O-GlcNAcylation, an important O-linked glycosylation of cellular glycoproteins with a single molecule of N-acetylglucosamine (GlcNAc), is involved in regulation of many cellular processes. Alteration of O-GlcNAcylation is associated with development and progression of many cancers. Here, we demonstrated aberrant O-GlcNAcylation in the cholangiocarcinoma (CCA) using immunohistochemistry of O-GlcNAc modified proteins (OGP), O-GlcNAc transferase (OGT) and N-acetylglucosaminidase (O-GlcNAcase or OGA). OGP expression was low in normal bile ducts corresponding with the low OGT and high OGA expression. In contrast, OGP was strongly expressed in CCA tissues together with the up-regulation of OGT and down-regulation of OGA. Moreover, elevation of O-GlcNAcylation was associated with non-papillary type CCA and poor survival outcome of CCA patients. Our study showed for the first time that O-GlcNAcylation is increased in CCA tissues and is associated with a poor patient outcome. The OGT expression level could be a useful prognostic indicator and inhibition of O-GlcNAcylation might be a therapeutic target for CCA.

Keywords: O-GlcNAcylation - O-linked β-N-acetylglucosaminyl transferase - N-acetylglucosaminidase - glycosylation

Asian Pacific J Cancer Prev, 13, 101-105

Introduction

O-GlcNAcylation is a reversible post-translational modification of the proteins with a single molecule of N-acetylglucosamine (GlcNAc) on serine (Ser) or threonine (Thr) (Comer and Hart, 2000; Hart et al., 2007). The modification is regulated by O-linked β-N-acetylglucosaminy transferase (OGT) and N-acetylglucosaminidase (O-GlcNAcase or OGA). OGT transfers GlcNAc from uridine diphospho-N-acetylglucosamine (UDP-GlcNAc) to Ser or Thr, while OGA is responsible for the GlcNAc removal (Comer and Hart, 2000; Hart et al., 2007; Butkinaree et al., 2010). O-GlcNAcylation appears to be important in many cellular processes such as transcription, translation, cell proliferation, apoptosis, signal transduction, etc. (Slawson et al., 2006; Zachara and Hart, 2006; Butkinaree et al., 2010; Hart et al., 2011). Alteration of O-GlcNAcylation was implicated in a number of human diseases such as Type II diabetes mellitus, neurodegenerative diseases, and cancers (Zachara and Hart, 2006; Butkinaree et al., 2010; Hart et al., 2011). Aberrant OGT, OGA expression and the level of UDP-GlcNAc were associated with alteration of O-GlcNAcylation and were reported to be involved in the development and progression of many cancers (Caldwell et al., 2010; Gu et al., 2010; Krzeslak et al., 2010; Krzeslak et al., 2011; Liu et al., 2011; Mi et al., 2011; Slawson and Hart, 2011; Zhu et al., 2011; Krzeslak et al., 2012a; Krzeslak et al., 2012b; Lynch et al., 2012).

Cholangiocarcinoma (CCA) is the malignancy of biliary epithelium which has high prevalence in the Northeast Thailand (Patel, 2006; Sripa and Pairojkul, 2008). CCA is a heterogeneous, slowly growing cancer with high metastatic potential (Morise et al., 2010; Nakanuma et al., 2010). According to the gross appearance, CCA can be classified into mass-forming (MF), periductal infiltrating (PI), and intraductal growth (IG) type (Nakanuma et al., 2010). Based on histopathological features, CCA is classified into papillary and non-papillary type (Nakanuma et al., 2010). CCA is difficult to diagnose at an early stage, and most of CCA patients were detected at the late stage when tumors have metastasized to other organs, resulting in the poor survival after diagnosis (Blechacz and Gores, 2008).

Several glycans and glycoproteins are aberrantly expressed in CCA and are possibly used as biomarkers for diagnosis and prognostic prediction. Some of such examples are, carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA 19-9), biliary alkaline phosphatase, mucin-1 (MUC1), mucin-5AC (MUC5AC), etc.
p53, retinoblastoma protein (pRb), epidermal growth factor receptor (EGF-R) (Higashi et al., 1999; Khan et al., 2005; Blechacz and Gores, 2008; Briggs et al., 2009; Park et al., 2009; Sawanyawisuth et al., 2011; Silsirivanit et al., 2011). Some of those proteins such as p53, pRb, EGF-R can be modified by O-linked β-N-acetylgalcosamine (O-GlcNAc) and their functions and stability are potentially controlled by the modification (Zachara and Hart, 2006).

This study is aimed to explore the status of O-GlcNAcylation in CCA and determine the association of O-GlcNAcylation with the development and progression of CCA. The information obtained may fill the understanding of CCA development/progression and probably the improvement of therapy.

Materials and Methods

Formalin-fixed paraffin-embedded tissues

All formalin-fixed paraffin-embedded CCA tissues were obtained from the specimen bank of the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand. Informed consent was obtained from each subject and the study protocol was approved by the Ethics Committee for Human Research, Khon Kaen University (HE521209). All cancer tissues were from histologically proven intrahepatic CCA patients. Tumor staging was classified according to the 6th edition of American Joint Committee on Cancer (AJCC) classification and staging (Greene et al., 2002). CCA tissues microarray (TMA52-1) was constructed by the Department of Pathology, Faculty of Medicine, Khon Kaen University as previously described (Yonglitthipagon et al., 2012).

Immunohistochemistry of OGT, OGA, and OGP

Expression of OGT, OGA, and OGP were determined by a standard protocol of immunohistochemistry (IHC) staining. Briefly, after deparaffinization the antigen retrieval was performed in 0.1 M citrate buffer pH 6.0 followed by endogenous peroxidase neutralization by incubating with 0.3% H2O2 in methanol for 30 min at room temperature (RT). After blocking of non-specific binding by 5% fetal bovine serum (FBS) for 20 min, the sections were incubated with 20 µg/ml anti-O-GlcNAc (RL2, Santa Cruz, CA) or 20 µg/ml mouse anti-OGT (F12; Santa Cruz) or 2 µg/ml goat anti-OGA (L14, Santa Cruz) overnight at RT. After washing with PBS, the sections were incubated with EnVision-system-HRP (Dako, Glostrup, Denmark) or Histofine® Simple Stain MAX PO(G) (Nichirei, Tokyo, Japan) for 1 hour at RT. The sections were developed with diaminobenzidine and counter stained with Mayer’s hematoxylin (Bio-optica, Milano, Italy). Tissues incubated with PBS instead of primary antibody were used as negative controls. Fromowitz standard was used to semi-quantitatively assess the staining of OGT, OGA, and OGP, and expressed as the following positive range score (frequency): 0 = 0-5%; 1+ = 6-25%; 2+ = 26-50%; 3+ = 51-75%; 4+ = >75%; positive extent score (intensity): 0 = no staining; 1 = light yellow; 2 = brown; 3 = dark brown; and IHC index as frequency score plus intensity score (Fromowitz et al., 1987; Qin et al., 2003). For statistic analysis, the patients were divided into two groups according to the immunohistochemistry score, negative (IHC index=0) and positive (>1).

Statistical analysis

Data were analyzed using SPSS 16.0 software (SPSS, Chicago, IL). Association of OGT, OGA, and OGP immunoreactivities with age, sex, histological type, lymph node and gall balder metastasis were analyzed by χ2 or Fisher’s exact test. Survival analysis was performed using Kaplan-Meier plot and Log-Rank test. Cox regression was used to evaluate the association of several prognostic factors with the overall survival. P<0.05 was considered statistically significant.

Results

O-GlcNAcylation is elevated in CCA tissues

To investigate the role of O-GlcNAcylation in CCA, we firstly analyzed the level of O-GlcNAc modified proteins (OGP) in 20 CCA tissues and 10 adjacent normal liver tissues using immunohistochemistry. The weak immunoreactivity of OGP was observed in all bile duct epithelia of normal liver tissues but was strongly detected in the nucleus of 75% (15 of 20) of CCA bile ducts (P=0.036, Figure 1.).

The elevation of O-GlcNAcyltion in CCA in association with high OGT expression

To reveal the possible mechanisms underlying the elevation of O-GlcNAcylation in CCA, we further examined the expression of OGT, OGA and OGP in normal liver and CCA tissues by immunohistochemistry. Bile duct epithelia of normal liver tissues exhibited strongly positive OGA, but low OGT and OGP, immunostaining (Figure 2A). In CCA, OGT was seen as diffused cytoplasmic patterns, whereas OGP was mostly found in the nucleus. Tissue microarray consisting of 88 mass-forming CCA tissues revealed high expression of OGT (80.7%, 71/88) but low expression of OGA (85.2%, 75/88), resulting in high immunostaining of OGP in 54
CCA patients had no association with OGA and OGP expression (95%CI = 368-792 days) (P = 0.014). (B and C) The survival of CCA patients with positive OGT expression (71/88) exhibited the shorter survival than those with high OGT expression (P = 0.014; Table 1). Kaplan-Meier plot and Log-Rank test were used to determine the ability of OGT, OGA and OGP for the estimation of survival time of CCA patients. The expressions of OGT, OGA and OGP were not correlated with age, sex, histological type, and metastatic stage, all mass forming CCA patients (Figure 3). Patients with low OGT expression in CCA tissues showed longer survival time than those with high OGT expression (P = 0.014; Figure 3A). The survival of CCA patients with low OGT expression was observed in some CCA tissues, the immunoreactivities (n = 88). Original magnification×400.

High level of O-GlcNAcylation associates with aggressiveness of CCA

To elucidate the significance of O-GlcNAcylation in CCA, the associations of immuno-reactivity of OGT, OGA, and OGP in CCA tissues with clinicopathological data of CCA patients were analyzed. The expressions of OGT, OGA and OGP were not correlated with age, sex, lymph node and gall bladder metastasis (data not shown). However, the immuno-reactivity of OGT was significantly higher in non-papillary type CCA than in papillary type CCA (P = 0.035; Table 1). Kaplan-Meier plot and Log Rank analysis were used to determine the ability of OGT, OGA, and OGP for the estimation of survival time of mass-forming CCA patients (Figure 3). Patients with low OGT expression in CCA tissues showed longer survival time than those with high OGT expression (P = 0.014). However, the Cox regression analysis revealed that the ability of OGT in prognostic determination was not an independent factor (data not shown).
The enhancement of O-GlcNAcylation in CCA is associated with poor outcome of patients. High level of O-GlcNAcylation was observed more frequently in non-papillary type CCA than in papillary type CCA. Moreover, CCA patients who had high expression of OGT had a significantly shorter survival time than those who had low OGT expression. This result suggests the possible role of O-GlcNAcylation in the aggressiveness of CCA. As shown in many cancers, O-GlcNAcylation is involved in many steps of cancer progression including tumor growth (Caldwell et al., 2010; Mi et al., 2011), metastasis (Gu et al., 2010; Lynch et al., 2012), chemosensitivity (Pan et al., 2011), etc. High expression of inositol 1,4,5-triphosphate receptor-3 (InsP3R-3, an intracellular calcium channel) associated with O-GlcNAcylation in CCA cell line was reported (Bimboese et al., 2011). The modification by O-GlcNAcylation affected the channel open probability of InsP3R-3, resulted in the changes of intracellular calcium releasing following by the activation of downstream calcium dependent signaling cascades.

Many cellular proteins such as transcription factors, oncoproteins and tumor suppressors are modified by O-GlcNAc, and consequently their functions, interaction, and stability are affected (Ozcan et al., 2010). Several oncoproteins and tumor suppressors, are aberrantly expressed and involved in carcinogenesis, progression, and metastasis of CCA (Li et al., 2011; O’Dell et al., 2012). The elevation of O-GlcNAcylation in CCA reported here may modify these and other proteins, and, consequently alter their functions. To understand the molecular mechanisms underlying O-GlcNAcylation and CCA progression, the proteins which are aberrantly modified by O-GlcNAcylation should be identified and characterized.

In conclusion, our findings demonstrate that CCA exhibited the enhancement of O-GlcNAcylation via increasing of OGT expressions. The elevation of O-GlcNAcylation was associated with non-papillary type CCA and shorter survival of CCA patients. Our data suggested that O-GlcNAcylation may be an important mechanism involved in CCA development and progression.

Acknowledgements

This study was supported by grants from Khon Kaen University and the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, through the Center of Excellence in Specific Health Problems in Greater Mekong Sub-region cluster of Khon kaen University (SHeP-GMS). C. Phoomak is grateful to Khon Kaen University for the M.Sc scholar support via SHeP-GMS (H-2553-M-05). A. Silsirivanit was supported by the New Scholar Grant (MRG-5580031), which co-funded by the Office of the Higher Education Commission, Thailand Research Fund, and Khon Kaen University. We wish to acknowledge the Khon Kaen University Publication Clinic, Research and Technology Transfer Affairs, Khon Kaen University, for English-language presentation of the manuscript.

References


