*Original Research*

# Low reactivity of tumor MUC1-binding natural anti-α-galactoside antibody is a risk factor for breast cancer

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## Impact statement

This paper offers a molecular explanation for the positive correlation of individuals' lipoprotein(a) [Lp(a)] size with breast cancer incidence, found more pronounced using interference-free assays. It established unambiguously the marked affinity of human anti-Gal antibody towards cancer phenotype of the cell surface MUC1 and inertness towards normal cell MUC1. This selectivity enabled small Lp(a) molecules, known to produce higher specific reactivity anti-Gal by affinity maturation, to achieve more efficient immune defense so that women with specific reactivity lower than the mean value of normal subjects ran cancer risk with odds ratio (OR) above 3.2. However, increasing O-glycosylation and decreasing O-glycan length of MUC1 with tumor advance increased anti-Gal specific reactivity, indicating antigenic stimulation and/or affinity maturation of the antibody by tumor MUC1. Thus, pre-cancer anti-Gal specific reactivity should be lower than that measured on detection and the above OR actually higher. Results suggest small Lp(a) and high specific reactivity anti-Gal infusions as therapeutic options.

## Abstract

Natural plasma anti-α-galactoside antibody (anti-Gal) reactivity was reported to vary inversely with the individual's lipoprotein(a) [Lp(a)] size. Since MUC1 mucin overexpressed in tumors bear surrogate peptide ligands for anti-Gal, we examined if high anti-Gal reactivity in small size/high titer Lp(a) individuals correlated with lower incidence of breast cancer. Newer protocol for size determination revealed that Lp(a) in controls were significantly smaller than in breast cancer patients ($P = 0.0023$; $n = 46$ in either group). Activity per unit plasma volume and specific reactivity (reactivity per unit immunoglobulin) of anti-Gal were significantly lower in cancer patients ($P = 0.0033$). Specific reactivity lower than the mean of controls was a risk factor for breast cancer with odds ratio (OR) 3.2 (95% confidence interval [CI]: 1.368–7.557). Immunochemical staining using fluorescein isothiocyanate-labeled anti-Gal revealed absolute inactivity towards normal cells and strong recognition of cancer cells by the antibody. O-Glycosylation of MUC1, though more frequent than in normal cells, was incomplete in tumor cells as revealed by binding of the O-glycan-specific lectin jacalin, accounting for the access of anti-Gal to its peptide ligand in cancer MUC1. As tumor advanced and MUC1 with increasing affinity for anti-Gal was synthesized by the tumor, the specific reactivity of circulating anti-Gal also increased, apparently due to antigenic stimulation or affinity maturation by the proliferating MUC1, indicating that pre-cancer anti-Gal reactivity in patients should have been much lower than measured after detection of cancer and that lower reactivity of the antibody is a stronger risk factor for breast cancer than indicated by the OR above. Reactivity towards a given group of tumor MUC1 antigens increased in proportion to anti-Gal specific reactivity. Results suggested tumor-specific MUC1 as likely target for anti-Gal-mediated anti-cancer defense and offer infusion of small Lp(a) or high reactivity anti-Gal as possible immunopotentiation measures.

**Keywords:** Breast cancer, anti-α-galactoside antibody, lipoprotein(a), tumor-associated MUC1, affinity maturation, serine- and threonine-rich peptide sequence

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## Introduction

Immunotherapy is presently the fastest advancing therapeutic option in cancer and is predicted to constitute a major part of anti-cancer therapy in a few years.[1] While monoclonal antibodies against cancer-specific antigens are the most widely used biomolecules for anti-cancer therapy, the potential of naturally occurring host antibodies that recognize cancer-specific antigens in anti-cancer

defense is also increasingly being investigated.[2] MUC1, a cell membrane mucin family protein, is conspicuous by the changes in structure and function it undergoes during malignancy.[3] Expressed in epithelial cells such as in breast and lungs, MUC1 possesses a heavily O-glycosylated extracellular region and protects the epithelia by providing a charged and non-adhesive surface.[4] High serine and threonine content (~40%) due to dense distribution of these amino acids in the variable number tandem repeats of MUC1 accounts for its heavy O-glycosylation which increases remarkably during malignancy and contributes to 50–90% of its molecular weight which varies between 250 kDa and 500 kDa.[5] Malignancy shifts O-glycosylation, which is dominantly of core-2 type in normal MUC1, heavily towards core-1 type and releases more MUC1 to serum.[3] Tumor MUC1 destabilizes trans-membrane signaling by combining with mediators such as NF-kB.[6] Circulating naturally occurring anti-α-galactoside antibody (anti-Gal), an evolutionary milestone expressed only in primates has been reported to be involved in rejecting non-primate xenografts by recognizing the Gal-α(1–3) Gal-epitope in the latter.[7] It also recognizes the serine-and threonine-rich peptide sequences (STPS) such as in MUC1 by accommodating these sequences as surrogate ligands at the sugar-binding sites.[8]

We had reported that anti-Gal forms circulating immune complexes with endogenous lipoprotein(a) [Lp(a)] by recognizing the STPS in the heavily O-glycosylated region of the latter as surrogate ligand analogous to MUC1 peptide sequences.[9] High serum concentrations of Lp(a) had been reported to be associated with lower incidence of cancer,[10] though reasons were not investigated. High serum Lp(a) concentration which is generally associated with low molecular weight Lp(a) phenotype could only be a consequence of the latter since smaller Lp(a) molecules are likely to be synthesized in larger numbers. According to a more recent report, low molecular weight Lp(a) phenotype gives rise to anti-Gal molecules with relatively greater specific reactivities.[11] Since more reactive anti-Gal could be expected to bind faster to MUC1-bearing cancer cells during antibody-mediated anti-tumor defense, the above phenomenon offered a possible route by which small Lp (a) phenotype could bring about better anti-tumor defense, since Lp(a) *per se* could not be conceived to take part in this defense. It was pertinent therefore to examine the factors that modulate the serum concentration as well as reactivity of this antibody towards normal and tumor-derived MUC1 and thereby the susceptibility of individuals to cancer. In a randomized control study, the present work investigated whether the higher specific activity of anti-Gal brought about by smaller size Lp(a) phenotype offered protection from breast cancer in women. It also examined the differential affinities of normal and tumor-derived MUC1 for anti-Gal and changes in the affinity of MUC1 for anti-Gal and in the specific reactivity of anti-Gal, with tumor progression.

## Materials and methods

### Materials

Methyl α-D-galactopyranoside, galactose, melibiose, soybean trypsin inhibitor, soluble guar gum, Tween-20, horse radish peroxidase (HRP) type II, sulpho-NHS-biotin, avidin-HRP, orthophenylene diamine (OPD), Tris, phenyl methyl sulfonyl fluoride (PMSF), dimethyl sulfoxide, Lectin GS-IB4 from *Griffonia simplicifolia*, fluorescein isothiocyanate (FITC), Triton X-100, poly-L-lysine were purchased from Sigma-Aldrich (India), Bangalore. Glycine, boric acid, ammonium persulfate, disodium salt of ethylene diamine tetra acetic acid (EDTA) and hydrogen peroxide were purchased from Merck, India. Sepharose 6B was from Pharmacia Fine Chemicals, Uppsala, Sweden. Polystyrene 96-well flat bottom microplates (MAXISORB) were purchased from Nunc, Denmark. Antibodies to human IgA, IgG, and IgM (raised in goat) and to apo(a) and apoB (raised in rabbit) were purchased from Dako, Denmark. Lp(a) assay standard (99 mg/dL; International Reference SRM 2B Standardization) was obtained from APTEC Diagnostics nv, Belgium). Anti-MUC1 antibody (sc-6825, goat polyclonal IgG) and rabbit anti-goat IgG-HRP (sc-2768) were purchased from Santa Cruz Biotechnology, USA. BCA protein assay kit was purchased from Thermo Scientific, USA. All other chemicals used were of analytical grade. The seeds of *Artocarpus integrifolia* (jack fruit) were obtained locally.

### Collection of samples

The present study was conducted in the Department of Biochemistry, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), in collaboration with the Department of Surgical Oncology, Regional Cancer Centre, Trivandrum, Kerala, India, with prior approval by the Institutional Ethics Committee of the latter institute (RCC/HEC No. 08/2016). Written informed consent was obtained from all the participants from whom blood and tissue samples were collected. A total of 46 histologically confirmed cases of breast cancer and 46 normal subjects as controls were enrolled. The breast cancer patients were selected according to the following selection criteria: (i) cases of breast cancer patients, confirmed histologically after fine needle aspiration cytology (FNAC), who underwent surgery in the Department of Surgical Oncology, Regional Cancer Centre, Thiruvananthapuram, Kerala, India; (ii) no history or documentary evidence of hepatic and renal dysfunction; (iii) age <70 yrs; (iv) no history of neoadjuvant chemotherapy. Controls were selected from healthy volunteers outside the two institutions where the study was undertaken. The selection criteria for controls were as follows: (i) age- and sex-matched to the cases; (ii) no history of malignancy or cancer treatment; (iii) no history of any chronic disease such as tuberculosis; (iv) normal hepatic and renal functions. Further clinicopathological details of patients and controls are provided in Table 1.

**Table 1.** Clinicopathological characteristics of subjects.

| Characteristics | Controls (n=46) | Patients (n=46) |
|---|---|---|
| Age (mean) | 48 | 51 |
| Sex | Female | Female |
| Hypertension | 2/46 | 12/46 |
| Diabetes mellitus | 4/46 | 10/46 |
| Hypercholesterolemia | 4/46 | 5/46 |
| Menopausal status | Pre, peri, and post | Pre, peri, and post |
| Histological grade | | II (10/46) |
| | | III (36/46) |
| Metastases | | Absent |

Patients selected in this study did not receive any pre-operative treatment such as radiation or chemotherapy. Blood samples were collected from patients and controls by trained personnel. Ten milliliter of blood was collected from each subject and was transferred to appropriate vacutainer tubes and serum was isolated. Serum samples were aliquoted and stored at −20°C and parameters were analyzed within one month of sample collection. A total of 24 breast tumor samples (infiltrating ductal carcinoma) were collected from patients treated with surgical resection. Histologically normal breast tissue samples adjacent to the tumor tissues were obtained and they were included as controls. Tissue samples were collected immediately after surgical removal of the tumor. A piece of tissue was fixed in 10% neutral buffered formalin for immunofluorescence analysis, while another tissue sample was rinsed with fresh 20 mM phosphate-buffered saline (PBS), pH 7.4, stored at −20°C and subsequently processed for the isolation of MUC1 glycoprotein.

### Proteins and conjugates

The affinity chromatography matrix, cross-linked guar galactomannan (CLGG), was prepared by cross-linking soluble guar galactomannan (from *Cyamopsis tetragonolobus* beans) using epichlorohydrin to form an insoluble gel by a procedure described earlier.[12] The lectin, jacalin was prepared from the seeds of *A. integrifolia* (jack fruit) by affinity chromatography on CLGG.[13] Antibodies against apo(a), apoB, human IgA, IgG, IgM were labeled with HRP as described earlier.[14] Melibiose was covalently coupled to the non-glycosylated protein soybean trypsin inhibitor by reductive amination using sodium cyanoborohydride[15] to obtain the neoglycoconjugate trypsin inhibitor-melibiose (TIM). *G. simplicifolia* IB4 (GS-IB4) lectin was labeled with biotin as described earlier.[16] Soluble proteins were assayed by Bradford method using bovine serum albumin as standard.[17] Out-dated plasma of healthy volunteers (18 to 40 yrs) was collected from the Division of Blood Transfusion Services of SCT Institute for Medical Sciences and Technology, based on institutional ethics committee approval (No. SCT/IEC/926). Affinity-purified anti-Gal (APAG) from healthy volunteers or from patients' or control sera was isolated by chromatography on CLGG gel by a procedure reported earlier.[9] Electrophoretically purified anti-Gal from APAG was prepared by a procedure reported earlier[18] and labeled with FITC as described by Hudson and Hay.[19] Fluorescence was measured using excitation at 485 nm and emission at 525 nm in a BIOTEK FLx800 fluorescence reader employing a sensitivity level of 35 throughout.

### Lp(a) determination by jacalin-based enzyme immunoassay

Lp(a) in serum samples was determined by an apoB-independent jacalin-based enzyme immunoassay.[20] Briefly, polystyrene wells coated with jacalin (1 μg in 200 μL PBS) by incubating at 37°C for 3 h were washed with PBS containing 0.05% Tween-20 (PBST) and blocked with PBS containing 0.5% Tween-20 at 37°C for 30 min. After washing again with PBST, wells were incubated with 500 times diluted serum in 200 μL PBST for 2 h at 4°C. Washed wells were probed with 200 μL HRP-conjugated anti-human apo(a) (1.5 μg per mL) in PBST. After washing, the bound HRP was assayed by treating wells with 200 μL OPD (0.5 mg/mL) in 0.1 M citrate phosphate buffer, pH 5.0 containing 0.03% $H_2O_2$ for 15 min, followed by stopping the reaction by the addition of 50 μL of 12.5% $H_2SO_4$ and absorbance was read at 490 nm in ELISA reader (BioTek, USA).

### Isolation of Lp(a) from human serum

Lp(a) was prepared by affinity precipitation of serum proteins with the lectin jacalin followed by electrophoresis and elution of lipoprotein bands as described earlier.[21] Briefly, proteins precipitated from serum by jacalin were re-dissolved using lectin-specific sugar and subjected to ultracentrifugation whereby natural form of Lp(a) with adduct LDL bound to it (L1) sequestered in the top 20% volume. Pure Lp(a) and LDL were separated by electrophoresis of L1 in Tris-Borate-EDTA buffer at pH 8.7 and recovered by passive elution to PBS.

### Molecular size index of Lp(a) isoforms

While apo(a) size is the only variable factor in Lp(a) size, this subunit has been found to mask the apoB subunit to increasing extent as the size of apo(a) increases so that ratio of responses of microplate-coated Lp(a) to anti-apo(a) and anti-apoB antibodies was an index of apo(a) size.[21] Appropriate dilution of eluted Lp(a) isoforms from different individuals were directly coated on microtiter plate wells. After blocking and washing, wells were separately probed with HRP-labeled anti-human apo(a) and anti-human apoB (1.5 μg per mL of each antibody) and the bound HRP activity was assayed as described for Lp(a) assay above. The ratio of absorbance for anti-apo(a) (a) to that for anti-apoB (B) at 490 nm ([a]/[B]) was taken as an index of molecular size of Lp(a).

### Serum anti-Gal assay

Polystyrene wells were coated with melibiose-conjugated soybean trypsin inhibitor (TIM, 1 μg/well) and after blocking as described above, 50 times diluted serum in PBST was added and wells incubated for 2 h at 4°C. After washing the wells with PBST, bound anti-Gal was assayed by incubation with a mixture of HRP conjugates of anti-human IgG, IgA, and IgM (1.5 μg per mL PBST for each antibody) for 2 h at 4°C. The bound HRP was assayed using OPD as substrate as described above. Unmodified soybean trypsin inhibitor served as control for TIM.

### Determination of specific reactivity of anti-Gal antibody

Specific reactivity of anti-Gal was defined as the ratio of ligand-binding activity to immunoglobulin content of the same amount of antibody.[22] These parameters were measured by ELISA. ELISA response of binding of anti-Gal from a given sample to polystyrene well-coated TIM was measured as reactivity of anti-Gal and is a contribution of both the specific reactivity and the total amount of anti-Gal present. Polystyrene wells were coated with TIM (1 μg/well) and after blocking, wells were incubated for 2 h with anti-Gal samples (50 ng in 200 μL PBST). Following washing, bound antibody was treated with a mixture of HRP conjugates of anti-human IgG, IgM, and IgA (1.5 μg per mL of each antibody). HRP bound to the well was assayed by using OPD as substrate. To assay immunoglobulin content, anti-Gal (50 ng) directly coated on microwells were probed with the mixture of HRP conjugates of anti-human IgG, IgM, and IgA and bound HRP activity assayed. The ratio of responses (OD at 490 nm) in the above two ELISA protocols was taken as specific reactivity of the antibody.

### Extraction of MUC1 glycoprotein

Extraction of mucin was carried out by a modification of the procedure reported by Paszkiewicz-Gadek et al.[23] All steps were carried out at 4°C. Preliminary homogenate (10% w/v) was prepared in PBS pH 7.4 containing 1 mM EDTA and 1 mM PMSF with the use of a knife homogenizer. Homogenate was mixed with 1% Triton X-100 for mucin extraction at 4°C for 1 h. The extract was centrifuged for 20 min at 16,000 r/min and supernatant was collected. For partial purification of MUC1 glycoprotein, the above supernatant was passed through Sepharose 6B gel filtration column (1.8 × 60.0 cm) which was equilibrated and eluted as 2 mL fractions with PBS. To determine the proteins recognized by jacalin and anti-MUC1 antibody, each fraction was directly coated on microtiter plate wells. Wells blocked and washed as described above were then incubated separately with the specified concentrations of jacalin-HRP and anti-MUC1 antibody (goat polyclonal IgG) at 4°C for 2 h. After washing, wells with bound anti-MUC1 antibody were probed with rabbit anti-goat IgG-HRP. Bound HRP in each well was quantitated as described above. MUC1-positive fractions (which, as expected, were jacalin-reactive as well) were pooled and concentrated by membrane filtration and stored at –20°C. Protein content of MUC1 positive fractions was assayed with the use of Pierce® BCA protein assay kit.

### Measuring the inhibition of anti-Gal/GS-IB4 lectin by tumor-associated MUC1

Inhibition of binding of anti-Gal or GS-IB4 to microplate-coated TIM by MUC1 samples was checked. To polystyrene wells coated with TIM (1 μg/well) anti-Gal or biotinylated GS-IB4 lectin (75 ng in 200 μL PBS-T), pre-incubated at 4°C for 18 h with 2 μg tumor-associated MUC1 (TA-MUC1), was added and wells incubated at 4°C for 2 h. After washing, the plates were incubated in turn for 2 h at 4°C with 200 μL PBST containing, respectively, a mixture of HRP conjugates of anti-human IgG, IgA, and IgM (1.5 μg per mL of each antibody) for bound antibody or with avidin-HRP (60 ng avidin per mL) for bound lectin. Following washing, the bound HRP was estimated using OPD as substrate as described above. Untreated anti-Gal/biotinylated GS-IB4 added to TIM-coated wells served as control.

### Immunohistochemical detection of anti-Gal binding to tumor tissue

Immunofluorescence staining of formalin fixed tissue sections using FITC-labeled anti-Gal was examined. Transverse sections (5 μm) were cut from the paraffin embedded tissue and spread on to poly-L-lysine-coated slides. After deparaffinization in xylene, the slides were rehydrated with graded alcohol and washed with water. Antigen retrieval was done in boiling cooker for 3 min in citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0). Non-specific staining was blocked by incubating the tissue section with 1% human serum albumin (glycoprotein free) in TBST (Tris-Buffered Saline with 0.05% Tween 20) for 2 h. Sections were incubated overnight at 4°C in the dark with FITC-labeled anti-Gal (1 μg/mL) in TBST. Sections were washed with TBST and mounted in glycerol-PBS and viewed under a fluorescence microscope (Olympus BX43) with appropriate filters. Images were captured using QImaging MicroPublisher 5.0 RTV and analyzed using QCapture Pro7.

### Statistical analysis

All results were expressed as mean ± SEM. One-way ANOVA followed by Tukey's multiple comparison test or unpaired Student's t-test was used to determine the significance of difference between the groups where appropriate. Statistical significance of categorical variables was determined by Pearson chi-square test. Binary logistic regression analysis was used to estimate odds ratio (OR) and 95% confidence interval (CI) for group comparisons. A value of $P < 0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism version 5.0. Binary logistic regression analysis was performed using SPSS version 18.0 for Windows (SPSS Inc., Chicago, IL, USA).

## Results

### Smaller Lp(a) phenotype is strongly associated with lower incidence of breast cancer

Lp(a) size which differs among individuals due to genetic factors and determines the activity of the Lp(a)-binding

natural antibody, viz. anti-Gal,[11] could be the primary phenotypic variable that determines an individual's serum concentration of this lipoprotein as well since smaller phenotypes tend to be synthesized more. This antibody also binds to MUC1 antigen which undergoes structural changes and is overexpressed in cancer cells. Therefore, the correlation of Lp(a) size with cancer incidence was examined using a cohort of 46 cancer patients and as many controls. Direct measurement of Lp(a) size as ratio of availability of its apoB subunit relative to that of the apo (a) subunit was also reported recently.[21] The correlation of serum Lp(a) concentration with cancer incidence was also reappraised in the same subjects since reported results on this issue are contradictory. A recent study reported that low serum Lp(a) was a significant risk factor for all cancers, though this trait was protective against lung cancer.[24] In contrast, elevated serum Lp(a) was reported earlier in breast cancer.[25] Although a marginally significant protection from cancer death was reported for individuals with high serum Lp(a) concentration,[10] this study compared cancer incidence in very low Lp(a) concentrations (<8 mg/dL) with that in the rest of the study population. Further, the above trials employed Lp(a) assay methods involving probing with antibody to the apoB chain of Lp (a) and ran the risk of overestimating larger Lp(a) molecules which have been found to contain additional LDL molecules adhering to them in numbers proportionate to their apo(a) size.[26]

Using a method recently developed for determining Lp (a) concentration without the use of anti-apoB antibody[20], we found (Figure 1(a)) that controls had significantly smaller Lp(a) phenotype compared to patients ($P$ value = 0.0023, n = 46 in either group). This relationship was corroborated by the observation (Figure 1(b)) that controls possessed significantly higher serum Lp(a) concentrations than patients ($P$ value = 0.0067, n = 46 in either group) since smaller Lp(a) molecules are generally higher in serum titer.[27] Results show primarily that Lp(a) size is a much better determinant of cancer susceptibility than Lp(a) concentration. Secondly, the much more significant negative correlation of Lp(a) concentration with cancer incidence than in an earlier report ($P$ value = 0.03) could be due to the use of an Lp(a) assay protocol that does not depend upon the apoB subunits in Lp(a). To quantitate the association of low Lp(a) levels with an increased risk of cancer, Pearson chi-square test was used. Binary logistic regression analysis was carried out using group of participants (cases/controls) as the dependent variable and level of Lp(a) as independent variable, to estimate OR and 95% CI. Since the median Lp(a) level in healthy controls was around 30 mg/dl, contribution of Lp(a) values below this as a risk factor for breast cancer was determined. Analysis (Table 2) revealed that low Lp(a) level is associated with a risk for cancer with an OR of about 3.8 (95% CI: 1.487–9.554). This indicated that the chance of getting lower Lp(a) level (≤30 mg/dL) in cancer patients is around 3.8 times greater as compared to controls. That is, there is around 3.8 times greater risk of getting cancer in the group bearing lower than median Lp(a) level.

### Total reactivity per unit volume and specific reactivity of anti-Gal are significantly lower in cancer patients

It was recently reported from this laboratory that individuals carrying Lp(a) of relatively smaller size and higher plasma titer synthesized anti-Gal with higher specific reactivity.[11] Since both specific reactivity and concentration of anti-Gal could be expected to count in MUC1-dependent anti-tumor defense by this antibody, the combined effect of the two parameters, termed reactivity produced by defined sample volumes, and measured in terms of optical density response in ELISA for anti-Gal, was compared between patients and controls. Patients had decidedly less anti-Gal reactivity response than controls ($P$ value = 0.0033; n = 46 in either group; Figure 2(a)), indicating that small Lp(a)-mediated protection from cancer may be operating through the synthesis of anti-Gal in larger numbers and/or with

**Table 2.** Binary logistic regression analysis of low Lp(a) level as a risk factor for breast cancer with odds ratio (OR) and confidence interval (CI).

| Lp(a) level | Controls, n (%) | Patients, n (%) | *P* value | Unadjusted OR | 95% CI |
|---|---|---|---|---|---|
| >30 mg/dL | 22 (47.8) | 9 (19.6) | | 1 | |
| ≤30 mg/dL | 24 (52.2) | 37 (80.4) | 0.004 | 3.769 | 1.487–9.554 |

n: number of subjects with low and high Lp(a) concentration values.

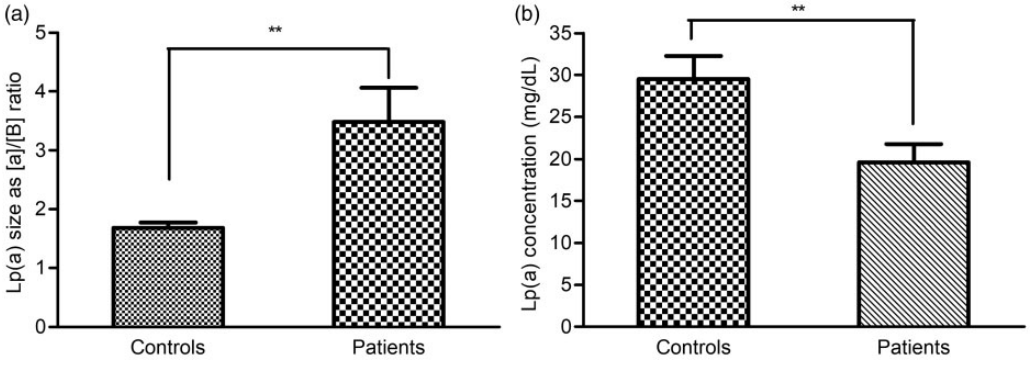


**Figure 1.** (a) Molecular size of serum lipoprotein(a) in individuals. Molecular size of Lp(a) as ([a]/[B]) ratio was measured in controls and patients as described in Materials and Methods section. **$P$ value = 0.0023; n = 46 in each group. (b) Lipoprotein(a) concentration of individuals. Lp(a) concentration in sera of controls and patients was assayed by an apoB-independent enzyme immunoassay as described in Materials and Methods section. **$P$ value = 0.0067; n = 46 in each group.

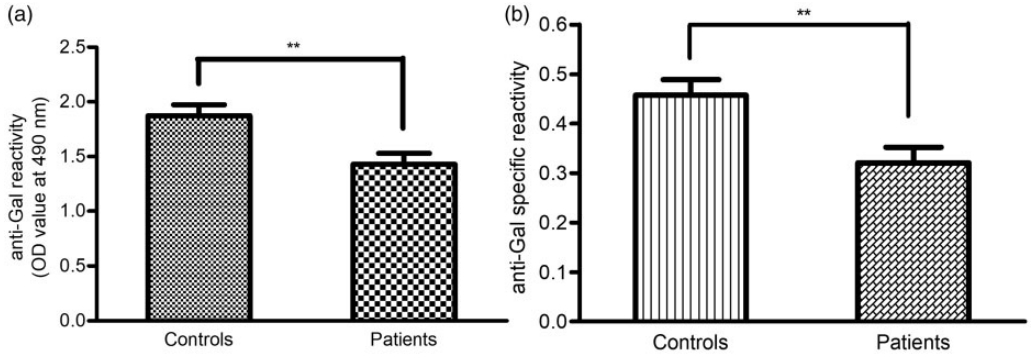


**Figure 2.** (a) Total anti-Gal activity in given serum volume in patients and controls. ***P* value = 0.0033; n = 46 in each group. (b) Specific activity of anti-Gal in individuals. ***P* value = 0.0033; n = 46 in each group.

higher specific reactivity since assay response shown in Figure 2(a) was a combined effect of both the number and the specific reactivity of anti-Gal molecules. The latter factor has been shown to be correlated negatively with the size and positively with the plasma concentration of Lp(a).[11] Figure 2(b) results show that cancer patients had anti-Gal with significantly lower specific reactivity (*P* value = 0.0033; n = 46 in either group) compared to controls indicating that specific reactivity of the antibody, dictated by Lp(a) size,[11] could by itself account for the increased incidence of cancer in low titer/high molecular weight Lp(a) individuals.

Correlation of anti-Gal specific reactivity with cancer incidence was examined by Pearson chi-square test. Binary logistic regression analysis was carried out using group of participants (cases/controls) on either side of mean specific activity value of normal subjects as the dependent variable and anti-Gal specific reactivity as independent variable, to estimate OR and 95% CI. Results (Table 3) revealed that low specific reactivity anti-Gal was associated with a risk for cancer with an OR of around 3.2 (95% CI: 1.368–7.557). These values indicated that the chance of getting low specific reactivity anti-Gal ($\leq$0.4) in cancer patients was around 3.2 times greater compared to controls.

### Reactivity per unit serum volume as well as specific reactivity of anti-Gal increased during the progress of tumor

Histologically, all breast cancer patients' tissue samples examined belonged to either grade II or grade III. Among cancer patients, serum Lp(a) levels did not change significantly from grade II to grade III (result not shown). However, both total anti-Gal reactivity and specific reactivity of anti-Gal increased substantially during the progress from grade II to grade III (Figure 3). Since MUC1 is a known anti-Gal ligand[8] and is increasingly synthesized and secreted as tumor progresses,[4] the above observation prompted us to examine the difference between normal and tumor-derived MUC1, and among tumor-derived MUC1 samples at various stages of tumor, in terms of their affinity towards the antibody.

**Table 3.** Binary logistic regression analysis of low anti-Gal specific activity as a risk factor for breast cancer with odds ratio (OR) and confidence interval (CI).

| Anti-Gal specific activity | Controls, n (%) | Patients, n (%) | *P* value | Unadjusted OR | 95% CI |
|---|---|---|---|---|---|
| >0.4 | 28 (60.9) | 15 (32.6) | | 1 | |
| ≤0.4 | 18 (39.1) | 31 (67.4) | 0.007 | 3.215 | 1.368–7.557 |

n: number of subjects with low and high anti-Gal specific activity values.

### Tumor-specific MUC1, but not normal MUC1 is anti-Gal-reactive

Synthesis of anti-Gal-binding MUC1 by breast cancer cells in contrast to normal cells was demonstrated by immunohistochemistry. Although immunohistochemical detection of anti-Gal binding to breast cancer tissue was reported earlier, the factors that are likely to have contributed to the non-specific staining observed therein[8] included the use of affinity-purified anti-Gal for test and anti-Gal-free human serum as control. Further, HRP-conjugated anti-human IgG which is likely to recognize non-anti-Gal immunoglobulin super family molecules on cells[28] was used for detection. Moreover, this study[8] employed affinity-purified anti-Gal which, as shown recently, contains only triplet-bound antibodies whose binding sites are only partially available unlike the electrophoretically purified anti-Gal used in the present study.[18] Immunohistochemistry using FITC-labeled purified anti-Gal revealed that the discrimination by anti-Gal between normal and cancer cells was absolute and that normal cells were totally spared by the antibody (Figure 4(a)). Although macromolecular antigens are superior to small sugar moieties as ligands for carbohydrate-specific antibodies, melibiose could reverse most of the anti-Gal binding to cancer cells, indicating the binding site dependence of attachment of FITC-labeled anti-Gal to cells. Result showed that MUC1 recognized by anti-Gal is an exclusively tumor-specific phenotype and indicated a unique role for anti-Gal in tumor surveillance and destruction. This result agrees with the observation above that individuals who possess more active anti-Gal antibodies are less susceptible to cancer (Figure 2, Table 3).

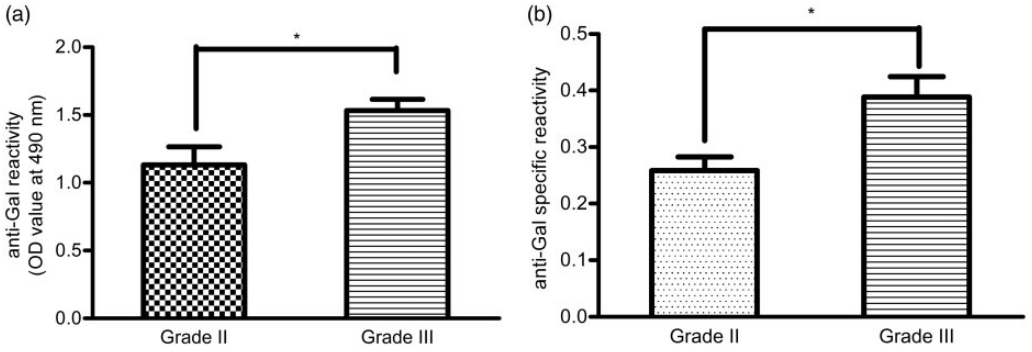


**Figure 3.** (a) Variation of serum anti-Gal titer in patients with different histological grades of tumor. *$P$ value = 0.0258 for grade II vs. grade III; n = 10 for grade II, n = 36 for grade III. (b) Variation of serum anti-Gal specific activity in patients with different histological grades of tumor. *$P$ value = 0.0286 for grade II vs. grade III; n = 10 for grade II, n = 36 for grade III.

Recognition of MUC1 in tumor-extracted proteins by anti-α-galactoside antibody was assayed by ELISA. Presence of MUC1 antigen in the protein samples isolated from tissues was confirmed by the ability of microplate-coated forms of these samples to capture jacalin as well as anti-MUC1 antibody (Materials and Methods section; data not shown). Reactivity of MUC1-containing samples as ligands for anti-Gal was examined in terms of their capacity to inhibit binding of the antibody to its ligand TIM coated on microplate. Results (Figure 4(b)) showed that tumor-associated MUC1 (TA-MUC1) samples were efficient anti-Gal ligands as they strongly inhibited anti-Gal binding to TIM, whereas normal tissue MUC1 (N MUC1) was hardly an inhibitor. GS-IB4 is a lectin from the plant *G. simplicifolia* with nearly identical specificity as anti-Gal and binds to terminal α-galactoside groups as well as to MUC1, by accommodating the STPS of the latter as surrogate for sugar ligands at its binding site.[8] Binding of biotinylated GS-IB4 to TIM, its specific ligand, was strongly inhibited by TA-MUC1 but poorly by normal MUC1 (N MUC1) (Figure 4(b)). Presence of MUC1 in proteins extracted from tumor was further confirmed using anti-MUC1 antibody. Inhibition of anti-Gal binding to microplate-coated TIM by TA-MUC1 was significantly reversed by the presence of anti-MUC1 in a dose-dependent manner (Figure 4(b)).

### TA-MUC1 is reactive towards anti-Gal since the latter's core-1 O-glycosylation, though greater in number, is incomplete

As tumor progresses, breast cancer cells are known to synthesize MUC1 with increasing numbers of O-glycans as well as to shift O-glycosylation to predominantly core-1 type.[3] However, incomplete glycosylation of individual core-1 O-glycans is characteristic of many tumors including breast cancer and leads to the absence of terminal or penultimate Gal-β1→3GalNAc disaccharide units on O-glycans. Since the latter moiety is ligand for the O-glycan-specific lectin jacalin, this lectin is often used for detecting the presence of non-truncated normal core-1 O-glycans with or without terminal sialic acid moiety.[29] Variation in anti-Gal reactivity towards TA-MUC1 at different stages of tumor and therefore possessing varying extents of truncation of their core-1 O-glycans was examined using the decline in jacalin reactivity as an index of the latter. Tumor-associated MUC1 samples (n = 24) were coated on polystyrene wells, and binding of HRP-conjugated form of jacalin to the coated samples was measured as described earlier. Percentage of inhibition of anti-Gal binding to TIM by TA-MUC1 samples in ELISA was also measured. Plot of jacalin-binding response of TA-MUC1 samples against percentage inhibition produced by these samples on anti-Gal-TIM interaction (Figure 5) revealed a convincing negative correlation with Pearson's correlation coefficient r= –0.7618 and $P$ value <0.0001 and suggested that anti-Gal reactivity to MUC1 increased as the content of non-truncated core-1 O-glycans decreased. This conclusion was further confirmed by a nearly identical pattern with the above when inhibition of GS-IB4 binding to TIM and jacalin reactivity of the MUC1 samples was plotted. (r= –0.7485, $P$ value <0.0001) (Figure 5). Even though core-1 type O-glycosylation increases following tumorigenesis, the response of microplate-coated MUC1 to jacalin (core-1 O-glycan-specific lectin) in enzyme-linked lectin assay was significantly lower for TA-MUC1 compared to N MUC1 (Figure 5 inset) indicating marked incomplete glycosylation in TA-MUC1, resulting in reduced density of peripheral Gal-β1-3GalNAc moiety essential for jacalin binding.[29] However, incomplete O-glycosylation which could cause greater exposure of serine and threonine residues is a possible reason for TA-MUC1 to be more accessible and reactive to anti-Gal, akin to the increase in affinity of this antibody to apo(a) following de-O-glycosylation of the latter.[9]

### MUC1 with increasing affinity for anti-Gal is synthesized as tumor progresses

Anti-Gal reactivity of MUC1 from tumors at different stages was measured in terms of their inhibition of anti-Gal binding to its ligand TIM. Results (Figure 6) showed that while TA-MUC1 from grade II and grade III significantly inhibited anti-Gal, the increase in inhibitory capacity from grade II to grade III was also significant. A non-O-glycosylated protein ovalbumin was not inhibitory. Inhibition of GS-IB4 lectin activity by TA-MUC1 in grade II and III cancer cells showed a pattern identical to that of

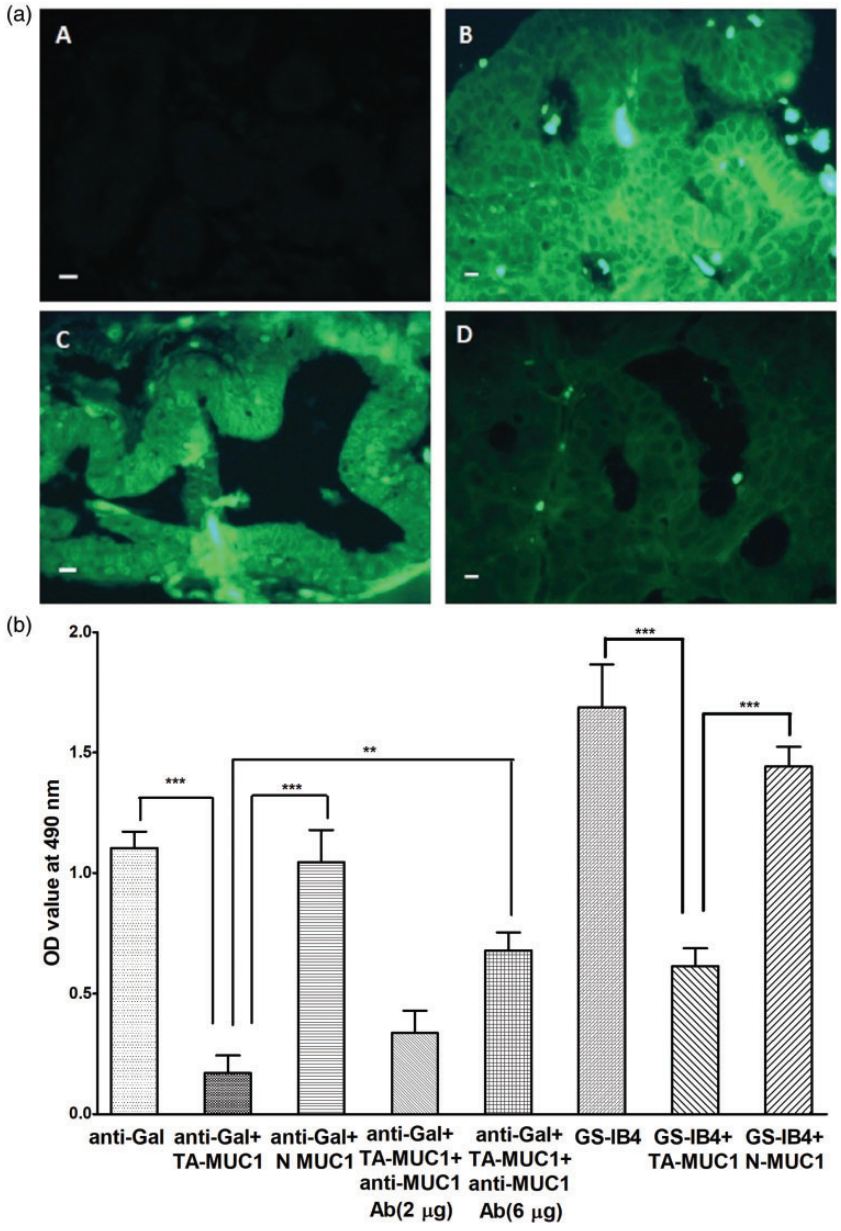


**Figure 4.** (a) Fluorescence immunohistogram of human breast tissue. FITC-labeled anti-Gal antibody was used on formalin fixed and paraffin embedded tissue. (A) Normal breast tissue showing acinar lobule with surrounding stromal tissue. (B) Moderately differentiated infiltrating breast carcinoma. (C) Poorly differentiated infiltrating breast carcinoma. (D) Moderately differentiated infiltrating breast carcinoma tissue treated with anti-Gal antibody which had been previously incubated with its specific sugar (25 mM melibiose). Scale bar represents 10 μm. (b) Recognition of tumor-associated MUC1 by anti-Gal antibody and its inhibition using anti-MUC1 antibody. Polystyrene wells were coated with TIM (1 μg/well). Anti-Gal (75 ng in 200 μL PBST) or biotinylated GS-IB4 lectin (75 ng in 200 μL PBST) pre-incubated at 4°C for 18 h with either 2 μg tumor-associated MUC1 (TA-MUC1) or 2 μg MUC1 from normal tissue (N MUC1) was added to the wells, followed by incubation at 4°C for 2 h. To confirm the recognition of TA-MUC1 by anti-Gal, 2 μg TA-MUC1 was incubated at 4°C for 6 h with either 2 or 6 μg anti-MUC1 antibody (goat) in 100 μL PBST. Anti-Gal (75 ng in 100 μL PBST) was added, incubation continued for 18 h and mixture added to the wells for incubation at 4°C for 2 h. After washing with PBST, well-bound anti-Gal and lectin were assayed as described (Materials and Methods section). Untreated anti-Gal or untreated GS-IB4-biotin added to TIM coated wells served as control. ***$P$ value $<0.001$ and **$P$ value $<0.01$. n = 12 in each group. (A color version of this figure is available in the online journal.)

anti-Gal (Figure 6). To demonstrate tumor stage-dependent TA-MUC1-mediated activation of anti-Gal, another parameter, namely increase in fluorescence of fluorescent-labeled anti-Gal (anti-Gal-FITC) by TA-MUC1 was also measured. Antigenic macromolecular ligands had been shown to increase the fluorescence of anti-Gal-FITC, largely due to conformational shift produced in the antibody's Fc region consequent to occupation of its binding site.[22] Result (Figure 7) showed that the increase produced in fluorescence of anti-Gal-FITC was twice as much by TA-MUC1 grade II and four times as much by TA-MUC1 grade III, as that by the same quantity of TIM alone, signifying the far greater binding affinity of TA-MUC1 than that of α-galactoside-bearing proteins. Result also underlined the cancer stage-dependent increase in TA-MUC1 affinity for anti-Gal. The increase in fluorescence of anti-Gal was exclusively owing to occupation of binding site of the antibody by

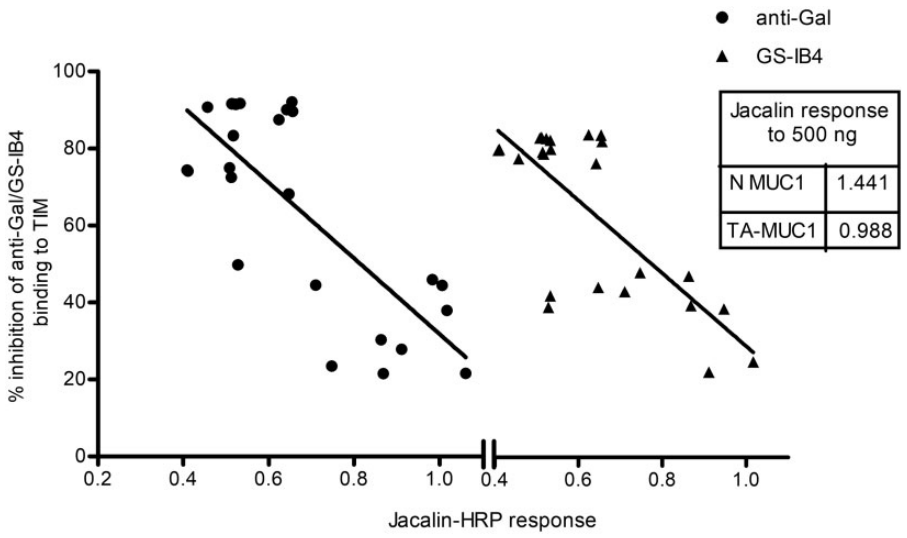


**Figure 5.** Correlation between O-glycosylation of tumor-associated MUC1 and percentage inhibition of anti-Gal/GS-IB4 binding to TIM. To assess the O-glycosylation status of different tumor-associated MUC1 samples, 500 ng of TA-MUC1 was directly coated on microplate wells and incubated with HRP conjugate of jacalin (75 ng lectin per mL) for 2 h at 4°C and bound HRP activity was assayed. Ability of tumor-associated MUC1 to inhibit binding of anti-Gal/GS-IB4 to its ligand, TIM was measured by incubating 2 μg tumor-associated MUC1 (TA-MUC1) from different tumor samples with anti-Gal (75 ng in 200 μL PBST) or biotinylated GS-IB4 (75 ng in 200 μL PBST) for 18 h at 4°C followed by addition to TIM-coated wells (1 μg/well) and incubation at 4°C for 2 h. After washing, well-bound anti-Gal and lectin were assayed as above. HRP response for anti-Gal or GS-IB4 alone added to TIM coated wells was used as a benchmark to calculate the percentage inhibition of anti-Gal/GS-IB4 binding to TIM by TA-MUC1 samples. n = 24. Pearson's correlation coefficient, r = −0.7618 for percentage inhibition of anti-Gal binding to TIM and r = −0.7485 for percentage inhibition of GS-IB4 binding to TIM. *P* value <0.0001 for both.

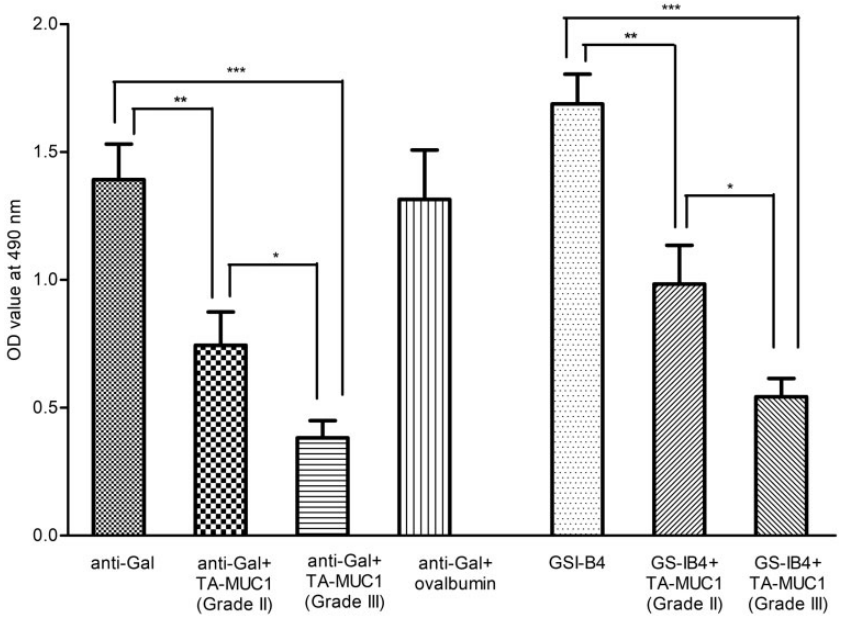


**Figure 6.** Inhibition of binding of anti-Gal or GS-IB4 to TIM by MUC1 from different grades of breast tumor. Polystyrene wells were coated with TIM (1 μg/well). Anti-Gal (75 ng in 200 μL)/biotinylated GS-IB4 lectin (75 ng in 200 μL) pre-incubated at 4°C for 18 h with 2 μg tumor-associated MUC1 (TA-MUC1) from different grades of tumor or ovalbumin (2 μg) was added to the wells, followed by incubation at 4°C for 2 h. Bound antibody and lectin were assayed using avidin-HRP. Untreated anti-Gal/GS-IB4-biotin added to TIM coated wells served as control. Ovalbumin was used as the non-specific protein to rule out any non-specific binding of anti-Gal. ***$P$ value <0.001, **$P$ value <0.01 and *$P$ value <0.05. n = 12 in each group.

the MUC1 samples since presence of melibiose totally abolished the enhancement of fluorescence.

Results in Figures 6 and 7, along with those in Figure 3, lead us to a reassessment of the contribution of specific reactivity of anti-Gal towards protection from breast cancer. While Figures 6 and 7 show that breast cancer cells produce MUC1 of increasing affinity for anti-Gal as cancer advances, Figure 3 shows that this advance is also accompanied by increase in specific reactivity of anti-Gal, most probably due to the tumor-associated MUC-1, which is strongly recognized by anti-Gal, acting as an affinity maturation antigen for the antibody, as done by low molecular weight Lp(a) molecules.[11] Since the patients studied were in stage II or III, their specific reactivity values used in Table

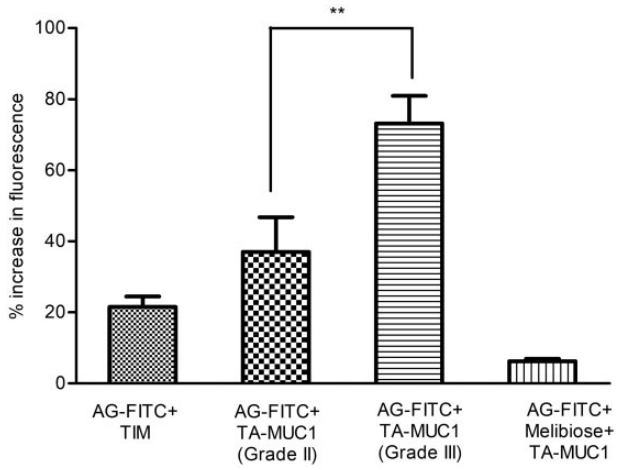


**Figure 7.** Binding of FITC-labeled anti-Gal to MUC1 from different grades of breast tumor. MUC1 (10 μg) isolated from different grades of tumor was incubated at 4°C for 18 h with FITC-labeled anti-Gal (3 μg). Volume was made up to 300 μL with PBS just before fluorescence measurement by excitation at 485 nm and emission at 520 nm. Positive control TIM (10 μg) was used in place of MUC1 sample. Fluorescence of 3 μg FITC-anti-Gal alone in PBS was used to calculate the percentage increase in fluorescence on addition of other ligands. To verify the involvement of sugar-binding site of anti-Gal in accommodating TA-MUC1, FITC-labeled anti-Gal pre-incubated with its specific sugar melibiose (25 mM) was incubated with TA-MUC1 sample. ***P* value = 0.0095 for difference in fluorescence enhancement of FITC-anti-Gal produced by TA-MUC1 from grade II and grade III. n = 8 in each group.

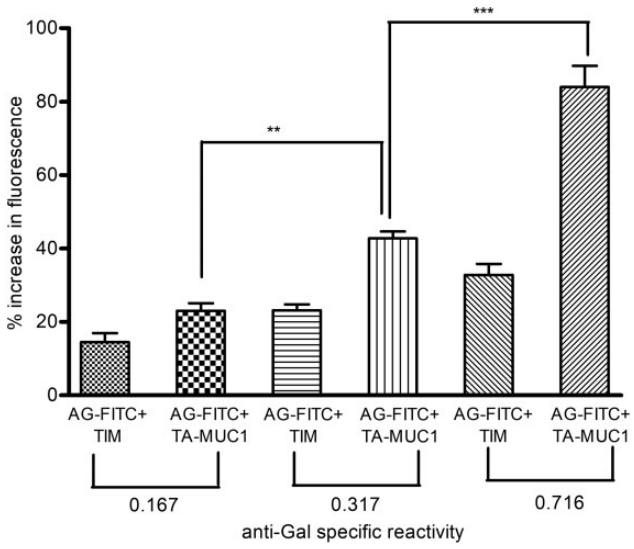


**Figure 8.** Comparison of binding of anti-Gal of varying specific activity to TA-MUC1.Three groups (n = 6 in each) of anti-Gal samples representing different ranges of specific activity with average specific activity values 0.167, 0.317, 0.716, respectively, were used. Anti-Gal samples with varying specific activity were then labeled with FITC. Same amount of three randomly selected TA-MUC1 samples (10 μg) or the known anti-Gal ligand (TIM, 10 μg) was separately mixed with each of the FITC-labeled anti-Gal (3 μg) of varying specific activities at 4°C for 18 h. Volume was made up to 300 μL with PBS just before fluorescence measurement by excitation at 485 nm and emission at 520 nm. Fluorescence of 3 μg FITC-anti-Gal alone in PBS was used to calculate the percentage increase in fluorescence on addition of other ligands. ***P* value <0.001 and **P* value <0.01.

3 are those that had already been enhanced from the respective pre-cancer values due to the presence of tumor-specific MUC1 in the body. Had pre-cancer anti-Gal specific reactivity values, which must have been lower than those recorded after the disease had set in, been available, the corresponding OR as per Table 3 would have been even higher. Thus, extrapolation and deduction from the above results tell us that low anti-Gal specific reactivity is an even more significant risk factor for breast cancer than indicated by the OR in Table 3.

### MUC1 binding increases with specific reactivity of anti-Gal

Fluorescence enhancement in FITC-labeled antibody increases with the size of macromolecular antigen and is also a measure of antigen binding affinity (specific reactivity) of the antibody. Three groups of purified anti-Gal samples, six in each group, were selected so that each group represented a different specific reactivity level and average specific reactivity in groups varied in geometric progression. Reactivity of each of three randomly selected grade II TA-MUC1 samples with all these antibody samples was measured in terms of increase in fluorescence of anti-Gal-FITC. Mean ± SEM of response in each antibody group was noted. TA-MUC1 was recognized with increasing affinity by anti-Gal samples of increasing specific reactivities (Figure 8). Since antigen-induced conformational shift in Fc region is the trigger for all events in antibody-mediated cytotoxicity, this result explains how anti-Gal molecules with relatively higher specific reactivities could bring about more effective tumor cell surveillance and how specific reactivity of anti-Gal decides the capacity of individual to resist tumor immunologically. Result also explains how smaller size/higher titer serum Lp(a) correlates with lower cancer incidence since smaller Lp(a) phenotypes are also accompanied by higher specific reactivity values for circulating anti-Gal.[11]

## Discussion

The suggested negative correlation of serum Lp(a) concentration and positive correlation of its size with susceptibility to cancer had been waiting for an explanation in molecular and pathobiological terms since no evidence linking these variables existed. We propose an immunobiological reason for this correlation suggesting that small Lp(a) phenotype, which generally accompanies high serum concentration of the lipoprotein, protects from tumors by enhancing the reactivity of anti-Gal antibody that differentiates between normal and tumor-specific cell surface MUC1 antigens. The hypothesis rested mainly on two assumptions: (i) Lp(a) size-dependent alterations in specific reactivity of anti-Gal and (ii) specific reactivity-dependent increase in binding of anti-Gal to tumor-specific antigen MUC1, which could result in improved tumor surveillance and destruction by pathways such as antibody-dependent cytotoxicity. The first assumption was verified by our recent report[11] that specific reactivity of anti-Gal varies inversely with Lp(a) size. Results in the present paper verify the second part of the above hypothesis mainly by two streams of observations. Firstly, women with higher specific reactivity as well as total reactivity of anti-Gal due to their possessing smaller Lp(a) molecules as shown recently[11] were protected from breast cancer so that those with

anti-Gal specific reactivity less than median values were at greater risk with an OR of around 3.2.

Secondly, in contrast to normal epithelial cell MUC1 that is inert to anti-Gal, tumor cell MUC1 is a potent anti-Gal ligand which is recognized more strongly as specific reactivity of anti-Gal increases and which also develops into stronger anti-Gal-binding ligand as tumor progresses. Correlation of plasma Lp(a) concentration to incidence of breast cancer reported earlier[10,24] needed to be redefined since later evidences pointed to interference of LDL, non-covalently adhering to Lp(a), on apoB-dependent assay of the latter and apo-B-independent Lp(a) assay protocols became available.[20] Lp(a) values of 46 breast cancer patients and as many controls showed (Figure 1(b)) that serum Lp(a) titer was more significantly lower among cancer patients ($P$ value = 0.0067) than was reported earlier ($P$ value = 0.03). On the other hand, association of small Lp (a) size with lesser incidence of cancer was stronger than that of high Lp(a) titer, possibly because qualitative rather than quantitative differences in Lp(a) could be expected to modulate the properties of anti-Gal better by mechanisms such as affinity maturation. Also, higher serum Lp(a) concentration could be expected as a consequence of smaller Lp(a) size. MUC1 molecules expressed on normal cell surfaces, despite possessing large numbers of variable number tandem repeats containing STPS, are inert towards anti-Gal, unlike other STPS-rich molecules such as Lp(a) or plasma albumin-associated O-glycoproteins.[9,18] The high affinity of tumor-associated MUC1 towards anti-Gal could be attributed to the reported unfolding, in tumor cell MUC1, of cryptic peptide sequences that possess serine and threonine residues as evidenced by the increased O-glycosylation in these MUC1 molecules.[4] Incomplete or truncated forms of these newly added O-glycans in tumor cells[4] is another factor that could possibly enhance anti-Gal access to these newly exposed sites. This conclusion is supported by the lower jacalin binding to TA-MUC1 than to normal MUC1 despite the former bearing more core-1 type O-glycans. The increase in anti-Gal-binding activity of a given amount of tumor-extracted MUC-1-containing protein with increase in tumor growth could arise either due to an overall increase in MUC1 synthesis or due to qualitative changes in MUC1 structure or both. This shows that once the tumor has set in, anti-Gal-reactive MUC1, which is a dominant tumor-specific and non-self-antigen, is likely to take over antigen-dependent modulation of antibody activity including affinity maturation. Since conformational change produced in the Fc part of antibody is trigger for initiation of steps in antibody-dependent cell cytotoxicity, increase in MUC1-induced conformational change in anti-Gal with tumor progression, reflected in fluorescence increase of the FITC-labeled antibody (Figure 7), also underscored the role of anti-Gal in defense against breast cancer.

A surprising observation (Figure 2) was that specific reactivity as well as total reactivity of anti-Gal, which are remarkably less than normal in cancer patients as a whole, start rising as cancer progresses from stage II to stage III. This could have happened due to the increase in synthesis and secretion of anti-Gal reactive MUC1 molecules which, as antigens, could stimulate anti-Gal synthesis. More importantly, this result confirmed that pre-cancer anti-Gal reactivity level in cancer patients should have been much lower than the values used in Figure 2 and Table 3 and suggested that the contribution of anti-Gal towards defense against breast tumor is much greater than what is indicated by the present data.

Among the three groups of anti-Gal samples representing three ranges of specific reactivity values, the conformational changes produced by the same set of three MUC1 molecules were in tandem with the increase in specific reactivity. Besides validating the use of specific reactivity of anti-Gal as determined above as an index of its affinity towards MUC1, these data reiterate the contribution of anti-Gal specific reactivity in natural immune defense against cancer.

The exclusive specificity of anti-Gal towards tumor-specific MUC1 could be a corollary to the reported high antigenicity of its surrogate, the α-galactoside epitope in humans and has been exploited in anti-cancer defense by incorporating this ligand in vaccines particularly for pancreatic cancer.[30,31] Cancer cells breaking away from primary sites travel through blood vessels and get lodged at distant locations before growing again,[32] emphasizing the relevance of a cancer antigen-selective and ubiquitous natural antibody in scavenging them. The dominance of IgG type in anti-Gal and the marked rise of MUC1 antigen in breast cancer cells are factors that could increase Fc density on antibody-bound cells and facilitate antibody-mediated cell destruction processes including antibody-dependent cell cytotoxicity, complement-mediated lysis and phagocytosis of cells and apoptosis.[33] Finally, anti-Gal is a target-selective natural antibody against cancer without the need for humanization.[34] Present results open therapeutic options of infusing small Lp(a) molecules, high reactivity anti-Gal, or plasma containing both in cancers of breast and possibly other organs.

**Authors' contributions:** JJ was involved in conceptualization, planning, bench work, data compilation, and writing. KC contributed to clinical assessment, surgical procedures, patient selection, and data interpretation. TA was involved in planning, facilitation of clinical biochemistry and FNAC data, inter-institutional co-ordination, and manuscript writing. PSA contributed to conceptualization, experiment design, interpretation of data, manuscript preparation, and correspondence.

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#### DECLARATION OF CONFLICTING INTERESTS

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