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Research Article

Hyper-O-GlcNAcylation of YB-1 affects Ser102 phosphorylation and promotes cell proliferation in hepatocellular carcinoma

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ABSTRACT

As an essential post-translational modification, O-GlcNAcylation has been thought to be able to modulate various nuclear and cytoplasmic proteins and is emerging as a key regulator of multiple biological processes, such as transcription, cell growth, signal transduction, and cell motility. Recently, authoritative glycomics analyses have reported extensive crosstalk between O-GlcNAcylation and phosphorylation, which always dynamically interplay with each other and regulate signaling, transcription, and other cellular processes. Also, plentiful studies have shown close correlation between YB-1 phosphorylation and tumorigenesis. Therefore, our study aimed to determine whether YB-1 was O-GlcNAc modified and whether such modification could interact with its phosphorylation during the process of HCC development. Western blot and immunohistochemistry were firstly conducted to reveal obvious up-regulation of YB-1, OGT and O-GlcNAc modification in HCC tissues. What is more, not only YB-1 was identified to be O-GlcNAcylated but hyper-O-GlcNAcylation was demonstrated to facilitate HCC cell proliferation in a YB-1 dependent manner. Moreover, we detected four specific O-GlcNAc sites and confirmed T126A to be the most effective mutant in HCC cell proliferation via close O-GlcNAcylation-phosphorylation interaction. Even more interestingly, we discovered that T126A-induced HCC cell retardation and subdued transcriptional activity of YB-1 could be partially reversed by T126A/S102E mutant. From all above, it is not difficult to find that glycosylated-YB-1 mainly enhanced cell proliferation through congenerous actions with YB-1 phosphorylation and thus played indispensable roles in fine-tuning cell proliferation and procession of HCC.

1. Introduction

Abnormal energy metabolism is widespread in cancer cells, especially the glucose metabolism [1,2]. Malignant tumors always need more glucose intake to promote tumor growth and proliferation [3]. Hepatocellular carcinoma (HCC), acting as the third leading cause of cancer-related death worldwide, has been proved to be closely related to glycometabolism disorder, which usually influence each other and are easy to form a vicious circle, finally leading to HCC progression [4,5]. So understanding the abnormal regulation of glucose metabolism in liver cells may help to comprehend the development and evolution of HCC, and thus provide a novel basis for the clinical diagnosis and treatment.

The hexosamine biosynthetic pathway (HBP) has been considered as a sensor of the cell nutrition status, for its roles in integrating sugar metabolism, lipid metabolism, amino acid metabolism, and nucleic acid metabolism [6]. Depending upon the cell type and metabolic state, about 2–5% of the glucose that enters the cell is directed to the HBP to make UDP-N-acetyl-D-glucosamine (UDP-GlcNAc), which is used as the substrate for O-GlcNAc modification [7,8]. O-GlcNAcylation, a post-translational modification, is thought to modulate a wide range of biological processes, such as transcription, cell growth, signal transduction, and cell motility [9]. O-GlcNAcylation is catalyzed by the nucleocytoplasmic enzymes, OGT and OGA, which respectively adds or removes O-GlcNAc moieties [10]. Abnormal O-GlcNAcylation has been implicated in a variety of human cancers including HCC [11].

Mounting evidence reveals that O-GlcNAcylation has extensive crosstalk with phosphorylation either on the same or adjacent sites of

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various proteins [12,13]. At first glance, interplay between O-GlcNAcylation and phosphorylation is proposed as a yin-yang model with a shared modification site in which O-GlcNAc on/off cycle is rapidly regulated in similar time scale and cellular state as phosphorylation [8]. Effects in several proteins are reciprocal, while others are not [14]. The interplay of these two post-translational protein modifications can work simultaneously and regulate protein function, stabilization, translocation, complex formation, and enzyme activity, subsequently affecting cellular signaling pathways.

YB-1 (Y-box binding protein 1), a well-known oncogenic protein, is reported to be a positive regulator in cell proliferation, differentiation, stress reaction, and malignant transformation [15]. How it works mainly depends on its localization. Under normal circumstances, YB-1 primarily expresses in the cytoplasm. By binding to mRNA, it participates in the control of protein translation. Nevertheless, it translocates into the nucleus in most cancers, including breast cancer, non-small cell lung cancer, prostate cancer, B-cell lymphoma and so on. Within the nucleus it serves as a positive regulator targeting downstream genes, such as cyclinD1, EGFR and MDR1, and ultimately promotes carcinogenesis. Remarkably, activation of YB-1 Ser102 phosphorylation accounts for such consequences most [16–20]. Although O-GlcNAcylation participates in HCC oncogenesis and dramatic interplay between O-GlcNAcylation and phosphorylation is identified [11], yet whether YB-1 is also O-GlcNAc modified and whether such modification can promote HCC progression via interaction with phosphorylation are still not clear.

In our study, higher YB-1, OGT and O-GlcNAc levels were detected in HCC tissues when in comparison with non-tumorous ones. Through further statistical analysis, we observed significant associations between YB-1 and OGT, as well as several clinicopathological features such as histological grade, tumor size, and Ki-67. What is more, YB-1 was verified to be O-GlcNAc modified and OGT-promoted HCC cell proliferation was discovered to be dependent on YB-1. In order to further investigate potential roles of YB-1 O-GlcNAcylation, we used electron transfer dissociation (ETD)-MS analysis and identified four specific glycosylation sites. Based on this, we constructed Myc-tagged YB-1 in which all Ser/Thr sites were individually replaced with Ala to abolish O-GlcNAcylation. On one hand, we observed most reduction of O-GlcNAcyaltion upon T126A transfection and clearly decreased phosphorylation was generated after transfecting T126A. On the other hand, O-GlcNAcylation of YB-1 was proven to occur in dependent of the status of Ser102 phosphorylation through detail experiments. Even more interestingly, we noticed obvious reverse in YB-1 transcriptional activity and HCC cell proliferation when the T126A/S102E mutant was introduced. Thus it can be seen that YB-1 O-GlcNAcylation could play a role in HCC cell proliferation and HCC development mainly via the coordinating effects on Ser102 phosphorylation.

2. Materials and methods

2.1. Patient samples

Paired samples of tumorous and adjacent non-tumorous tissues were obtained from 109 HCC patients who underwent surgery at the Affiliated Cancer Hospital of Nantong University. The diagnosis of all cases of HCC was confirmed through histological examination of H & E-stained biopsy sections. All HCC specimens were collected in accordance with the protocols approved by the Ethics Committee of Cancer Hospital of Nantong University, and every patient submitted written informed consent. The main clinical and pathological variables were shown in Table 1. The 109 HCC cases comprised 81 males and 28 females. Their ages ranged from 25 to 77 years, with an average age of 53.04 years. Tissue specimens were immediately processed after surgical removal. For histological examination, all tumorous and surrounding non-tumorous tissue portions were processed into 10% buffered formalin-fixed, paraffin-embedded blocks. Protein was analyzed in 8 snap-frozen tumorous and adjacent non-tumorous tissue samples that were stored at −80 °C.

Table 1
YB-1 and OGT expression and clinicopathological features in 109 HCC specimens.

Characteristics	Total	YB-1		P value	OGT		P value
		Low	High		Low	High	
Gender				0.229			0.767
Female	28	11	17		13	15	
Male	81	22	59		35	46	
Age (years)				0.994			0.445
< 45	43	13	30		17	26	
≥45	66	20	46		31	35	
HbsAg				0.298			0.483
Absent	26	10	16		13	13	
Present	83	23	60		35	48	
Serum AFP(ng/ml)				0.064			0.905
< 50	37	7	30		16	21	
≥50	72	26	46		32	40	
Histological grade				0.000*			0.000*
I-II	45	27	18		31	14	
III-IV	64	6	58		17	47	
Cirrhosis				0.751			0.991
Absent	34	11	23		15	19	
Present	75	22	53		33	42	
Tumor size (cm)				0.009*			0.002*
< 4.5	13	8	5		11	2	
≥4.5	96	25	71		37	59	
Tumor metastasis				0.600			0.969
Absent	57	16	41		25	32	
Present	52	17	35		23	29	
Ki−67				0.000*			0.000*
Low expression	33	26	7		28	5	
High expression	76	7	69		20	56	
OGT				0.000*			
Low expression	48	31	17				
High expression	61	2	59				

Statistical analyses were carried out using Pearson's χ^2 test.
* $P < 0.05$ was considered significant.

2.2. Cell culture

The HCC cell line HepG2 was obtained from the Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 atmosphere at 37 °C.

2.3. Antibodies

Antibodies used were as follows: antibodies against the Myc epitope (mouse monoclonal anti-Myc antibody, Sigma), O-GlcNAc motif (mouse monoclonal RL2 antibody, Affinity Bioreagents), YB-1 (rabbit polyclonal antibodies, Santa Cruz Biotechnology), p-YB-1 (Ser102) (rabbit polyclonal antibodies, Santa Cruz Biotechnology), OGT (mouse monoclonal antibodies, Santa Cru Biotechnology) and Ki-67 (rabbit polyclonal antibodies, Santa Cruz Biotechnology), CyclinD1 (mouse monoclonal antibodies, Santa Cru Biotechnology), MNK2 (rabbit polyclonal antibodies, Abcam).

2.4. Immunohistochemistry and immunohistochemistrical evaluation

Immunohistochemistry was performed in accordance with previous reports [21]. In brief, paired tissue sections were dewaxed, washed, and blocked. Afterwards, tissue sections were incubated overnight at 4 °C with primary antibodies: anti-YB-1 (1:100), anti-OGT (1:100), or anti-Ki-67 (1:100), followed by horseradish peroxidase (HRP)-conjugated secondary antibodies. After rinsing in water, the sections were counter-