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Glycogenes mediate the invasive properties and chemosensitivity of human hepatocarcinoma cells

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ABSTRACT

Aberrant cell-surface glycosylation patterns are present on tumors and have been linked to tumor progression. This study aimed to identify the alterations of glycogene and N-glycan involved in tumor invasion, tumorigenicity and drug resistance in MHCC97-H and MHCC97-L human hepatocarcinoma cell lines, which have high, low metastatic potential, respectively. Using real-time PCR for quantification of glycogene and FITC-lectin binding for glycan profiling, we found that the expression of glycogenes and glycan profiling were different in MHCC97-H cells, as compared to those in MHCC97-L cells. We silenced the expression levels of glycogenes MGAT3 and MGAT5, which were over-expressed in MHCC97-L and MHCC97-H cells. Knockdown of MGAT3 expression promoted MHCC97-L cells invasion and increased resistance to 5-fluorouracil in vitro. The silencing of MGAT5 in MHCC97-H cells inhibited invasion and increased sensitivity to 5-fluorouracil in vitro. Further analysis of the N-glycan regulation by tunicamycin application or PNGase F treatment in MHCC97-H and MHCC97-L cells showed partial inhibition of N-glycan glycosylation, decreased invasion, tumorigenicity and increased sensitivity to 5-fluorouracil both in vitro and in vivo. These findings suggest that alterations of glycogene and N-glycan in human hepatocarcinoma cells correlate with tumor invasion, tumorigenicity and sensitivity to chemotherapeutic drug, and have significant implications for the development of treatment strategies.

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1. Introduction

Glycosylation plays an important role in regulating properties of proteins on the cell surface. Glycan interactions mediate biological events such as trafficking, signaling and adhesion, in addition to processes such as development, immunity and diseases (Varki, 1993; Dove, 2001; Rudd et al., 2001; Haltiwanger and Lowe, 2004). Furthermore, glycans are considered to have great potential as therapeutic targets or clinical biomarkers for diagnosis of various malignant diseases. Aberrant glycosylation has been also known to be associated with carcinogenesis, invasion and metastasis (Bertozzi and Kiessling, 2001; Gu and Taniguchi, 2008). Thus, alterations to glycans found in glycoproteins derived from cancer are a common feature and can be used as a convenient biomarker.

Metastasis of tumor cells and the development of resistance to antitumor therapies are the main causes of morbidity and mortality from malignancy. These two properties of malignant tumors have been studied extensively, and some investigations have addressed the linkage between the two phenotypes (Jia et al., 2008a, 2008b; Zhao et al., 2012). Specific changes in the glycosylation pattern of cell surface glycoproteins have been shown to correlate with the enhancement of the metastatic efficiency of tumor cells. In human hepatocarcinoma cells, the alteration of N-glycan structure in surface glycoproteins resulting from the activity change of MGAT5 contributes to the alterations in metastasis-associated phenotypes (Guo et al., 2001). Cell surface sialylation as well as β1–6 branching of N-linked glycans are strongly correlated with metastatic potential of cancer cells (Dennis et al., 1982, 1987). Furthermore, a number of studies have aimed to identify those glycans that are expressed specifically by chemo resistant tumor cells (Nakahara et al., 2003; Kudo et al., 2007). Lattová et al. reported that N-glycomic changes in human breast carcinoma MCF-7 and T-lymphoblastoid cells occurred after treatment with herceptin

Abbreviations: 5-FU TM, 5-fluorouracil tunicamycin; PBS, phosphate-buffered saline; PBST, PBS containing 0.1% Tween 20; TBS, tris buffered saline; DMSO, dimethyl sulfoxide; PNGase F, peptide N glycosidase F; IR, tumor inhibition rate.

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and herceptin/Lipoplex (Lattová et al., 2010). Kudo et al. have also described that N-glycan alterations are associated with drug resistance in human hepatocellular carcinoma (Kudo et al., 2007). These findings suggest that glycosylation alterations may be involved in the regulation of multiple aspects of tumor metastasis and drug resistance.

Glycogenes, which encode proteins, are involved in glycan synthesis and modification and almost the entire human glycogenes have been cloned, including those encoding glycosyltransferases, sulfotransferases, and sugar–nucleotide transporters (Mantelli et al., 2009). Real-time PCR, being both sensitive and accurate, is one of the most popular validation protocols for mRNA quantification analysis (Kudo et al., 2007), and we used it in this study to analyze the expression levels of glycogenes.

Lectins have long been used as useful tools to characterize cell surface glycans because of their substantial selectivity in terms of branching, linkage, and terminal modifications of complex glycans (Elloway et al., 2004). In this study, FITC-lectin binding was used for glycan profiling of cell membrane glycoproteins in MHCC97-H and MHCC97-L cell lines.

The objective of the present study was to investigate alterations of glycogene and N-glycan between the MHCC97-H and MHCC97-L cell lines by using real-time PCR and FITC-lectin binding, in order to examine whether glycogenes and N-glycans participate in the regulation of tumor invasion, tumorigenicity and drug resistance. Meanwhile, we mainly focused on the regulation of N-glycans of cell surface and knockdown of glycogene expression to further address the important roles of glycomics in mediating the invasive properties and chemosensitivity of human hepatocarcinoma cells.

2. Materials and methods

2.1. Cell culture

Human hepatocarcinoma cell lines MHCC97-H and MHCC97-L were obtained from the Liver Cancer Institute Zhongshan Hospital, Fudan University (China). Two cell clones of the same genetic background but with different metastatic potential were established from parental hepatocellular carcinoma (HCC) cell line MHCC97 (obtained from the Liver Cancer Institute Zhongshan Hospital, Fudan University, China). The parental cell line MHCC97 (Tian et al., 1999) is a human HCC cell line performed on the animal model of human HCC LCI-D20 (Sun et al., 1996). Compared with MHCC97-L, MHCC97-H had a high metastasis rate (Li et al., 2001). The two cell lines were cultured in 90% DMEM (Gibco) supplemented with antibiotics (1× penicillin/streptomycin 100 U/ml, Gibco) and 10% heat-inactivated fetal bovine serum (Gibco). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Analysis of glycogenes

To investigate the expression profiles of genes related to glycan synthesis, a real time RT-PCR analysis was performed. Total RNA were isolated from two cell lines using an RNeasy Mini Kit (QIAGEN) and cDNA was synthesized using QuantiTect Reverse Transcription Kit (QIAGEN) from 5 μg of total RNA according to the manufacturer's instruction. Real-time PCR amplification and analysis were performed on 7500 fast Real-time PCR System (Applied Biosystems) for 40 cycles (15 s at 95 °C, 15 s at 60 °C and 30 s at 72 °C). All reactions were performed with QuantiTect SYBR Green PCR Kit (QIAGEN) according to the manufacturer's instruction. Primer sequences were summarized in Table 1. Expression levels of each glycogene were normalized using either the expression level of GAPDH mRNA and compared between MHCC97-H and MHCC97-L cell lines. Real-time RT-PCR analysis was performed in triplicate.

2.3. RNAi assay

MHCC97-L or MHCC97-H cells were incubated in appropriate antibiotic-free medium with 10% fetal bovine serum (Gibco), transferred to a 6 well tissue culture and incubated at 37 °C, in a CO_2 incubator to obtain 60–80% confluens. The cell cultures were transfected with MGAT3 or MGAT5 specific siRNA Transfection Reagent Complex, respectively (Santa Cruz Biotech, Inc., sc-44469; sc-40642), which was prepared according to the protocol. Scrambled siRNA was used as the negative control. Transfer cells were cultured and incubated at 37 °C for 6 h, followed by incubation with complete medium for additional 24 h. Then cells were harvested and experimented as described for Western blot analysis, in vitro ECM invasion assay and in vitro drug sensitivity assay. The cell transfection efficiency was 80%, 78% and the survival rate was 91%, 90%, respectively.

2.4. Western blot analysis

Whole cell proteins were electrophoresed under reducing conditions in 10% polyacrylamide gels. The separated proteins were transferred to a polyvinylidene difluoride membrane. After blocking with 5% skimmed milk in PBS containing 0.1% Tween 20 (PBST), the membrane was incubated with antibody (anti-CD147, 1/200 diluted, Santa Cruz Biotech; anti-integrin β1, 1/200 diluted, Santa Cruz Biotech; anti-MGAT3, 1/500 diluted, Abcam; anti-MGAT5, 1/500 diluted, Abcam) and then with peroxidase-conjugated anti-rabbit IgG (1/10,000 diluted; GE Healthcare UK Ltd., Little Chalf-ont, UK). A GAPDH antibody (1/200 diluted; Santa Cruz Biotech) was used as a control. All bands were detected using ECL Western blot kit (Amersham Biosciences, UK), according to the manufacturer's instruction. The bands were analyzed with LabWorks (TM ver4.6, UVP, BioImaging systems).

2.5. Coimmunoprecipitation

Coimmunoprecipitation was carried out to detect whether glycosylation interfered the interaction of CD147 with the integrin β1. MHCC97-H cells were seeded into culture dishes and incubated with the tunicamycin (0, 1, 5, or 10 μg/ml) and PNGase F (0, 8, 16, 24 h), respectively. The cells were then lysed using 1% NP40 Lysis buffer with protease inhibitors. Prepared cell lysates (400 μg) were incubated with CD147 antibody for 4 h at 4 °C on a rotator prior to incubation with 50 μl of a 50% slurry of immobilized protein A agarose (Invitrogen) overnight at 4 °C. Immunoprecipitates were collected by centrifugation, washed three times with coimmunoprecipitation buffer, eluted with SDS sample buffer, and subjected to Western blot analyses using integrin β1 antibody.

2.6. Flow cytometry assay

MHCC97-H and MHCC97-L cell lines were washed thrice with fluorescence-activated cell sorting (FACS) buffer (PBS containing 20 g/L bovine serum), and then centrifuged at 1000 r/min for 5 min in a 1.0 ml eppendorf tube for collecting cells. The cells were blocked for 30 min 37 °C in 5% powdered skim milk and then were washed between each step with FACS buffer. Cells were placed in sterile conical tubes in aliquots of 500,000 cells each and stained with one of the 8 FITC-lectins at a final concentration of 10 μg/ml for 40 min at 4 °C in the dark. Residual unbound FITC-lectin was then discarded by repeat centrifugation of samples at 1000 r/min. After removal of supernatant, cells were resuspended in 0.2 ml PBS. The

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