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Clinical significance of GalNAcylated glycans in cholangiocarcinoma: Values for diagnosis and prognosis

Waraporn Saentaweesuk[a,d], Atit Silsirivanit[a,d,*], Kulthida Vaeteewoottacharn[a,d], Kanlayanee Sawanyawisuth[a,d], Chawalit Pairojkul[b,d], Ubon Cha'on[a,d], Somsiri Indramanee[a,d], Somchai Pinlaor[c,d], Thidarut Boonmars[c,d], Norie Araki[e], Chaisiri Wongkham[a,d,*]

[a] Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand
[b] Department of Pathology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand
[c] Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand
[d] Liver Fluke and Cholangiocarcinoma Research Center, Khon Kaen University, Khon Kaen 40002, Thailand
[e] Department of Tumor Genetics and Biology, Graduate School of Medical Sciences, Kumamoto University, Kumamoto 860-8556, Japan

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ABSTRACT

Cancer cells exhibited the aberrant cancer-associated glycans that are potential biomarkers for diagnosis and monitoring of the cancer. In this study, *Sophora japonica* agglutinin (SJA) was used to detect SJA-specific *N*-acetylgalactosamine-associated glycans (SNAG) in liver tissues and sera from cholangiocarcinoma (CCA) patients. Whether SNAG could be the diagnostic and prognostic markers for CCA was evaluated. SJA-histochemistry revealed that SNAG was undetec2 in normal bile ducts but was highly expressed in hyperplastic/dysplastic bile ducts and CCA. SNAG was negative in hepatocytes and hepatoma tissues indicating SNAG as a differential marker of CCA and hepatoma. SJA-histochemistry of CCA hamster tissues revealed the involvement of SNAG in the early pathogenesis of bile duct epithelia and CCA development. A SJA-based ELISA was successfully developed to determine SNAG in serum. Serum-SNAG from CCA patients was significantly higher than those of non-CCA control groups with the diagnostic values of 59.5% sensitivity and 73.6% specificity, comparable to those of serum CA19-9. High levels of serum SNAG (≥ 69 AU/ml) indicated poor survival of CCA patients. Taken together, SNAG was first demonstrated here to be a glycobiomarker for diagnosis and prognosis of CCA. Association of SNAG with pathogenesis of bile ducts and CCA development were suggested. (198).

1. Introduction

Glycosylation, an enzymatic posttranslational modification with carbohydrates, is an important process for maturation of glycoproteins and consequently achieving the appropriate functions. Membranous glycans and glycoproteins play important roles in many biological processes such as cell adhesion and cell-cell/matrix communications, etc. [1–3]. Alteration of glycosylation was frequently observed in cancer cells, resulting in neoexpression of cancer-associated glycans. Not only being biomarkers for cancers, cancer-associated glycans also plays crucial roles during tumor development and metastasis [4–8].

The incidence of cholangiocarcinoma (CCA), a malignancy of bile duct epithelium, is high in Khon Kaen province, Thailand [9] and has increased globally in the past decades [9–11]. There are many risk factors reported for CCA. Primary sclerosing cholangitis, hepatitis, choledochal cysts, etc., are considered as potential risk factors for CCA in western countries, whereas infection with liver flukes (*Opisthorchis viverrini*, OV, and *Clonorchis sinensis*) are strongly associated with CCA in Southeast and East Asia [12]. The epidemiological studies in OV infected persons, however, indicated that fewer than 1% of infected people developed CCA [13,14], suggesting the involvement of other personal factors in CCA genesis in individuals. Generally, CCA is difficult to diagnose due to the lack of early and specific markers. Most of CCA patients are diagnosed at the late stage that is incapable of curative treatment, resulting in the unfavorable prognosis and short survival of the patients [15,16]. Although complete resection provides the highest

Abbreviations: (AU), arbitrary unit; (BBD), benign biliary diseases; (BSA), bovine serum albumin; 19-9 (CA19-9), carbohydrate antigen; (CCA), cholangiocarcinoma; (GalNAcTs), GalNAc-transferases; (HE), healthy controls; (HCC), hepatoma or hepatocellular carcinoma; (HP/DP), hyperplastic/dysplastic bile ducts; (MUC), mucin; (GalNAc), *N*-acetylgalctosamine; (NDMA), *N*-nitrosodimethylamine; (OCA), other gastrointestinal cancers; (ROC), receiver operating characteristic; (SNAG), SJA specific *N*-acetylgalactosamine-binding glycan; (SJA), *Sophora japonica* agglutinin; (TMB), tetramethylbenzidine

* Corresponding authors at: Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand.
E-mail addresses: atitsil@kku.ac.th (A. Silsirivanit), chaisiri@kku.ac.th (C. Wongkham).

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survival rate and curative treatment for CCA patients, however, accurate preoperative diagnosis is still the most problematic.

Previous studies indicated the aberrant glycosylation in CCA [17–20], e.g., carbohydrate antigen 19-9 (CA19-9), mucin (MUC) 5AC, MUC1, CCA-CA, and CA-S27 [19,21–23]. Many of these were not only applicable for diagnosis and prognostic prediction of CCA, but were also shown to mediate migration and invasion of CCA cell lines [20,22,24,25]. A previous study by the present authors of CCA, using lectin histochemistry of 14 commercially available lectins, revealed the aberrant glycosylations in CCA compared with normal bile ducts [18]. *Sophora japonica* agglutinin (SJA), an *N*-acetylgalactosamine (GalNAc)-binding lectin from the Japanese pagoda tree was marked as one of the potent lectins for discriminating CCA from normal bile ducts.

In this report, the aberrant expression of SJA specific *N*-acetylgalactosamine-associated glycan (SNAG) in CCA tissues was demonstrated using SJA-histochemistry. The clinical significance of SNAG expression in the pathogenesis and carcinogenesis of CCA was determined in CCA patient tissues and liver fluke-induced CCA hamster tissues. A SJA-based enzyme linked immunosorbent assay (ELISA) was successfully developed to detect SNAG in serum. The possibilities of using SNAG as diagnostic and prognostic markers for CCA were evaluated.

2. Materials and methods

2.1. Tissue and serum samples

Tissues and sera from histologically-proven CCA patients without any pre- or post-operative chemotherapy or radiotherapy ($n = 89$), hepatoma (HCC, $n = 38$), benign biliary diseases (BBD, $n = 17$; including 3 biliary cysts, 3 cholangitis, 2 cirrhosis, 2 inflammatory duct dilations, 3 inflammatory pseudotumors, and 4 papillary adenomas), and other gastrointestinal cancers (OCA, $n = 52$; including 5 ampullary carcinomas, 12 colon carcinomas, 4 cystadenocarcinomas, 1 gastrointestinal tract metastatic cancer, 14 HCC, 8 pancreatic cancers and 8 stomach carcinomas) were obtained from the Liver Fluke and Cholangiocarcinoma Research Center, Khon Kaen University, Thailand. Tumor staging of CCA was categorized according to the 7th American Joint Committee on Cancer classification [26]. *Opisthorchis viverrini* (OV) infected persons with the positive examination for OV-eggs in feces and had apparently normal livers by ultrasonography ($n = 25$) were included in this study. Sera from healthy controls (HE, $n = 50$) who had normal fasting blood sugar, complete blood count, and liver function tests; were obtained from persons who had an annual check-up at Srinagarind Hospital, Khon Kaen University. Serum CA19-9 levels of each group were obtained from the medical records of patients, Srinagarind Hospital. Serum CA19-9 were analyzed using ELISA (Roche Diagnostic GmbH) according to the International Federation of Clinical Chemistry and Laboratory Medicine. Informed consents were obtained from the subjects and the research protocol was approved by the Ethics Committee for Human Research of Khon Kaen University (HE571283 and HE591308).

Liver sections of experimental hamsters were obtained from a previous study [27], under a protocol approved by the Animal Ethics Committee of Khon Kaen University (AEKKU 17/2552). Samples were collected at 1, 3, and 6 month post-treatment from 4 groups of hamsters; including 1) non-treated, 2) OV-infected, 3) *N*-nitrosodimethylamine (NDMA; 12.5 ppm in water ad libitum)-treated, and 4) CCA (OV + NDMA-treated) groups.

2.2. Lectin histochemistry staining

SJA-lectin histochemistry was performed as previously described [18]. Briefly, after blocking with BSA, sections were incubated with 20 μg/ml biotinylated-SJA (Vector Laboratories, Inc., Burlingame, CA) for 2 h at room temperature followed by 1:100 streptavidin-conjugated horseradish peroxidase (streptavidin-HRP, Invitrogen, Camarillo, CA) for 40 min. Signal was developed with diaminobenzidine and the sections were counter-stained by Mayer's hematoxylin (Bio-Optica, Milano, Italy). Sections that were probed with PBS instead of biotinylated-SJA were used as a negative control. The intensity of SNAG was scored as 0, negative; 1 = weakly positive; 2 = moderately positive and 3 = strongly positive. The frequency of positive cells was scored based on the percentage of SJA-positive biliary cells; 0–10% = negative, 11–25% = + 1, 26–50% = + 2, and > 50% = + 3. The expression of SNAG was semi-quantified as SNAG-score as intensity x frequency. SNAG-score = 0 was considered as SNAG negative and SNAG-scores > 0 were considered as SNAG positive.

2.3. Double-SJA sandwich ELISA

Serum SNAG was determined using double-SJA sandwich ELISAs. 96-well EIA/RIA plates (Corning, NY) that were pre-coated overnight with 1.25 μg (50 μl, 25 μg/ml) of SJA in PBS (Vector Laboratories). After washing 5 times with 0.1% Tween-20 in PBS (PBST), non-specific binding was blocked using 3% BSA, followed by 1 h incubation with 50 μl of 1:10 serum in PBS. Antigens were decanted and the reaction plate was washed with PBST. The plate was incubated with 50 μl of 4 μg/ml biotinylated-SJA and 1:5000 streptavidin-HRP, respectively. Signal was developed with 100 μl of TMB substrate, room temperature, 15 min. The reactions were stopped by adding 50 μl of 1 N H_2SO_4 and the absorbance were measured at 450 nm. Pooled-CCA serum samples diluted to 12.5, 25, 50 and 100 arbitrary units of (AU)/ml of SNAG were used as standard controls. Levels of serum SNAG were calculated as AU/ml regarding of standard controls.

2.4. Statistical analysis

Statistical analysis was performed using SPSS version 16.0 software (SPSS, Chicago, IL). Differences of serum-SNAG level between CCA and controls were compared using Student's *t*-test. The paired t-test was used to compare serum-SNAG levels between pre- and post-operative sera of CCA patients. The power of serum-SNAG in discriminating CCA from the control groups was evaluated using the receiver operating characteristic (ROC) curve. Chi-square (χ^2) was used to analyze the correlation of SNAG levels in sera and tissues as well as the clinicopathological data of CCA patients. Survival time was measured from the time of surgical resection to death. Survival analysis was performed using Kaplan-Meier plot and Log-Rank test.

3. Results

3.1. SNAG was aberrantly expressed in precancerous bile duct epithelia and CCA.

Expression of SNAG in CCA tissues ($n = 89$) was determined using SJA lectin histochemistry. SNAG was positively stained in hyperplastic/dysplastic bile ducts (HP/DP) and CCA with SNAG signal observed in lumina of CCA bile ducts, while it was negative in normal bile ducts in the adjacent non-tumor area, inflammatory cells, hepatocyte and HCC (Fig. 1A). SNAG was differentially expressed in CCA with SNAG-scores varying from 0 to 9 (Fig. 1B). Among 89 CCA cases, 67.4% (60/89) had positive SNAG scores of moderate to high levels (Fig. 1C). All normal bile duct epithelia ($n = 79$) had negative staining, while 77.6% (52/67) of HP/DP strongly expressed SNAG. In contrast to CCA, 97.4% (37/38) had negative for SNAG (Fig. 1C). The clinical relevance of SNAG on the clinicopathological features and survival of CCA patients were analyzed based on the median value of SNAG scores = 6. The univariate and survival analyses using Kaplan-Meier plot and Log-Rank test showed no association between SNAG score with clinicopathological data as well as survival of the patients (Table 1).