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Caveolin-1 up-regulates integrin α2,6-sialylation to promote integrin α5β1-dependent hepatocarcinoma cell adhesion

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ABSTRACT

The alterations of integrin glycosylation play a crucial role in tumor metastasis. Our previous studies indicated that caveolin-1 promoted the expression of the key α2,6-sialytransferase ST6Gal-I and fibronectin-mediated adhesion of mouse hepatocarcinoma cell. Herein, we investigated the role of α2,6-sialylated α5-integrin in the adhesion of mouse hepatocarcinoma H22 cell. We demonstrated that caveolin-1 up-regulated cell surface α2,6-linked sialic acid via stimulating ST6Gal-I transcription. Cell surface α2,6-sialylation was required for integrin α5β1-dependent cell adhesion to fibronectin, and an increase in α2,6-linked sialic acid on α5-subunit facilitated fibronectin-mediated focal adhesion kinase phosphorylations, suggesting that α2,6-sialylated α5-subunit promoted integrin α5β1-dependent cell adhesion.

1. Introduction

Extracellular matrix (ECM) is an important regulator of cell behaviours and microenvironment and consists of many molecules, such as fibronectin (Fn), collagen (Col), laminin (Ln), proteoglycans and non-matrix proteins [1]. Enhanced tumor cell adhesion to ECM is a key step to induce cell invasion in tumor metastasis [2]. Integrins are transmembrane glycoproteins that form non-covalent heterodimers composed of α- and β-subunits. Members of integrin family are the major cell surface receptor for ECM and play a crucial role in mediating cell–ECM interactions during cell proliferation, development and tumor malignant behaviours [3]. Glycosylation, as a post-translational modification reaction, is tissue specific and developmentally regulated by the activity of glycosyltransferases and glycosidases. Although integrin-dependent cell adhesion is based on the binding of integrin to defined peptide sequence of ECM protein, this interaction is regulated by various factors including glycosylation modification [4].

Caveolin-1 (Cav-1) is identified as a major structural protein of caveolae and is implicated in lipid transport, signal transduction and tumor progression [5]. Current studies demonstrated that Cav-1 positively regulated tumor growth and metastatic ability in hepatocellular carcinogenesis (HCC) [6–9]. There is accumulating evidence that Cav-1 may participate in the regulation of glycosylation modification. Cav-1 could mediate the subcompartmental localization of glycosyltransferase N-acetylglucosaminyltransferase III in Huh6 cell [10]. 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 a α2,6 linkage. Elevated levels of ST6Gal-I and α2,6-linked sialic acid had been observed in carcinomas of cervix, brain and liver [11–13]. However, the regulation mechanisms and roles of aberrant α2,6-sialylation in HCC progression are poorly understood.

H22 is a mouse hepatocarcinoma cell line with high metastasis potential. Our present study found that knockdown of Cav-1 in H22 cells down-regulated cell surface α2,6-linked sialic acid through inhibiting ST6Gal-I transcription, which could be restored by the reintroduction of wild-type Cav-1. Cell surface α2,6-sialylation-induced enhancement of cell adhesion to Fn was strongly suppressed by function-blocking antibody against α5- or β1-subunit. Furthermore, an increase in α2,6-linked sialic acid on α5-subunit up-regulated the Fn-mediated focal adhesion kinase (FAK) phosphorylations.

2. Materials and methods

2.1. Cell culture

Mouse hepatocarcinoma cell line H22 was obtained from Cell Center of Peking Union Medical University (Beijing) and maintained in Rosewell Park Memorial Institute (RPMI) 1640 medium

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(Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco) under a humidified atmosphere of 95% air and 5% CO_2.

2.2. Construction of RNA interference vector and transfection

Three Cav-1-specific small hairpin RNA (shRNA) sequences used in the construction of RNA interference (RNAi) vector were as follows, shRNA-1: 5′-CACCGTACCTGAGTCTCCAGAAATTCAAGAGATTTCTGGAGACTCAGGTACTTTTTTG-3′ and 5′-GATCCAAAAAAGTACCTGAGTCTCCAGAAATCTCTT GAATTTCTGGAGACTCAGGTAC-3′; shRNA-2: 5′-CACCGCCACTCAGCAACTGAATGA TTCAAGAGATCATTCAGTTGCTGAGTGGTTTTTTG-3′ and 5′-GATCCAAAAAAACCACTCAGCAACTGAATGATCTCTTGAATCATTCAGTTGCTGAGTGGC-3′; shRNA-3: 5′-CACCGTACCTGAGTCTCCAGAAATT CAAGAGATTTCTGGAGACTCAGGTACTTTTTTG-3′ and 5′-GATCCAAAAAAGTACCTGAGTCTCCAGAAATCTCTTGAATTTCTGGAGACTCAGGTAC-3′. Three shRNAs were annealed and ligated into pGPU6 vector (GenePharma) to generate shRNA constructs (shCav-1-1, shCav-1-2 and shCav-1-3), respectively. Negative control shRNA construct (shNC) was used as a control. H22 cells were transfected with the mixture of plasmids and Lipofectamine™ 2000 (Invitrogen) according to manufacturer's recommendation.

2.3. Construction of rescue vector and transfection

Construction of expression vector encoding wild-type Cav-1 or ST6Gal-I had been described previously [14]. Rescue vectors were transfected into Cav-1 knockdown H22 cells, respectively. After 48 h transfection, Cav-1- and ST6Gal-I-rescued cells were used to perform the following assays.

2.4. Construction of luciferase reporter vector, transfection and promoter activity assay

The transcriptional start site for mouse ST6Gal-I gene was retrieved from UCSC Genome Browser (http://genome.ucsc.edu). A 2000-bp region upstream of the transcriptional start site was subcloned into pGL3-basic vector (firefly luciferase reporter vector) (Promega) to generate pGL3-basic/ST6Gal-I vector. The pGL3-basic/ST6Gal-I construct and control plasmid pRL-TK (Promega), which expresses *Renilla* luciferase under the control of TK promoter, were cotransfected into negative control, three Cav-1-shRNA and wild-type Cav-1-rescued transfectants by using Lipofectamine™ 2000 (Invitrogen), respectively. After 24 h transfection, firefly luciferase and *Renilla* luciferase activities were measured using Dual-Luciferase Assay Kit (Promega) as described by manufacturer. Firefly luciferase activities were normalized against the *Renilla* luciferase activities.

2.5. Real-time PCR analysis

Real-time PCR was performed as described previously [14]. Relative Cav-1 and ST6Gal-I mRNA levels were normalized with GAPDH and calculated using $2^{-\Delta\Delta CT}$ method.

2.6. Western blot analysis

Protein concentration was measured with BCA assay kit (Pierce). Equal amounts of denatured proteins were subjected to 10% SDS–PAGE and blotted onto nitrocellulose membranes (Pall Corporation). Antibodies against α5-subunit, β1-subunit, Cav-1, ST6Gal-I, FAK, phosphorylated FAK (p-FAK), paxillin, p-paxillin, ERK1/2, p-ERK1/2 and GAPDH (Santa Cruz Biotech Inc.) were used as the primary antibodies. The detection was performed using ECL kit (Amersham Biosciences) according to manufacturer's instructions. Relative amount of protein was determined by densitometry using LabWorks software.

2.7. Lectin blot analysis

Cells were harvested, rinsed with PBS, and lysed with ProteoPrep® Membrane Extraction Kit (Sigma–Aldrich). The lysates containing equal amounts of denatured proteins were subjected to 8% SDS–PAGE and transferred to nitrocellulose membranes. The identification was performed with 2 μg/ml biotinylated *Sambucus nigra* (SNA) lectin (Vector Laboratories, Inc.), which preferentially recognizes sialic acid attached to terminal galactose in a α2,6-linkage. The blots were developed using ECL detection system (Amersham Biosciences).

2.8. Flow cytometry analysis

Cells were blocked with PBS containing 1% bovine serum albumin (BSA), and then incubated with 2 μg/ml FITC-conjugated SNA lectin for 30 min on ice. For the analysis of α5- or β1-subunit, cells were treated with the primary antibody against α5- or β1-subunit for 1 h on ice, and then incubated with FITC-conjugated goat anti-rabbit IgG for 1 h on ice. After washing thrice with PBS, cells were analyzed using a FACScan instrument (BD Biosciences).

2.9. Immunoprecipitation and Western blot

Cells were lysed with ProteoPrep® Membrane Extraction Kit (Sigma–Aldrich). Protein concentration was measured with BCA assay kit (Pierce). The lysates containing equal amounts of proteins were incubated with 2 μg of anti-α5 subunit antibody and then with 50 μl protein A-agarose beads (Invitrogen). Immunoprecipitates were subjected to 8% SDS–PAGE and transferred to nitrocellulose membranes. The blots were probed with biotinylated SNA lectin and anti-α5 subunit antibody, respectively, and developed using ECL detection system (Amersham Biosciences).

2.10. Cell adhesion assay

Cell adhesion assay was performed as described previously [14]. For adhesion inhibition assays, cells were pre-incubated with control IgG (5 μg/ml), EDTA (5 mM), function-blocking anti-α5 or anti-β1 subunit antibody (2.5 μg/ml or 5 μg/ml) for 20 min at room temperature before inoculation.

2.11. 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.

3. Results

3.1. Knockdown of Cav-1 in H22 cells reduces cell surface α2,6-linked sialic acid via down-regulating ST6Gal-I transcription

To investigate a possible relationship between Cav-1 and cell surface α2,6-sialylation, we developed three shRNA interference vectors (shCav-1-1, shCav-1-2 and shCav-1-3) to silence Cav-1 expression in H22 cells, respectively. Cav-1 and ST6Gal-I expression were suppressed in different Cav-1-shRNA transfected cells (Fig. 1A and B), and shCav-1-1 transfectant exhibited a weaker