Original Article
B4GALNT2 knock-down suppresses proliferation and induces apoptosis in non-small cell lung cancer cells

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Abstract: B4GALNT2 was reported as a cognate biosynthetic enzyme of the histo blood group carbohydrate Sda antigen. Previous reports showed that B4GALNT2 might be involved in tumorigenesis. However, its role in lung cancer remains unclear. Here we report that B4GALNT2 might be associated with tumorigenesis of NSCLC. We found knock-down of B4GALNT2 suppressed cell growth and proliferation in A549 cells with Celigo Cell Counting application, MTT and colony formation assay. Flow cytometry results showed knock-down of B4GALNT2 induced significant apoptosis in A549 cells. Therefore, B4GALNT2 serves an important role in the growth of lung cancer cells, and may be considered as a potential biomarker and therapeutic target for lung cancer.

Keywords: Lung cancer, NSCLC, B4GALNT2, cell growth, cell apoptosis

Introduction

Lung cancer is the leading cause of cancer deaths worldwide with 1.6 million deaths annually [1]. Histological subtypes analysis show approximately 85% of patients are identified as non-small cell lung cancer (NSCLC), of which could be divided into lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) [2]. Smoking is proved to be an independent risk factor which is associated with all major histological subtypes of NSCLC, as well as small cell lung cancer (SCLC) [3]. However, lung cancer in never smokers is more common in women and in East Asia. Despite environmental exposures including second-hand smoking, pollution, and occupational carcinogens, inherited genetic susceptibility are regarded as important factors for tumorigenesis of lung cancer [4]. A hallmark of personalized medicine, with subsets of patients treated according to the genetic alterations of their tumor is much better promising in the treatment of lung cancer than the empirical use of cytotoxic therapy based on a physician’s preference. Therefore novel tumor related genes and therapeutic approaches are still urgently needed.

B4GALNT2 is encoded from B4GALNT2 (formerly GALGT2, NM_153446) gene [5]. The human B4GALNT2 gene is on chromosome 17q21.33 and consists of 11 coding exons. Two different isoforms of human B4GALNT2 are identical in their sequence with the exception of their cytoplasmic tail. In humans, B4GALNT2 is responsible for transfer of N-acetyl-D-galactosamine (GalNAc) to form the Sda antigen [5-10]. For B4GALNT2, Byrne et al. identified that B4GALNT2 may participate in antibody-dependent rejection in xenotransplantation. They found expression of 6 cDNAs, including B4GALNT2, separately increased the antibody binding level, and the other 5 genes are PROCR, CD9, CD46, CD59, and ANXA2. Based on these results, the Tector group demonstrated that disruption of these B4GALNT2 and 2 other genes reduced human IgM/IgG binding to pig peripheral blood mononuclear cells [9, 11]. However, whether if B4GALNT2 involved and the physiological role of B4GALNT2 in lung cancer especially in NSCLC remained unidentified.

In this study, we investigated expression of B4GALNT2 in NSCLC cell lines. Then taking advantage of lentivirus mediated shRNA knock-down of B4GALNT2, the expression of B4GALNT2 was decreased in A549 cells. The inhibited effects of B4GALNT2 on cell proliferation was investigated with the Celigo system,
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MTT assay and colony formation. Furthermore, flow cytometry results indicated down regulation of B4GALNT2 induced apoptosis in A549 cells. Together, our results may reveal a novel function and mechanism towards better understanding the role of B4GALNT2 in lung cancer.

Methods and materials

Cell lines and cell culture

Four lung cancer cell lines including A549, NCI-H1299, 95D, and NCI-H460 were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). A549 cells were cultured in F-12K medium supplemented with 10% bovine serum. NCI-H1299, 95D and NCI-H469 cells were cultured in RPMI-1640 medium supplemented with 10% bovine serum. Both kinds of medium were supplemented with penicillin (100 U/ml) and streptomycin (100 U/ml). Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO2.

Construction of B4GALNT2 knock-down lentivirus.

The target DNA sequence (GAAGCTGTTGAA-GTTGATTCATT) of B4GALNT2 was selected from the full-length (NM_153446) by GeneChem Co. Ltd. (Shanghai, China). According to the sequence of B4GALNT2, two vectors shRNA S1 and shRNA S2 were designed. The sequences are as follows: shRNAS1: 5'-CCG GCG GAA GCT GTT GAA GTT CAT TCT CGA GAA TGA ACT TCA ACA GCT TCC GTT TTT G-3'; shRNA S2: 5'-AAT TCA AAA ACG GAA GCT GTT GAA GTT CAT TCT CGA GAA TGA ACT TCA ACA GCT TCC GTT TTT G-3'; The shRNAs were annealed and ligated to the linearized GV115 lentivirus vector to transform DH5α competent cells. The plasmid was extracted and verified by enzymatic digestion and sequencing. A549 cells were infected with the lentivirus. Cells infected with a lentivirus carrying an empty vector were used as a control. Fluorescence expression was measured after 72 hours when the achieved infection efficiency was 80%. The expression of B4GALNT2 was analyzed by qRT-PCR and Western blotting.

Quantitative RT-PCR

Total RNA from the 4 cell lines, A549, NCI-H1299, 95D, and NCI-H460, was extracted using the TRIzol reagent (Invitrogen, Shanghai, China), according to the manufacturer's instructions and was then used for RT reaction. Briefly, 2 μg of total RNA from each sample was reverse transcribed to single-stranded cDNA. One microliter of cDNA was used as a template for the following PCR. The primers used were as follows: for B4GALNT2 forward, 5'-TTT TTC TAC GAT GGA ATC TGG C-3' and reverse, 5'-CAG CCT GTC TTC TCG CTT TC-3'; and for GAPDH forward, 5'-TGG TTT CAA CAG CGA CAC CCA-3' and 5'-CAC CCT GTT GCT GTA GCC AAA-3'. The quantitative RT-PCR comprised an initial denaturation at 95°C for 15 sec, then 45 cycles at 95°C for 5 sec and 60°C for 30 sec. The PCR products of B4GALNT2 and GAPDH were 241 and 121 bp, respectively. All samples were examined in triplicate.

Colony formation assay

B4GALNT2-shRNA transfected and control A549 cells were trypsin-digested and resuspended in standard medium after achieving logarithmic growth phase. Cells were seeded into six-well plates at a density of 500 cells/well. The cells were incubated and observed over a period of 10 days with half of the medium being changed every 3 days. The cells were washed with PBS and then fixed with paraformaldehyde (1 ml/well; Shanghai Sangon, China) for 30-60 min. All cell wells were washed with PBS and then stained with 500 μL Giemsa (ECM550 Chemicon) for 20 minutes. The cells were then washed with ddH2O for three times. Cell colonies were photographed by fluorescence microscopy (MicroPublisher 3.3RTV; Olympus, Japan).

Cell growth assay

3-(4, 5-Dimethyl-thiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT, Sigma) assay was performed to assess cell proliferation after drug exposure as described previously [12]. Briefly, lentiviral infected A549 cells were seeded in 96-well plates at an inoculation density of 3,000 cells/well. At different time points after incubation (1, 2, 3, 4, and 5 days), MTT 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide solution was added to each well and incubated at 37°C for 4 hours. Then 100 μL acidic isopropanol (10% SDS, 5% isopropanol and 0.01 mol/L HCl) was added into each
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Figure 1. B4GALNT2 expression in four cell lines including A549, NCI-H1299, 95-D, and NCI-H460.

well after the medium were carefully removed. Plates were then read with an Automated Microplate Reader (Molecular Device, US) at 490 nm.

Plate analysis with the adherent cell cytometry system Celigo®

In brief, shB4GALNT2 or control shRNA transfected A549 cells were trypsin-digested and resuspended in standard medium after achieving logarithmic growth phase. Cells were seeded into 96-well plates at a density of 2000 cells/well. The fluorescence expression was collected everyday with the Celigo® system for 5 days continuously. With adjustments in the parameters in the Analysis Setting, fluorescence expression was converted to cell numbers.

Flow cytometric analysis of apoptosis

Apoptosis assessment was performed by using an Annexin-V/FITC apoptosis detection kit (Invitrogen, USA) as described by the manufacturer’s instruction. Briefly, cells were transfected with shB4GALNT2 or control shRNA. Harvested cells were stained with Annexin-V/FITC and PI in binding buffer for 15 minutes at room temperature in the dark as previously reported. The samples were analyzed by FACScan flow cytometry (Becton Dickinson, USA) to determine the percentage of cells displaying Annexin-V and PI staining.

Western blot analysis

Cells were transfected with shB4GALNT2 or control shRNA. Protein was abstracted and Western blotting were performed as previously described [13]. Protein concentrations of cell lysates were determined using the Bradford method. Briefly, certain quantized proteins were separated in SDS-PAGE and then were transferred to PVDF membrane. After blocking at room temperature, membranes were incubated with different primary antibodies before visualized and photographed. The antibodies used in the experiments were: anti-B4GALNT2 and anti-GAPDH which were obtained from Epitomics (US).

Statistical analysis

All experiments were performed in triplicate and were repeated at least three times. The results are presented as mean value ± SD. Statistical significance was performed by using one-way analysis of variance (ANOVA) test. The significance level was set as *P<0.05, **P<0.01 and ***P<0.001.

Results

The mRNA expression of B4GALNT2 in different lung cancer cell lines

qRT-PCR was used to investigate the mRNA level of B4GALNT2 in four lung cancer cell lines including A549, NCI-H1299, 95-D and NCI-H460 cells. B4GALNT2 was found expressing in all four cell lines with different mRNA level. Compared with the GAPDH expression, the highest mRNA level fold change was found in NCI-H460 cells with 20.02 fold. The lowest mRNA level fold change was found in A549 cells with 12.94 fold. The upregulated mRNA fold change in NCI-H1299 and 95-D cells were 15.50 fold and 18.40 fold separately (Figure 1).

Lentivirus-mediated knock-down of B4GALNT2 in non-small cell lung cancer A549 cells

Lentivirus-mediated gene transfection was used to further explore the function of B4GALNT in lung cancer. The Figure 2A results showed most A549 cells presented EGFP positive signals after infected by lentivirus recombined with shRNA targeting B4GALNT2 (shB4GALNT2) or control scrambled shRNA (shCtrl), indicating that the recombinant lentivirus could infect A549 cells with high efficiency. Further qRT-PCR results (Figure 2B) showed the mRNA level was significantly down-regulated in A549 cells with shB4GALNT2 treatment (p<0.05). The calculated data indicated that the reduction efficiency reached 64.6% after the infection of shRNA lentivirus. Western-blot analysis (Figure 2C) also supported that no B4GALNT2 protein
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band was detected in shB4GALNT2 infected cells. All these data together confirmed that recombinant lentivirus taking shB4GALNT2 could effectively suppress expression of endogenous B4GALNT2 in lung cancer cells.

Knocking down of B4GALNT2 inhibited cell proliferation in A549 cells

The Celigo Cell Counting application was used to directly image and count the proliferation in A549 cells. The cells were infected with shB-4GALNT2 or shCtrl which could be identified with EGFP positive signals (Figure 3A). After 5 days, the green signals in cells infected with shB4GALNT2 were much weaker than those cells infected with shCtrl significantly (P<0.05). The calculated cell counting result also supported the inhibition in knocking down of B4GALNT2 using shB4GALNT2 (Figure 3B and 3C).

MTT assay was then used to further investigate the potential effects of knocking down of
Figure 3. The inhibited effects of down-regulated B4GALNT2 in A549 cell growth with shB4GALNT2 transfection. A. Unprocessed raw images of cell growth with Celigo Cell Counting application assay for 5 days. B. Cell counts of A549 cells expressing shCtrl lentivirus and shB4GALNT2 lentivirus were seeded in 96-well plates and cell growth was assayed every day for 5 days. C. Cell counts fold of A549 cells expressing shCtrl lentivirus and shB4GALNT2 lentivirus on 1st day of 2nd, 3rd, 4th and 5th days by Celigo Cell Counting application assay. All experiments were performed at least three times and independently. Significant differences from untreated control were indicated as *P<0.05; **P<0.01; ***P<0.001.
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Figure 4. Downregulation of B4GALNT2 inhibits the proliferation in A549 cells identified by MTT assay. A. Cell viability of A549 cells expressing shCtrl lentivirus and shB4GALNT2 lentivirus was seeded in 96-well plates and cell growth was assayed every day for 5 days. B. Cell viability fold of A549 cells expressing shCtrl lentivirus and shB4GALNT2 lentivirus on 1st day of 2nd, 3rd, 4th, and 5th days by Celigo Cell Counting application assay. All experiments were performed at least three times and independently. Significant differences from untreated control were indicated as *P<0.05; **P<0.01; ***P<0.001.

Figure 5. Colony formation abilities of A549 lung cancer cells were inhibited with downregulation of B4GALNT2. A. Representative microscopic images of colonies were stained by crystal violet in A549 cells. B. Statistical analysis of the number of colonies in A549 cells. All experiments were performed at least three times and independently. Significant differences from untreated control were indicated as *P<0.05; **P<0.01; ***P<0.001.

B4GALNT2 on cell proliferation in A549 cells.

As indicated in Figure 4A and 4B, the growth was much slower in cells transfected with shB4GALNT2 than that in cells transfected with shCtrl (P<0.05), which showed that the proliferation rate was significantly inhibited.

The colony formation results also showed that knocking down of B4GALNT2 inhibited cell proliferation in A549 cells consistently. As shown in Figure 5A, the size was relatively smaller and colonies were fewer in shB4GALNT2 groups compared with shCtrl groups in A549 cells.

Statistical analysis further confirmed that knock-down of B4GALNT2 significantly reduced colonies formed in A549 cells (Figure 5B, P<0.001). Furthermore, all these results suggested that suppression of B4GALNT2 could inhibit cell proliferation of A549 cells.

**Knocking down of B4GALNT2 induced apoptosis in A549 cells**

Flow cytometry assay was performed to identify apoptosis in A549 cells transfected with shB4GALNT2. As shown in Figure 6A and 6B, the
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apoptosis proportion was increased in A549 cells transfected with shB4GALNT2. The total average percentage of apoptotic cells was 8.21% with shB4GALNT2 treatment, which was significantly different (P<0.001) to that in cells transfected with shCtrl (4.64%). These data suggest that knocking down of B4GALNT2 induced apoptosis in A549 cells.

Discussion

In this study, we report that B4GALNT2, a GalNAc-transferase, might be closely associated with tumorigenesis of NSCLC. We found B4GALNT2 expression in different NSCLC cell lines. Functional analysis demonstrated that knock-down of B4GALNT2 suppressed NSCLC cancer cell growth and proliferation. Flow cytometry results showed knock-down of B4GALNT2 induced significant apoptosis in A549 cells.

B4GALNT2 was reported as a cognate biosynthetic enzyme of the histo blood group carbohydrate Sda antigen [14]. Previous research showed a higher B4GALNT2 activity in the poorly differentiated cells of the colonic crypt, which indicated the oncogenic potential of B4GALNT2 [15]. Caco-2 cells, one of the few in vitro models of intestinal differentiation, showed an increased B4GALNT2 expression concomitantly with a differentiated phenotype [16]. In human colonic cancer, B4GALNT2 activity exhibits a dramatic down-regulation, compared with the normal surrounding mucosa, due to down-regulated expression of the mRNA [17]. B4GALNT2 expression was found in lung cancer cells. As Figure 1 shows, different mRNA levels were found in 4 lung cancer cells including A549, NCI-H1299, 95-D, and NCI-H460. These data first suggest that B4GALNT2 might be associated with the lung cancer lung tumorigenesis.

Functional studies indicated that B4GALNT2 regulated oncogenic development and cell differentiation which was indicated by the observation that the guinea-pig kidney enzyme showed a five-fold increase after birth [18]. In rat colon, B4GALNT2 was practically absent at birth, increased slowly in the first days of life and then rapidly after weaning [19]. However, the function of B4GALNT2 in lung cancer remained unknown. After effectively knocking down of B4GALNT2 with shRNA B4GALNT2 lentivirus (Figure 2A-C), Celigo Cell Counting application, MTT and colony formation assay results further confirmed that knock-down of B4GALNT2 with shB4GALNT2 lentivirus transfection effectively inhibited the growth and proliferation in non-small cell lung cancer A549 cells. Compared to the control, the growth was much slower in cells transfected with shB4GALNT2 (P<0.05) using Celigo Cell Counting application (Figure 3A-C). MTT assay also supported the inhibited effects with shB4GALNT2 consistently (Figure 4A and 4B). Knock-down of B4GALNT2 significantly reduced colonies formed in A549 cells (Figure 5A and 5B). Our study supports involvement of B4GALNT2 in cancer cell function and indicates that suppression of B4GALNT2 could inhibit cell proliferation of A549 cells.

Forced expression of B4GALNT2 in cancer cell lines of gastrointestinal origin has revealed molecular mechanisms involved in cancer prog-

Figure 6. Depletion of B4GALNT2 arrests cell cycle progression in A549 lung cancer cells. A. Cell cycle progression was analyzed by flow cytometry. B. Statistical analysis of the percentage of cells in GO/G1, S, and G2/M phases in Cal-62 and 8305C cells after B4GALNT2 knock-down. All experiments were performed at least three times and independently. Significant differences from untreated control were indicated as *P<0.05; **P<0.01; ***P<0.001.
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ress by 2 independent groups [17]. One group expressed the B4GALNT2 in gastric cancer KATO III cells and in colon cancer HT29 cells, observing a striking reduction of their metastatic ability. The other study showed that both the long and the short protein isoforms of B4GALNT2 were able to induce the expression of the Sda antigen and the inhibition of sLex in LS174T cells. These data reported intrinsic regulation of B4GALNT2 in cancer. As aberrant apoptosis is one important feature of cancer cells, flow cytometry assay identified apoptosis in A549 cells transfected with shB4GALNT2. As shown in Figure 6A and 6B, the apoptosis proportion in A549 cells transfected with shB4GALNT2 was increased significantly (8.21%) (P<0.001) to that in cells transfected with shCtrl (4.64%). These data suggest that knocking down of B4GALNT2 induced apoptosis in A549 cells.

In summary, we found that B4GALNT2 expressing in NSCLC cells. Meanwhile, inhibition of B4GALNT2 suppressed NSCLC cancer cell proliferation and induced apoptosis in A549 cells. This new information of B4GALNT2 involved in NSCLC cancer cell proliferation and apoptosis will be essential to identify a potential biomarker or treatment target that will have predict value in detection, therapy and predisposition of NSCLC.

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Disclosure of conflict of interest

None.

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