Knockdown of Mgat5 inhibits CD133⁺ human pulmonary adenocarcinoma cell growth in vitro and in vivo

Abstract

Purpose: In spite of many therapeutic advances, the prognosis of lung cancer remains poor. Therefore, understanding the molecular mechanisms underlying cancer progression, invasion and metastasis is needed. Accumulating evidence indicate that N-acetylglucosaminyltransferase V (Mgat5 or GnT-V) is involved in cancer development. The purpose of this study was to characterize the expression and function of Mgat5 in CD133⁺ pulmonary adenocarcinoma cells.

Methods: CD133⁺ pulmonary adenocarcinoma cells were separated by magnetic activated cell sorting (MACS) from excised pulmonary adenocarcinoma specimens from 10 patients. Expression of Mgat5 in CD133⁺ cells was detected by fluorescent quantitative RT-PCR (FQRT-PCR) and Western blot. Subsequently, CD133⁺ cells were transfected with specific siRNA of Mgat5 to evaluate the effects of Mgat5 inhibition on cancer cell growth in vitro and in vivo.

Results: Expression of Mgat5 was 1.2-fold and 1.4-fold higher in CD133⁺ cells than in CD133⁻ cells detected by FQRT-PCR and Western Blot, respectively (p<0.05). The L-PHA binding assay also showed higher reactivity in CD133⁺ cells than in CD133⁻ cells. In addition, Mgat5-specific siRNA efficiently knocked down the expression of Mgat5 in CD133⁺ cells. Interestingly, downregulation of Mgat5 resulted in significant inhibition of cancer cell growth in vitro and in vivo.

Conclusion: Mgat5 is expressed at a relatively high level in CD133⁺ lung adenocarcinoma cells, and knockdown of Mgat5 in CD133⁺ cells inhibits cancer cell growth both in vitro and in vivo. These findings suggest Mgat5 may play an important role during oncogenesis, identifying a potential therapeutic target for pulmonary adenocarcinoma.
Lung cancer is often incurable and remains the leading cause of death due to cancer worldwide. Histologically, lung cancer can be divided into four major subtypes: squamous cell carcinoma, adenocarcinoma, small cell carcinoma and large cell carcinoma. Adenocarcinoma is the most common subtype, accounting for almost half of all non-small cell lung carcinomas. Despite continuous efforts to improve the therapeutic response, the overall five-year survival rate for such tumors is lower than 15% [1, 2].

CD133 is a stem cell marker for cancerous tissues, and it is currently being used, alone or in combination with other markers, for identification and isolation of the putative cancer stem cell population from malignant tumors [3-5]. In lung cancer, there is a rare population of CD133+ cancer stem-like cells able to self-renew and capable of generating unlimited progeny of the differentiated cells that constitute the major tumor population6. Molecular and functional characterization of such a tumorigenic population may provide valuable information to the development of effective therapies [7].

N-acetylglucosaminyltransferase V (Mgat5), a key enzyme in the formation of branching of asparagine-linked oligosaccharides, is strongly linked to tumor invasion and metastasis of colon and breast cancers [8]. Several studies have demonstrated the association between Mgat5 activity and increased tumor invasiveness[9-11]. Hirotsoshi et al. suggested that expression of Mgat5 is associated with prognosis and histology in non-small cell lung cancers [12]. Oncogenesis increases β1,6-N-acetylglucosaminyltransferase V expression, and its high-affinity galectin ligands promote surface retention of growth receptors with a reduced dependence on UDP-GlcNAc. Of note, Mgat5−/− tumor cells are less metastatic in vivo and less responsive to cytokines in vitro [13]. Because the function of Mgat5 overlaps with the role of tumor stem cells, we conjectured that Mgat5 may play a role in the activity of tumor stem cells, such as CD133+ lung cancer cells. This study was designed to investigate the expression of Mgat5 and its function in tumor cells isolated from lung adenocarcinomas, which can be separated into two subpopulations by the expression of CD133.

Materials and Methods

Patients and tissue specimens

Ten surgically-resected adenocarcinoma specimens were obtained from patients diagnosed with pulmonary adenocarcinoma who had not undergone radiotherapy or chemotherapy, between 2009 and 2010 at Wuhan University Zhongnan Hospital (Wuhan, China) and Union Medical Hospital (Wuhan, China). The patients ranged in age from 35 to 72 years with an average of 62.3 years. Six patients (60%) were male and four (40%) were female. All studies were performed with the patients’ informed consent and approved by the Institutional Review Board of School of Medicine.

Magnetic cell separation (magnetic activated cell sorting, MACS)

Surgical specimens were washed three times and left overnight in DMEM–F12 medium supplemented with antibiotics to avoid contamination (200 IU/mL penicillin, 200µg/mL streptomycin and 5µg/mL amphotericin B). The tumour tissue specimens were mechanically disrupted and cells were separated by density centrifugation as described previously [7]. Tissue dissociation was carried out by enzymatic digestion using 20µg/mL collagenase II (Gibco-Invitrogen, Carlesbad, CA) for 2h at 37ºC. Recovered cells were cultured in serum-free medium containing 50µg/mL insulin, 100µg/mL apo-transferrin, 10µg/mL putrescine, 0.03mM sodium selenite, 2µM progesterone, 0.6% glucose, 5mM HEPES, 0.1% sodium bicarbonate, 0.4% BSA, glutamine and antibiotics, dissolved in DMEM–F12 medium (Gibco-Invitrogen) and supplemented with 20µg/mL EGF (Epidermal Growth Factor) and 10µg/mL bFGF (basic Fibroblast Growth Factor). After three subcultures, single cells were prepared with enzymatic digestion, magnetically labeled with anti-CD133 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) in the dark at 4ºC for 30 min and applied to the prepared MS Column (Miltenyi Biotec). CD133+ cells were collected in the flow-through of the column; CD133+ cells bound to the beads were flushed out by applying the plunger supplied with the column.

Flow cytometry

The percentage of CD133 expressing cells in the original cell populations, the flow-through fraction, and the flushed-out fraction were analyzed by flow cytometry. Cells were washed twice with phosphate-buffered saline (PBS) and then were stained at the concentration of 1×106 cells per 90µL PBS buffer and 10µL control or specific antibody. PE-conjugated anti-CD133 was used as the antibody (Miltenyi Biotec). After 30 min incubation at 4ºC, cells were washed twice with PBS and analysed with a FACScan (Beckman Coulter, Brea, CA).

L-PHA binding assay

CD133+ cells transfected with wild type Mgat5 siRNA or mutant Mgat 5 siRNA were harvested 60 h post-transfection and then washed twice with PBS containing 1% BSA. FITC-conjugated leukoagglutinin (L-PHA, Vector Laboratories, Peterborough, UK) was added to a final concentration of
2.5\mu g/mL in PBS-BSA, and cells were incubated for 30 min at 4\degree C. The cells were collected by centrifugation and then suspended in 1mL PBS-BSA for FACS analysis (Beckman coulter).

**RNA Isolation and Real Time Fluorescence Quantitation RT-PCR (FQ RT-PCR)**

Total RNA was extracted from the cell lysates using TRIZOL (Gibco-Invitrogen). RNA was quantitated by the real-time reverse transcription polymerase chain reaction using the QuantiTect\textsuperscript{TM} SYBR Green PCR Handbook Kit (QIAGEN, Valencia, CA). The samples were heated to 95\degree C for 10 min, followed by 30 cycles of 95\degree C for 10 sec, 56\degree C for 20 sec, and 72\degree C for 20 sec. The oligonucleotide primers used were as follows: Mgat5 forward: 5'-AGCAGCTCATGTTCAGG-3', reverse: 5'-GACCAAGATTTCCACTT-3'; Beta-actin forward: 5'-TACTGAGGCGTGGAC-3', reverse: 5'-CC TCGTGCTCAGTACA-3' (Shanghai Sangon Biological Engineering Technology & Services, Shanghai, China). The relative expression quantities of Mgat5 in each sample were normalized by comparing with that of Beta-actin.

**Western blotting**

Two million cells were lysed with 500mL SDS-PAGE 1× loading buffer, after boiling for 5 min, samples were separated by 12.5% SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. Skim milk powder solution (10%, w/v) was used in blocking. Monoclonal mouse anti-Mgat5 antibody (Abcam, Cambridge, UK) was used at a 1:2000 dilution at RT in TBST buffer (50 mM Tris–HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20) with 3% fetal bovine serum (Hyclone). The membrane was incubated with primary antibody for 1h at RT, washed three times and then incubated with 1:3000-fold diluted HRP-conjugated goat anti-mouse IgG. Signals were visualized using enhanced chemiluminescence (ECL).

**Stable Transfection**

As reported, wild-type Mgat5 siRNA and mutant Mgat5 siRNA used in our studies were as follows: siRNA forward: 5'-UCGGGCUUCAAGAUGCGG-3', siRNA reverse: 5'-CC GCAUUUUGAGCCCGA-3'; siRNA mut forward: 5'-UGGGCUUCAAGAUGCGG-3'; siRNA mut reverse: 5'-CCGCAUUUUGAGCCCGA-3' [14] (Shanghai Sangon Biological Engineering Technology & Services, Shanghai, China). Transfection of siRNAs were performed with LipofectamineTM 2000 (Gibco-Invitrogen) at a final concentration of 100nmol/L. Cells were cultured for 48h in complete medium and transferred to complete medium containing 300\mu g/mL zeocin (Gibco-Invitrogen).

**Cell proliferation assay**

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) proliferation assay was used to determine the anti-growth effects of Mgat5 siRNA to CD133\textsuperscript{+} cells. The cells were resuspended in fresh medium, diluted to 1.25×10\textsuperscript{5} cells/mL, and seeded into four 96-well plates at 100\mu L per well. After transfection of siRNA, cells were grown for up to 4 days and one plate was used for MTT assay each day. Twenty microliters of MTT (5mg/mL; Sigma, St. Louis, USA) was added to each well, and the cells were incubated at 37\degree C for another 4h. Then the culture medium was replaced with 150\mu L dimethyl-sulfoxide (Sigma). After shaking for 10 min, the plate was read for the absorbance at 490nm on a plate reader. The proliferation rate was determined as follows: absorbance of transfected group/absorbance of untransfected group.

**Generation of subcutaneous lung cancer xenografts into SCID mice**

For mice xenografts, CD133\textsuperscript{+} cells 48h after transfected with indicated siRNA, diluted in growth factor-containing medium before subcutaneous injection. Four-week-old female SCID mice were used. Cells (100 µl of 1×10\textsuperscript{5}/mL) were injected to the single cell suspensions were separated into CD133\textsuperscript{+} and CD133\textsuperscript{-} cells by magnetic cell separation and then analyzed by flow cytometry for CD133 expression. CD133\textsuperscript{+} cells were in-
FIGURE 1. *CD133 expression in pulmonary adenocarcinoma cells as analysed by flow cytometry.* CD133 was expressed in 1.2% of the unsorted pulmonary adenocarcinoma cells (left panel). Cells expressing CD133 constituted 0.3% of the CD133− subpopulation after sorting (middle panel). Cells expressing CD133 constituted 92.1% of the CD133+ subpopulation after sorting (right panel). The gray shadow represents the isotype control.

FIGURE 2. *Mgat5 expression in CD133+ or CD133− pulmonary adenocarcinoma cells.* FQRT-PCR (A) showed that Mgat5 expression was significantly higher in CD133+ cells than CD133− cells (*p<0.05), with an average of 1.33-fold and 1.13-fold based on the expression of Beta-actin, which was confirmed by Western blot (B). Data from six patients is shown and the expression of Mgat5 in 10 patients were normalized based on the expression of Beta-actin by gray scale scanning (C). (D) CD133+ cells and CD133− cells were stained with L-PHA and then analysed by flow cytometry. The gray shadow represented isotype control. (E) Statistical analysis of the mean fluorescence intensity (MFI) of L-PHA binding assay is shown. The data shown were calculated as the mean ± s.e.m, and data are from three independent experiments.
frequent, but consistently, detectable in all the patients analyzed (Fig. 1), which is in accordance with prior reports [7,15]; however, there is a good degree of enrichment of the CD133+ subpopulation sorted by magnetic cell separation (Fig. 1).

The expression of Mgat5 was increased in CD133+cells

To determine the expression of Mgat5 in different subpopulations of lung cancer cells, FQRT-PCR and Western blotting were performed as described above. These two separate assays gave rise to similar results, indicating that Mgat5 expression was significantly higher in CD133+ cells than CD133- cells. The FQRT-PCR and Western blot showed the expression of Mgat5 in CD133+ cells was 1.2-fold and 1.4-fold higher than in CD133- cells, respectively (Fig. 2A, 2B, 2C, *p<0.05). These results indicated that Mgat5 was highly expressed in CD133+ cells compared with CD133- cells.

The plant lectin, leukoagglutinin (L-PHA), binds specifically to mature Mgat5 products and has been used as a probe for Mgat5-modified glycans [16]. CD133+ cells and CD133- cells were stained with L-PHA (2.5 μg/mL) in PBS-BSA and then analyzed by flow cytometry. The results show that L-PHA binding to Mgat5-modified glycans on CD133+ cells (99.1%, MFI=72±5) was significantly increased compared with CD133- cells (89.2%, MFI=12±3) (Fig. 2D, 2E, *p<0.05). The highly expressed Mgat5-modified glycans on CD133+ cells is probably due to the highly expressed Mgat5 in CD133+ cells, which is consistent with the results of FQRT-PCR and Western blot analyses.

Mgat5 siRNA down-regulates the expression of Mgat5 in CD133+cells in vitro

As previously reported, wild-type Mgat5 siRNA and mutant Mgat5 siRNA have been synthesized [14] but the ability of siRNA to knockdown the expression of Mgat5 in CD133+ cells has not yet been demonstrated. Here we confirm the interference efficiency by FQRT-PCR, Western blot and L-PHA binding assay. The results of FQRT-PCR show that siRNA was able to interfere with Mgat5 with an interference efficiency of 82%, consistent with the 80% reported previously [14] (Fig. 3A). The Western blot and L-PHA binding assay show that siRNA efficiently reduces the Mgat5 protein levels and the Mgat5-modified glycans production (Fig. 3B, 3C). The mutant siRNA had no effect on Mgat5 as shown in Fig. 3. The siRNA specific for Mgat5 could efficiently knockdown the expression of Mgat5 in CD133+ cells in vitro.

Knockdown of Mgat5 Inhibits CD133+ cells growth in vitro

To determine whether the inhibition of Mgat5 by siRNA affects the growth of CD133+ cell post-transfection, the proliferation of CD133+ cell post-transfection was evaluated by MTT test as described above. The MTT assay showed that the proliferation rate of CD133+ cell treated with siRNA was decreased compared with the untransfected cells (Fig. 4). At 48h after transfection, the proliferation rate of CD133+ cell treated with siRNA was significantly lower than that of CD133+ cell treated with siRNA mutant (*p<0.05), whereas there was no significant difference between the proliferation rate with siRNA or siRNA mutant at the point 24h after transfection (Fig. 4). These data indicated that specific siRNA for Mgat5 influence the proliferation of CD133+ cell in vitro.

Knockdown of Mgat5 Inhibits CD133+ cells growth in vivo

Finally, we performed experiments aiming at evaluating the effect of Mgat5 siRNA on the growth of the CD133+ cells in vivo. As shown in Fig. 5, the Mgat5 specific siRNA significantly suppresses the growth of CD133+ cells in SCID mice, in comparison with all other groups (Fig. 5, p<0.05). These data indicated that specific siRNA for Mgat5 influenced the growth of CD133+ cell in vivo.

Discussion

In the last decade, data have been lending support to the idea that tumors contain a small population of cancer stem cells that are responsible for tumor maintenance and spreading. The growing understanding of cancer biology has led to the clear concept that tumors are hierarchically organized and are composed of a heterogeneous population of cells in which a rare population referred to as tumor stem cells (TSC) are at the top of this hierarchy, and play a critical role in the development of cancer. The same phenomena exist in lung cancer; therefore, the identification and characterization of the cell populations responsible for lung cancer will contribute significantly to the development of new therapeutic strategies and may improve the prognosis of this malignancy.

Although recent evidence suggests the likely presence of cancer stem cells in pulmonary tumors, the phenotype of lung cancer stem cells remains controversial. According to a recent report by Eramo et al., only CD133+ cells serve as a tumorigenic population in lung cancer tissue, being able to reproduce the original tumor in immunocompromised mice, while the CD133+ cancer cell population was completely devoid of tumor-initiating activity [6]. In this study, the rare population of CD133+ cancer stem-like cells were separated from excised
pulmonary adenocarcinoma specimens of 10 patients for further analysis. Subsequent study found Mgat5 to be differentially expressed in pulmonary adenocarcinoma cells and predominantly in CD133+ cells (Fig. 2). Our data indicate that the distribution of Mgat5 in pulmonary adenocarcinoma tissue was asymmetrical, which was similar to the distribution of galectin-3 in pulmonary adenocarcinoma tissue as reported previously [7].Taken together, these data indicate that both Mgat5 and galectin-3 are highly expressed in the CD133+ cell and it seems likely that the gene expression pattern is very different between the CD133+ cells and CD133− cells, which is understandable given their different roles in the tumor progress.

Li et al. previously reported that knockdown of Mgat5 inhibited murine breast cancer cell growth in vitro and in vivo, and indicated that tumor growth inhibition in vivo might be due to the direct effect of Mgat5 specific shRNA on tumor cells in the early stage and T-cell and macrophage activities at the later stage of tumor development [14]. In this study, Mgat5 specific siRNA can efficiently knockdown the expression of Mgat5 in CD133+ cells (Fig. 3), and knockdown of Mgat5 inhibited CD133+ cells growth both in vitro and in vivo (Fig. 4, Fig. 5). The immune cells activities were not involved in the reason of inhibited growth of Mgat5 siRNA treated CD133+ cells in vivo in this study because the mice employed were SCID mice. Of note, the MTT assay showed that knockdown of Mgat5 not only decreased the expression of Mgat5 but also caused defective beta-1,6-GlcNAc branched N-glycan on the cell (Fig. 3). Hamaguchi et al. reported that swainsone, an N-glycan inhibitor that inhibits golgi alpha-mannosidase II and ultimately causes the inhibition of N-
linked beta-1,6-oligosaccharide expression upstream of the action of Mgat5 [17], at the concentration of 5 mg/mL had no cytotoxicity on murine colorectal cancer cells [18]. These data suggest that the defective beta-1, 6GlcNAc branched N-glycan expression on the cell has no effect on cell growth. Taken together, these data suggest that down-regulated expression of Mgat5 in CD133+ cells, but not the defective beta-1, 6-GlcNAc branched N-glycan on the cell, may be the major reason for the decreased proliferation ratio of siRNA-treated CD133+ cells.

Mgat5, and its glycan products beta-1,6-GlcNAc branched N-glycans, are tumor-associated glycoproteins commonly increased in malignancies, and their high level expression correlates with disease progression [8,9,11]. Furthermore, many studies have demonstrated the association of increased L-PHA binding with increased tumor cell invasiveness. Likewise, cancers of breast, colon, and melanomas show increased levels of beta-1,6-GlcNAc branched N-glycan measured by L-PHA immunohistochemistry [8,10]. L-PHA reactivity is also increased in atypical hyperplasia and carcinomas of breast compared with normal and benign lesions [10]. In our study, the results of L-PHA binding assay also showed higher reactivity in CD133+ cells than in CD133- cells (Fig.2D, 2E) and suggest increased L-PHA reactivity of CD133+ cells, is in accordance with the state of CD133+ cells as cancer stem-like cells.

In conclusion, this study provides concrete experimental evidence that Mgat5 is highly expressed in CD133+ cells from lung adenocarcinoma tissue and contributes to the growth of CD133+ cells. Therefore, understanding the role of Mgat5 in the CD133+ cell will yield important insights into such malignancies and delineate novel strategies for disease intervention.

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List of Abbreviations
MACS magnetic activated cell sorting
FCM flow cytometry
FQRT-PCR fluorescent quantitative real time polymerase chain reaction
ELISA enzyme linked immunosorbent assay
CD133+ cell  CD133+ human pulmonary adenocarcinoma cells
CD133- cell  CD133- human pulmonary adenocarcinoma
GlcNAc  N-acetylgalactosamine
L-PHA  leukoagglutin
Mgat5  N-acetylgalactosaminyltransferase V

References