

# Loss of L-PHA-, PNA-, or ConA-reactive oligosaccharides is associated with a poor prognosis in human Burkitt's lymphoma

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**Abstract.** The expression of cell surface oligosaccharides is associated with various biological phenomena. To clarify the relationship between lectin binding and the survival of patients with Burkitt's lymphoma, tumor samples from nine patients with Burkitt's lymphoma were analyzed by lectin histochemistry. Kaplan-Meier analysis showed that survival is significantly shorter for patients with negative reactivity for lectins from *Phaseolus vulgaris* (L-PHA), *Arachis hypogaea* (PNA), or *Canavalia ensiformis* (ConA) than for those with positive reactivity for these lectins. Immunohistochemistry for N-acetylglucosaminyltransferase V, which synthesizes  $\beta$ 1,6-branched oligosaccharides such as L-PHA-reactive oligosaccharides, was positive in 8 of 9 patients, but there was no correlation between its expression and that of L-PHA-reactive oligosaccharides. Collectively, a loss of L-PHA-, PNA-, or ConA-reactive oligosaccharides is closely associated with a poor prognosis in patients with Burkitt's lymphoma.

## Introduction

Cell surface N-glycosylation of tumor cells affects various biological processes such as metastasis (1-4), carcinogenesis (5-7), prognosis (8), malignant potential (9-11), cell adhesion and invasion (12-14). Demetriou *et al* reported that *Phaseolus vulgaris* lectin (L-PHA)-reactive oligosaccharides synthesized by N-acetylglucosaminyltransferase V (GnT-V) modify T cell receptor signaling by interacting with galectin-3, a galactose-specific lectin (15). In addition, inhibition of cell surface N-glycosylation in  $\alpha$ -mannosidase II gene knockout mice is reported to be closely associated with development of autoimmune disease (16). These data suggest that L-PHA-reactive oligosaccharides regulate T lymphocyte activation.

Previously, Dennis *et al* reported that L-PHA-reactive oligosaccharides regulate adhesion of murine T cell lymphoma

cells to the extracellular matrix (ECM) and reduce their adhesion to fibronectin and collagen type IV (1). N-glycosylation also affects the adhesion of  $\beta_1$  integrin to ECM (17). Furthermore, studies with the  $\alpha$ -mannosidase II inhibitor swainsonine show that cell surface N-glycosylation closely correlates with tumor cell invasiveness on laminin-1 (13). These findings suggest that cell surface N-glycosylation is closely linked to the adhesion or invasion to ECM by tumor cells.

The expression of L-PHA-reactive oligosaccharides, which are N-glycans, is closely associated with the clinical outcome of human diffuse large B cell lymphoma (18,19), and a loss of L-PHA-reactive oligosaccharides corresponds with a poor prognosis in non-small cell lung cancer (20). In the present study, we report that a loss of cell surface reactivity for L-PHA, *Arachis hypogaea* lectin (PNA), or *Canavalia ensiformis* lectin (ConA) is closely associated with a poor prognosis in human Burkitt's lymphoma.

## Materials and methods

**Patients.** Nine cases of Burkitt's lymphoma from the files of the First Department of Pathology, Fukushima Medical University were examined. In all cases, a diagnosis of Burkitt's lymphoma was made based on the WHO classification (21). The cases included five male and four female patients, with ages ranging from 4 to 42 years (median, 32). The survival time ranged from 2 to 24 months.

**Reagents.** Biotinylated lectins from *Arachis hypogaea* (PNA), *Phaseolus vulgaris* (L-PHA), *Canavalia ensiformis* (ConA), *Maclura pomifera* (MPA), *Artocarpus integrifolia* (AIA), *Triticum vulgare* (WGA), *Ulex europaeus* (UEA-1), and *Glycine max* (SBA) were purchased from EY Laboratories (San Mateo, CA, USA). The monoclonal antibody that recognizes GnT-V was kindly provided by Drs N. Taniguchi and E. Miyoshi (8).

**Lectin staining.** Formalin-fixed, paraffin-embedded sections of biopsy materials were prepared according to standard procedures. The paraffin-embedded sections were deparaffinized in xylene and rehydrated with a graded series of ethanol. After washing twice for 5 min in 0.01 M phosphate-buffered saline, pH 7.4 (PBS), the sections were incubated with 0.3% hydrogen peroxidase in methanol for 20 min at room temperature to eliminate the endogenous peroxidase activity.

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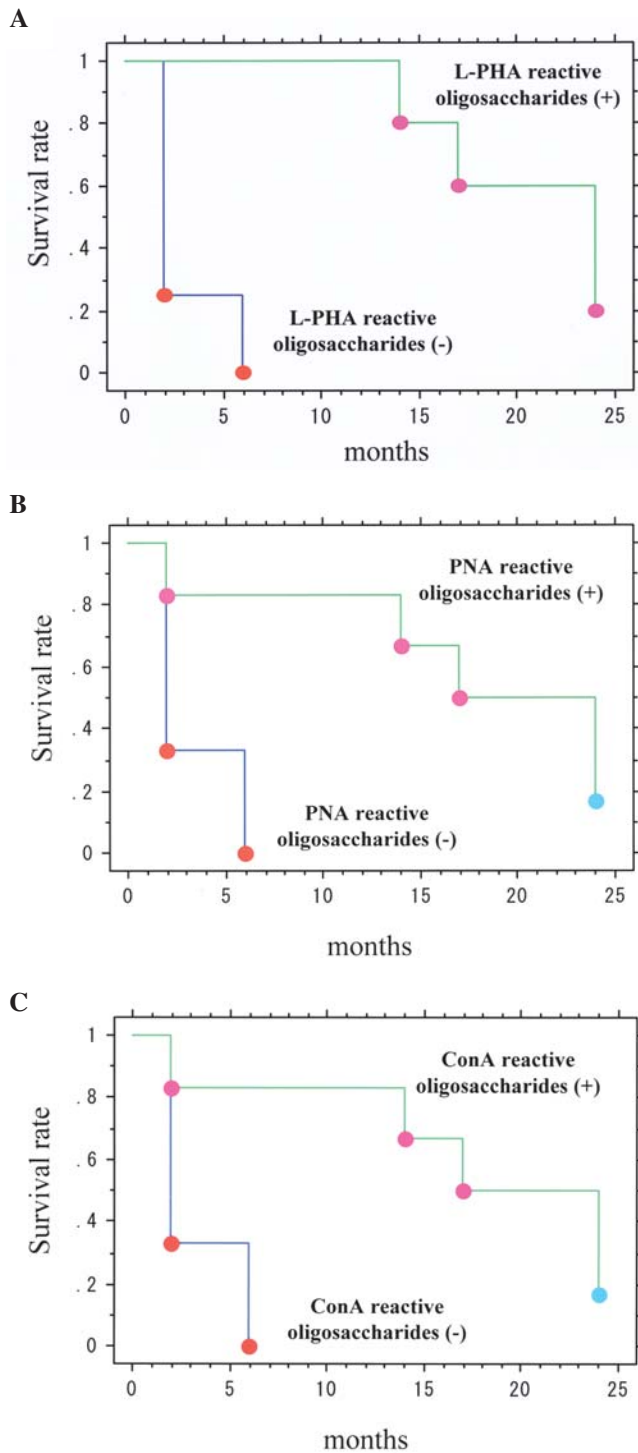


Figure 1. Relationship between survival and lectin binding to tumor samples from patients with Burkitt's lymphoma. The survival of patients negative for binding of L-PHA, PNA, or ConA was significantly shorter than that of patients positive for binding of these lectins. Survival curves were established according to the Kaplan-Meier method, and P-values were calculated based on Wilcoxon's test. The horizontal line represents months of survival, and the vertical line represents the survival rate. (A)  $P=0.0033$ , L-PHA-positive cases vs. L-PHA-negative cases. (B)  $P=0.0271$ , PNA-positive cases vs. PNA-negative cases. (C)  $P=0.0271$ , ConA-positive cases vs. ConA-negative cases.

To remove sialic acids from oligosaccharides, the sections were treated for 1 h at 37°C with 0.1 units/ml neuraminidase from *Vibrio cholerae* (Roche, Tokyo, Japan) in sodium acetate

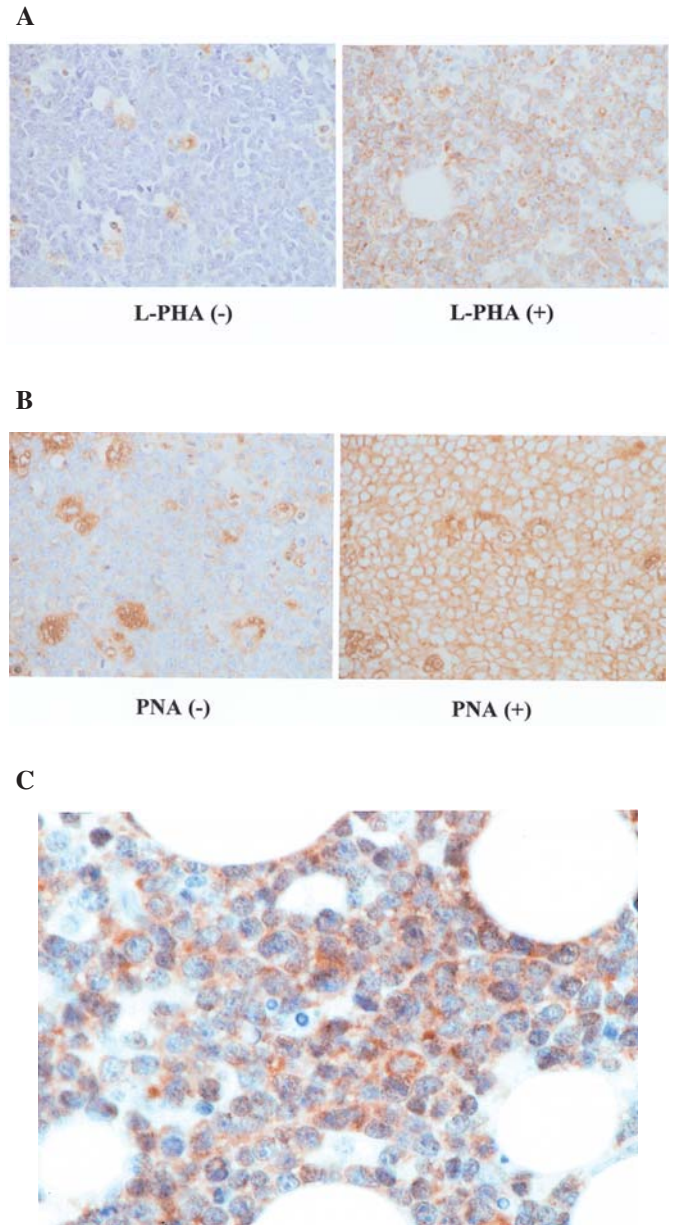


Figure 2. Staining for L-PHA or PNA-reactive oligosaccharides and immuno-histochemical staining for GnT-V in Burkitt's lymphoma. (A) L-PHA-reactive glycoconjugates are expressed on the surface or in the cytoplasm of lymphoma cells. (B) PNA-reactive glycoconjugates are expressed on the surface of lymphoma cells. (C) GnT-V is found throughout the cytoplasm of lymphoma cells.

buffer (pH 5.6) containing 0.04 M  $\text{CaCl}_2$  (20). Next, the sections were incubated with 5% skim milk in PBS for 20 min at room temperature to reduce nonspecific staining. After washing 3 times for 5 min in 0.05 M Tris (pH 7.6), the sections were incubated overnight at 4°C with biotinylated lectins at various concentrations, followed by 20 min at room temperature with streptavidin-biotin-peroxidase complex (Nichirei, Tokyo, Japan) and then 5 min in diaminobenzidine (DAB)- $\text{H}_2\text{O}_2$  solution (30  $\mu\text{g}$  DAB in 0.05 M Tris buffer). After each incubation step, the sections were washed 3 times for 5 min in PBS. Finally, the sections were counterstained with hematoxylin and mounted (Table I).

Lectins	Dilution	Carbohydrate specificity
<i>Arachis hypogaea</i> (PNA)	1:300	$\beta$ Gal/GalNAc
<i>Maclura pomifera</i> (MPA)	1:300	$\alpha$ Gal/GalNAc
<i>Artocarpus integrifolia</i> (AIA)	1:500	
<i>Glycine max</i> (SBA)	1:300	
<i>Canavalia ensiformis</i> (ConA)	1:200	$\alpha$ Man
<i>Triticum vulgare</i> (WGA)	1:200	$\beta$ GlcNAc/ $\alpha$ NeuAc
<i>Ulexeuropaeus</i> (UEA-1)	1:100	$\alpha$ Fuc
<i>Phaseolus vulgaris</i> leukoagglutinating lectin (L-PHA)	1:500	GlcNAc $\beta$ 1-6Man $\alpha$ 1-6R-Asn

Gal, galactose; GalNAc, N-acetylgalactosamine; Fuc, fucose; Man, mannose; GlcNAc, N-acetylglucosamine; NeuAc, N-acetylneuraminic acid; Asn, aspartic acid.

**Immunohistochemical staining for GnT-V.** Paraffin-embedded sections were deparaffinized in xylene and rehydrated with a graded series of ethanol. After washing the sections twice for 5 min in 0.01 M PBS, they were incubated for 20 min at room temperature with 0.3% hydrogen peroxidase in methanol to eliminate the endogenous peroxidase activity. After washing 3 times for 5 min in PBS, the sections were incubated overnight at 4°C with 1:1000 anti GnT-V monoclonal antibody, followed by 1:1000 biotinylated anti-mouse immunoglobulin antibody (Dako) for 20 min at room temperature and then for 20 min with avidin-biotin-peroxidase complex (Dako). Next, the sections were incubated for 5 min with DAB-H<sub>2</sub>O<sub>2</sub> solution (30  $\mu$ g DAB in 0.05 M Tris buffer). After each incubation step, the sections were washed 3 times for 5 min in PBS. The sections were counterstained with hematoxylin and mounted.

**Evaluation of lectin-binding reactivity and immunohistochemical findings.** All lectin- and immunohistochemically stained sections were examined using a light microscope. Lectin-binding reactivity and GnT-V expression was assessed as follows: negative, <10% positive tumor cells; positive,  $\geq$ 10% positive tumor cells.

## Results

**Expression of L-PHA-, PNA-, or ConA-reactive oligosaccharides and survival curves.** We examined the binding of eight lectins to tumor samples from nine patients with Burkitt's lymphoma. We found that the survival of the patients that were negative for L-PHA, PNA, or ConA binding was significantly shorter than that of patients that were positive for the binding of these lectins (Fig. 1A-C). There was significant correlation between the reactivity for L-PHA, PNA, and ConA and prognosis ( $P=0.0033$  for L-PHA binding and  $P=0.0271$  for PNA or ConA binding), but no significant correlation between the binding of other lectins (from MPA, AIA, WGA, UEA-1, and SBA) and prognosis.

**Lectin staining.** In lymphoma cells positive for L-PHA binding, the L-PHA-reactive oligosaccharides were expressed on the cell surface or in the cytoplasm. In all samples that were positive for PNA binding, PNA-reactive oligosaccharides were expressed on the surface of lymphoma cells (Fig. 2A and B).

**Expression of GnT-V.** Of the 9 cases of Burkitt's lymphoma, lymphoma cells from 8 expressed GnT-V. Except in one of these cases, GnT-V was expressed in the cytoplasm (Fig. 2C). There was no significant correlation between the expression of L-PHA-reactive oligosaccharides and GnT-V expression.

## Discussion

It has been reported that expression of L-PHA-reactive oligosaccharides is closely associated with distant metastasis of murine lymphoma (1). In contrast, we show herein that a lack of L-PHA-reactive oligosaccharides corresponds to a worse prognosis in Burkitt's lymphoma. Therefore, L-PHA-reactive oligosaccharides may have different functions in human and mouse lymphomas.

N-glycosylation on the cell surface is regulated by several glycosyltransferases including GnT-V, which synthesizes  $\beta$ 1,6-branched oligosaccharides such as L-PHA-reactive oligosaccharides. GnT-V is known to play important roles in metastasis (3,4), carcinogenesis (5-7), cell adhesion (12,13) and invasion (14), and the expression patterns of GnT-V-synthesized N-glycans appear to correlate with the patient's clinical outcome (19,20). Alteration in the activity of some glycosyltransferases that synthesize N-glycans results in the remodeling of the N-glycan structures on several glycoproteins, affecting their function (22). In the present study, however, we did not find a significant correlation between the expression of L-PHA reactive oligosaccharides and the expression of GnT-V. This suggests that the differences in the level of L-PHA-reactive oligosaccharides between the different

patients is not due to altered expression of GnT-V but rather to changes in the expression of other enzymes that regulate N-glycan biosynthesis. The production of  $\beta$ 1,6-branched oligosaccharides by GnT-V is reported to be regulated by the concentration of the donor, UDP-GlcNAc, as well as the level of GnT-V (23). Thus, the loss of L-PHA-reactive oligosaccharides may be due to a reduction in the amount of UDP-GlcNAc.

In the present study, we showed that ConA-reactive oligosaccharides were present in the cytoplasm of lymphoma cells. ConA reacts with mannose residues, such as high mannose-type N-linked oligosaccharides, which are synthesized in the Golgi and are precursors of mature complex-type N-linked oligosaccharides including L-PHA-reactive oligosaccharides (24). There is a tendency of a positive correlation between the binding of L-PHA and ConA, suggesting that the loss of L-PHA-reactive oligosaccharides may be due to a loss of precursors, such as ConA-reactive oligosaccharides, but not a loss of GnT-V.

N-glycans such as L-PHA-reactive oligosaccharides are known to be ligands for galectin-1, and L-PHA-reactive oligosaccharides regulate cell death by interacting with galectin-1 (25,26). Ligands of galectin-1 are known to be N-glycans of CD45 (27), and L-PHA reactive oligosaccharides are reported to be expressed on CD45 (28). It has been reported that N-glycans synthesized by GnT-V regulate cell adhesion to ECM (29,30). Further investigations are needed to determine whether alteration of cell surface N-glycans such as L-PHA-reactive oligosaccharides modulate galectin-induced cell death or cell adhesion to ECM in Burkitt's lymphoma.

We have previously shown that loss of L-PHA reactive oligosaccharides is associated with a poor prognosis in human diffuse large B cell lymphoma (DLBCL) (18,19). Although there is a slight difference in sialylation of the terminal residues of oligosaccharides, relationship between L-PHA reactive oligosaccharide expression and prognosis in DLBCL is correlated to that in Burkitt's lymphoma in the present study. These data suggest that L-PHA reactive oligosaccharides may play important and common roles in malignant behavior in both types of lymphoma.

The current results suggested that a loss of PNA-reactive oligosaccharides is associated with a poor prognosis in human Burkitt's lymphoma. PNA is known to react with O-glycans or glycolipids, and cell surface O-glycans have been shown to regulate galectin-1-induced cell death in human B cell lymphoma (26). Therefore, further studies should examine whether a loss of PNA-reactive oligosaccharides prevents galectin-1-induced cell death in Burkitt's lymphoma. PNA reactive O-glycans are known to be linked to CD45 (31), and these O-glycans of CD45 regulate homodimerization and phosphatase activity of CD45 isoforms (32). Therefore, further study is needed to clarify whether alteration of CD45 O-glycans modulate functional activity of phosphatase of CD45 in human B cell lymphoma.

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