筑　波　大　学

博 士 （医 学） 学 位 論 文

Glycan profile of signet ring cell gastric cancer and potential applicability of lectin drug conjugate therapy

（シグネットリングセル胃癌細胞表面糖鎖の発現解析と糖鎖を標的する治療法の開発）

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Abbreviations:

BPL	Bauhinia Purpurea Lectin
BSA	bovine serum albumin
Con A	Concanavalin A lectin
FBS	fetal bovine serum
GC	gastric cancer
HER2	human epidermal growth factor receptor 2
IC_{50}	50% inhibitory concentration
IMDM	Iscove's Modified Dulbecco's Medium
LDC	lectin-drug conjugate
NSRC	non-signet ring cell
PBS	phosphate-buffered saline
rBC2LCN	recombinant N-terminal domain of BC2L-N lectin
RPMI 1640	Roswell Park Memorial Institute medium
SRC	signet ring cell carcinoma

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Chapter 1. Glycan expression profile of signet ring cell gastric cancer

1-1. Background

1-1-1. Present situation of signet ring cell gastric cancer

Gastric cancer (GC) is the 2nd cancer leading death cause worldwide, even the incidence has decreased in last decades, it remains the 4th common malignancy in the world, especially in Eastern Asia [1][2][3]. There are several subtypes of GC, in which, signet ring cell carcinoma (SRC) is a typical histological subtype, accounts for 16–32% of all GC [4]. In Japan, 10% of all gastric cancers are SRC, even the rates of mortality have declined but the incidence rates are continuing to be high [5] [6].

According to Bormann classification, most of SRC is categorized as Bormann type 4, also known as linitis plastica (LP), but not all LPs are SRC, 10% ~ 20% of LP patients are not diagnosed as SRC [7] [8]. The WHO system defines 5 main types of GC, SRC is included in one of them, poorly cohesive gastric carcinoma [9]. Compare with other subtypes, SRC is more aggressive, infiltrating, and more metastatic. The age distribution of SRC patients is younger, also, SRC occurs more often in female [10].

Detection of gastric cancer usually relies on examination through endoscope [11], as for SRC, histologic appearance of SRC is a solid diagnosis. Whereas there are some limitations. First, endoscopic examination may ignore tiny cancer foci leads to the inaccurate detection [12] [13]. Second, an important characteristic of SRC, aggressive behavior, also results in detection is usually at an advanced stage [14] [15].

Benefit from the development of novel cytotoxic medicaments and surgical techniques, the prognosis for GC has been improved. However, the majority of patients with SRC who are diagnosed during late stages are more likely resistant to chemotherapy [16][17].

1-1-2. Glycosylation and lectins

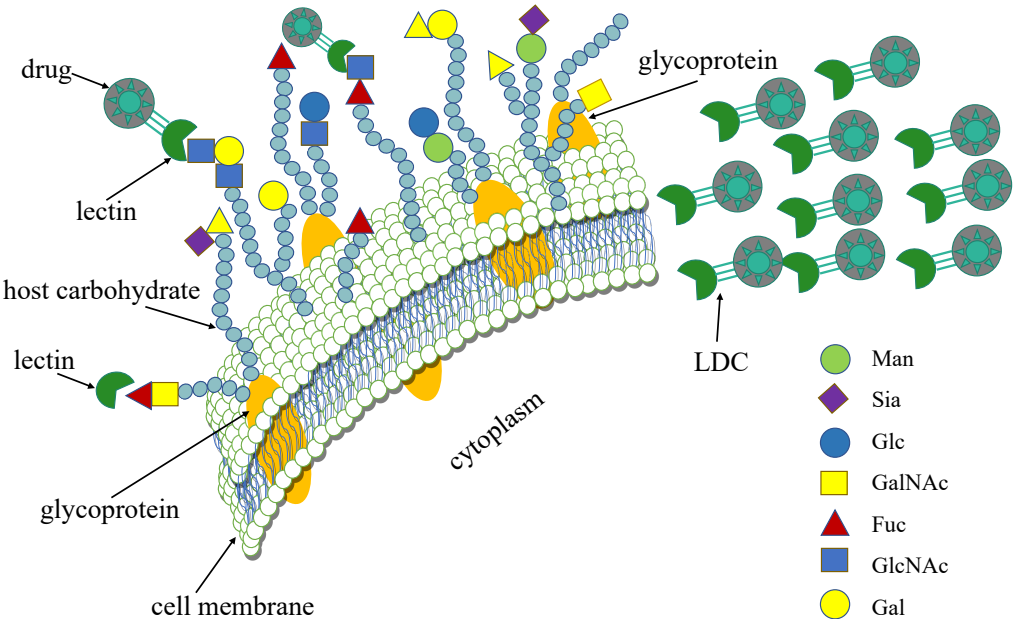
Glycans are covering the most outside layer of cell membrane, and glycosylation is a common modification of proteins in human bodies, aberrant glycosylation plays a role of tumor invasiveness, metastasis and prognosis by formatting sialylation or other formations which will lead to glycans associated to tumor [18][19][20].

Several studies were reported for glycosylation in GC and some lectins, which were bind to specific structure of glycosylation, were associated with GC and its prognosis. In the comparison between GC and normal tissue or gastric ulcer tissues, GalNAc glycans were highly expressed in GC and its binding lectin (VVA) was associated with lymph node metastases [21] [22].

However, it remains unclear that the difference of glycan expression and glycosylation of GC cells between each histological subtypes including SRC. I therefore investigated that the glycan profile of SRC by the comparison with NSRC using lectin microarray.

1-2. Aim of research

To explore the glycan expression of SRC and find out the specific lectin with intensive affinity.



1-3. Materials and methods

1-3-1. Cell lines

Human gastric adenocarcinoma cell lines, KATO-III, SNU-1, and NCI-N87 were purchased from ATCC (Manassas, VA, USA), NUGC-4 was purchased from RIKEN BioResource Research Center (Tsukuba, Ibaraki, Japan), and MKN-45 was purchased from the Japanese Collection of Research Bioresources cell bank (Sennan, Osaka, Japan). KATO-III and NUGC-4 are signet ring cell GC cell lines, which I defined as SRC cell lines. NCI-N87 is a well differentiated, and SNU-1 and MKN-45 are poorly differentiated gastric adenocarcinomas without signet ring cells, which I defined as NSRC. The attributes of the aforementioned cell lines were confirmed using the Cancer Cell Line Encyclopedia. SNU-1 and NCI-N87 cells were cultured in RPMI 1640 (ATCC), KATO-III cells in IMDM (ATCC), and MKN-45 and NUGC-4 cells in RPMI 1640 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The media were supplemented with 10% or 20% FBS (GIBCO, Paisley, RF, UK) and 1% penicillin-streptomycin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). All cells were incubated at 37 °C in a 5% CO_2 atmosphere and were passaged directly or via trypsinization.

1-3-2. Clinical tissue samples

Thirty-two pairs of formalin-fixed and paraffin-embedded (FFPE) human GC tissue sections and frozen adjacent normal tissues were obtained from the Department of

Pathology, University of Tsukuba Hospital, Tsukuba, Japan. All clinical samples were obtained with written informed consent from the patients. All the protocols followed the regulations relating to ethics of human subject research at the University of Tsukuba, Tsukuba, Japan. This study was approved by the Tsukuba Clinical Research and Development Organization (T-CReDO), Tsukuba, Japan.

1-3-3. Protein extraction

A total of 5×10^4–10^7 live cells, or frozen tissue sections were lysed with CelLytic MEM Protein extraction kit (Sigma-Aldrich, St. Louis, Missouri, USA) the protein concentrations were determined using the Micro BCA™ Protein Assay kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and γ-globulin was used as the standard (Bio-Rad Laboratories, Hercules, California, USA). The procedures were performed according to the manufacturer's instructions.

1-3-4. Lectin micro-array

A lectin microarray was constructed and analyzed as previously described [23][24][25]. In brief, 96 lectins were immobilized (Fig. 1). Protein samples were labeled with fluorescent dye Cy3 (GE Healthcare, Cat#:PA13104), then, the Cy3-labelled lysates were applied to each well of the microarray slides, after incubating at 20°C overnight, the chips were scanned with an evanescent field activated fluorescence scanner (Bio-REX Scan 200, Rexxam Co., Ltd, Osaka, Japan). Automatic intensity analysis was performed after high–speed scan, the quantified signal was analyzed using Cluster 3.0

(yellow: high; blue: low; black: intermediate), mean-normalized, log-transformed and analyzed via the average linkage were performed.

1-3-5. Statistical analysis

Cluster 3.0 and Java tree view were used to perform cluster analysis, and the results were exported as heat maps. Student's t-test in SPSS Statistics 21.0 (SPSS Inc.) was used to evaluate the differences in lectin array signals between the two data sets. Differences were considered statistically significant at $p<0.05$.

1-4. Results

1-4-1. High affinity lectins and its binding glycan structure in SRC cell lines

Glycan expression in SRC cell lines was compared with that in NSRC cell lines using high-density lectin microarrays. I compared the signal intensity and heat map, several lectins showed different affinities between SRC and NSRC cell lines and demonstrated separate branch of a tree diagram (Fig. 2A). A total of 19 lectins showed significantly different binding affinity; 11 lectins (BPL, rDiscoidin II, rPALa, GSL-I A4, SBA, ECA, rSRL, DSA, rBC2LCN, CCA, and rBC2LA) showed higher affinity to SRC cell lines. Ratio of SRC signal intensity to NSRC, showed highest ratio in the case of the rBC2LCN lectin (1.930-fold), and next was BPL lectin (1.786-fold) (Fig. 2B, C). GalNAc binding lectins have a tendency towards SRC than NSRC. Signal intensity of

rBC2LCN lectin in each cell lines showed that higher signal was observed in both SRC cell lines: KATO-III (75.4 ± 28.5), and NUGC-4 (57.7 ± 15.8) (Fig. 2C). In the NSRC cell lines, two cell lines showed lower signal intensity: SNU-1 (25.5 ± 7.9), and MKN-45 (24.0 ± 6.2), but NCI-N87 (53.9 ± 7.7) showed higher signal similar as SRC.

1-4-2. Low affinity lectins and its binding glycan structure in SRC cell lines

There were 8 lectins (TJAI, SNA, SSA, rACG, rPSL1a, PVL, rLSLN, and STL) showed lower affinity (Table 1). As the lectins which can recognize GalNAc structures have the tendency towards SRC than NSRC. Contrary, lectins which binds to α2-6Sia or other sialylation structures are bind tighter to NSRC (Fig. 2A, B).

1-4-3. Glycan expression in clinical tissue samples

To get to the bottom of whether the BPL and rBC2LCN lectin's affinity to SRC cell lines can rolling out to the clinical GC samples, I analyzed 9 pairs clinical GC specimens by lectin microarray, 4 SRC and 5 NSRC samples (Fig. 3A). Based on the definition of Japanese Classification of Gastric Carcinoma, I took the tissues which were diagnosed with sig and por2 as SRC group, while tub or por1 or pap were regarded as NSRC group, I also analyzed the paired adjacent normal tissues as negative control. The result didn't achieve the desired expectations. There were no statistical differences between the SRC and NSRC groups, or even between the tumor and normal samples of BPL lectin or rBC2LCN lectin (Fig. 3B, C).

1-5. Discussion

The present study reported the characteristics of glycan expression profiles in SRC cells and identified 11 high-affinity and eight low-affinity lectins, but the analysis of clinical specimens didn't support the results from cell lines. As we all know, the clinical samples were more complicated, it may be the reason why the 2 analyses were different. The SRC samples usually obtain abundant fibrous tissue that could cause strong noises, and the relative amount of cancer cells are fewer relevantly, which would make weak signals. Considering the uncontrollability of clinical samples, I did not abandon the current results directly, but decided to seek validation through other methods following with the data from glycan expression profile in cells.

Glycan structures which high-affinity lectins bind to varied, but a common glycan structure, α2-6 Sia, was identified among the low-affinity lectins. Therefore, relative downregulation of α2-6 Sia might be related to the specific properties of SRC cells. Among the high-affinity lectins, rBC2LCN lectin showed the highest SRC/NSRC affinity ratio and has potential applicability as an lectin-drug conjugate (LDC) targeted therapy for SRC.

The typical difference in glycan expression profiles between SRC and NSRC cells was the relative downregulation of α2-6 Sia in SRC, which could be detected by TJAI, SNA, SSA, rACG, and rPSL1a lectins. Sialic acids are acidic sugars that are typically located at the outer ends of cell surface glycan chains [23], and an increase in α2-6 sialylation

is frequently observed in tumor cells [24]. Expression of α2-6 Sia is mediated by β-galactoside α2-6-sialytransferase (ST6Gal-I), and its increase is correlated with cancer progression, metastatic spread, and poor prognosis [25]. Also, the sialyl Thomsen-nouvelle antigen (sTn) which is a known tumor-associated carbohydrate antigen is an α2-6 sialylation of Tn antigen. Several studies have highlighted that sTn participates in adverse outcomes in gastric cancer [26]. Therefore, higher sialylation is associated with malignant properties of cancer cells.

SRC has been historically recognized as a GC subtype with worse prognosis when compared with other GC subtypes, because of its characteristic “diffuse-type” growth and frequent peritoneal metastases in advanced stages [27]. However, recent studies have shown that the prognosis of early stage SRC is similar to [4, 28], or even better [29,30] than that of the common type of adenocarcinoma, while advanced stage SRC is associated with poor prognosis [29, 31]. These results suggest that the signet-ring cells are not intrinsically malignant, but gain malignant properties following submucosal invasion through cell-cell interactions with stromal cells, such as cancer-associated fibroblasts [31]. This potentially explains the lower sialylation in SRC seen in my study. In addition, α2-6 sialylation of N-glycans is known as an important modulator of β1 integrin, which affects tumor metastasis by modulating cancer cell adhesion and migration [32-34]. Increased α2-6 sialylation of β1 integrin is frequently observed in colon adenocarcinoma through increased activity of ST6Gal-I, which augments tumor progression and metastases [32]. These findings seem to correlate with

a higher frequency of hematogenous metastases in colon cancer, and provides an explanation for the low frequency of hematogenous metastases in SRC [28]. Upregulation of ST6Gal-I is a consequence of the oncogenic *ras* gene [35]; however, *ras* mutations are less frequent in SRC. Therefore, the lower level of α2-6 sialylation in SRC has a genetic basis. Additionally, α2-6 sialylation regulates the cell surface platelet endothelial cell adhesion molecule (PECAM) [36]. Although further investigation is required, this characteristic finding of low α2-6 Sia may also play a role in cell-cell interactions of poorly cohesive SRC by modulating cell adhesion molecules.

Among the 11 high-affinity lectins, rBC2LCN showed the highest SRC/NSRC ratio, followed by BPL. The binding structures of glycans for these two lectins are not the same, but share a common motif, Galβ1-3GlcNAc. This motif is a type-1 glycan chain and is classified as a mucin-type-O-glycan [37]. Mucinous proteins are decorated by hundreds of O-linked glycans and glycosylation patterns, making them important in many cancer-associated biological processes [38]. Therefore, our results showing high affinity for these O-glycans in SRC are understandable.

There are some limitations in this work. For one thing, only small number of clinical samples were contained. It's needful to enlarge the cohort. In addition, the proteins were extracted from whole tumor tissues, there may have different tumor types inside it. It's necessary to use micro dissection in further studies. In general, I expect BPL or rBC2LCN lectin can conduce to diagnosis of SRC.

1-6. Conclusion

In conclusion, this study reported that specific glycan expression in SRC cells and lower expression levels of α2-6 Sia might be key features for understanding the biology of SRC cells. In addition, SRC cells showed higher affinity for rBC2LCN lectin and BPL lectin. The result of lectin array gave me these 2 candidates. With the analysis of clinical samples in mind, it's necessary to confirm their affinities through other methods.

Chapter 2: Glycan targeting therapy for signet ring cell gastric cancer using lectin drug conjugate

2-1. Background

At present, antibody-drugs are still the mainstream of cancer treatment. With the deepening of the understanding of glycosylation and lectins, more researchers are beginning to explore whether lectin-drug conjugate could be a new cancer treatment option. However, the use of lectins in drug targeting is still a fledgling subject, on account of their characteristic of causing agglutination. Despite of that, efforts are being made to overcome the barriers behind lectin-drug delivery. Several studies reported that high levels of galectins can be used as a therapeutic target to fight colon carcinoma [39] and lectin-mediated drug delivery improved intracellular availability of conjugated drugs for colon cells [40-42], which supported the lectin-drug delivery is an expected open filed. Indeed, in my lab, we already succeeded in applying rBC2LCN-PE38 conjugate as a treatment strategy in pancreatic cancer and colorectal cancer [43, 44].

2-2. Aim

I investigated SRC-specific glycans using lectin microarray, it's requisite to verify the result and then evaluate the potential applicability of developing a glycan-targeting therapy using LDC.

2-3. Material and methods

2-3-1. Lectin staining

2-3-1-1. Immunohistochemistry

FFPE tissue blocks were prepared as 3 μm sections and autoclaved for antigen retrieval. Endogenous hydrogen peroxide activity was blocked with 3% hydrogen peroxide in methanol. The lectins, BPL and rBC2LCN, was labelled with horseradish peroxidase and applied to the blocked sections, and 3,3′-diaminobenzidine (Histofine SAB-PO(M) kit, Nichirei Bioscience, Tokyo, Japan) was added. The stained slides were scored according to average staining intensity and positive area percentage (staining area) of positive cells, which were categorized as highly positive (3+), positive (2+), slightly positive (1+), and negative (0).

2-3-1-1. Live cell staining

Live cells (3×10^5 cells) were seeded in 35 mm glass dishes. After 48 hours of seeding, the dishes were washed with PBS three times prior to the addition of 1 μL of rBC2LCN-FITC (AiLecS1-FITC) (Wako Pure Chemical Corporation, Osaka, Japan) in 1 mL of medium. The cells were incubated for 1 hour at 37 °C in a CO_2 incubator, and then the medium was replaced with PBS. Images were captured using a BIOREVO BZX-710 fluorescence microscope (Keyence, Osaka, Japan).

2-3-2. Protein blotting assay

Extracted protein samples have been adjusted to 1 μg/10 μl/well, heated at 95°C, 5 minutes, and cooled on ice, 2 minutes, then applied the prepared proteins into an acrylamide gel (Perfect NT Gel, 5-20%, 20 well or 12 well, DRC, NTH-676HP), run electrophoresis at 20 mA/gel for 90 min.

2-3-2-1. Lectin blotting

After running electrophoresis, the gels were transferred to PVDF membranes (Bio-RAD, 162-0716) constant volt 50 V, 60 min at 4°C, then incubated the membranes in lectin-HRP mixture solution 1-hour. Western lighting plus (PerkinElmer, NEL104001EA) was used for substrate reaction.

2-3-2-2. Silver blotting

The gels were prepared like I described previously. A silver blotting MS kit (Wako, 299-58901) was used, and followed the manufacturer's protocol to conduct this blotting.

2-3-3. Flow cytometry

Lectins were labelled with phycoerythrin (PE) and adjusted to 1 mg/mL. Cells were harvested and washed twice with 1% BSA/PBS, and 1 μL of the pretreated lectins was added to each tube. After one hour incubation and washing, I obtained cells that were bound to the lectin BPL or rBC2LCN. The cells were analyzed using Cell Sorter

SH800s (SONY, Tokyo, Japan), and fluorescence signals were determined using FlowJo (v10.5, BD Biosciences, New Jersey, USA).

2-3-4. Cell viability assay

Commercially available rBC2LCN-PE38 conjugate, stem secure human pluripotent stem cell (hPSC) Remover was purchased (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). BPL-saporin conjugate was synthesized in the lab. BPL was labelled with Biotin and adjusted to 1 mg/ml, the toxin which I used was Streptavidin-ZAP (Advanced Targeting Systems, IT-27, Lot: 132-16, 2.5mg/ml, 127 kDa). The viability of cells treated with rBC2LCN-PE38 or BPL-saporin was determined using the CCK-8 kit (Dojindo Molecular Technologies, Kumamoto, Japan). The cells were seeded at 5×10^3 cells/100 μL/well in 96- well plates and cultured for 48 h. On the third day after seeding, the rBC2LCN-PE38 or BPL-saporin conjugate was added, diluted to multiple concentrations ranging from 1×10^6 to 0 pg/mL. and incubated for 48 h at 37 °C in a CO_2 incubator. On day 5, 10 μL of CCK-8 was added per well, and after 2 h of incubation, the absorbance was measured at 450 nm using a SpectraMax M3 spectrophotometer (Molecular Devices LLC, San Jose, California, USA). Data were analyzed using GraphPad Prism 9.0.

2-3-5. Hemagglutination assay

Blood samples were collected from healthy volunteers including type A, B, O and AB. Each volunteer was taken 5 ml blood. The blood samples were washed 3 times by 20

ml PBS, centrifuged 2000 rpm for 5 minutes, the upper layers had been discarded. The lower layer was erythrocytes and then mixed with an equal volume of 1 unit/ml neuraminidase from clostridium perfringens (C. welchii, Sigma, Lot#: SLBP4674V, Darmstadt, Germany) and incubated at 37°C for 1 hour with shaking gently. At the same time, Concanavalin A (Sigma, Darmstadt, Germany) and BPL were adjusted to 100 μg/ml with PBS. Con A was used as positive control. All the samples were washed 3 times again and prepared as 2% suspension in PBS. 50 μl PBS was added from the 2nd column to the 12th column on a U shaped 96 well plate, 100 μl Con A or BPL was applied to the 1st column. 50 μl lectin was taken from the 1st column to the 2nd column, mixed solution was transferred 50 μl to next column except the 12th column so that the concentration of lectins was diluted by half column by column. Then 50 μl of the 2% erythrocytes suspension was applied to each well to observe aggregation.

2-3-6. Animal models

Subcutaneous cell xenograft model: A total of 2×10^6 cells of NCI-N87 and NUGC-4 were suspended in 200 μl PBS and subcutaneously injected into nude mice (BALB/cA-nu, female, 5 weeks old, CLEA, Japan). Animal experiments were kept to the ethical rules approved by the Animal Care Committee, University of Tsukuba, Tsukuba, Ibaraki, Japan.

2-3-7. *in vivo* treatment assay using lectin-drug conjugate

Tumors were allowed to grow to close to 100 mm^3 or 14 days as the initiation of LDC treatment. After the nodules were established, nodules from one mouse in each group were excised and were prepared as FFPE sections to examine the morphology and perform lectin staining to confirm the affinity to BPL and rBC2LCN, and the remaining mice were randomly allocated to two groups (5 mice per group). The rBC2LCN-PE38 was diluted into 1 μg/300 μL/mouse in PBS and administered by intraperitoneal injection on days 1, 3, 5, 8, 10, and 12, and PBS was administered to the control group in the same way. A digital caliper was used to measure the width and length of the tumor on the day of injection. Tumor volume was determined using the following formula: $0.5 \times (\text{width})^2 \times (\text{length})$. On day 15, the mice were sacrificed, and the tumor nodules were excised, followed by weight measurement.

2-3-8. Statistical analysis

The analyses were performed using GraphPad Prism 9.0. The cell viability curve has accepted goodness of fit.

2-4. Results

2-4-1. Lectin selection for glycan targeting therapy

As I described previously, I got 2 candidate lectins, the BPL and rBC2LCN lectin, from glycan expression profile experiment in SRC cells. BPL showed most significant signal intensity difference between SRC and NSRC, and rBC2LCN which showed highest SRC/NSRC ratio. I also used clinical samples to perform the same array, however, the 2 candidates didn't show significant difference. Considering the complexity of the clinical samples, I thought it's better to confirm the affinity using more methods. Hence, I still focused on the BPL and rBC2LCN lectin and tried to apply them to a LDC treatment.

2-4-2. BPL lectin

2-4-2-1. Lectin affinity assay

The affinity between BPL lectin and GC cells was confirmed by blotting assays (Fig. 4A). Silver blotting was conducted to understand the presence of proteins in each cell line, and lectin blotting told us which molecular weight proteins would bind to BPL. Compared lectin blotting using BPL with silver blotting, I got to know that even there were abundant membrane proteins of the GC cell lines, BPL can only bond to a particular part of them, and the high reactivity were more happened in KATO-III and NUGC-4 cells. In other words, the SRC cells had more specific glycan structures for BPL to recognize. The same experiment was done for 9 clinical specimens (Fig. 4D).

In 5 samples, BPL showed strongly positive to tumor lysate. Another 4 samples' normal lysates were more positive to BPL lectin, the rest 1 sample has similar positivity to BPL both tumor and normal. The flow cytometry revealed all 5 GC cell lines have positive affinity to BPL, but NUGC-4 and KATO-III had better affinity than NSRC cell lines, among them, NUGC-4 showed the consistently tightest affinity to BPL (Fig. 4B). I also performed immunohistochemistry staining with clinical samples to evaluate the affinity of BPL (Fig. 5A). A total 60 samples were scored. Only 3 samples had negative parts but there wasn't a sample showed completely negative, all sections were positive to BPL lectin. The adjacent normal tissues also had been stained positively (Fig. 5B, C).

2-4-2-2. Cytotoxicity of lectin drug conjugate

Since BPL could recognize the glycans on the SRC cells, makes it's a potential therapeutic target for SRC. I tried to conjugate BPL with a drug or toxin to achieve the goal of kill the cancer cells. Saporin is a protein has N-glycosidase activity to make it able to enzymatically inactivate the ribosomes, shutting down protein synthesis and resulting in cell death, and eventually causing death of the victim [45]. Because saporin has no chain capable of inserting it into the cell, it needs to attach to some antibodies or molecules to recognize cell surface antigens to increase its cytotoxic activity [46, 47]. Based on this, I decided to make BPL-saporin conjugate. Due to NUGC-4 cells showed stable and consistently strong affinity to BPL, I performed MTT assay with it to measure the cytocidal effective of BPL-saporin conjugate (Fig. 4C). I also tested the

BPL-saporin cytotoxicity in a NSRC cell line, NCI-N87. It turned out both NUGC-4 and NCI-N87 cells were sensitive to BPL-saporin.

2-4-2-3. Hemagglutination assay

Because lectins are considered have ability to agglutinate red blood cells which termed hemagglutination, I wanted to know if the candidate lectin BPL also has this feature and whether it could be administered to GC patients safely. I evaluated the reactivity of BPL to red blood cells of 4 types by an in vitro hemagglutination assay (Fig. 6). The lectin's concentration was reduced by half respectively, BPL exhibited obvious aggregation like Con A in A-, B- and AB-type erythrocytes. But it's notable that the BPL lectin did not cause hemagglutination in O-type even at the highest concentration of 50 μg/ml, this interesting phenomenon demonstrated the BPL lectin is hazardous for all crowds but safe for particular population.

2-4-3. rBC2LCN lectin

2-4-3-1. Lectin affinity assay for rBC2LCN

I confirmed the affinity between rBC2LCN and GC cell lines by live-cell staining (Fig. 7A) KATO-III, NUGC-4, and NCI-N87 were strongly stained by rBC2LCN, while SNU-1 was not. I also confirmed the affinity of rBC2LCN by histochemical staining of cell line derived xenograft models, excepting KATO-III which could not establish xenograft tumor (Fig. 7B, C). NUGC-4 and NCI-N87 showed strong reactivity to the rBC2LCN lectins, MKN-45 showed weakly positive, but SNU-1 showed negative.

Flow cytometry analysis also showed same affinity tendency with staining results (Fig. 7D).

To strength our findings, I evaluated the rBC2LCN affinity with 32 pairs clinical gastric specimens (Fig. 8A, B, C). Of the 32 clinical cases, all SRC cases (n=6) showed highly positive staining (greater than 2+) for rBC2LCN (Fig. 8A). In addition, 15 (71.4%) of the 21 poorly differentiated, 8 (88.9%) of 9 moderately differentiated, and 4 (57.1%) of 7 well differentiated adenocarcinomas were strongly positive for rBC2LCN (Fig. 8B, C). Only five (15.6%) of the 32 clinical samples had a focal negative staining area, and none of the samples stained completely negative.

2-4-3-2. Cytotoxicity of rBC2LCN-PE38 conjugate

The rBC2LCN affinity for pancreatic ductal adenocarcinoma (Shimomura), and colorectal cancer (Kitaguchi) was confirmed previously, and potential applicability as a cancer targeting therapy using lectin drug conjugate (LDC), rBC2LCN-PE38; which is a conjugate of rBC2LCN lectin with a 38 kDa domain of pseudomonas exotoxin A containing domains Ib and II in addition to domain III (PE38) [48]. Cytocidal effect of LDC was measured using an MTT assay. KATO-III showed lowest dose (46.2 pg/mL) for reaching IC_{50}, and NUGC-4 (89.7 pg/mL) followed. NSRC cell lines required more higher dose to reach IC_{50}; NCI-N87 (104.0 pg/mL) was similar as SRC cell lines, but MKN-45 (1411 pg/mL) and SNU-1 (489944 pg/mL) required higher dose (Fig. 7E).

2-4-3-3. *in vivo* treatment assay using lectin drug conjugate

Treatment effect of LDC was evaluated using subcutaneous xenograft models by local injection of NUGC-4 and NCI-N87 cells (Fig. 9A), because both cell lines showed higher affinity to rBC2LCN lectin. NUGC-4 derived xenograft models showed significantly smaller tumor volume on Day 15 in the LDC group (292.2 ± 223.2 mm^3) than that in the control group (564.3 ± 218.6 mm^3) (p=0.0294, Fig. 9B). NCI-N87 derived xenografts also showed smaller tumor volume on Day 15 in the LDC group (161.3 ± 110.5 mm^3) than that in the control group (689.5 ± 190.3 mm^3) (p=0.0025, Fig. 9C). Significant LDC drug toxicity was not observed between LDC group and control group in terms of body weight transition (Fig. 9D, E).

2-5. Discussion

In this study, I reported that BPL and rBC2LCN lectin have specific affinity to the surface glycans of SRC cell lines after screening 5 GC cell lines.

The BPL lectin showed to be safe for particular population of GC patients and enhanced the efficiency of pharmacodynamics. The analysis of lectin micro-array revealed that the expression of glycan ligands of Galβ1-3GlcNAc(GalNAc), α/βGalNAc which identified by BPL showed significantly increased in SRC group compared to NSRC group.

Galβ1-3GlcNAc(GalNAc), α/βGalNAc is regulated by beta-1,3-galactosyltransferase, it makes the β3-glycosyltransferase family very vital, many glycosyltransferase genes can encode β3GT motifs, but the most essential are β3GALT5, β3GALT1, β3GALT2 which will form Galβ1,3GlcNAc- structure [49], what's remarkable about this is that the β3GALT5 is involved in the synthesis of the cancer-associated glycan CA19-9 and SSEA3 [50], while β3GALT1, β3GALT2 are important to the expression of other type 1 chain Lewis antigens [51].

In this study, lectin blotting and flow cytometry both revealed the same result that the SRCs expressed more glycans which the BPL lectin would bound to, suggesting that these glycans are related to the progression of gastric cancer, it also stands for the B3GT motifs are critical to the development of malignancy in gastric cancer. It was reported that BPL had been used as anti-dysenteric, astringent and a poison antidote with molecular mass of 197 kDa [52]. I hoped the BPL lectin can be utilized as a drug carrier like rBC2LCN in pancreatic cancer by Shimomura et al [43]. However, the aggregation had been observed in human blood. I must acknowledge that even the BPL-saporin conjugate illustrated a good cytocidal effect in vitro, but the applicable people are only limited to O-type. Nevertheless, take the advantage of the BPL's different affinities to gastric cancer patients may make it's a promising usage of using BPL as a biomarker for signet ring cell gastric cancer.

The rBC2LCN-PE38 inhibited the growth of cancer cells both in vitro and in vivo. Moreover, immunohistochemical staining of rBC2LCN lectins using clinical GC

samples showed strongly positive (score 2 or more) staining in all SRC samples, but the staining pattern was not specific for SRC. An attempt to classify SRC based on mucin expression level has been reported: gastric type (MUC1, MUC5AC, and MUC6), intestinal type (MUC2 and CDX2), and mixed gastrointestinal phenotypes [53]. However, a recent review concluded that there is no immunohistochemical mucin stain unique to SRC cancer nor are their differences with other GC subtypes [54] thus, these results where most NSRC samples stained positive, were not surprising. In addition, discrepancies in glycan expression among human GC tissue samples and GC cell lines were reported previously [22]. Yamashita, et al reported that VVA lectin which was not expressed in seven GC cell lines was expressed in clinical tissue samples. VVA lectin has a binding affinity for the Tn antigen, which is also a mucin-type glycan. My results were consistent with their report; all GC cell lines used in this study showed low VVA lectin expression. They suggested that the discrepancy was caused by carcinoma-stroma interactions, and this hypothesis seems acceptable because glycans and glycosylation are dynamically modulated by cell-cell interactions [25].

In our lab, we have previously shown that the rBC2LCN lectin has specificity for pancreatic ductal adenocarcinoma (PDAC) [43] and colorectal cancer (CRC) [44] cell surface glycans (Fuc α1-2Galβ1-3GlcNAc(GalNAc)- epitopes, such as H type 1/3/4). This finding was validated for SRC in the present study. Increased levels of fucose and fucosylation, resulting in enhanced expression of fucosyltransferase (FUT), are frequently observed in cancer patients [55]. The elevated expression of FUT4 in GC is correlated with tumor progression, and carcinogenesis of GC induced by CagA,

following *Helicobacter pylori* infection [56]. rBC2LCN lectin was originally identified as a probe specific for human embryonic stem cells and human induced pluripotent stem cells [57-59], and rBC2LCN-positive cancer cells are associated with cancer stem-like characteristics in prostate cancer [60]. Therefore, higher expression of rBC2LCN in SRC, a poorly differentiated GC subtype, is an understandable finding. rBC2LCN-PE38 is a conjugate of rBC2LCN lectin with the *Pseudomonas* exotoxin (PE)-A that we previously developed [43]. This LDC showed cytotoxic activity against rBC2LCN-positive PDAC and CRC without remarkable toxicity, including hemagglutination [44, 48]. The efficacy of rBC2LCN-PE38 against SRC and rBC2LCN-positive NSRC was reproduced in this study. These results shed new light on GC treatment, including that of SRC. Molecular targeting agents have improved survival outcomes in various cancers; however, many phase III trials have failed to demonstrate a survival benefit in GC. SRC is classified as a GS subtype in TCGA [61] and has few specific mutations limiting biomarkers for targeted therapy approaches. Historically, lectins have been recognized as toxins that cause hemagglutination in animals; rBC2LCN lectin, on the contrary, has unique properties and lacks this typical drawback. In my lab, we are now developing a novel LDC drug targeting rBC2LCN for pancreatic cancer in the preclinical trials; thus, it might be a novel treatment option for patients with SRC in the future.

This study has several limitations. First, we classified and compared SRC and NSRC based on the origin of the cell lines, but the clinical definition of SRC is controversial. The histological definition of SRC is inconsistent; the WHO definition of SRC has

changed several times between the 1st edition (1977) and the 4th edition (2010) [54]. Therefore, these results may not be applicable to clinical patients with SRC. Second, the biological mechanisms of low α2-6 Sia expression in SRC cells were not explored in this study. Therefore, further investigation on this front is required. Third, the anti-tumor effect of the LDC was observed only in mouse ectopic xenograft models. One more, the time point of inject LDC in vivo may influence the drug potency. For my further research, I need to try more schedules with varies start timing and cutoff timing. As the final aim is clinical application, animal administration is not enough to reach this goal, my compound must undergo the preclinical and clinical studies.

2-6. Conclusion

I demonstrated that the BPL and rBC2LCN lectin both can recognize the glycans on surface of the gastric cancer cells especially in the SRC. The follow-up experiments indicated the BPL lectin has limitations that couldn't apply in every human being. But it still has the potential for diagnosing. Conclusively, taking the advantage of the rBC2LCN's different affinities to gastric cancer cell lines may make it not just a promising usage of using it as a detecting probe for SRC cancer but also an effective drug carrier. Taken together, no hemagglutination and ability in the cancer cell differentiation articulate the brilliant biomedical future of rBC2LCN.

Chapter 3: Summary and plan of future

This research was focused on signet ring cell gastric cancer, I tried to identify the lectins which can recognize the surface glycans of SRC, not only the cell lines but also the clinical specimens. In this study, I investigated SRC-specific glycans using lectin microarray and evaluated the potential applicability of a glycan-targeting therapy. Among the 96 lectins tested, 11 high-affinity and 8 low-affinity lectins were identified for SRC. Glycan binding motifs varied in the high-affinity lectins, but 5 (62.5%) low-affinity lectins bound the same glycan structure, α2-6 linked sialic acids. The ratio of signal intensity in SRC to NSRC (SRC/NSRC) was highest in the rBC2LCN lectin (1.930-fold), followed by the BPL lectin (1.786-fold). BPL lectin showed good affinity toward SRC cell lines compared to NSRC cell lines. rBC2LCN lectin showed high affinity for both SRC cell lines and one of the three NSRC cell lines (NCI-N87). Neither BPL nor rBC2LCN showed statistical difference in clinical samples. Given the complexity of clinical samples, the result was acceptable. The therapeutic effects of the LDC, BPL-saporin and rBC2LCN-PE38 (rBC2LCN, and Pseudomonas exotoxin A), both showed cytocidal effects in vitro. Due to the ability of BPL to cause aggregation in human blood, it's not safe to administrate in vivo. Whereas rBC2LCN is safe and rBC2LCN-PE38 could assist tumor regression in in vivo mouse xenograft models.

To validate the claims in this study, I need to do more experiments to complete it and try to eliminate the drawbacks maximumly. The most important thing is increasing number of samples, including more cell lines and cohorts. It also needed to verify the

cytotoxicity effect and therapeutic efficacy in other species which are more resemble human before clinical application. Moreover, I should try not only PE-38, but also other toxins to conjugate with rBC2LCN. To date, I have not explored why these aberrant glycosalytions happened, but I assume the genes must have changed. In this point, further research is essential.

Figure and tables

Table 1: Differential glycan analysis between SRC and NSRC cell line array of 96 lectins that showed significant difference

		Mean signal intensity			
Lectins	Complement sugar chain	SRC	NSRC	SRC/NSRC ratio	P value
Increased signals					
BPL	Galβ1-3GlcNAc (GalNAc)	174.6 ± 3.0	97.8 ± 23.0	1.786	<0.001
rDiscoidin II	LacNAc, Galβ1-3GalNAc(T), GalNAc (Tn)	53.4 ± 5.1	39.9 ± 3.8	1.340	<0.001
rPALa	Man5, biantenna	168.4 ± 9.3	139.7 ± 11.9	1.206	<0.001
GSL-I A4	a GalNAc (A, Tn)	41.4 ± 7.7	26.2 ± 5.8	1.578	<0.001
SBA	a, βGalNAc (A, Tn, LacDiNAc)	37.6 ± 6.3	25.4 ± 5.1	1.482	0.001
ECA	βGal	88.7 ± 7.9	72.9 ± 7.3	1.216	0.002
rSRL	Core1,3,agalacto N-glycan	197.2 ± 9.5	173.0 ± 13.5	1.140	0.002
DSA	GlcNacβ1-6Man (Tetraantenna)	276.9 ± 64.6	181.1 ± 41.5	1.529	0.004
rBC2LCN	Fuc a1-2Galβ 1-3GlcNAc (GalNAc)	66.5 ± 22.8	34.5 ± 15.9	1.930	0.007
CCA	Galactosylated N-glycans up to trian	110.4 ± 17.9	75.6 ± 22.5	1.462	0.007
rBC2LA	a Man, High-man	267.2 ± 13.5	238.1 ± 19.5	1.122	0.008
Decreased signal					
TJAI	a 2-6Sia	59.1 ± 6.5	103.6 ± 11.7	0.570	<0.001
SNA	a 2-6Sia	33.1 ± 7.1	79.7 ± 12.6	0.415	<0.001
SSA	a 2-6Sia	43.5 ± 9.6	101.4 ± 20.3	0.429	<0.001
rACG	a 2-3Sia	57.6 ± 3.3	74.7 ± 10.1	0.771	<0.001
rPSL1a	a 2-6Sia	75.4 ± 6.0	101.0 ± 16.4	0.746	0.001
PVL	Sia, GlcNAc	105.1 ± 7.5	129.5 ± 15.4	0.811	0.001
rLSLN	LacNAc, polylactosamine	200.3 ± 21.4	257.1 ± 33.0	0.779	0.003
STL	Polylactosamine, (GlcNAc)n	167.8 ± 50.1	353.7 ± 125.6	0.474	0.005

Figure 1.

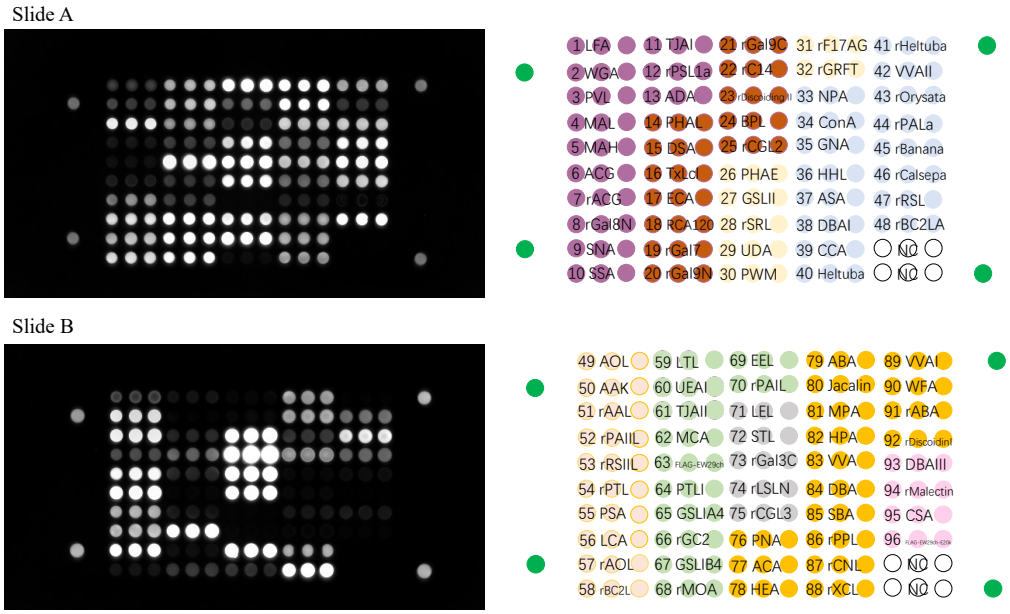


Figure 1. The location of 96 lectins in lectin micro-array. 48 lectins located at slide A, and 48 lectins located at slide B.

Figure 2.

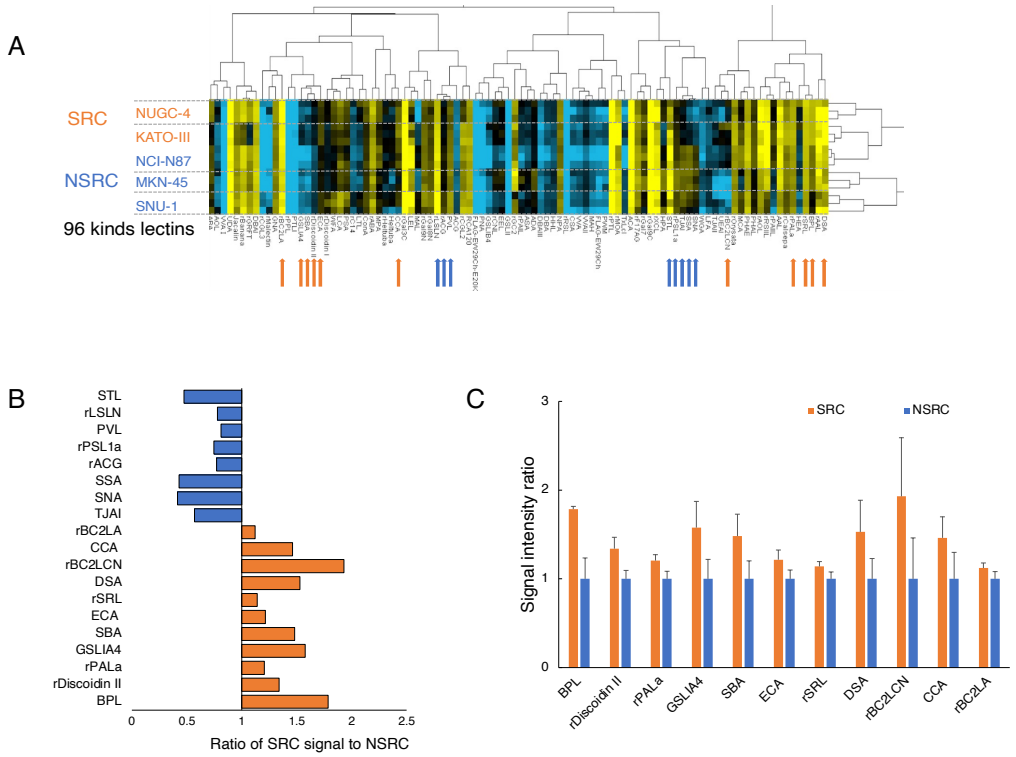


Figure 1. Lectin microarray analysis of GC cell lines. A. 96 lectins were analyzed, specific lectins showed different affinities to the glycan on the surface of SRC or NSRC cells (yellow: high; black: intermediate; blue: low). B. 19 lectins were significantly different ($p<0.05$) to 2 groups, 8 lectins (blue) had good affinity to NSRC cells, 11 lectins (orange) showed better binding to SRC cells. C. Compared 11 lectins which have better affinities to SRC cells than NSRC cells, rBC2LCN showed the biggest difference, followed by BPL lectin. (*:$p<0.05$; **:$p<0.01$).

Figure 3.

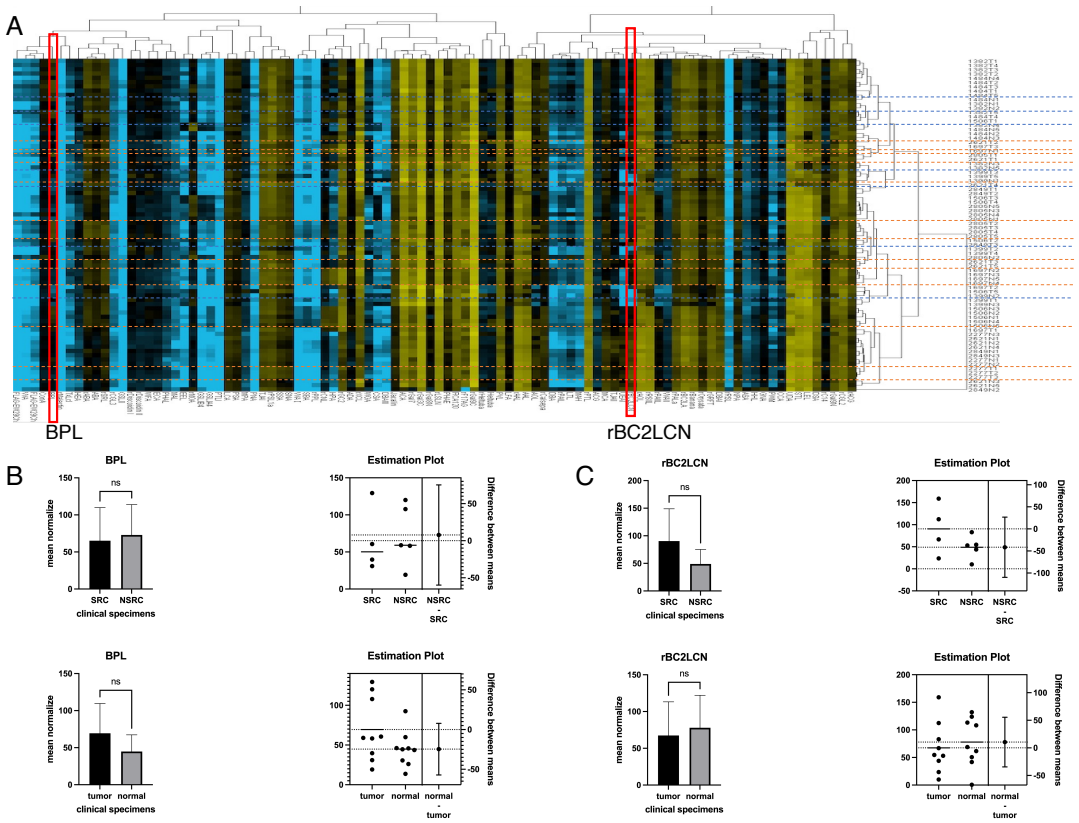


Figure 3. Lectin microarray analysis of 9 pairs GC clinical samples. A. The affinity of BPL and rBC2LCN lectin was confirmed (yellow: high; black: intermediate; blue: low). B. BPL didn't show statistical difference between SRC and NSRC, or between tumor and normal. C. rBC2LCN didn't show statistical difference between SRC and NSRC, or between tumor and normal. (ns: $p>0.05$)

Figure 4.

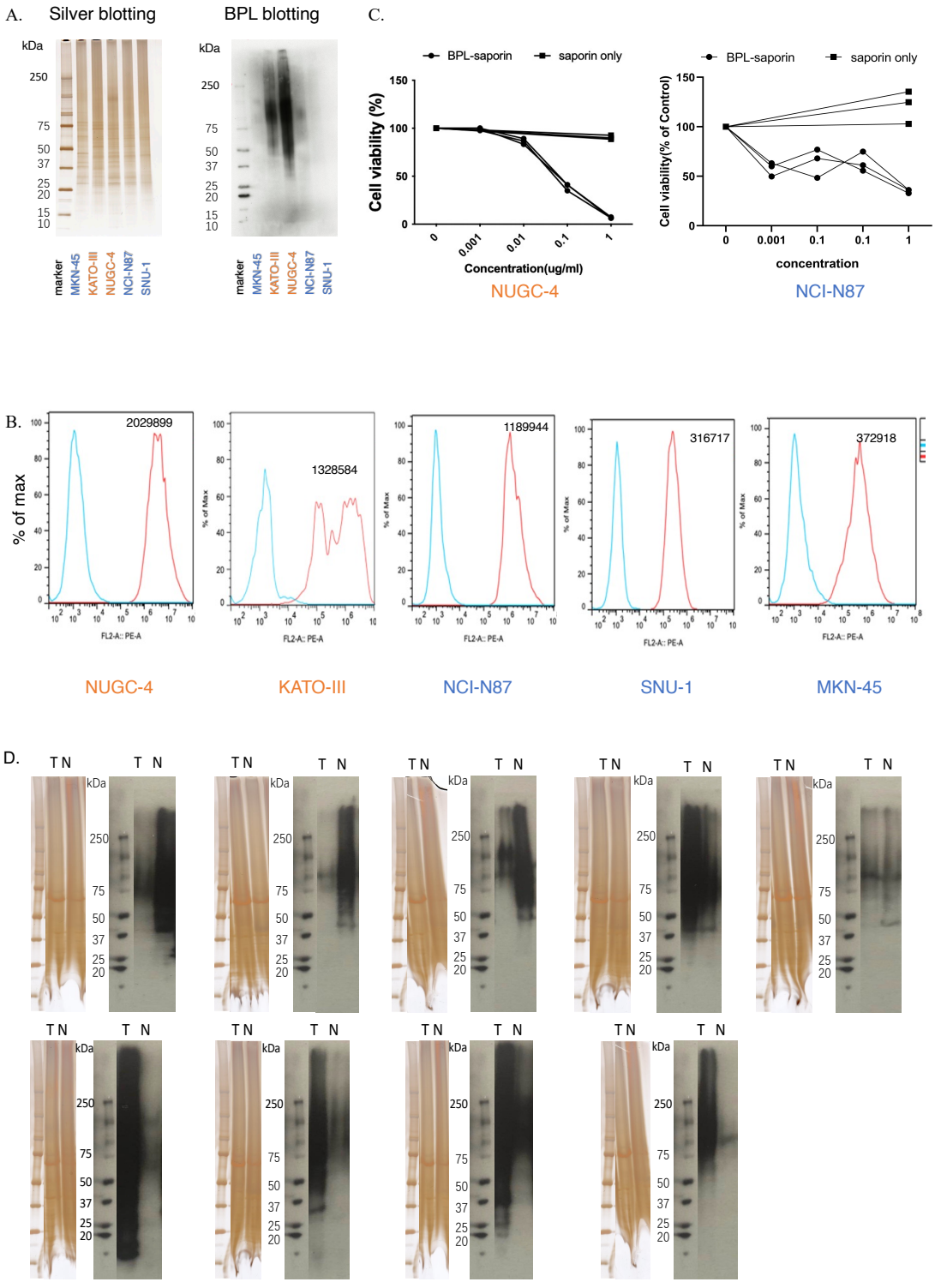
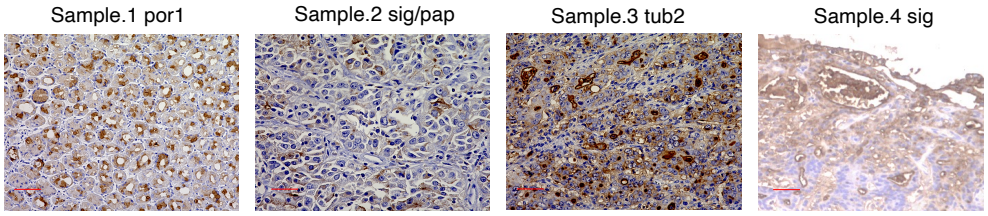


Figure 4. A. Sliver blotting and lectin blotting with GC cell lysates. Left: silver blotting demonstrated the presence of proteins of various molecular weights. Right: BPL

blotting. The staining result showed KATO-III and NUGC-4 have better affinity to BPL, both 2 cell lines are SRC cells. B. Flow cytometry analysis using PE labeled BPL. All cells were positive. C. Cytotoxicity of BPL-saporin conjugate in vitro was evaluated using CCK-8 assay kit. The curves were accepted goodness of fit. D. Silver blotting and BPL blotting with 9 clinical specimens.

Figure 5.

A.



B.

Histology	Negative (-)	Weakly positive (+)	Positive (++)	Strongly positive (+++)	Total
Tub 1	1	8	2	0	11
Tub 2	0	9	6	2	17
Por 1	0	2	1	0	3
Por 2	2	7	9	5	23
Sig	0	3	3	0	6

C.

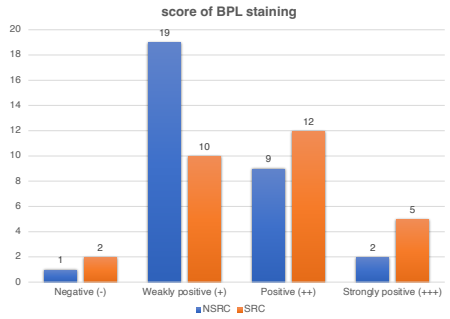


Figure 5. A. BPL staining showed all positive to the 4 cases. Scale bar: 50μm. B. 60 cases were stained with BPL, Tub1, Tub2 and Por1 were regarded as NSRC. Por2 and Sig were regarded as SRC. C. Scoring of BPL staining.

Figure 6.

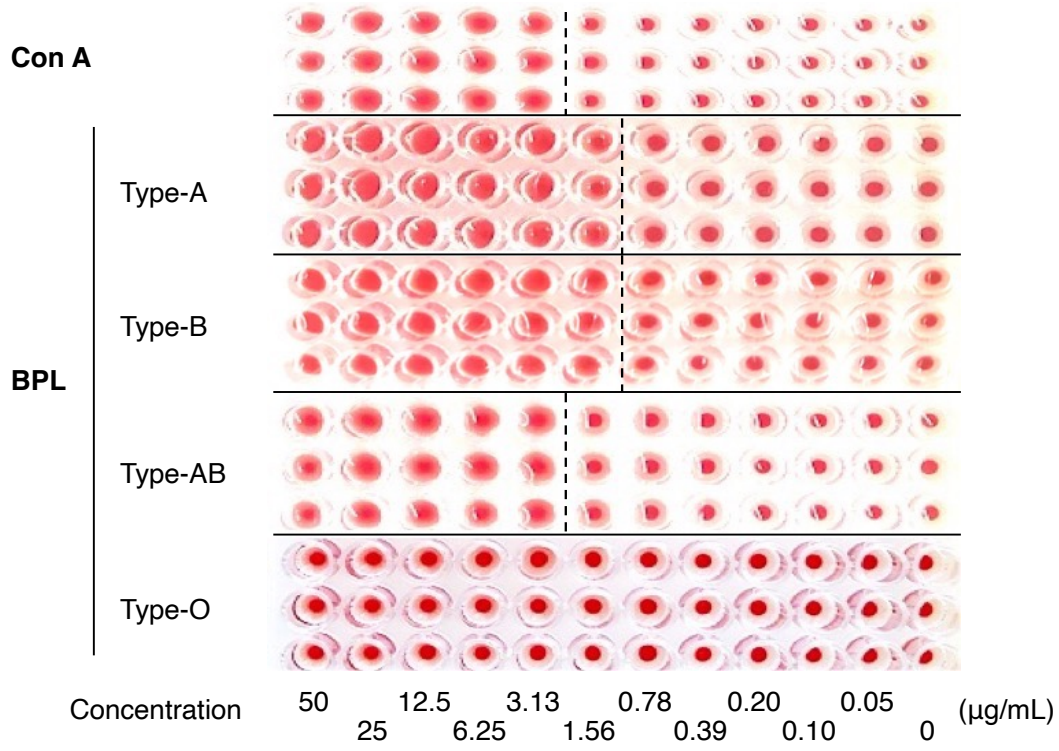


Figure 6. Hemagglutination assay. The BPL induces erythrocyte aggregation from a human volunteer of blood type-A, type-B, and type-AB. Only blood type-O did not show hemagglutination even in high concentrations (50 μg/mL). A positive control with concanavalin A (Con A) lectin demonstrated aggregation at 3.13 μg/mL. Each test contained n=3 technical replicate.

Figure 7.

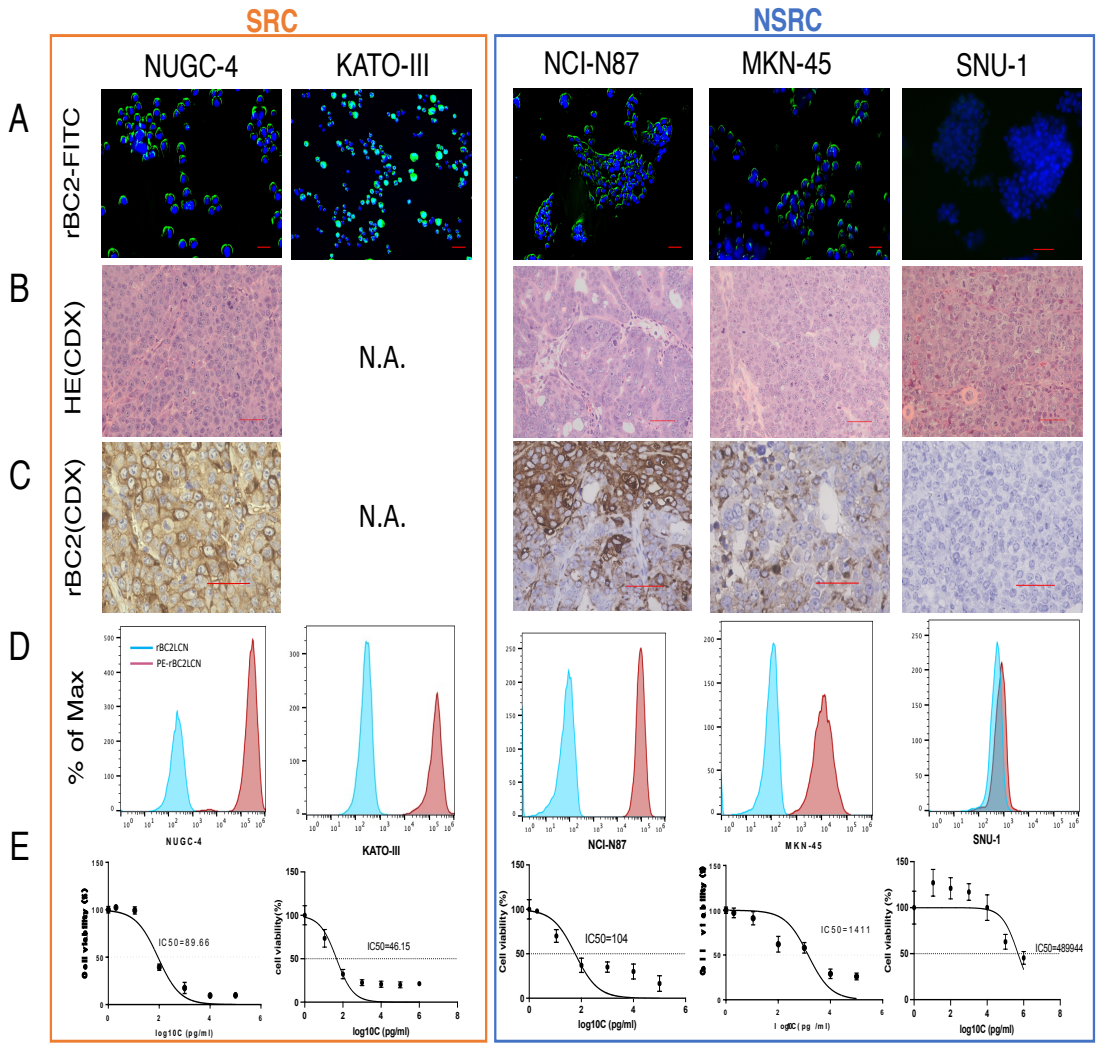


Figure 7. Lectin affinity confirming for rBC2LCN. A. Microscope images of live cell staining using FITC-rBC2LCN 1ug/ml, scale bar 50um. B. HE staining of cell derived subcutaneous xenografts, (NUGC-4, signet ring cell, poorly differentiated; KATO-III, signet ring cell, poorly differentiated/NA; NCI-N87, well differentiated; MKN-45, poorly differentiated; SNU-1, poorly differentiated), scale bar 50um. C. rBC2LCN lectin staining of cell derived xenografts, NUGC-4/strongly positive, KATO-III/NA, NCI-N87/positive, MKN-45/weakly positive, SNU-1/negative. scale bar 50um. D.

Flowcytometry analysis of live cells, blue represents cells labeled with rBC2LCN, red represents cells labeled with PE-rBC2LCN, 1ug/ml respectively, 2 experiments have been overlaid. E. Cytotoxicity of rBC2LCN-PE38 conjugate in vitro was evaluated using CCK-8 assay kit. The curves were accepted goodness of fit.

Figure 8.

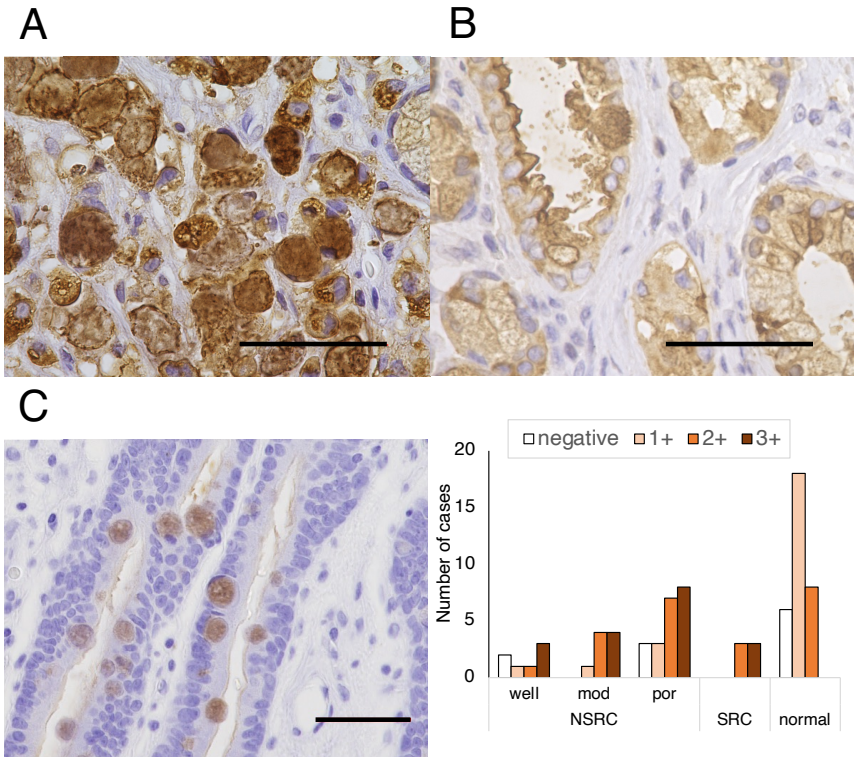


Figure 8. Evaluation of rBC2LCN affinity towards clinical samples of gastric cancers by immunohistochemistry (rBC2LCN-HRP staining). A. Signet-ring cell cancer; B. differentiated adenocarcinoma; C. Immunohistochemical staining results among each histological type. − : negative; + : slightly positive; 2 + : positive; 3 + : highly positive. Scale bar 50 μm.

Figure 9.

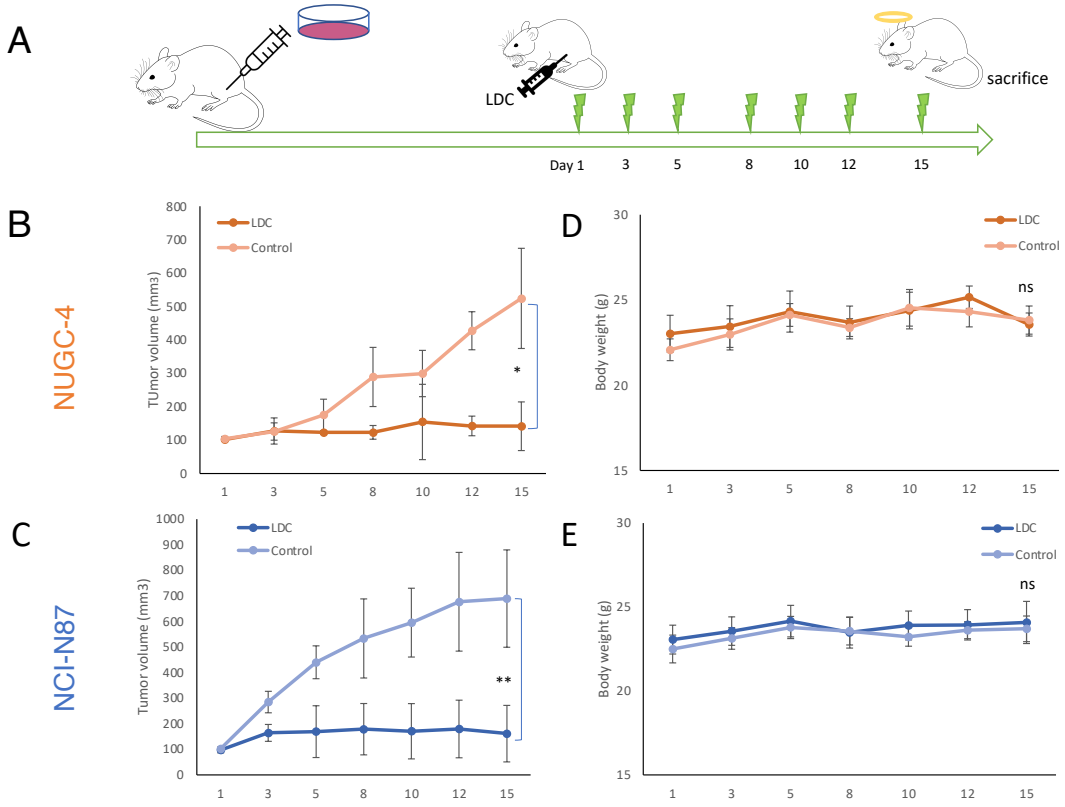


Figure 9. Therapeutic efficacy evaluation of rBC2LCN-PE38 conjugate in vivo. A. Schematic of treatment, subcutaneous xenografts were allowed to grow to close to 100 mm^3 or 14 days as the initiation of LDC treatment. Local injection was administered 6 times, on day 15, all the mice were sacrificed. N=5 each. B-E. Changes of tumor volume and body weights during the treatment period. Size were calculated as $0.5\times width^2\times$ length. Weights were measured after excising. *:$p<0.05$; **:$p<0.01$; ***:$p<0.001$; ns: not significant.

Supplementary

Figure S1.

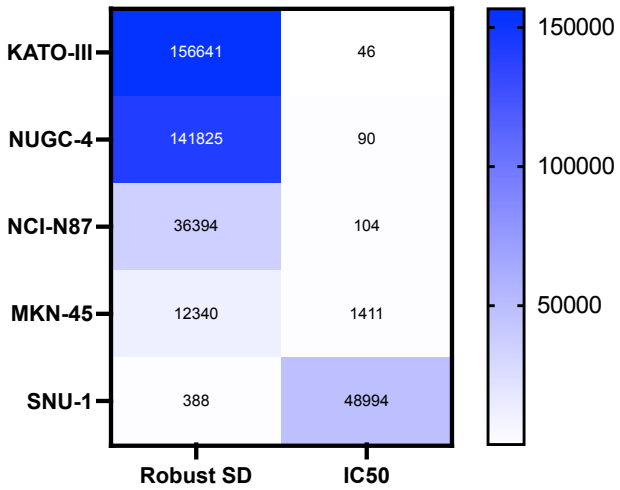


Figure S1. rBC2LCN. The robust SD of flow cytometry and IC50 is interrelated. Cell lines with low robust SD have higher IC50. With the increase of robust SD, the IC50 decreases gradually.

Figure S2.

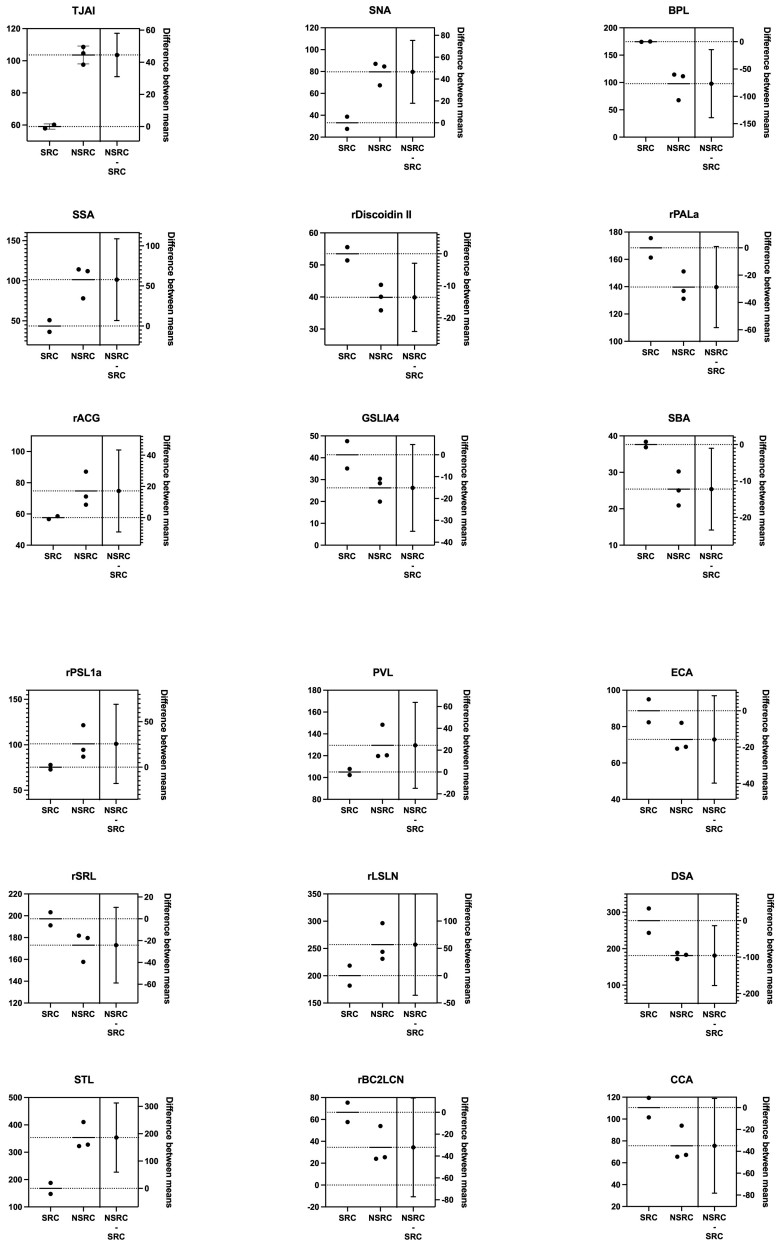


Figure S2. Estimation plots of top 18 p-valued lectins selected by lectin microarray from cell lysate. The left axis is scaled to show the data. The right axis is the effect size, precision of the calculated effect size as a 95% confidence interval.

Figure S3.

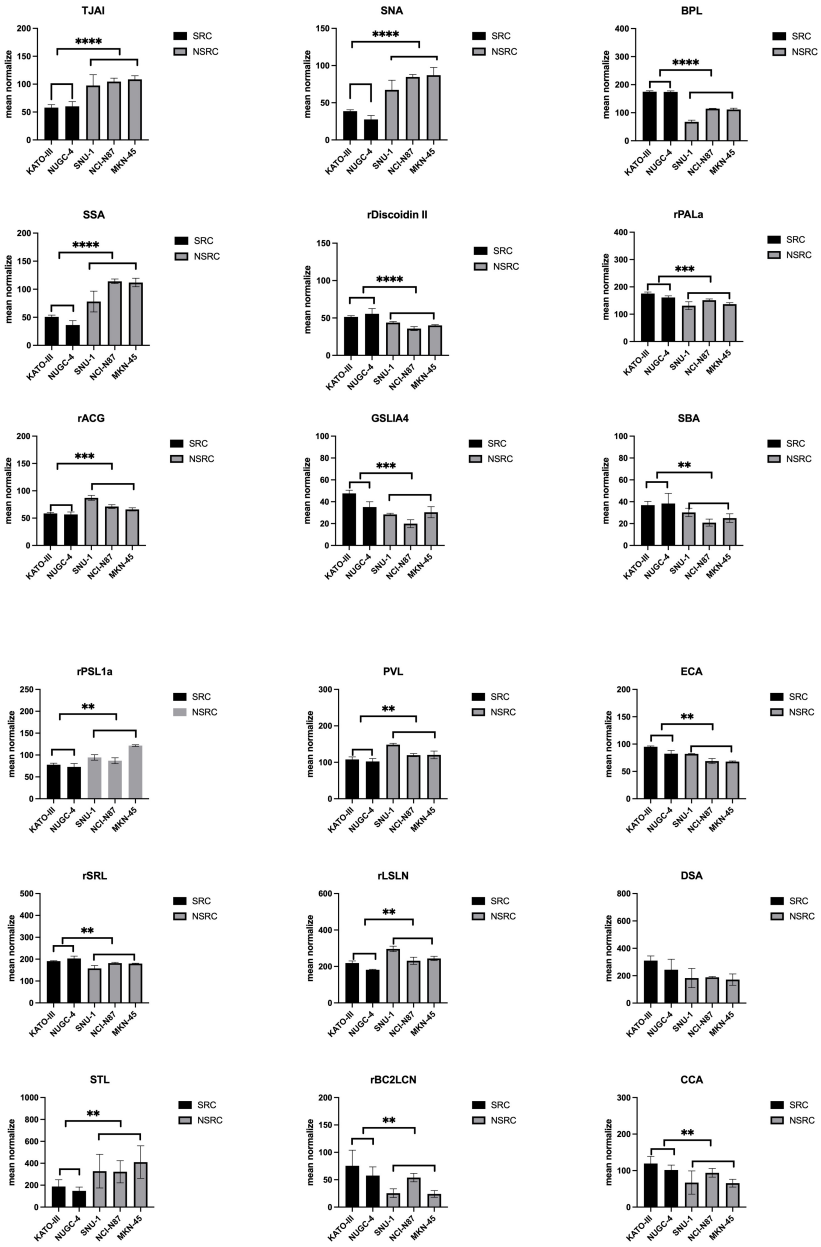


Figure S3. Top 18 p-valued lectins selected by lectin microarray expressed different affinities to each cell lines. *: p<0.05; **: p<0.01; ***:p<0.001; ****:p<0.0001.

Figure S4.

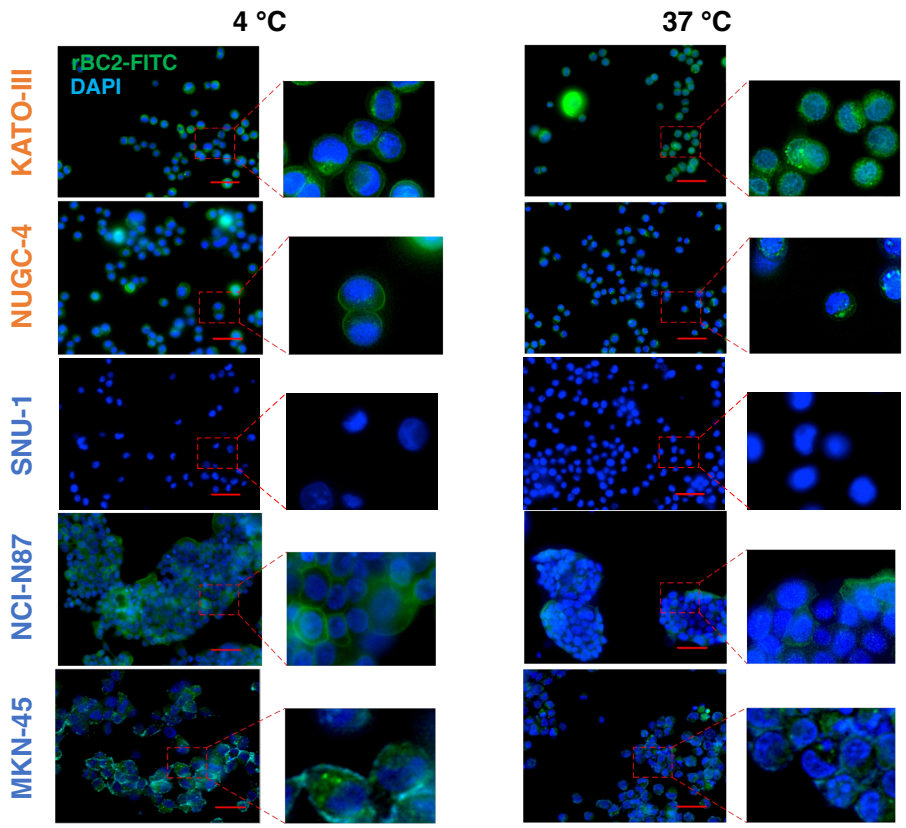


Figure S4. rBC2LCN-FITC binds to surface membrane of cancer cells in the condition of endocytosis inhibition (4°C, left column), and is internalized by endocytosis at 37°C temperature (right column).

Figure S5.

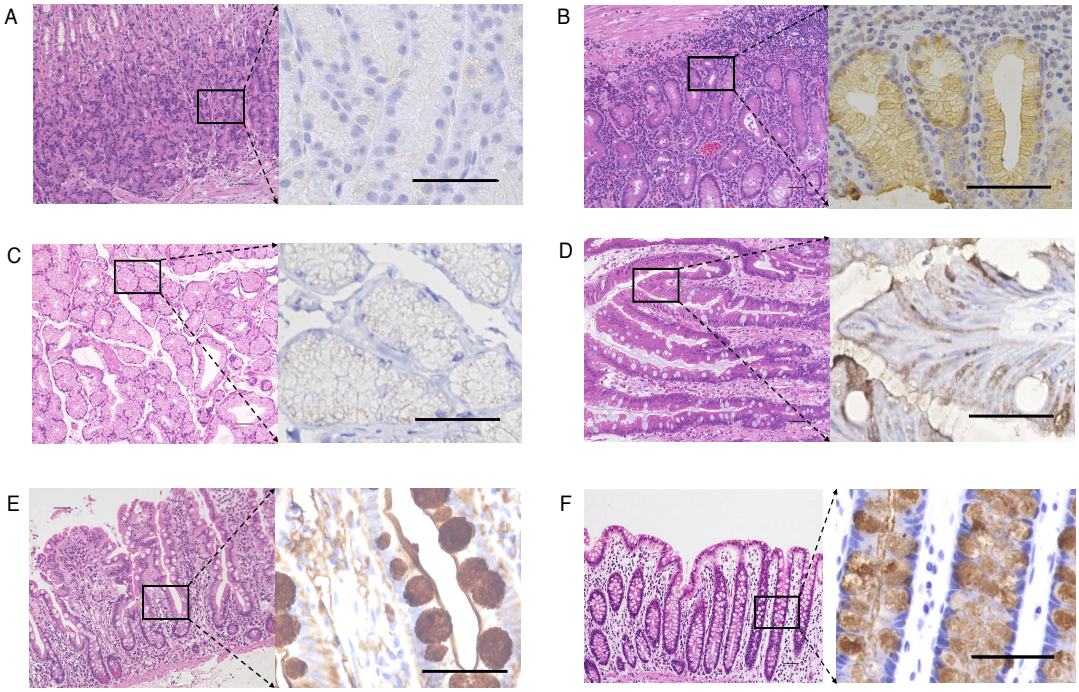


Figure S5. Lectin staining of rBC2LCN in clinical human normal tissue samples. Left panels show low magnification hematoxylin-eosin (HE) staining and right panels show high magnification lectin staining. A. Gastric fundic mucosa, B. Spasmolytic polypeptide-expressing metaplasia (SPEM), C. Gastric pyloric mucosa, D. Intestinal metaplasia, E. Small intestine, F. Large intestine. Scale bar 50 μm.

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