RESEARCH ARTICLE

α2,6-linked sialic acids on N-glycans modulate the adhesion of hepatocarcinoma cells to lymph nodes

Shujing Wang · Xixi Chen · Anwen Wei · Xiao Yu · Bachir Niang · Jianing Zhang

Abstract The alterations of cell surface sialylation play a key role in tumor metastasis. Enhanced α2,6-sialylation on N-glycans results from overexpression of the sialyltransferase ST6Gal-I. Hca-F and Hca-P cells are murine hepatocarcinoma cell lines which metastasize only to the lymph nodes. Our previous study revealed that ST6Gal-I was involved in the adhesion of Hca-F cells to fibronectin. However, the roles of sialic acids in the adhesion of Hca-F cells to lymph nodes still remain poorly understood. In this study, we observed that expression levels of α2,6-linked sialic acids on Hca-F cells were higher compared to Hca-P cells. Knockdown of ST6Gal-I by small hairpin RNA (shRNA) transfection decreased the expression of α2,6-linked sialic acids and inhibited the adhesion of Hca-F cells to lymph nodes. The adhesion ability was reported to be mediated by siglec-2 which preferentially binds to α2,6-linked sialic acids, and in addition, ST6Gal-I knockdown inhibited the phosphorylated levels of focal adhesion kinase (FAK) and paxillin when cells were treated with siglec-2. Taken together, these results suggest that interaction of α2,6-linked sialic acids with siglec-2 might modulate the adhesion of hepatocarcinoma cells to lymph nodes through the FAK signaling pathway.

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Introduction

Sialic acids are acidic sugars typically terminating the outer ends of cell surface glycan chains, which can mediate many biological functions such as innate and adaptive immune responses [1–3]. Sialic acid may be linked either through an α2,3- or an α2,6-bond to subterminal galactose; through an α2,6-bond to N-acetylgalactosamine (GalNAc); or through an α2,8-bond to another sialic acid, forming polysialic acid. Changes in terminal sialylation of N-glycans have been reported to be closely associated with cellular adhesion, migration, and metastasis in tumor cells [4, 5].

α2,6-linked sialic acid is catalyzed by β-galactoside:α2-6-sialyltransferase 1 (ST6Gal-I), which adds sialic acid attached to Galβ1-4GlcNAc in an α2,6 linkage. ST6Gal-I is upregulated in colon adenocarcinoma and its expression positively correlates with tumor cell invasiveness and metastasis [6–8]. Elevated levels of ST6Gal-I and α2,6-linked sialic acid have also been observed in carcinomas of the cervix, ovary, and liver [9–11]. However, the roles of sialic acids in liver tumorigenesis and development still remain poorly understood.

Siglecs (sialic acid-binding immunoglobulin-type lectins) are cell surface proteins that bind sialic acid. They are found primarily on the surface of immune cells and play different functions based on cell surface receptor-ligand interactions. Siglec-2, also called CD22, is a lymphocyte-restricted glycoprotein involved in cell adhesion and signaling. The cross-species reactivity of siglec-2 with its ligands underscores the potential physiologic importance of siglec-2-mediated lymphocyte interactions [12, 13].

Hca-F and its syngeneic cell line Hca-P are mouse hepatocarcinoma cell lines with high and low potential of

lymphatic metastasis, respectively. Hca-F and Hca-P cells metastasize only to the lymph nodes and do not disseminate to other organs [14]. Our previous study found that ST6Gal-I overexpression is positively correlated with the adhesive potential of mouse hepatocarcinoma cells to fibronectin [15]. However, the relationship between cell surface sialic acids and specific adhesion of Hca-F cells to lymph nodes still remains unknown.

In this study, we found that highly adhesive potential of tumor cells is correlated with the high expression levels of $\alpha 2,6$-linked sialic acids and the decreased expression of $\alpha 2,6$-linked sialic acids by downregulation of ST6Gal-I resulted in decreased cell adhesive potential to lymph nodes. Here, we also reported that $\alpha 2,6$-linked sialic acid-mediated adhesion of tumor cells to lymph nodes was dependent on siglec-2, and ST6Gal-I knockdown inhibited the focal adhesion kinase (FAK)-mediated adhesion signaling pathway. These results indicate that the interaction of $\alpha 2,6$-linked sialic acids with siglec-2 might modulate the adhesion of hepatocarcinoma cells to lymph nodes through the FAK adhesion signaling pathway.

Materials and methods

Cell culture and mice

Mouse hepatocarcinoma cell lines Hca-F and Hca-P, which have high and low metastatic potential, respectively, in the lymph nodes (established and stored by the Department of Pathology, Dalian Medical University), were maintained in 90 % RPMI 1640 (Gibco) supplemented with 10 % heat-inactivated fetal bovine serum (Gibco), 1× penicillin/streptomycin (Gibco), and 1 g/l sodium bicarbonate (Gibco). All cells were cultured in a humidified incubator at 37 °C with 5 % CO_2. A total of 615-mice (Hca-F and Hca-P tumor host) were obtained from the Animal Facility of Dalian Medical University. Inbred 615 mice, weighing 20 to 23 g, were used for this study.

Lectin immunocytochemistry for sialic acid expression

Cells were grown on sterile glass coverslips, washed three times in ice phosphate-buffered saline (PBS), and fixed for 5 min at −20 °C with acetone. After fixation, cells were blocked with Avidin/Biotin Blocking Kit (Vector, SP-2001) and then incubated with biotin-labeled lectins Sambucus nigra agglutinin (SNA) or Maackia amurensis agglutinin (MAA) (Vector, 1:1000 dilution) for 1 h, washed several times with PBS, and incubated for 30 min with fluorescein isothiocyanate (FITC)-avidin. After being washed, the coverslips were mounted onto slides for photography, set for appropriate fluorophore excitation emission viewing and storage.

Flow cytometry analysis for sialic acid expression

Cells were blocked with PBS containing 1 % bovine serum albumin (BSA) and then incubated with 2 μg/ml FITC-conjugated SNA or MAA lectin for 30 min on ice. After washing thrice with PBS, cells were analyzed using a FACScan instrument (BD Biosciences).

Real-time PCR analysis

Real-time PCR was performed with ABI PRISM 7900 detection system (Applied Biosystems) using SYBR Premix DimerEraser Kit (TaKaRa). Total RNA was extracted using TRIzol reagent (Invitrogen), and complementary DNA (cDNA) synthesis was performed using PrimeScript RT reagent Kit (TaKaRa) according to the manufacturer's instructions. Specific primers for ST6Gal-I, ST6Gal-II, ST6GalNac-I, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased (GenePharma). Relative changes in messenger RNA (mRNA) expression were normalized with GAPDH and calculated using the $2^{-\Delta\Delta CT}$ method.

Western blot analysis

Protein concentration was measured with BCA assay kit (Pierce). Equal amounts of denatured proteins were subjected to 10 % sodium dodecyl sulfate (SDS)-PAGE and blotted onto nitrocellulose membranes (Pall Corporation). Antibodies against ST6Gal-I, ST6Gal-II, ST6GalNac-I, FAK, phosphorylated FAK (p-FAK), paxillin, p-paxillin, ERK1/2, p-ERK1/2, and GAPDH (Santa Cruz Biotechnology, Inc.) were used as the primary antibodies. The detection was performed using ECL kit (Amersham Biosciences) according to the manufacturer's instructions. The relative amount of protein was determined by densitometry using LabWorks software.

Construction of vectors and transfection

ST6Gal-I-specific small hairpin RNA (shRNA) sequences used in the construction of RNA interference (RNAi) vector were as follows: 5′-CACCGAAAGGGAGCGACTATGAGT CAAGAGCTCATAGTCGCTCCCTTTCTTTTTTG-3′ and 5′-GATCCAAAAAAGAAAGGGAGCGACTATGAGCT CTTGA-CTCATAGTCGCTCCCTTTC-3′. The oligonucleotides targeting ST6Gal-I or the negative control were annealed and ligated into pGPU6/neo vector (GenePharma), respectively. Hca-F cells were transfected with the mixture of plasmids and Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's recommendation. Silencing level was determined by real-time PCR (RT-PCR) and Western blot assays 48 h after transfection. Construction of expression vector encoding ST6Gal-I cDNA had been described previously [15].

Lectin blot assay

Cells were harvested, rinsed with PBS, and lysed with Membrane Protein Extraction Reagent Kit (Sigma-Aldrich). Cell lysates containing 30 μg of protein were boiled in SDS sample buffer with β-mercaptoethanol, loaded on 10 % SDS-polyacrylamide gels, and then transferred onto a PVDF membrane. After being blocked with 5 % BSA, the membrane was incubated with 1:1000 dilution of biotin-labeled SNA for 2 h at room temperature. The blots were developed using ECL detection system (Amersham Biosciences).

Adhesion assay to lymph nodes

In vitro adhesion assay was performed as previously described [16]. The cell suspension (1×10^6) of 200 μl was overlaid onto the frozen sections of lymph nodes (type mouse 615), and the

sections were incubated for 16 h at 37 °C with 5 % CO_2. Then, the sections were washed one time with cold normal saline to residual. After a 30-min static incubation at 4 °C, followed by three washes with cold normal saline, the sections were fixed in 95 % alcohol for 5 min and stained with hematoxylin and eosin (HE). Adherent cells were calculated and analyzed with Image-Pro Plus 4.5 software (Media Cybernetics, USA).

Siglec binding assay

The wells were coated with 100 μl per well of 5 μg mouse siglecs (siglec-1, 2, 3, 5, R&D) in PBS, followed by blocking with 2 % BSA in PBS. BSA and poly-L-lysine-coated wells were used as negative and positive controls, respectively. Cells were harvested by centrifugation at 2000 rpm for 5 min and washed extensively in PBS. Then, the cells were seeded onto each well in serum-free media and incubated for 2 h at 37 °C. After 2 h of incubation, cells were washed with

Fig. 1 Surface expression of α2,6-linked sialic acids positively correlates with the adhesive potential of mouse hepatocarcinoma cells to lymph nodes. a An adhesion assay was performed to measure Hca-F and Hca-P cells binding to lymph nodes. The frozen sections were stained with hematoxylin and eosin (HE) (×200). Arrow represents tumor cell and empty lymph node as a control. b The number of adhesive Hca-P cells is represented as a percentage of adhesive Hca-F cells, which was taken as 100 %. c, e Lectin cytochemistry by using SNA and MAA lectins for analyzing the expression of α2,6- or α2,3-linked sialic acids on Hca-F and Hca-P cell surface (×200). NC negative control. d, f The expression levels of α2,6- or α2,3-linked sialic acids. The values are mean±SD of three independent experiments carried out in triplicate. Asterisk indicates significance by Student's t test ($P<0.05$). g FACS analysis for the expression of α2,6- or α2,3-linked sialic acids
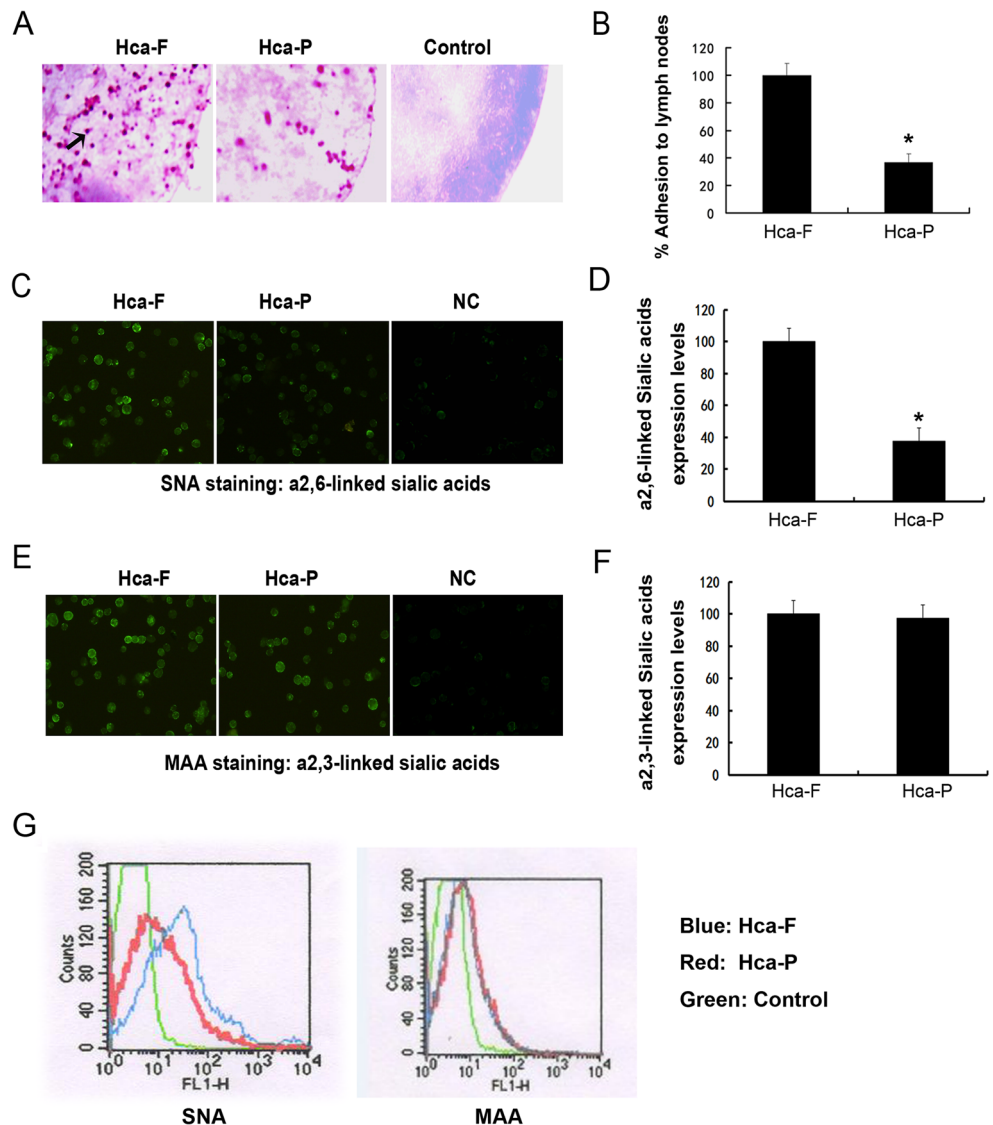
PBS twice and labeled with crystal violet for 1 h at room temperature. The number of crystal violet-labeled cells was measured by VersaFluor Fluorometer (Bio-Rad, Hercules, CA, USA) at 570 nm.
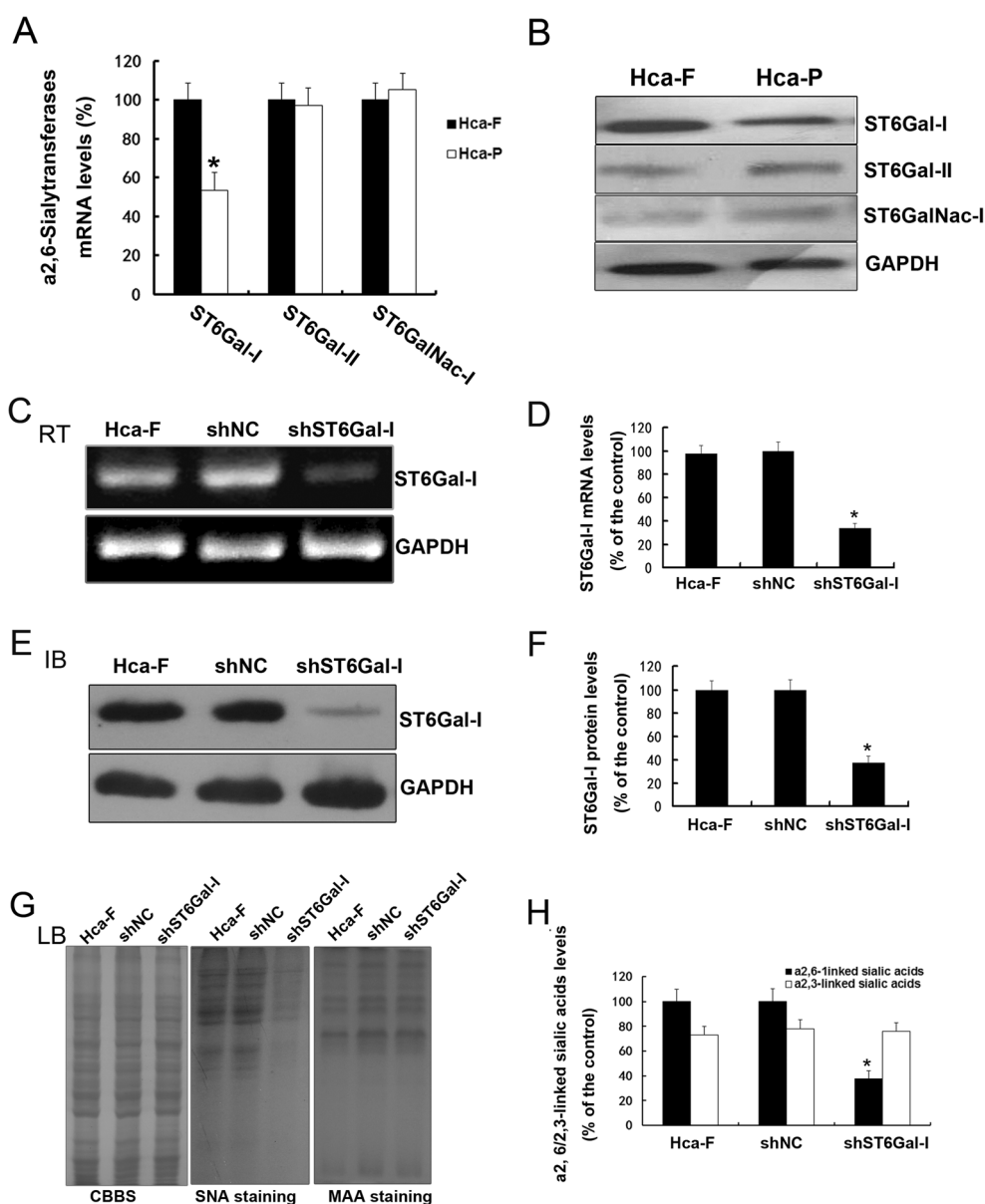
Statistical analysis

The data were expressed as the mean±S.E. Statistical analysis was performed with SPSS 13.0 software. One-way ANOVA with post hoc Tukey's test was performed for experiments that involved more than two groups, and Student's t test was performed for comparisons between two groups. $P<0.05$ was considered to be statistically significant.

Results

Differences in the expression levels of sialic acids between Hca-F and Hca-P cells

The mouse hepatocarcinoma cell lines Hca-F and Hca-P have been derived from mouse ascites-type hepatocarcinoma cell line H22, and they metastasize only to the lymph nodes and do not disseminate to other organs. Hca-F cells exhibited higher adhesive capability to lymph nodes than Hca-P cells (Fig. 1a, b). To investigate the difference in the expression of sialic acids on Hca-F and Hca-P cell surface, lectin cyto-chemistry and fluorescence-activated cell sorting (FACS) analysis were performed by using SNA and MAA lectins recognizing α2,6- and α2,3-linked sialic acids, respectively.

Fig. 2 Downregulation of ST6Gal-I in Hca-F cells by ST6Gal-I-shRNA. Hca-F cells were transfected with ST6Gal-I-specific shRNA and negative control shRNA, as described under "Materials and methods." **a** The mRNA levels of sialyltransferases were measured using real-time PCR with RNA extracted from the Hca-F and Hca-P cells. **b** Cell extracts were assessed by Western blot with anti-ST6Gal-I, anti-ST6Gal-II, anti-ST6GalNAc-I, and anti-GAPDH antibodies. **c, e** Knockdown efficiency on Hca-F cells was analyzed by RT-PCR and Western blot assays (*shNC* negative control, *shST6Gal-I* ST6Gal-I-specific short hairpin RNA); GAPDH was used as an internal control. **d, f** Relative signal intensities of ST6Gal-I mRNA and protein levels as compared with GAPDH were analyzed by LabWorks (TM ver4.6, UVP, BioImaging Systems), respectively (*$P<0.05$). **g, h** Lectin blot analysis of the α2,6-linked sialic acid levels from the cell membrane lysates using SNA lectin staining. Coomassie Brilliant Blue staining of gels (CBBS) was used to normalize the protein amounts and MAA staining as an unrelated control. The data were obtained from three independent experiments (*$P<0.05$)

The results showed that Hca-F cells expressed significantly higher levels of α2,6-linked sialic acids compared to Hca-P cells (Fig. 1c, d). However, there was no apparent difference in the expression of α2,3-linked sialic acids between Hca-F and Hca-P cells (Fig. 1e, f). These results indicate that the expression of α2,6-linked sialic acids might be correlated with the adhesive potential of hepatocarcinoma cell lines to lymph nodes.

Knockdown of ST6Gal-I inhibits the levels of α2,6-linked sialic acids on Hca-F cell surface

To further determine the difference in the expression of α2,6-linked sialic acids between Hca-F and Hca-P cells, three sialyltransferases (ST6Gal-I, ST6Gal-II, and ST6GalNac-I) were analyzed by using real-time PCR and Western blot assays. ST6Gal-I levels were higher in Hca-F cells than in Hca-P cells, and no significant changes in ST6Gal-II and ST6GalNAc-I expression were observed between the two cells (Fig. 2a, b). A Hca-F cell line stably silencing ST6Gal-I was established, and RT-PCR results showed that ST6Gal-I-shRNA transfection inhibited the expression of ST6Gal-I mRNA by 67 % as compared to untransfected cells and the cells transfected with negative control shRNA (Fig. 2c, d). ST6Gal-I protein levels were suppressed by up to 62 % in ST6Gal-I-shRNA-transfected cells compared with negative control cells (Fig. 2e, f). ST6Gal-I knockdown effectively inhibited the expression of α2,6-linked sialic acids as revealed by SNA staining (Fig. 2g, h). Thus, ST6Gal-I-shRNA transfection could effectively inhibit the ST6Gal-I mRNA and protein expression and suppress α2,6-linked sialic acid levels on Hca-F cell surface.

### Downregulation of α2,6-linked sialic acids attenuates the adhesive capability of Hca-F cells to lymph nodes

To investigate the effects of cell surface α2,6-linked sialic acids on the cell adhesive capability to lymph nodes, the adhesion assay to lymph nodes was performed. The result showed that the number of ST6Gal-I-shRNA-transfected cells adhering to frozen lymph node sections was markedly lower than that of negative control cells (Fig. 3a, b). To further explore the role of α2,6-linked sialic acids in mediating tumor cell adhesion to lymph nodes, cells were incubated with SNA, which can block the roles of α2,6-linked sialic acids. The result showed that the adhesion of SNA-incubated Hca-F cells to lymph nodes was decreased in a concentration-dependent manner (Fig. 3c). These observations indicate that α2,6-linked sialic acids on cell surface might positively mediate the adhesion of Hca-F cells to lymph nodes.
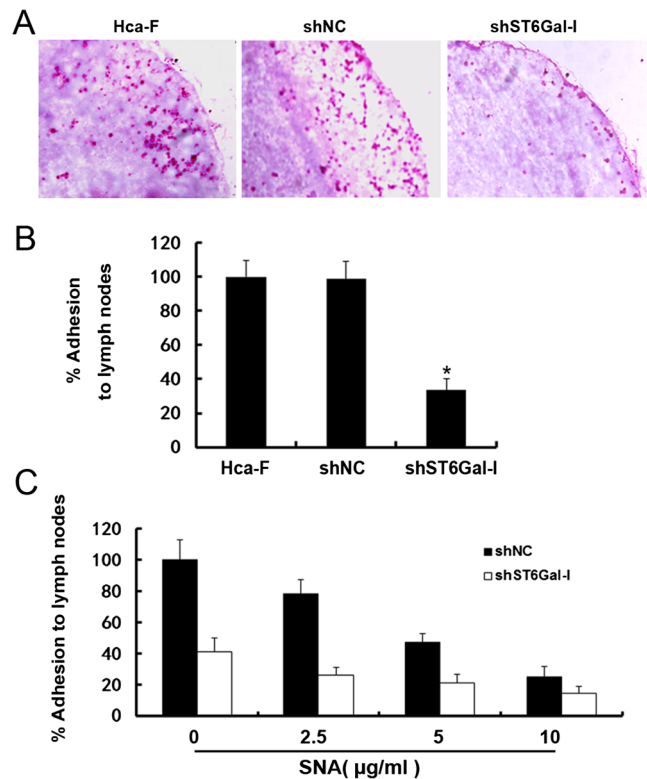
Fig. 3 Downregulation of α2,6-linked sialic acids attenuates the adhesive ability of Hca-F cells to lymph nodes. **a** An adhesion assay was performed to measure the binding of Hca-F cells and shNC- and shST6Gal-I-transfected cells to lymph node sections. The frozen sections were stained with HE (×200). **b** The number of adhesive shST6Gal-I-transfected Hca-F cells is represented as a percentage of adhesive Hca-F cells, which was taken as 100 %. The values are mean±SD of three independent experiments carried out in triplicate (*$P<0.05$). **c** The number of adhesive Hca-F cells treated with SNA was decreased in a concentration-dependent manner (0, 2.5, 5, and 10 μg/ml). The data were obtained from three independent experiments (*$P<0.05$)

α2,6-linked sialic acid-mediated tumor cell adhesion is dependent on siglec-2 in lymph nodes

Here, negative control shRNA- and ST6Gal-I-shRNA-transfected Hca-F cells were evaluated for cell binding ability to siglecs (siglec-1, 2, 3, 5). The result showed that Hca-F cells exhibited the highest binding capability to siglec-2 (CD22) rather than to other siglecs, and the binding was significantly limited after silencing ST6Gal-I expression (Fig. 4a). Sialic acid-dependent binding of siglec-2 was further confirmed using neuraminidase as shown in Fig. 4b. Cell adhesive capability to lymph nodes was reduced gradually in the presence of anti-siglec-2 antibody, and the inhibition percentage was dependent on the concentration of the antibody added (Fig. 4c). In contrast, no apparent change was found in the adhesion of the cells treated with anti-siglec-3 antibody (Fig. 4d). This indicates that α2,6-linked sialic acid-mediated tumor cell adhesion to lymph nodes might be dependent on siglec-2 in lymph nodes.
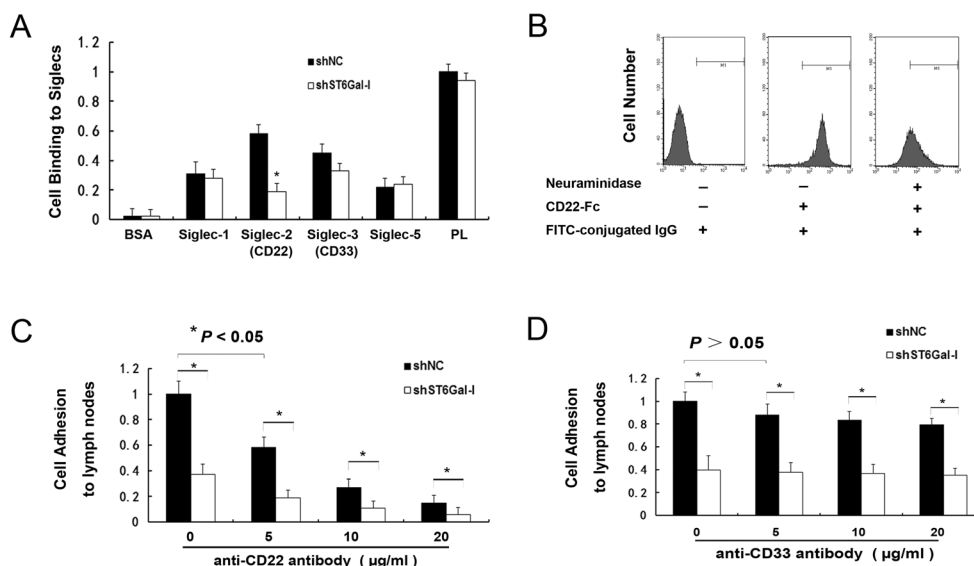
Fig. 4 α2,6-linked sialic acid-mediated adhesion is dependent on siglec-2 in lymph nodes. **a** shNC- and shST6Gal-I-transfected Hca-F cells were seeded onto cell culture plates coated with siglecs, and binding was quantified by measuring the absorption of crystal violet-stained cells. *Asterisk* indicates significance by Student's *t* test (*P*<0.05). **b** Characterization of glycans recognized by CD22 using neuraminidase. CD22 binding reduced after treatment with neuramidase (200 mU/ml) in Hca-F cells. Definite reduction in the binding intensities of CD22-Fc was observed by flow cytometry (MFI, mean fluorescence intensity: 316.82 vs. 61.89). **c**, **d** The frozen lymph node sections were treated with rabbit anti-mouse CD22 or CD33 polyclonal antibody at a dose-dependent manner (0, 5, 10, and 20 μg/ml), and then an adhesion assay was performed to measure cells binding to lymph nodes

Downregulation of α2,6-linked sialic acids inhibits FAK-mediated adhesion signaling in Hca-F cells

To further analyze the effect of ST6Gal-I downregulation on the related signaling pathways, Western blot assay was used to detect FAK and ERK signaling pathways in control and shST6Gal-I cells, with or without CD22 stimulation. The results showed that ST6Gal-I knockdown inhibited the phosphorylated levels of FAK and paxillin, and the phosphorylation of ERK1/2 was not be altered by the downregulation of α2,6-linked sialic acids on Hca-F cells (Fig. 5a, b). In addition, we used PF-562271, a specific inhibitor of the FAK signaling pathway, to determine the role of FAK in α2,6-linked sialic acid-mediated cell adhesion. The result demonstrated that the increased adhesion caused by ST6Gal-I overexpression was inhibited by PF-562271 treatment (Fig. 5c). These results indicate that α2,6-linked sialic acids moderate the adhesion of Hca-F cells to lymph nodes through the FAK signaling pathway.

### Discussion

The increase in sialylation is often manifested by specific increases in α2,6-linked sialic acids attached to the outer of *N*-acetyllactosamine (Galβ1–4GlcNAc units). In this study, we firstly compared the level of α2,6-linked sialic acid expression in two hepatocarcinoma cell lines by lectin cytochemistry using SNA lectin and found a higher level in Hca-F cells compared to Hca-P cells. This result is the same as that we got from our previous study by lectin microarray (data not shown). Increased expression of ST6Gal-I is reported in carcinomas of the colon and breast, in gastric cancer, and in some brain tumors [17–20]. Here, we found that the expression of ST6Gal-I in mRNA and protein levels was higher in Hca-F cells. Therefore, the altering expression of ST6Gal-I and α2,6-linked sialic acids in the two cell lines may be very important as indicators and functional contributors of tumor lymphatic metastasis.

Changes in terminal sialylation of N-glycans have been reported to be closely associated with cellular adhesion, migration, and metastasis in tumor cells [21–24]. For example, Seales et al. reported that upregulation of α2,6-linked sialic acids by ST6Gal-I overexpression may contribute to metastasis by regulating invasiveness and cell motility [25]. Hedlund et al. demonstrated that ST6Gal-I promoted tumor growth and inhibited differentiation of spontaneous mammary cancers in mice [26]. Our previous study showed that altered expression of ST6Gal-I mediates the adhesive capability of Hca-F cells to fibronectin [15]. Here, we observed that ST6Gal-I knockdown could effectively decrease the levels of α2,6-linked sialic acids and significantly inhibited the adhesion of Hca-F cells to lymph nodes. These observations clearly indicate that the changes in ST6Gal-I expression levels may have impact in the remodeling of cell surface sialylation, which may consequently affect the biological functions of tumor cells such as adhesion and invasion.

The molecular mechanisms regulating the lymphatic metastasis are still poorly understood. Siglecs are one type of
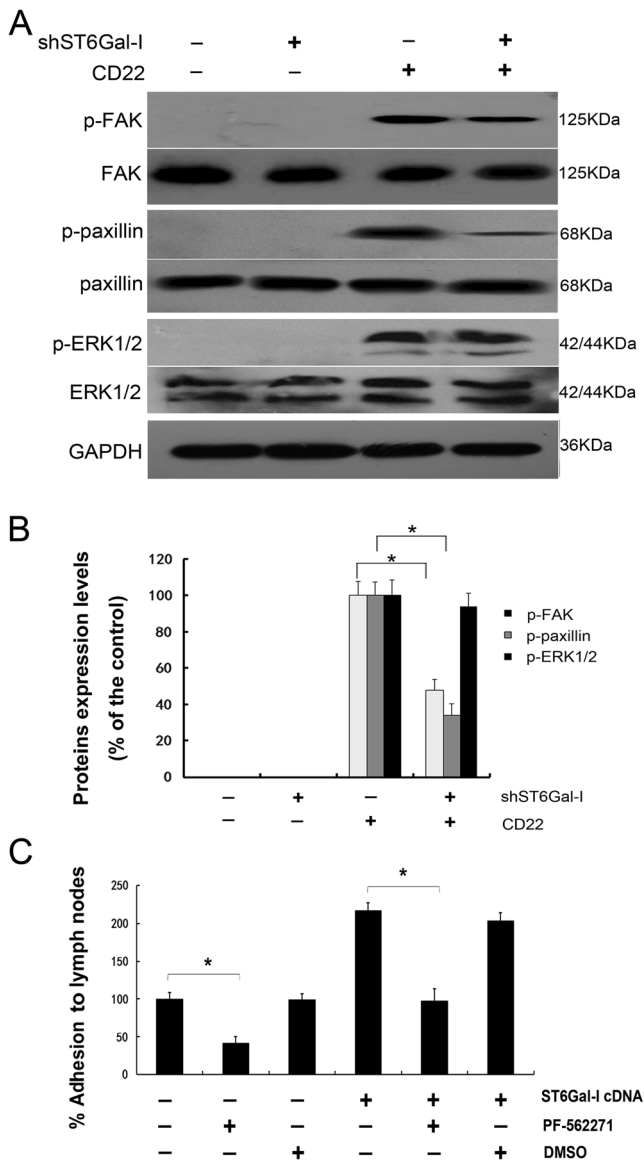
Fig. 5 Downregulation of ST6Gal-I inhibits FAK-mediated adhesion signaling in Hca-F cells. **a** The phosphorylation of FAK, paxillin, and ERK1/2 was estimated by Western blot assay. **b** Relative signal intensities of proteins as compared with GAPDH were analyzed by LabWorks (TM ver4.6, UVP, BioImaging Systems), respectively. Data shown is representative of three independent experiments (*$P < 0.05$). **c** Cells (Hca-F, Hca-F/ST6Gal-I) were treated with or without PF-562271 (10^{-5} M) and then used for adhesion assay to lymph nodes. Data shown is representative of three independent experiments (*$P < 0.05$)

adhesion receptors which can interact specifically with sialic acids. Siglecs are mainly expressed in immune cells, and the potential function of siglecs in tumor biology has received minimal attention [27, 28]. Since siglec-2 is likely to play an important role in interactions between lymphocytes and other cells, and in regulating signaling thresholds [29, 30], we hypothesize that siglec-2 can also be involved in the adhesion of tumor cells to lymph nodes. In agreement with these data, our results showed a direct correlation between α2,6-linked sialic acid levels on hepatocarcinoma cells and their adhesion

to siglec-2. In addition, we found that the adhesive capability of tumor cells to lymph nodes was reduced gradually in the presence of anti-CD22 antibody. Therefore, the tumor lymphatic metastasis might be regulated by the interaction of α2,6-linked sialic acids on tumor cell surface with siglec-2. However, the interaction of α2,6-linked sialic acids with siglec-2 in mediating in vivo lymph node metastasis still deserves further study.

Cell-ECM interaction can lead to intracellular phosphorylation, and focal adhesion kinase (FAK) activity and tyrosine phosphorylation will be preferentially upregulated in response to cell-matrix contact [31]. In agreement with this notion, we found that the decrease of α2,6-linked sialic acids inhibited FAK-mediated cell adhesion signaling pathways, and the increase of adhesion caused by ST6Gal-I overexpression was inhibited by PF-562271 treatment (a specific inhibitor of the FAK signaling pathway). These results indicate that ST6Gal-I promotes the adhesion of Hca-F cells to lymph nodes through the FAK signaling pathway.

Taken together, these results represent the first report indicating that α2,6-linked sialic acids on tumor cell surface modulate their adhesion to lymph nodes via siglec-2, suggesting a new mechanism of tumor lymphatic metastasis.

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