

RESEARCH ARTICLE

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

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Abstract

Background: *Agrocybe aegerita* Lectin (AAL) has been identified to have high affinity for sulfated and α 2-3-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

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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 (GalNAc α 1-Ser/Thr), sialosyl-Tn and TF antigen (Gal β 1-3GalNAc α 1-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

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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]Gal β 1-3GalNAc α -Sp8 (sulfated TF disaccharide fixed to a spacer arm Sp8-CH₂CH₂CH₂NH₂), [3OSO3]Gal β 1-3GalNAc α -Sp8, Neu5Ac α 2-3Gal β 1-3GalNAc α -Sp8 (Sialyl-TF disaccharide) and Gal β 1-3GalNAc α -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) H₂O₂ 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 μ g/ml at 37°C for 1 h and with 12.5 μ g/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 cases 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 exact 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 clinicopathological 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

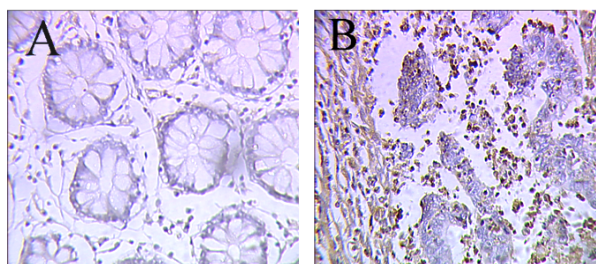
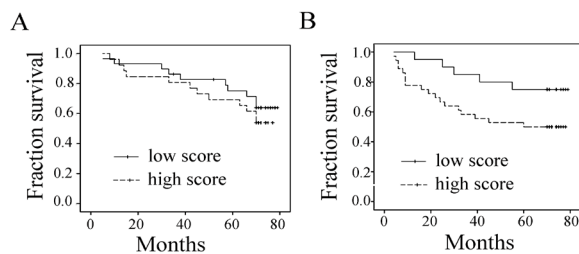


Figure 1. Representative Immunohistochemistry Staining of AAL (200 \times). A. Adjacent normal colorectal tissues stained with AAL. B. Colorectal cancer epithelial cells stained with AAL.

Table 1. Clinicopathologic Correlation with AAL-Specific Glycoconjugate Expression

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

*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]Gal β 1-3GalNAc α -Sp8 (Sulfated TF disaccharide)>Neu5Ac α 2-3Gal β 1-3GalNAc α -Sp8 (Sialyl-TF disaccharide)>[3OSO3]Gal β 1-4GlcNAc β -Sp8>Gal β 1-3GalNAc α -Sp14 (TF disaccharide), which shows that besides crystallization with TF antigen (Feng et al., 2010), AAL can permit selective detection of sulfated and α 2-3-linked sialic acid glycoconjugates, especially 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 (Balduş 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 Gal β 1-3GalNAc α - and the Gal β 1-4GlcNAc 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 glycoconjugates 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 α 2,3-

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 Gal β 1-4GlcNAc 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.

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