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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 vivo* and *in vitro*.

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.

Manuscript submitted 17th January, 2011

Manuscript accepted 28th March, 2011

Clin Invest Med 2011; 34 (3): E155-E162.

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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 cell invasiveness [9-11]. Hirotoshi *et al.* suggested that expression of Mgat5 is associated with prognosis and histology in non-small cell lung cancers [12]. Oncogenesis increases β 1,6-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, 200 µg/mL streptomycin and 5 µg/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 20 µg/mL collagenase II (Gibco-Invitrogen, Carlesbad, CA) for 2h at 37°C. Recovered cells were cultured in serum-free medium containing 50 µg/mL insulin, 100 µg/mL apo-transferrin, 10 µg/mL putrescine, 0.03mM sodium selenite, 2 µM 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 20 µg/mL EGF (Epidermal Growth Factor) and 10 µg/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 4°C 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 1×10^6 cells per 90 µL PBS buffer and 10 µL control or specific antibody. PE-conjugated anti-CD133 was used as the antibody (Miltenyi Biotec). After 30 min incubation at 4°C, 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 leucoagglutinin (L-PHA, Vector Laboratories, Peterborough, UK) was added to a final concentration of

2.5 µg/mL in PBS-BSA, and cells were incubated for 30 min at 4°C. The cells were collected by centrifugation and then suspended in 1 mL 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™ SYBR Green PCR Handbook Kit (QIAGEN, Valencia, CA). The samples were heated to 95°C for 10 min, followed by 30 cycles of 95°C for 10 sec, 56°C for 20 sec, and 72°C for 20 sec. The oligonucleotide primers used were as follows: Mgat5 forward: 5'-AGCAGCTCCATGTTACGG -3', reverse: 5'-GACCAGATTGTCCACCTTT-3'; Beta-actin forward: 5'-TACTGAAGGCCGTGGAC-3', reverse: 5'-CC TCGTGGCTCCAGTACA-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 500 µL 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 1 h 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'-UCGGGCUUCAAGAUUGCGG-3', siRNA reverse: 5'-CC GCAAUCUUGAAGCCCGA-3'. siRNAmut forward: 5'-UGCGGCUUCAAGAUUGCGG-3', siRNAmut reverse: 5'-CCGCAAUCUUGAAGCCGCA -3' [14] (Shanghai Sangon Biological Engineering Technology & Services, Shanghai, China). Transfection of siRNAs were performed with Lipofectamine™ 2000 (Gibco-Invitrogen) at a final concentration of 100 nmol/L. Cells were cultured for 48 h in complete medium

and transferred to complete medium containing 300 µg/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⁺ cells. The cells were resuspended in fresh medium, diluted to 1.25 × 10⁵ cells/mL, and seeded into four 96-well plates at 100 µL 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 (5 mg/mL; Sigma, St. Louis, USA) was added to each well, and the cells were incubated at 37°C for another 4 h. Then the culture medium was replaced with 150 µL dimethylsulfoxide (Sigma). After shaking for 10 min, the plate was read for the absorbance at 490 nm 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⁺ cells 48 h 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 × 10⁵/mL) were injected to evaluate the tumorigenic activity of CD133⁺ cells transfected with Mgat5 specific siRNA. Mice were monitored to check for the appearance of signs of disease, such as subcutaneous tumors or weight loss due to potential tumor growth in internal sites. The tumor size was measured with calipers and the tumor volume was calculated according to the formula (length × width²)/2.

Statistical analysis

Data were expressed as the mean ± standard error of the mean (SEM) of at least three independent experiments performed in triplicate. All statistical analyses were performed in the SPSS 11.0 statistical software package. Experimental data were analyzed by ANOVA followed by the Student-Newman-Keuls method for multiple comparisons among means. Values of *p* < 0.05 were considered statistically significant.

Results

MACS efficiency detected by FCM

The single cell suspensions were separated into CD133⁺ and CD133⁻ cells by magnetic cell separation and then analyzed by flow cytometry for CD133 expression. CD133⁺ 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 $\pm$ 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, leucoagglutinin (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 $\mu\text{g/mL}$) 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 $\pm$ 5) was significantly increased compared with CD133⁻ cells (89.2%, MFI=12 $\pm$ 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 decreases 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

FIGURE 3. *Mgat5* siRNA efficiently down-regulates the expression of *Mgat5* in CD133⁺ cells *in vitro*. (A) The expression of *Mgat5* in CD133⁺ cells, treated with wild-type siRNA, mutant siRNA or untreated 48 h after transfection, were detected by FQRT-PCR. (B) Western blot was applied to visualize the expression of *Mgat5* in CD133⁺ cells treated with wild-type siRNA, mutant siRNA or untreated 72 h after transfection. (C) The percentage of L-PHA positive cells in CD133⁺ cells treated with siRNA 72 h after transfection were analysed by FCM. The data shown were calculated as mean $\pm$ s.e.m. (* $p < 0.05$) and data were from three independent experiments (A,C).

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* influence the proliferation of CD133⁺ cells *in vitro* (Fig. 4). The amount of cells xenografted into SCID mice was the same, but the proliferation ratio was different among each group, which may be the one of the reasons for the inhibition of *Mgat5* siRNA treated CD133⁺ cells growth *in vivo*.

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 swainsonine, an N-glycan inhibitor that inhibits golgi alpha-mannosidase II and ultimately causes the inhibition of N-

FIGURE 4. The proliferation rate of CD133⁺ cell treated with indicated siRNA. Proliferation rate was determined as follow: absorbance of transfected CD133⁺ cells/absorbance of untransfected CD133⁺ cells. The data shown were calculated as mean±s.e.m. (**p*<0.05) and data were from three independent experiments with CD133⁺ cells were from three individual patients.

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 lesions¹⁰. 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

FIGURE 5. Knockdown of Mgat5 Inhibits CD133⁺ cells growth *in vivo*. CD133⁺ cells from a single patient were transfected with indicated siRNA and xenografted into SCID mice as described in the methods. The tumor size was measured with calipers and the tumor volume was calculated according to the formula (length×width²)/2. There were six mice in each group and the data shown were calculated as mean±s.e.m. (**p*<0.05).

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.

Acknowledgments

This work has been funded by grants to Dr. Zhao from the Natural Science Foundation of Hubei Province (No.2008 CHB418) and grants to Xuefeng Zhou from the Annual Research Project of Health Department of Hubei Province (NX2011-6).

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	<i>N</i> -acetylglucosamine
L-PHA	leukoagglutinin
Mgat5	<i>N</i> -acetylglucosaminyltransferase V

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