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Elevated activity of *N*-acetylglucosaminyltransferase V in human hepatocellular carcinoma

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Abstract Cell-surface glycoproteins are regarded as candidates for involvement in the spread of tumor cells. N-linked β1-6 branched oligosaccharides may contribute directly to the malignant or metastatic phenotypes of tumor cells. Increased β1-6 branching has been associated with an increased level of *N*-acetylglucosaminyltransferase V (GlcNAc transferase V), the glycosyltransferase that initiates the β1-6 branching. In this report, 33 pathologically verified hepatocellular carcinoma (HCC) specimens, six non-cancerous tissues surrounding HCC and five normal liver specimens have been studied. We have quantified N-linked β1-6 branched oligosaccharides indirectly by measuring GlcNac transferase V activity. The average GlcNac transferase V activities in hepatocellular carcinoma (HCC), noncancerous tissues surrounding HCC and normal liver tissues were 324.2 ± 269.8, 84.8 ± 20.7 and 7.0 ± 6.2 pmol product h^{-1} mg protein^{-1} ($P < 0.05$) respectively. In addition, the activity was correlated with the TNM classification of HCC. The average activities of GlcNAc transferase V in stages T1, T2–3 and T4 were 77.6 ± 57.8, 369.0 ± 294.7 and 329.9 ± 205.9 pmol product h^{-1} mg protein h^{-1} respectively ($P < 0.05$), showing that the activity of the enzyme in advanced HCC was higher than that in early HCC. Our preliminary results indicated that GlcNAc transferase V activity increased in human HCC and was correlated with its progression.

Key words *N*-Acetylglucosaminyltransferase · Hepatocellular carcinoma

Abbreviations *HCC* hepatocellular carcinoma

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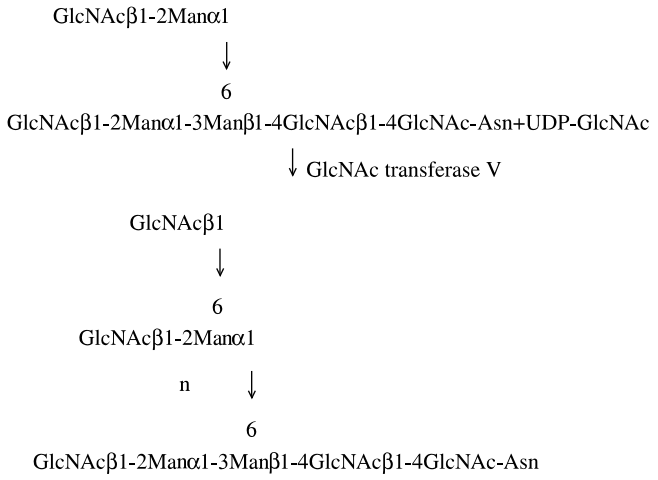
Introduction

Hepatocellular carcinoma (HCC) is one of the major malignancies in China, Japan, and Southeast Asia. During the past 20 years, owing to advances in the early detection of subclinical HCC, growing knowledge of the biological features of HCC and the introduction of new surgical techniques, rapid progress has been made in the treatment of this disease. The long-term prognosis, however, is not yet satisfactory, even for the resectable HCC, the main reason being high recurrence rates. The 1-, 3-, 5-, and 10-year recurrence rates are 9.2%, 38.8%, 54.9%, and 85.0% respectively (Zhou et al. 1994). High recurrence rates and poor prognosis mainly result from more malignant phenotypes of HCC, such as poor differentiation and highly invasive potentials.

The process of cancer progression and metastasis constitutes a complex series of sequential, biochemical interactions that enable a primary tumor to spread to secondary sites and colonize (Liotta 1986). Cell-surface glycoproteins are regarded as one of the principal candidates for involvement in tumor cell spread since they generally serve to mediate interactions of metastasis cells with their environment. Malignant transformation is commonly associated with the acquisition of larger asparagine (*N*)-linked oligosaccharides (Smets et al. 1984). This phenomenon has been widely observed in rodent tumor cells transformed by activated oncogenes (Dennis et al. 1989), DNA tumor viruses (Takasai et al. 1980) and chemical carcinogenesis (Rachesky et al. 1982). The larger N-linked oligosaccharides are generally more branched at the trimannosyl core (-GlcNAcβ1-6Manα1-6Man-) because of an increased *N*-Acetylglucosaminyltransferase V activity in malignant cells (Yamashita et al. 1985).

N-Acetylglucosaminyltransferase V (GlcNAc transferase V, EC 2.4.1.155) is a key enzyme in the processing of N-linked sugar chains during the synthesis of glycoproteins. It catalyzes the transfer of an *N*-acetylglucosamine (GlcNAc) residue from UDP-GlcNAc to the α1-6

mannoside arm in acceptor *N*-glycans to form a β1-6 branched structure:



N-linked β1-6 branched oligosaccharides may contribute directly to the malignant or metastatic phenotypes of tumor cells (Dennis et al. 1989; Dennis and Laferte 1989). Increased β1-6 branching was associated with an increased level of GlcNAc transferase V, the glycosyltransferase that initiates the β1-6 branching. Reduction of β1-6 branches in metastatic cells leads to a reduction in metastatic ability (Yoshimura et al. 1985). In this report, we have quantified N-linked β1-6 branched oligosaccharides indirectly by measuring GlcNAc transferase V activity in human hepatocellular carcinoma.

Materials and methods

Patients and tumor specimens

Thirty-three pathologically verified HCC and 6 corresponding noncancerous specimens were obtained and stored in liquid nitrogen immediately after removed from patients. Of the 5 normal liver specimens, 1 was from a patient who died suddenly and 4 from tissues surrounding a hepatic hemangioma.

Of the 33 patients with HCC, 29 were male and 4 female. The average age was 47 ± 9 years. The average tumor diameter was 7.3 ± 4.6 cm; 16 of the 33 tumors were small (less than 5 cm in diameter), 11 were large (5–10 cm), and 6 very large (more than 10 cm).

GlcNAc transferase V activity assay

Microsome extracts

All procedures were carried out at 4°C. Specimens were homogenized in four volumes of homogenizing solution in a glass homogenizer tube after being cleansed with 0.9% sodium chloride three times. After centrifugation at 2000 rev/min for 10 min, the supernatants were centrifuged at 36 000 rev/min for 1 h. The pellets were collected, resuspended in homogenizing solution as the crude enzyme preparations, and used for assaying GlcNAc transferase activity.

Assay of GlcNAc transferase V

The substrate for GlcNAc transferase V, the fluorescently labeled biantennary sugar chain GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNacβ1-4GlcNac-PA (where PA represents a pyridylamine group) was prepared as described (Hase et al. 1984). A previously described method to assay the activity of GlcNAc transferase-V was adopted (Wang et al. 1997). The product peak was collected and confirmed by comparing it with standard sugar chains by HPLC. The 50-µl reaction mixture contained 100 mM MES (pH 6.25), 100 µM substrate, 50 mM UDP-GlcNAc, 0.2 M GlcNAc, 1% Triton X-100 and 100 µg crude enzyme protein. After incubation at 37°C for 5 h, the reaction was stopped by heating at 100°C for 3 min and the samples were centrifuged at 5000 rev/min in an Eppendorf tube for 15 min. An aliquot (20 µl each sample) was applied to an HPLC column (CLC-ODS C18 column, Shimadza). Elution was performed at 55°C with 10 mM ammonium acetate, containing 0.125% *n*-butanol for the first 10 min, followed by a concentration gradient of *n*-butanol (0.125%–0.500%) in 10 mM ammonium acetate for 40 min and 0.500% *n*-butanol in the same buffer for the final 10 min. The flow rate was 1 ml/min. The product was quantified as: (amount of the standard peak) × (area under product peak)/(area under standard peak). The specific activity of GlcNAc transferase V was expressed as pmol product h^{-1} mg $protein^{-1}$ (see Fig. 1).

Protein determination

Protein was determined according to Lowry et al. (1951) taking bovine serum albumin as the standard.

Statistical analysis

Comparison of values of each group was made by *t* test. $P < 0.05$ was considered statistically significant.

Results

The average GlcNAc transferase V activities in hepatocellular carcinoma (HCC), noncancerous tissues surrounding HCC and normal liver tissues were 324.2 ± 269.8, 84.8 ± 20.7, 7.0 ± 6.2 pmol product h^{-1} mg $protein^{-1}$ respectively. The enzyme activity was significantly higher in HCC than in the noncancerous

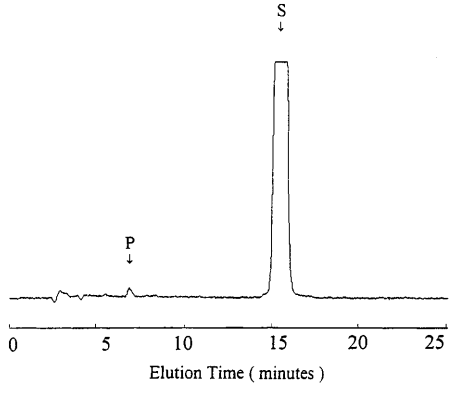


Fig. 1 Product pattern of *N*-acetylglucosaminyltransferase V. A 100-µg sample of protein homogenate was added to the reaction cocktail described in the text. The reaction time was 5 h (Wang et al. 1997). *S* substrate: GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNac-PA. *P* product of *N*-acetylglucosaminyltransferase V: GlcNAcβ1-2(GlcNAcβ1-6)Manα1-6(GlcNAcβ1-2Manα1-3) Manβ1-4GlcNAcβ1-4GlcNAc-PA (where PA represents a pyridylamine group)

Table 1 GlcNAc transferase V activity in heptatocellular carcinoma (*HCC*) and liver tissues. *NCT* noncancerous tissues surrounding HCC, *NLT* normal liver tissues

Sample	*n*	Enzyme activity (pmol product h^{-1} mg protein)
HCC	33	324.2 ± 269.8*
NCT	6	84.8 ± 20.7
NLT	5	7.0 ± 6.2

* $P < 0.05$ compared to NTC and NLT

Table 2 Correlation of GlcNAc transferase V activity and TNM classification of HCC

TNM classification	*n*	GlcNAc transferase-V activity (pmol product h^{-1} mg $protein^{-1}$)
T1	4	77.6 ± 57.8
T2–T3	21	369.0 ± 294.7*
T4	8	329.9 ± 205.9**

* $P < 0.05$ compared to T1
** $P < 0.05$ compared to T1

surrounding tissue and in normal liver ($P < 0.05$, see Table 1).

GlcNAc transferase V activity was correlated with the TNM classification of HCC. The average activities of the enzyme in stages T1, T2–3 and T4 were 77.6 ± 57.8, 369.0 ± 294.7 and 329.9 ± 205.9 pmol product h^{-1} mg $protein^{-1}$ respectively (Table 2), showing that GlcNAc transferase V activity in advanced HCC T2–4 was higher than that in early HCC (T1). There were no significant differences between GlcNAc transferase V activity and clinical-pathological parameters such as tumor size, age, α-fetoprotein level, hepatitis B virus infection or severity of liver cirrhosis.

Discussion

In several experimental tumor models, an increase in complex-type asparagine-linked oligosaccharides was correlated with invasiveness and metastatic potential. In support of this hypothesis, both somatic mutations and drugs that inhibit N-linked processing and reduce sialylation or branching of complex-type oligosaccharides also severely reduce the metastatic potential of MDAY-D2 lymphoreticular tumor cells, B16 melanoma cells, and human MeWo melanoma cells. Dennis et al. (1987) reported that an increase in β1-6 branched oligosaccharides is directly related to the metastatic potential of the cells. SP_1, a tumorigenic but nonmetastatic mouse mammary carcinoma cell line, expresses very small amounts of β1-6 branched oligosaccharides. Following transfection of SP_1 with activated H-*ras*, the tumor cells showed enhanced expression of GlcNAc transferase V activity and β1-6 branched oligosaccharides, as well as an increased metastatic potential in mice. The level of β1-6 branching was dependent on competition for substrate between GlcNAc transferases III and V, which play a role in invasion and cell attachment in the extravasation stage of metastasis (Yoshimura et al. 1995). Similarly in human uroepithelial cell lines, increased branching of complex-type asparagine-linked oligosaccharides was correlated with the degree of transformation and invasiveness (Debray et al. 1986). Loss or truncation of β1-6 branched oligosaccharides has multiple effects on cellular phenotypes, including decreased adhesion to vascular endothelial cells, increased adhesion to extracellular matrix proteins (Dennis et al. 1982), reduced cellular motility and invasion of the extracellular matrix (Yagel et al. 1990), reduced cellular response to autocrine growth stimulation (Dennis et al. 1990; VanderElst and Dennis 1991) and increased susceptibility to natural killer cells (Ahrens and Ankel 1987; Dennis and Laferte 1985).

β1-6 branched oligosaccharides were quantified either directly by L-PHA phaseless vulgaris agglutinin binding or indirectly by measuring GlcNAc transferase V activity. All breast carcinomas and epithelial hyperplasia with atypia showed significantly increased L-PHA staining compared to fibroadenomas and hyperplasia without atypia (Fernandes et al. 1991). GlcNAc transferase V activity correlated with the levels of L-PHA-reactive oligosaccharides (Dennis et al. 1989; Dennis and Laferte 1989). In this report, the activity of β1-6-*N*-acetylglycosaminyltransferase in human HCC specimens was significantly higher than that of noncancerous tissues surrounding HCC and normal liver tissues, implying that GlcNAc transferase V possibly contributes to malignant transformation of HCC and is a relative specific biological maker of HCC.

In several clinical investigations, increased β1-6 branching helped cancer cells to invade surrounding tissues of esophageal carcinomas (Takano et al. 1990). In colon carcinoma, Dukes stage C tumors presented higher levels of L-PHA staining than did stage A tumors, indicating that β1-6 branched oligosaccharides play a role in colon carcinoma progression (Fernandes et al. 1991). The latest results in our Research Center also show elevated GlcNAc transferase V activity, consistent with its mRNA levels, in pancreas carcinoma compared to those in normal pancreatic tissues (data not shown). Intrahepatic spreading and/or intraportal vein involvement are characteristic features signifying the highly metastatic potential of HCC. The TNM classification of HCC by UICC (UICC 1987) accurately reflects the progression of the disease on the basis of tumor size, number and blood vessel involvement. According to the clinical and pathological data, we found that GlcNAc transferase V conversely correlated with the TNM classification of HCC. The activity of *N*-acetylglycosaminyltransferase V in stage T1 (less than 2 cm in size and no blood vessel involvement) was significantly lower than that in stages T2–4 (more than 2 cm and/or blood vessel involvement). It seems primarily that the level of β1-6 branched oligosaccharides increased as a result of the elevated activity of *N*-acetylglycosaminyl-

transferase V during the progression of HCC, which possibly enhanced tumor cell adhesion and cell motility to vascular endothelial cells and extracellular matrix proteins. This hypothesis was partially confirmed by the fact that retinoids, promising modulators of tumor differentiation, invasion and metastasis (Lotan 1991), can change the structure of *N*-glycans on the surface of human hepatocellular carcinoma cells and decrease their GlcNAc transferase V activity (Chen et al. 1995). Preclinical and clinical trials for prevention and treatment of human HCC are under investigation.

Our preliminary results indicated that elevated activity of GlcNAc transferase V correlates with human HCC and its progression.

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