that the mAb T2-2 and its unknown antigen may be candidates for diagnosis and therapy of HCC and other cancers.

MATERIALS AND METHODS

Cells, tissues, and animals

Human hepatoma cell line SMMC 7721 and colorectal carcinoma cell line LS174T were from American Type Culture Collection (Rockville, MD). Cells were maintained as a monolayer in Dulbecco's modified Eagle's medium supplemented with 10 percent fetal bovine serum, penicillin-streptomycin and incubated at 37°C with 5 percent CO_2. Human tumor tissues were obtained from the tissue bank of the 301 Hospital in Beijing.

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Human normal tissues were obtained from the Beijing Legal Medical Institute. Athymic BALB/c nude mice were purchased from the Animal Center of the Chinese Academy of Medical Science.

Generation of monoclonal antibody

Hepatoma cell line LS174T (1×10^7) were injected intraperitoneally with Freund complete adjuvant into 6-week-old BALB/c mice and boosted 4 times weekly. Spleens were taken for hybridoma preparation as described Köhler and Milstein (11).

Screening of antibody

The enzyme-linked immunosorbent assay (ELISA) was used to select antibodies binding to LS174T cells. Briefly, LS174T cells were grown to confluence in 96-well plates, fixed with 1:1(v/v) methanol:acetone, washed, and incubated with hybridoma culture supernatants at 25°C for one hour. Plates were then rinsed 3 times in washing buffer (phosphate-buffered saline [PBS] with 0.05 percent Tween 20), and the bound antibodies were detected by incubation with HRP-conjugated anti-mouse IgG (Pierce, Rockford, IL) at 25°C for another hour. After careful washing, 3,3′,5,5′-tetramethylbenzydine (Sigma, Deisenhofen, Germany) was added as substrate. The color reaction was measured at 450 nm with a BioRad ELISA reader (Richmond, CA).

Immunohistochemistry

Specimens from normal and pathologic tissues were cryosectioned, fixed in acetone at −20°C for 10 minutes, and then preincubated with 0.3 percent H_2O_2 in methanol for 30 minutes to quench endogeneous peroxidases. After each incubation step the sections were washed with PBS. Next the sections were blocked with 5 percent goat serum in PBS at room temperature for one hour, following by incubation with primary antibodies in PBS for 2 hours. mAb T2-2 hybridoma culture supernatant was used at a 1:5 dilution. biotin-conjugated goat antimouse IgG was then applied followed by HRP-conjugated streptavidin (Vector, Hertfordshire, UK). For negative controls, the primary antibodies were omitted or isotype-matched control IgG was used. All sections were finally counterstained with hematoxylin or hematoxylin and eosin.

Flow cytometry

Cells were detached with 0.03 percent EDTA and incubated with T2-2 hybridoma culture supernatant at a 1:5 dilution or isotype-matched mouse IgG for one hour, followed by incubation with FITC-conjugated goat antimouse $F(ab)_2$ (Sigma, Deisenhofen, Germany), All incubations were conducted on ice. Following each step, cells were washed 3 times with PBS/1% BSA before the next incubation. After the final incubation, cells were washed, resuspended in PBS, and immediately analyzed on a FACSCalibur flow cytometry system (Becton & Dickinson, San Jose, CA). A total of 10,000 cells were analyzed per sample.

Immunofluorescence

To determine T2-2 antigen expression, cells were washed gently with PBS twice and and fixed in mathanol/acetone (1:1) for 2 minutes, then incubated with T2-2 hybridoma culture supernatant for one hour at 37°C, followed by 3 washes with PBS. FITC-conjugated goat anti-mouse $F(ab)_2$ was then added and incubated at 37°C for 30 minutes. Samples were observed under videomicroscopy.

Immunoblotting

Tissue extracts were prepared by lysis of 1 g tumor tissues in 5 ml RIPA buffer (1 percent Triton X-100, 1 percent sodium deoxycholate, 0.1 percent SDS, 150 mM NaCl, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.14 mg of aprotinin per ml, Tris-HCl; pH 7.5) on ice for 30 minutes. The extracts were centrifuged at 4°C at 12,000 g for 15 minutes and separated by SDS-PAGE (12 percent resolving gel, 4 percent stacking gel), electrically transferred to nitrocellulose filters (Invitrogen) and held in PBS plus 5 percent milk powder for one hour at room temperature. Antibodies were added, and the filter was held for one hour at RT. After washing with PBS plus 0.05 percent Tween 20 once and PBS twice, the blot was detected with T2-2 or PBS followed by HRP-conjugated Goat-anti-mouse IgG for one hour at RT. Protein bands were visualized with enhanced chemiluminescence reagent (Pierce, Rockford, IL)

Cell proliferation assay

Cells were seeded in 96-well plates (3000 SMMC7721 cells/well) and grown in the presence of mAb T2-2 at 10 mg/L or isotype matched control mIgG for 48 hours and then analyzed for viability by MTT assay (which determines relative cell numbers based on the conversion of MTT to formazan in viable cells). 20 μl MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltertrazolium bromide) (5 mg/ml) were added to each well and incubated for 4 hours at 37°C. The medium was removed and 150 μl DMSO were added to lyse cells and solublize formazan. Absorbance was determined on a Bio-Rad microplate reader at 595 nm.

Animal experiments

Male athymic 6-week-old BALB/c nude mice with a body weight of approximately 20 g were used and kept under specific pathogen-free conditions. Xenografts of human hepatoma were produced by subcutaneously injecting hepatoma cell line SMMC 7721 cells (1×10^7 resuspended in PBS) into the back of mice. Mice were randomly grouped as 7 mice per group and administered intratumorally with purified mAb T2-2 at a dose of 10 mg/kg, or PBS, twice per week for 7 weeks. Tumor size was measured twice per week and determined according to the equation: tumor size = $\text{width}^2 \times \text{length} \times (\pi/6)$.

Adhesion to extracellular matrix proteins

The cell adhesion assay was performed as described previously (12). Briefly, 50 ng of vitrogen, fibronectin, laminin, or

vitronectin diluted in PBS (pH 7.4) were adsorbed on each well of the microtiter plate overnight at 4°C. After blocking with 2 percent BSA, cells (10^5) were added to the coated wells in the presence of either purified mAb T2-2 (25 μg/10^6 cells) or control mIgGl. After a further incubation at 37°C for 60–90 minutes, nonadherent cells were removed by gently washing the wells. Adherent cells were fixed with 1.5 percent polyformaldehyde and stained by 0.1 percent crystal violet, and further solublized by 0.5 percent Triton X-100 and measured at 595 nm with a BioRad ELISA reader (Richmond, CA).

Cell migration assay

Migratory ability of SMMC 7721 cells was analyzed using wound assays. Cells were grown at confluence and the cell layer was wounded with a yellow tip. Adherent cells were washed twice with medium and incubated with 10 μg/ml purified T2-2 antibody or isotype matched mIgG at 37°C. 5 percent CO_2 for 24 hours. Cells were then washed with PBS twice and fixed in 3 percent paraformaldehyde. After staining with 0.25 percent Coomassie blue in 50 percent methanol-10 percent acetic acid for 15 minutes at room temperature. Cells were washed with 50 percent methanol-10 percent acetic acid and observed under videomicroscopy.

Annexin-V assay

This assay measures early and late events in apoptosis (13). SMMC 7721 cells were plated at 2.5×10^5/ml in 24-well Falcon plates, allowed to adhere overnight, and then treated with either 100 μg/ml of the mAb T2-2 or isotype-matched mIgG. After a 48 hour incubation, the cells were harvested by trypsinization, washed, and stained with Annexin-V-FITC apoptosis detection kit (Beijing Biosea Biotechnology Co, Ltd). A Becton Dickinson FACS was used to quantify Annexin and PI^- cells.

Statistical analysis

The 2-tailed Student/test was used for statistical analysis. A P value less than 0.05 was considered statistically significant.

RESULTS

Generation of mAb T2-2

LS174T cells were used as an immunogen in order to generate mouse monoclonal antibodies. Approximately 300 clones were screened for the production of antibodies that bound to the LS174T cells. From this screen, The hybridoma clone T2-2 was found to produce an IgG1/κ antibody that binds to LS174T with high affinity.

Characterization and specificity of mAbT2-2

We next investigated the specificity of the mAb T2-2 for a panel of normal and tumor tissues by immunohistochemistry. A significant difference of T2-2 binding pattern was found in normal and tumor tissues. As represented in Figure 1, mAb T2-2 specifically recognized tumor cells in hepatoma (Figure 1A) but did not stain normal liver tissue (Figure 1B), which indicates the overexpression of T2-2 antigen on hepatocellular carcinoma cells.

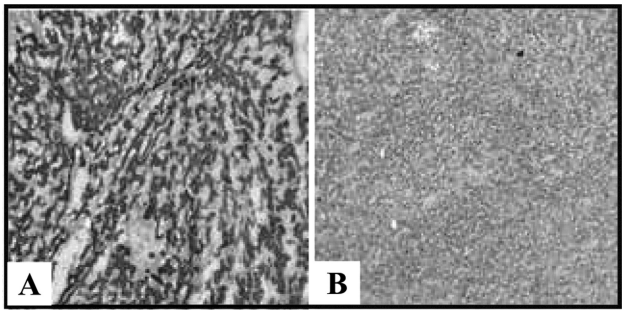

Figure 1. Immunohistochemistry showing that mAb T2-2 recognizes human hepatoma (A) but not normal liver tissues (B).

For further evaluation of the antitumor effect of mAb T2-2, we adopted a human hepatocellular carcinoma cell line SMMC 7721 as an in vitro model. FACS analysis showed that mAb T2-2 bound to SMMC 7721 cells (Figure 2). However, the binding activity was different between the fixed and unfixed cells. In the unfixed cells. In the unfixed cells, 17.98 percent of the cells were stained positively with T2-2, whereas 57.87 percent positive cells were found in the cells pre-fixed with polyformaldehyde, which reflects the antigen distribution and property.

In order to confirm the localization of the T2-2 antigen in SMMC 7721 cells, we performed immunofluorescence assay with mAb T2-2 followed by secondary staining with FITC conjugated anti-mouse $F(ab)_2$. The results demonstrate that the T2-2 antigen localized to the cell periphery with high labeling intensity appearing on polarized area and projections of many cells (Figure 2B, arrows). Further immunofluorescence staining with mAb T2-2 and anti-cytokeratin indicates the similarity of the mAb T2-2 antigen distribution with keratin in cells (refer to Figure 6).

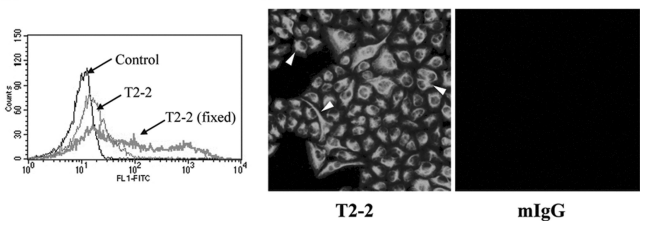

Figure 2. mAb T2-2 specifically binds to SMMC 7721 cells. (A) Flow cytometric analysis of mAb T2-2 binding to the prefixed SMMC 7721 cells much stonger than the unfixed SMMC 7721 cells. (B) Immunofluorescence assay shows the specific binding of mAb T2-2 to SMMC7721 cells, while the control mIgG did not. Arrows indicate polarized area and cell projection.

Effect of Mab T2-2 on SMMC 7721 cell proliferation, adhesion to matrix proteins, migration, and apoptosis

To determine the function of mAb T2-2 on the target cells, we performed a MTT assay to assess its effect on cell proliferation of SMMC 7721. After 48 hours treatment, the cellular growth was 18.53 percent inhibited with 40 μg/ml mAb T2-2 and 46.24 percent with 60 μg/ml antibody in a dose-dependent manner compared to control mIgG treatment (Figure 3A).

We further examined the mechanisms allowing T2-2 to affect tumor cell growth by investigating the properties of T2-2 in the cell adhesion to matrix proteins, migration and apoptosis. The effect of T2-2 on cell adhesion to matrix substrates such as vitrogen, vitronectin and laminin was tested. As shown in Figure 3B, T2-2 significantly inhibited the adhesion of SMMC 7721 cells to Laminin ($P < 0.05$), but did not affect the attachment of cells to vitrogen and vitronectin compared with an irrelevant mouse IgG.

We next analyzed the ability of mAb T2-2 to affect migration of SMMC 7721 cells in the wound assay. Figure 3C displays that the migration of SMMC 7721 cells was significantly inhibited in the presence of mAb T2-2, while the wound in the control dish with mIgG treatment almost closed up.

In the apoptosis assay, the SMMC 7721 cells were incubated with either mAb T2-2 or control mIgG for 48hr and then stained with both Annexin-V and PI. No significant apoptotic activity was found in either treated cell cultures (data not shown).

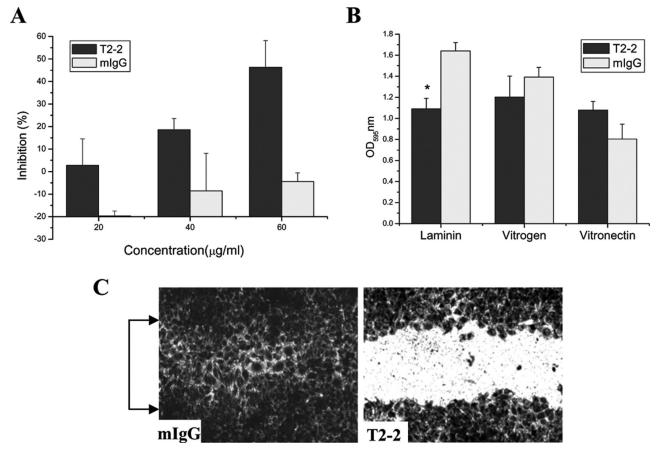

Figure 3. Effect of T2-2 on SMMC 7721 cells in vitro. A) In vitro inhibition of T2-2 on SMMC 7721 cell proliferation. SMMC 7721 cells were treated with 20, 40, 60 μg/ml T2-2 or control mIgG for 48 hours Cell proliferation was determined by MTT assay. The inhibition by mAb T2-2 (60 μg/ml) compared with control IgG (60 μg/ml) indicated that T2-2 had a significant inhibitory effect on the proliferation of SMMC 7721 cells. B) Effect of mAb T2-2 on SMMC 7721 cell adhesion to various matrix proteins. SMMC 7721 cells incubated with mAb T2-2 or control mouse IgG (25 μg/10^6 cells) were added to wells coated with 0.5 μg of Laminin, vitrogen, or vitronectin and let adhered for 40 minutes at 37°C. Nonadherent cells were removed and cell adhesion was measured using a BioRad ELISA reader after stained with crystal violet. T2-2 represented a significant inhibition of SMMC 7721 cells to Laminin ($P<0.05$). *Bars*. SEM of triplicate samples. C) T2-2 reduced SMMC 7721 cell migration at the wound edge. SMMC 7721 cells were grown at confluence and cell layers wounded by a yellow tip. Adherent cells were washed twice and incubated with 10 μg/ml mAb T2-2 or control IgG for 24 hours. Arrows indicated the size of the wound. Cells were fixed and stained with Coomassie blue before analysis.

Mab T2-2 suppress human hepatoma xenografts in mice

In order to investigate whether mAb T2-2 inhibits tumor growth in vivo, we established a hepatoma model by injection of SMMC 7721 cells into athymic BALB/c nude mice. mAb T2-2 or PBS was administered intratumorally to the mice on the day of tumor cell implantation. The treatment was performed twice a week for a total of 49 days. The mean tumor volume in T2-2-treated animals was reduced by 95 percent compared with control group (5.72 ± 0.96 mm^3 versus 114.62 ± 26.72 mm^3, respectively; $P < 0.001$). As shown in Figure 4, tumor nodules of mAb T2-2 treated mice grew slowly in early the phase but showed regression between Day 21 and Day 28. However, the slight increase in tumor volume during this period resulted from the slowly developing tumor burden on 2 mice, while the other 5 mice showed continuous tumor regression until tumors became nonpalpable for a total of 5 weeks. In contrast, the tumor burden of PBS treated group sustained rapid growth rate and exceeded 260 mm^3 at the end of the experiment.

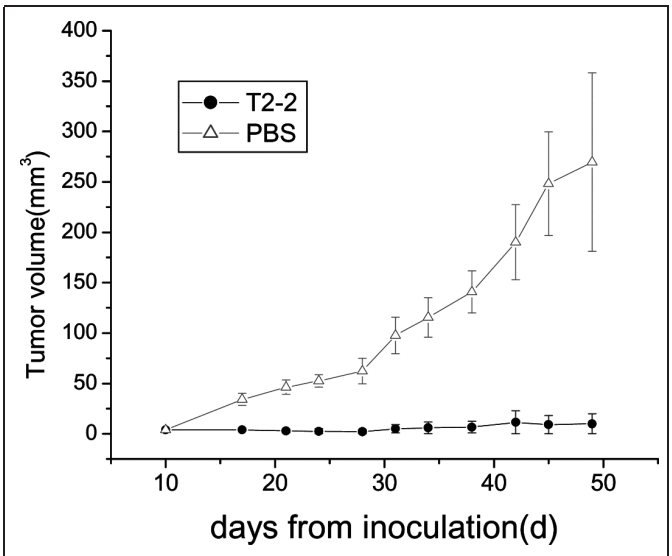

Figure 4. Inhibition of human hepatoma growth in nude mice by mAb T2-2. SMMC 7721 cells (6×10^6) were injected subcutaneously into nude mice ($n = 7$), and treated with mAb T2-2 (100 μg/ml) or control mIgG intratumorally twice a week. The volume of the s.c. tumors were determined every 3–5 days. mAb T2-2 represented a significant inhibitory effect ($P < 0.001$) on the growth of SMMC 7721 cells in nude mice.

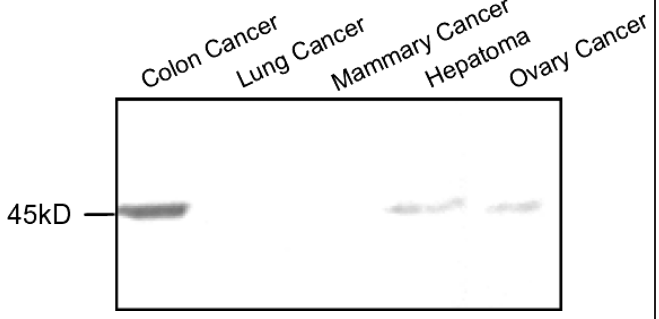

Figure 5. Western blot analysis of the antigen recognized by mAb T2-2. Extracts of human hepatocellular carcinoma tissues were separated on a SDS-10 percent polyacrylamide gel, transferred to nitrocellulose membrane and detected by T2-2 followed by HRP-conjugated antimouse IgG.

Western analysis of the mAb T2-2 antigen

To identify the antigen recognized by T2-2 mAb. Western blot analysis was performed. As shown in Figure 5, the mAb T2-2 immunoblotted a unique Mr 45,000 band from the extracts of human hepatocellular, colorectal and rectal carcinoma tissues of clinical cases, while no signal was detected from mammary cancer, melanoma or the corresponding normal tissues (not shown).

DISCUSSION

Monoclonal antibodies have been widely applied for the treatment of various types of tumors (4). The screening of mAb against human hepatocellular carcinoma (HCC) is still in the early phase. Effective mAb therapies require characterization of many factors (14) such as antibody specificity, affinity and notably the access to new tumor-associated antigen as effective target for successful mAb-mediated cancer therapy.

The mAb T2-2 originally raised against a colorectal carcinoma cell line LS174T recognize an antigen on colon cancer cells but not normal colon tissue. We have demonstrated that expression of the antigen bound by mAb T2-2 is not restricted to colorectal carcinoma. This tumor antigen is also selectively expressed on hepatoma and other tumors such as ovary cancer, but not present on corresponding normal tissues, which implies that the antigen might be a marker for these tumor progression.

In this study we have presented the specificity and function of the mAb T2-2 on hepatoma. In vivo studies demonstrated that the mAb T2-2 significantly inhibited the growth of hepatoma. Tumor volumes in T2-2-treated animals reduced significantly ($P < 0.001$) compared with PBS controls. Antibody treatments were well tolerated. We did not observe any signs of antibody toxicity or organ abnormalities at autopsy. As isotype-matched mIgG was found no effect on tumor growth in colon cancer animal model, we applied PBS as the only control in the in vivo hepatoma experiment.

Tumor growth is a complex process, which includes interaction between tumor cells and their surrounding connective tissue components (15). As mAb T2-2 significantly inhibited the proliferation of SMMC 7721 cells, potential mechanisms that might contribute to the efficacy of mAb T2-2 in vivo were investigated. We observed an inhibitory effect of T2-2 on SMMC 7721 cells proliferation and migration in vitro, suggesting that the inhibition of tumor growth is caused by the blockade of a key antigen on the tumor cell. However, the mAb alone is unlikely to be efficient in inducing cell apoptosis, indicating the inhibitory roles of mAb T2-2 are apoptosis independent. Further study on T2-2's effect on cell adhesion to extracellular matrix proteins reveals that T2-2 decreases the adhesion of SMMC 7721 cells to laminin, but not to vitrogen or vitronectin. As a major component of basement membranes, laminin mediates interactions between cells and the extracellular matrix. Besides its fundamental role in organizing the basement membrane network, laminin contributes to several cellular activities—namely, adhesion, growth, polarization, and differentiation (16).

mAb T2-2 was used to immunoblot a Mr 45,000 antigen from various human tumor tissues (Figure 5). Interestingly, characterization of T2-2 antigen expression by FACS reveals that the antigen represents much higher fluorescence intensity with a pre-fixation step, compared to direct incubation with T2-2 in sample preparation. The distribution of p45 reflected by further immunofluorescence staining indicates that the antigen localized to the cell periphery and polarized areas, so that many cells represent an asymmetric fluorescence intensity (Figure 2B). This staining profile suggests the functional role of p45 in cytoskeleton rearrangement, implying the similarity of p45 with cytoskeleton proteins such as actin and keratin. We performed an immunofluorescence staining to compare the distribution of mAb T2-2 antigen and cytokeratin and found the stained areas are exactly overlayed (Figure 6). This can be also inferred by the finding that T2-2 inhibits adhesion to laminin and cell migration. Laminin functions through its receptors, mainly integrins (17, 18) to mediate interactions with actin, keratin or intermediate proteins (19, 20) to trigger cytoskeletal rearrangements and intracellular signalling events, thus, control immediate-early gene expression, cell cycle machinery, and cell survival (21–23). Induction of keratin expression by laminin through its receptors on cell surface also was observed in HCC cells (24). Together with the finding that released keratin may cause basement membrane damage through binding to laminin (25), we hypothesize that the mAb T2-2 functions as an inhibitor of tumor cell migration and

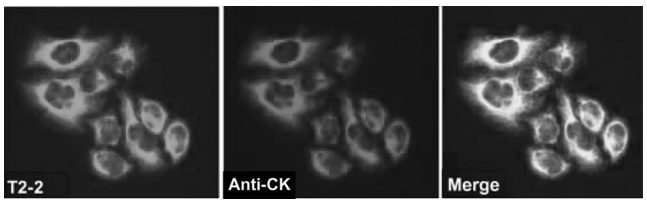

Figure 6. Distribution of mAb T2-2 antigen and cytokeratin in SMMC 7721 cells. SMMC 7721 cells were incubated with mAb T2-2 and rabbit anti-cytokeratin, followed with secondary antibody FITC-anti-mouse F(ab)2 and Cy3-anti-rabbit IgG respectively. Cells were photographed under fluorescence microscope (x 400).

expansion through a laminin-mediated pathway that prevents matrix degradation and controls cell survival. Importantly, more details in cell signaling will need to be elucidatd.

Taken together, we have shown that treatment with mAb T2-2 against a Mr 45,000 protein leads to a significant decrease in HCC SMMC 7721 cell proliferation both in vitro and in vivo. We hypothesize that the mechanism to explain this efficacy relates to effective inhibition of cell migration and adhesion to the matrix substrates, Laminin. The Mr 45,000 antigen represents cytoskeleton-associated distribution and may involve in skeleton rearrangement and cell migration. It will be interesting to identify the T2-2 antigen and to further investigate the antitumor mechanisms of mAb T2-2 and especially to find out whether T2-2 will be applicable to other tumor types. mAb T2-2 may become a new diagnostic tool and a therapy of great importance to HCC and other cancers.

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