RESEARCH ARTICLE

Lectin from *Agrocybe aegerita* as a Glycophenotype Probe for Evaluation of Progression and Survival in Colorectal Cancer

Yi Liang[1,3&], Hua Chen[2,3&], Han-Bin Zhang[2,3&], Yan-Xia Jin[4], Hong-Qiang Guo[5], Xing-Gui Chen[6*], Hui Sun[4*]

Abstract

Background: *Agrocybe aegerita* **Lectin (AAL) has been identified to have high affinity for sulfated and α2-3-linked sialic acid glycoconjugates, especially the sulfated and sialyl TF (Thomsen-Friedenreich) disaccharide. This study was conducted to investigate the clinicopathological and prognostic value of AAL in identifying aberrant glycosylation in colorectal cancer (CRC). Materials and Methods: Glycoconjugate expression in 59 CRC tissues were detected using AAL-histochemistry. Clinicopathological associates of expression were analyzed with chi-square test or Fisher's exact test. Relationships between expression and the various clinicopathological parameters was estimated using Kaplan-Meier analysis and Cox regression models. Results: AAL specific glycoconjugate expression was significantly higher in tumor than corresponding normal tissues (66.1% and 46.1%, respectively, *p*=0.037), correlating with depth of invasion (*p*=0.015) and TNM stage (*p*=0.024). Patients with lower expression levels had a significantly higher survival rate than those with higher expression (*p*=0.046 by log rank test and *p*=0.047 by Breslow test for overall survival; *p*=0.054 by log rank test and P=0.038 by Breslow test for progress free survival). A marginally significant association was found between AAL specific glycoconjugate expression and overall survival by univariate Cox regression analysis (*p*=0.059). Conclusions: Lower AAL specific glycoconjugate expression is a significant favorable prognostic factor for overall and progress free survival in CRC. This is the first report about the employment of AAL for histochemical analysis of cancer tissues. The binding characteristics of AAL means it has potential to become a powerful tool for the glycan investigation and clinical application.**

Keywords: Lectin histochemistry - glycoconjugates - colorectal cancer

Asian Pac J Cancer Prev, **15 (14)**, 5601-5605

Introduction

Colorectal cancer (CRC) is a major health problem worldwide, with global increases in incidence and death due to an expanding and aging population. Based on the World Health Organization cancer statistics in 2013, there is over 1.36 million new cancer cases and 694,000 deaths to have occurred (Ferlay et al., 2013).

Exceptional amount of high-throughput experiments have been performed for well validated prognostic markers (Balog et al., 2012). However, the clinical useful markers based on genomics and proteomics remain limited. In recent years, aberrant glycosylation has been recognized as a cellular hallmark event during carcinogenesis (Reis et al., 2010; Adamczyk et al., 2011), and it has been a topic for well characterization of tumor and better understanding the mechanism (Meany et al., 2011). Both N- and O-linked glycoproteins have been shown to be altered in a wide range of cancers, including CRC. Examples include increased oligosaccharide chains Tn (GalNAcα1-Ser/Thr), sialosyl-Tn and TF antigen (Galβ1-3GalNAcα1-Ser/Thr) of colonic mucins in colorectal carcinoma (Itzkowitz et al., 1989; Baldus et al., 2000); increased sialylated Lewis-type, sulfated and pauci-mannosidic structures, decreased bisecting GlcNAc structures in cancer tissue (Balog et al., 2012); higher levels of sialylation and fucosylation of plasma glycoprotein from patient with colorectal cancer or adenomas (Qiu et al., 2008); increased bisecting N-acetylglucosamine in metastatic CRC cell line (Sethi et al., 2013); increased O-GlcNAcylation in metastatic CRC clone (Yehezkel et al., 2012). Identifying changes in glycosylation in tumor development may help monitor disease progression and guide therapy.

Lectins are proteins or glycoproteins that can recognize specific glycan structures. To investigate the significance of distinct glycosylation states, it is crucial to identify new

*[1]Department of Clinical Immunology, [2]Sino-American Cancer Research Institute, Guangdong Medical College, Dongguan, [3]Key Laboratory for Medical Molecular Diagnostics of Guangdong Province, Dongguan, [4]The College of Life Sciences, Wuhan University, Wuhan, [5]The Affiliated Cancer Hospital of Zhengzhou University, [6]Cancer Center, the Affiliated Hospital of Guangdong Medical College, Zhanjiang, China [&]Equal contributors *For correspondence: sunhui@whu.edu.cn, xingguichen@hotmail.com*

lectins that discriminate unique sugar structures among kinds of oligosaccharides. AAL (Agrocybe aegerita lectin) from the edible mushroom A. aegerita is an antitumor protein that exerts its tumor-suppressing function via apoptosis-inducing activity in cancer cells and animal models (Zhao et al., 2003). We demonstrate that AAL belongs to galectin family with a unique carbohydrate recognition domain that specifically recognizes β-galactose (Liang et al., 2009). The recent glycan-array analysis reports that glycans with high affinity to AAL contain [3OSO3]Galβ1-3GalNAcα-Sp8 (sulfated TF disaccharide fixed to a spacer arm Sp8-CH2CH2CH2NH2), [3OSO3] Galβ1-3GalNAcα-Sp8, Neu5Acα2-3Galβ1-3GalNAcα-Sp8 (Sialyl-TF disaccharide) and Galβ1-3GalNAcα-Sp14 (TF disaccharide) etc (Feng et al., 2010). The observations lead to this assessment of using AAL for understanding the glycosylation changes associated with the development of colorectal cancer.

Materials and Methods

Patients and tissue samples

CRC and corresponding non-tumor colorectal tissues were obtained from 59 patients as described previously (Huang et al., 2012). All samples were graded histopathologically by two experienced pathologists. Tumor staging was established according to 7th edition of the Cancer Staging Manual of the American Joint Committee on Cancer (AJCC) (Gunderson et al., 2010). Clinical data related to the samples used in this study, including age, gender, tumor stage, and original tumor site were summarized in Table 1. The patients were followed up annually with a median follow-up time of 70 months. Informed consent was obtained from all subjects, and this study was approved by the Institutional Ethics Committee.

Lectin-histochemistry detection

Purified AAL with a His6 tag was biotinylated performed by Beijing Biosynthesis Biotechnology CO. LTD. All paraffin-embedded tissues were deparaffinized in xylene and rehydrated in a graded ethanol series according to routine histochemistry. The sections were boiled in EDTA (1 mM; pH8.0) for 15 mins in a microwave oven for antigen retrieval, and cooled down in phosphate-buffered saline (pBS), pH 7.2 at room temperature. Endogenous peroxidase activity was eliminated using 0.3% (v/v) H_2O_2 in methanol for 30 mins. The non-specific glycoconjugate binding was blocked using 0.5% (v/v) periodate treated BSA in PBS for 30 mins at room temperature. Sections were incubated with biotinylated AAL 20 μg/ml at 37°C for 1 h and with 12.5 μg/ml horseradish peroxidase-conjugated streptavidin (proteintech group) for 40 mins. After additional washes, bound lectin was visualized with diaminobenzidine tetrahydrochloride (DAB), and then specimens were counterstained with hematoxylin. A positive control and a negative control (without lectin) were included in each run.

Each section was scored under a light microscope according to frequency of presence in cells as <5%=0; <25%=1; 25-50%=2; 50-75%=3; >75%=4 and intensity of the stain as 0=no staining; 1=weak staining; 2=moderate staining; 3=strong staining. The lectin-histochemistry score was calculated from 'frequency'×'intensity': '-' stands for score 0-1, '+' for score 2-3, '++' for score 4-6 and '+++' for score >6. For statistical analysis, we combined the cased scored as '-' and '+' (low score) to compare with the cases with scored as '++' and '+++' (high score).

Statistical analysis

Statistical analysis was performed using the SPSS software package (version 16.0). The chi-square test or Fisher's extract test were used to analyze the correlations between clinicopathological features and AAL histochemistry score of CRC patients. Survival analysis was performed using the Kaplan-Meier method with the log-rank test and Breslow test. The prognostic significance of each clinicopathological feature was determined using the univariate and multivariate Cox regression analyses. In a multivariate Cox proportional hazard model, the independent prognostic factors were identified from the significant predictors in univariate analysis with an enter mode.

Results

AAL specific glycoconjugate expression and clinicopthological associations

The glycoconjugate expression recognized by AAL in the colorectal cancer tissues and corresponding adjacent normal counterparts were examined using immunohistochemistry staining. The representative results are shown in Figure 1. Various levels of immunoreactivity for AAL were found in cancer and adjacent non-tumor regions. The AAL specific glycoconjugate expression was significantly higher in tumor tissues than corresponding normal tissues (66.1% and 46.1%, respectively, p=0.037). The associations between lectin-histochemistry score and clinicopathological parameters were assessed using chi-square test or Fisher's exact test. The results indicated that AAL specific glycoconjugate expression was correlated with depth of invasion (p=0.015) and TNM stage (p=0.024, Table 1).

Associations between AAL specific glycoconjugate expression and patient survival

Kaplan-Meier analysis with the log-rank and Breslow test were performed to analyze the correlation between AAL specific glycoconjugate expression and patient

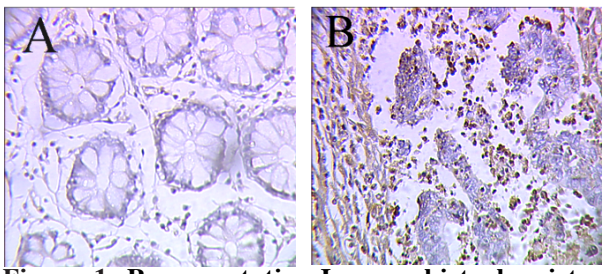

Figure 1. Representative Immunohistochemistry Staining of AAL (200×). A. Adjacent normal colorectal tissues stained with AAL. B. Colorectal cancer epithelial cells stained with AAL

Table 1. Clinicopathologic Correlation with AAL-Specific Glycoconjugate Expression

Parameter		Tumor AAL Low	Tumor AAL High	*p* value
Gender	Female	11	18	
	Male	9	21	0.52
Age(years)	<50	7	12	
	>=50	13	27	0.747
Depth of invasion	T1+T2	7	4	
	T3+T4	12	35	0.015 *
Lymph node status	Positive	16	24	
	Negative	3	15	0.08
Metastasis	No	17	27	
	Yes	2	12	0.091
TNM stage	I+II	16	21	
	III+IV	3	18	0.024 *
Operative Site	Colon	17	25	
	Rectum	3	14	0.093
Pathological stage	I+II	17	24	
	III+IV	3	15	0.064

*p<0.05

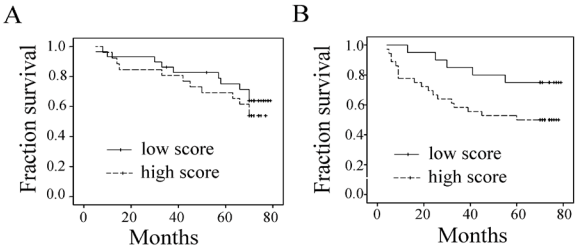

Figure 2. Kaplan-Meier Survival Analysis of CRC Patients According to AAL-specific glycoconjugate expression. A. overall survival, log rank test p=0.046, Breslow test p=0.047. B. progress free survival, log rank test p=0.054, Breslow test p=0.038

survival. According to the Kaplan-Meier survival curves, the higher expression of AAL specific glycoconjugate is a significant prognostic factor for poor overall survival and progress free survival in CRC patients. Patients with lower AAL specific glycoconjugate expression levels have a significantly higher survival rate than that with higher expression (78.9% for lower expression group and 50.0% for higher expression group, respectively, p=0.046 by log rank test and p=0.047 by Breslow test for overall survival, Figure 2A; 75% for lower expression group and 50% for higher expression group, respectively, p=0.054 by log rank test and p=0.038 by Breslow test for progress free survival, Figure 2B).

To identify the AAL specific glycoconjugate expression and other variables of potential prognostic significance in all of the patients with CRC, univariate and multivariate Cox regression models were performed. However, only a marginally significant association was found between AAL specific glycoconjugate expression and overall survival or progress free survival by Univariate Cox regression analysis (p=0.059 for overall survival, Table 2; and p=0.064 for progress free survival, Table 3). The univariate Cox regression analysis indicated that clinical variables including lymph node status, metastasis and pathological stage were significantly associated with overall survival (p=0.001, p=0.002, and p=0.001, respectively, Table 2) and with progress free survival (p=0.001, p=0.004, and p=0.002, respectively, Table 3). Furthermore, multivariate Cox regression analyses were performed to evaluate the potential of AAL specific glycoconjugate expression as an independent predictor for overall survival or progress free survival of CRC. The results showed that no independent predictor was found with or without AAL's addition.

Table 2. Univariate and Multivariate Analyses of Individual Parameters for Correlations with Overall Survival Rate: Cox Proportional Hazards Model

Parameters	Univariate analysis			Multivariate analysis			Multivariate analysis (AAL's addition)		
	HR	CI(95%)	*p* value	HR	CI(95%)	*p* value	HR	CI(95%)	*p* value
AAL	2.824	0.961-8.405	0.059				1.956	0.639-5.988	0.24
Gender	0.763	0.381-1.704	0.474						
Age(years)	2.197	0.743-6.492	0.155						
Depth of invasion	5.394	0.725-40.133	0.101						
Lymph node status	5.006	2.133-11.751	0.001*	4.586	0.565-37.239	0.154	5.011	0.617-40.728	0.132
Metastasis	3.841	1.616-9.127	0.002*	2.026	0.599-6.860	0.256	1.937	0.574-6.536	0.287
TNM stage	4.356	1.845-10.283	0.001*	0.762	0.69-8.384	0.824	0.624	0.056-6.957	0.702
Operative Site	0.604	0.222-1.639	0.322						

*p<0.05

Table 3. Univariate and Multivariate Analysis of Individual Parameters for Correlations with Progress Free Survival Rate: Cox Proportional Hazards Model

Parameters	Univariate analysis			Multivariate analysis			Multivariate analysis (AAL's addition)		
	HR	CI(95%)	*p* value	HR	CI(95%)	*p* value	HR	CI(95%)	*p* value
AAL	2.555	0.947-6.891	0.064				2.209	0.807-6.052	0.123
Gender	0.806	0.356-1.827	0.606						
Age(years)	1.329	0.524-3.373	0.549						
Depth of invasion	6.59	0.887-48.959	0.065						
Lymph node status	4.32	1.885-9.901	0.001*	4.129	0.521-37.737	0.179	5.154	0.642-41.390	0.123
Metastasis	3.445	1.499-7.917	0.004*	1.87	0.505-6.927	0.349	1.927	0.52-7.146	0.327
TNM stage	3.711	1.614-8.530	0.002*	0.699	0.061-7.992	0.773	0.511	0.044-5.958	0.592
Operative Site	0.598	0.222-1.614	0.31						

*p<0.05

Discussion

At present, lectin-based strategies are the popular techniques to observe glyco-alteration in various cancers (Indramanee et al., 2012; Rambaruth et al., 2012). The binding preference of AAL can be summarized as follows: [3OSO3]Galβ1-3GalNAcα-Sp8 (Sulfated TF disaccharide)>Neu5Acα2-3Galβ1-3GalNAcα-Sp8 (Sialyl-TF disaccharide)>[3OSO3]Galβ1-4GlcNAcβ-Sp8>Galβ1-3GalNAcα-Sp14 (TF disaccharide), which shows that besides crystallization with TF antigen (Feng et al., 2010), AAL can permit selective detection of sulfated and α2-3-linked sialic acid glycoconjugates, expecially the sulfated and sialyl TF disaccharide.

Glycoconjugate markers for colon cancer include aberrant mucins, cadherins, selectins and Ig-like adhesion molecules, glycoconjugate components of ECM, etc (Szajda et al., 2008). Expression of TF antigen on glycoconjugates of the cell surface has been correlated with tumor prognosis and metastasis (Baldus et al., 2000; Szajda et al., 2008). Lately, sulfated and sialylated glycans have been detected to be increased in tumor tissues or plasma samples from colorectal cancer patients (Qiu et al., 2008; Balog et al., 2012). High level of sialylation has been observed on core 1 type glycan (TF structure) from breast cancer patient (Storr et al., 2008) and colorectal cancer patient (Schneider et al., 2001). According to sulfated glycoconjugates, only few studies have correlated the increase in sulfated glycans with cancer in general. Gal-3-O-sulfotransferase is expressed in most breast cancer cells and colon cancer cells, which is specific for T-hapten Galβ1-->3GalNAcα- and the Galβ1-4GlcNAc terminal unit in O-glycans respectively (Chandrasekaran et al., 2006). These results suggest that sialic acid and sulfated glycoconjugates, especially the sialyl-TF and sulfated TF may act as potential tumor-related biomarker in cancer. Some of our recent findings show that AAL specifically recognizes the sulfo-TF antigen expressed on malignant human hematopoietic cells resulting in apoptosis with the activation of caspase-8, -9, and -3 (data not published). The present study finds the significant relationship between prognosis and staining characteristics for AAL in colorectal cancer tissues (Figure 1 and 2), which suggests that sialoglycoconjugates and sulfoglycoconjugates recognized by AAL are related to worse prognosis for colorectal cancer patients. Multivariate Cox regression analyses have not found the potential of AAL specific glycoconjugate expression as an independent predictor, but there is a marginally significant association between its expression and overall survival or progress free survival by Univariate Cox regression analysis (Table 2 and 3), which may due to the limited amount of tumor tissues.

Although the study has discussed the importance of aberrant glycoconjugats histochemically detected by AAL, the nature of these glycoconjugates remains unknown. Carbohydrate moieties of glycoconjugates are constructed by complex interaction involving a series of glycosyltransferases (Essentials of Glycobiology, 2009). Differences in AAL staining intensity might be due to quantitative differences in the expression level of glycosyltransferases. Enzyme activity of alpha2,3-sialyltransferase (ST3Gal-I) has been found to be significantly increased in carcinoma specimens compared with normal mucosa (Schneider et al., 2001), and Gal-3-O-sulfotransferase with preference for the Galβ1-4GlcNAc terminal unit in O-glycans is expressed in colon cancer cells (Chandrasekaran et al., 2006). These finding is consistent to the levels of sialoglycoconjugates and sulfoglycoconjugates expression recognized with AAL.

There have been specific monoclonal antibodies recognizing sialyl TF glycan antigens (Essentials of Glycobiology, 2009), but to our knowledge, no lectin or antibodies have been found to bind sulfo TF. Sulfation of TF has been reported to lead to modulation of galectin-1 interaction with glycoconjugate and play a role in galectin functions (Allen et al., 1998). SB1a with terminal sulfo TF disaccharide on the cell surface of human colon adenocarcinoma cells has been reported to be the important ligand for galectin-4 and promote cell adhesion (Ideo et al., 2002; Ideo et al., 2005). AAL, with high affinity with sulfo TF, could be a useful tool for the investigation of the glycan.

The prognostic information provided by AAL lectin histochemistry may be used clinically to inform the physician and aid treatment decisions; far more interesting is the challenge of further understanding the precise nature of the AAL-binding ligands, and defining their role in the progress of the disease.

Acknowledgements

This work was supported by the Natural Science Foundation of China (program no. 81102850), Scientific Research Program for Educational Commission of Guangdong Province (LYM11070), Medical Scientific Research Foundation of Guangdong Province (A2011434), Higher Education Institutions of Dongguan (program no. 2011108102049), and Science and Technology Foundation of Zhanjiang (2011C3109015). The author(s) declare that they have no competing interests.

References

Adamczyk B, Tharmalingam T, Rudd PM (2011). Glycans as cancer biomarkers. *Biochim Biophys Acta*, **1820**, 1347-53.

Allen HJ, Ahmed H, Matta KL (1998). Binding of synthetic sulfated ligands by human splenic galectin 1, a beta-galactoside-binding lectin. *Glycoconj J*, **15**, 691-5.

Baldus SE, Zirbes TK, Hanisch FG, et al (2000) Thomsen-Friedenreich antigen presents as a prognostic factor in colorectal carcinoma: A clinicopathologic study of 264 patients. *Cancer*, **88**, 1536-43.

Balog CI, Stavenhagen K, Fung WL, et al (2012). N-glycosylation of colorectal cancer tissues: a liquid chromatography and mass spectrometry-based investigation. *Mol Cell Proteomics*, **11**, 571-85.

Chandrasekaran EV, Xue J, Neelamegham S, Matta KL (2006). The pattern of glycosyl- and sulfotransferase activities in cancer cell lines: a predictor of individual cancer-associated distinct carbohydrate structures for the structural identification of signature glycans. *Carbohydr Res*, **341**, 983-94.

Essentials of Glycobiology (2009). Cold Spring Harbor Laboratory Press, Cold Spring Harbor (NY).

Feng L, Sun H, Zhang Y, et al (2010). Structural insights into the recognition mechanism between an antitumor galectin AAL and the Thomsen-Friedenreich antigen. *FASEB J*, **24**, 3861-8.

Ferlay J, Soerjomataram I, Ervik M, et al (2013). GLOBOCAN 2012 v1.0, Cancer incidence and mortality Worldwide: *IARC CancerBase*, No. 11.

Gunderson LL, Jessup JM, Sargent DJ, et al (2010). Revised TN categorization for colon cancer based on national survival outcomes data. *J Clin Oncol*, **28**, 264-71.

Huang GL, Guo HQ, Yang F, et al (2012) Activating transcription factor 1 is a prognostic marker of colorectal cancer. *Asian Pac J Cancer Prev*, **13**, 1053-7.

Ideo H, Seko A, Ohkura T, et al (2002). High-affinity binding of recombinant human galectin-4 to SO(3)(-)-->3Galbeta1-->3GalNAc pyranoside. *Glycobiology*, **12**, 199-208.

Ideo H, Seko A, Yamashita K (2005). Galectin-4 binds to sulfated glycosphingolipids and carcinoembryonic antigen in patches on the cell surface of human colon adenocarcinoma cells. *J Biol Chem*, **280**, 4730-7.

Indramanee S, Silsirivanit A, Pairojkul C, et al (2012). Aberrant glycosylation in cholangiocarcinoma demonstrated by lectin-histochemistry. *Asian Pac J Cancer Prev*, **13**, 119-24.

Itzkowitz SH, Yuan M, Montgomery CK, et al (1989). Expression of Tn, sialosyl-Tn, and T antigens in human colon cancer. *Cancer Res*, **49**, 197-204.

Liang Y, Feng L, Tong X, et al (2009). Importance of nuclear localization for the apoptosis-induced activity of a fungal galectin AAL (Agrocybe aegerita lectin). *Biochem Biophys Res Commun*, **386**, 437-42.

Meany DL, Chan DW (2011). Aberrant glycosylation associated with enzymes as cancer biomarkers. *Clin Proteomics*, **8**, 7.

Qiu Y, Patwa TH, Xu L, et al (2008). Plasma glycoprotein profiling for colorectal cancer biomarker identification by lectin glycoarray and lectin blot. *J Proteome Res*, **7**, 1693-703.

Rambaruth ND, Greenwell P, Dwek MV (2012). The lectin Helix pomatia agglutinin recognizes O-GlcNAc containing glycoproteins in human breast cancer. *Glycobiology*, **22**, 839-48.

Reis CA, Osorio H, Silva L, et al (2010). Alterations in glycosylation as biomarkers for cancer detection. *J Clin Pathol*, **63**, 322-9.

Schneider F, Kemmner W, Haensch W, et al (2001). Overexpression of sialyltransferase CMP-sialic acid:Galbeta1, 3GalNAc-R alpha6-Sialyltransferase is related to poor patient survival in human colorectal carcinomas. *Cancer Res*, **61**, 4605-11.

Sethi MK, Thaysen-Andersen M, Smith JT, et al (2013). Comparative N-glycan profiling of colorectal cancer cell lines reveals unique bisecting GlcNAc and alpha-2,3-linked sialic acid determinants are associated with membrane proteins of the more metastatic/aggressive cell lines. *J Proteome Res*, **13**, 277-88.

Storr SJ, Royle L, Chapman CJ, et al (2008). The O-linked glycosylation of secretory/shed MUC1 from an advanced breast cancer patient's serum. *Glycobiology*, **18**, 456-62.

Szajda SD, Jankowska A, Zwierz K (2008). Carbohydrate markers in colon carcinoma. *Dis Markers*, **25**, 233-42.

Yehezkel G, Cohen L, Kliger A, et al (2012). O-linked beta-N-acetylglucosaminylation (O-GlcNAcylation) in primary and metastatic colorectal cancer clones and effect of N-acetyl-beta-D-glucosaminidase silencing on cell phenotype and transcriptome. *J Biol Chem*, **287**, 28755-69.

Zhao C, Sun H, Tong X, Qi Y (2003). An antitumour lectin from the edible mushroom Agrocybe aegerita. *Biochem J*, **374**, 321-7.