MODULATION OF TUMOR ANTIGEN-REACTIVE ANTI-GAL ANTIBODIES BY LIPOPROTEIN(a) CONCENTRATION AND ITS EFFECT ON TUMOR SUSCEPTIBILITY

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A THESIS PRESENTED BY

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DECLARATION

I, **Jessy John**, hereby declare that I had personally carried out the work depicted in the thesis entitled **"Modulation of tumor antigen-reactive anti-Gal antibodies by lipoprotein(a) concentration and its effect on tumor susceptibility"**. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

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This is to certify that Mrs. Jessy John, in the Department of Biochemistry of this Institute, has fulfilled the requirements prescribed for the Ph.D degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum.

The thesis entitled **"Modulation of tumor antigen-reactive anti-Gal antibodies by lipoprotein(a) concentration and its effect on tumor susceptibility"** was carried out under my direct supervision. No part of thesis was submitted for the award of any degree or diploma prior to this date.

Clearance was obtained from the Institutional Ethics Committee for carrying out the study.

27 July 2018 **Dr. P. S. Appukuttan**

The thesis entitled

MODULATION OF TUMOR ANTIGEN-REACTIVE ANTI-GAL ANTIBODIES BY LIPOPROTEIN(a) CONCENTRATION AND ITS EFFECT ON TUMOR SUSCEPTIBILITY

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for the degree of

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Jessy John

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LIST OF ABBREVIATIONS

ABG	Anti-β-glucoside antibody
AG-FITC	Anti-Gal-FITC
ANOVA	Analysis of variance
Anti-Gal	Anti-α-galactoside antibody
APC	Antigen presenting cell
Apo(a)	Apolipoprotein(a)
ApoB	Apolipoprotein B100
CHD	Coronary heart disease
CLGG	Cross-linked guar gum
CNBr	Cyanogen bromide
CVD	Cardiovascular vascular disease
DIg	Dextran-binding immunoglobulin
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
ELLA	Enzyme-linked lectin assay
FITC	Fluorescein isothiocyanate
GalNAc	N-acetyl galactosamine
GC	Germinal centers

GlcNAc	N-acetyl glucosamine
GS-IB4	*Griffonia simplicifolia* isolectin B4
h	Hour
HRP	Horse radish peroxidase
Ig	Immunoglobulin
IgA1	Immunoglobulin A1
IgG	Immunoglobulin G
IgM	Immunoglobulin M
JL1	Lipid fractions of jacalin precipitate of plasma
kDa	Kilodaltons
L1	Lipoprotein layer
LacNAc	N-acetyl lactosamine
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LIg	Lactose-binding immunoglobulin
Lp(a)	Lipoprotein(a)
Man	Mannose
MHC	Major histocompatibility complex
min	Minute
N MUC1	Normal MUC1
OD	Optical density

OPD	Ortho-phenylenediamine
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline (20 mM) pH 7.4
PBS-T	PBS containing 0.05 % Tween-20
RBC	Red blood cell
SEM	Standard error mean
STPS	Serine- and threonine-rich peptide sequence
T/TF antigen	Thomsen-Friedenreich antigen (Galβ1→3GalNAc-)
TA-MUC1	Tumor-associated MUC1
TBS-T	Tris-buffered saline, 0.05% Tween 20
TEMED	N,N,N',N'- tetramethyl ethylene diamine
Th	T-helper cells
TI	Typsin inhibitor
TIC	Trypsin inhibitor-cellobiose
TIM	Trypsin inhibitor-melibiose
TLR	Toll-like receptor
VLDL	Very low density lipoprotein
VNTR	Variable number tandem repeat
α1,3GT	α1,3-Galactosyl transferase
α-gal epitope	Galα1-3Galβ1-4GlcNAc-R

SYNOPSIS

Background

A plasma lipoprotein unique to animals with higher cognitive ability is lipoprotein(a) [Lp(a)] which contains an apolipoprotein(a) [apo(a)] covalently attached to an LDL molecule. Plasma concentrations of Lp(a) vary over a wide range among individuals due to variations in the size of apo(a) and in general an inverse relationship exists between plasma Lp(a) concentration and Lp(a) size. High circulating concentration of Lp(a) has been established as a positive risk factor for several cerebrovascular disorders including atherosclerosis, stroke, Alzheimer's disease and aneurysm. Recently various epidemiological studies reported that people with higher plasma Lp(a) concentration which is usually accompanied by a dominance of low molecular weight Lp(a) are better protected from cancer than low plasma titre/ high molecular weight Lp(a) individuals. This laboratory has reported that naturally occurring plasma anti-α-galactoside antibody (anti-Gal) present only in the animals that synthesize Lp(a) recognizes this lipoprotein through the binding site of the former. Devoid of α-galactosyl epitopes specific for anti-Gal, Lp(a) offers its serine- and threonine-rich peptide sequences (STPS) as surrogate ligands for the antibody. Notably STPS are also present at very high levels in the hypoglycosylated tumor-associated MUC1 molecules in cancer cells. Analysis of different plasma samples has revealed a strong inverse relationship between the size of Lp(a) synthesized and specific activity of anti-Gal in circulation, suggesting that the structure of apo(a) in Lp(a) affects the specific activity of anti-Gal. Sporadic antigenic stimuli generally produce IgM type antibodies whereas continued presence of antigen in the system is known to affect the affinity maturation of antibodies and direct its class-switching to

IgG-domination. It appears that although anti-Gal antibody synthesis is proposed to be triggered by α-galactoside-containing antigens synthesized by gut microbes as happens in the case of most other anti-carbohydrate antibodies, its affinity maturation is affected by Lp(a) structure. Indeed unlike most other carbohydrate-specific circulating antibodies anti-Gal is predominantly IgG with IgA and IgM in traces.

The reason for larger Lp(a) individuals possessing relatively less sharp (low affinity) anti-Gal may be that larger Lp(a) molecules, though carrying longer STPS-containing regions are relatively more likely to have this region occupied by extra LDL molecules so that net availability of STPS epitopes to effect affinity maturation of anti-Gal is less. Smaller Lp(a) molecules are relatively less engaged with additional LDL molecules and therefore act as an antigenic stimulus in affinity maturation process leading to sharper anti-Gal molecules. In addition generally smaller Lp(a) phenotype is accompanied by higher plasma concentration of Lp(a).

Hypothesis

Hypoglycosylated variant of MUC1 antigen recognized as a tumor-associated antigen has also been shown to be an anti-Gal ligand. Since Lp(a) molecule per se does not seem to possess features capable of affecting cancer incidence it is hypothesised that high specific activity anti-Gal antibodies seen in individuals with small size/high plasma titre lipoprotein(a) could provide protection against cancer possibly by recognition of tumor-associated MUC1.

Objectives

1. To find out the association between lipoprotein(a) size and concentration on one hand and anti-Gal antibody specific activity and affinity maturation on the other, using plasma samples from healthy individuals.

2. To find out the association between incidence of cancer and specific activity of anti-Gal antibody and lipoprotein(a) concentration of the individual.
3. To compare anti-Gal antibody binding to MUC1 from different grades of tumor.
4. To compare the reactivity of anti-Gal antibody samples with differing specific activity towards tumor-associated MUC1

Methods

Plasma samples from Department of Blood Transfusion Services, SCTIMST was used for isolation of Lp(a), apo(a), LDL, and anti-Gal antibody. Lp(a) concentration of the individual was assayed by apoB-independent enzyme immunoassay by capturing Lp(a) on microplate-coated lectin jacalin. Anti-Gal titre of the individual was assayed by capturing anti-Gal on microplate-coated trypsin inhibitor conjugated to melibiose (TIM), and bound antibody was assayed by probing with HRP-conjugated anti immunoglobulins. The lectin jacalin, anti-glycan antibodies (anti-Gal, anti-β-glucoside antibody (ABG) and dextran-binding immunoglobulin (DIg) were isolated by affinity chromatography. Lp(a) was isolated from human plasma by affinity precipitation with the lectin jacalin, followed by tris-borate EDTA electrophoresis and passive elution of lipoprotein bands. Purified apo(a) was prepared by reduction of plasma lipoprotein-rich fraction with dithiothreitol followed by ultracentrifugation.

Specific activity of anti-Gal was defined as activity per unit immunoglobulin. Activity was measured by binding the antibody to a specific ligand and measuring the bound antibody using HRP-labeled anti-immunoglobulins. Immunoglobulin content of antibody sample was measured by direct coating to polystyrene microwells and measuring the bound antibody as above. For characterization of

Lp(a) isoforms anti-apo(a) and anti-apoB responses of plate- coated purified Lp(a) was measured and the ratio of anti-apo(a) response to anti-apoB response was taken as an index of molecular size since larger the Lp(a), the more its apo(a) masked the apoB from getting recognized by anti-apoB.

For verification of hypothesis blood samples and surgically removed tumor and normal tissues were collected from breast cancer patients. As controls blood samples of healthy subjects were used. For the partial purification of MUC1 glycoprotein, preliminary tissue homogenate (10 % w/v) was prepared and mixed with 1%Triton X-100 for mucin extraction followed by centrifugation. Supernatant after centrifugation was subjected to gel filtration chromatography on Sepharose-6B column and MUC1-containing fractions identified by their recognition by jacalin were pooled and concentrated. Presence of MUC1 in extracted proteins was confirmed by the use of MUC-1-reactive lectin *Griffonia simplicifolia* IB4 (GSIB4) labeled with marker enzyme and anti-MUC1 antibody. Anti-Gal recognition of MUC1 was assayed by ELISA and by using FITC-labeled anti-Gal employing ligand-induced fluorescence increase in antibodies reported earlier from this laboratory. Anti-Gal binding to tumor tissue was examined by immunofluorescence analysis using FITC-labeled anti-Gal. FITC-labeled anti-Gal molecules possessing varying specific activities were used to determine difference in their capacity to bind to the same MUC1.

Results

In circulation low molecular weight Lp(a) are more available for recognition by anti-Gal because of low LDL adduct index defined as the ratio of responses to anti-apoB and ant-apo(a) and indicates the extent of non-covalently attached LDL on Lp(a).

Presumably because they are either not or poorly covered by adduct LDL low molecular weight Lp(a) offer better antigenic stimuli for affinity maturation and class-switching to IgG-domination for anti-Gal than high molecular weight Lp(a). This results in high specific activity anti-Gal in individuals with small size/high concentration Lp(a).

Larger Lp(a) in lower concentration along with low specific activity anti-Gal in lower concentration was present in breast tumor patients compared to controls. Specific activity of an antibody non-reactive with Lp(a) did not differ between patients and controls. No stage-wise difference in Lp(a) concentration was found in breast cancer patients whereas level and specific activity of anti-Gal antibody increased with stage of tumor.

Both anti-Gal and GSIB4 lectin bind to MUC1 expressed on the surface of tumor cells but not on normal cells. Decrease in jacalin reactivity of tumor-associated MUC1 (TA-MUC1) shows the reduced O-glycosylation of MUC1 in tumor cells and consequent increased exposure of STPS which are ligands for anti-Gal. Anti-Gal binding as well as GSIB4 binding to MUC1 increased with stage of tumor. Recognition of MUC1 in tumor by anti-Gal antibody was confirmed by immunofluorescence analysis. Most significantly anti-Gal binding of MUC1 increases exponentially with specific activity of antibody.

Significance

The above results underline the feasibility of developing therapeutic strategies aimed at cancer-specific immunopotentiation of individuals by vaccination or antibody infusion in order to achieve surveillance and destruction of tumor cells through recognition of tumor associated MUC1 antigen. Development of cancer-specific

immunotherapy either by administration of high specific activity anti-Gal or inducing the production of high specific activity anti-Gal by infusion of small apo(a)/Lp(a) as an antigenic stimulus is also a possibility thrown up by the present results.

1. INTRODUCTION

Lipoprotein(a) [Lp(a)] is a unique lipoprotein which is present only in humans, apes and old world monkeys. It is similar to low density lipoprotein (LDL) in structure. Lp(a) particle contains a lipid core covered by an apoB subunit as in LDL with an additional more hydrophilic glycoprotein apo(a) attached by a disulfide bridge to the apoB chain (Koschinsky and Marcovina, 2004). Apo(a) consists of several loop-like structures (kringles) mainly of class IV and V. Among kringle IV loops types 1 and 3-10 are present in single copies whereas kringle IV type 2 is present in multiple copies varying in number from 3 to >40 among individuals, leading to apo(a) size variation between 300-800 kDa. Overall size of Lp(a) varies between 4.5-5.5x10^6 Da. A notable consequence of protein-protein interaction between apo(a) and apoB chains in Lp(a) is prevention of apoB chain from being recognized by LDL receptors, so that Lp(a) clearance from circulation hardly depends on the latter. Every inter-kringle region contains an average of 6 potential O-glycosylation sites (rich in serine and threonine residues). This O-glycosylation that varies in density widely among individuals is unique to Lp(a) among lipoproteins (Garner *et al.*, 2001). Plasma concentrations of Lp(a) vary over a wide range among individuals, but are remarkably stable in any given individual. In healthy subjects Lp(a) levels are mainly dependent on the isoform type, with those with low molecular weight isoforms having high levels and those with high molecular weight isoforms having lower levels of circulating Lp(a) (Siekmeier *et al.*, 2010).

Numerous epidemiological studies have identified elevated plasma Lp(a) concentrations which is usually accompanied by dominance of low molecular weight Lp(a) isoforms, as an independent risk factor for several cerebrovascular disorders including atherosclerosis, stroke, Alzheimer's disease and aneurysm

(Berglund and Ramakrishnan, 2004, Malaguarnera *et al.*, 2013, Solfrizzi *et al.*, 2002). Though Lp(a) concentration in circulation is nearly one twentieth of that of LDL, atherosclerotic plaques have been found to contain more Lp(a) than LDL (Pepin *et al.*, 1991). However the molecular mechanisms of Lp(a) sequestration into vascular tissue remains unclear. On the other hand a curious observation has been made that individuals with high circulating Lp(a)/small Lp(a) phenotype are relatively less prone to cancer than their large Lp(a) counterparts (Lippi *et al.*, 2007, Sawabe *et al.*, 2012).

In the search for molecular mechanisms for Lp(a)-mediated pathology, immunoglobulins that recognize Lp(a) demand prime consideration. In this context this laboratory has reported (Mandagini *et al.*, 2013) that all samples of a circulating naturally occurring antibody, namely anti-α-galactoside antibody (anti-Gal) isolated by affinity chromatography contained Lp(a) but no other lipoprotein as co-purified molecule and treatment with antigen specific to the antibody (α-galactosides) could reverse Lp(a)-anti-Gal association. The content of Lp(a)-associated antibody increased with Lp(a) concentration of plasma. Later it was found out that anti-Gal binds to Lp(a) on the apo(a) subunit of the latter utilizing serine- and threonine-rich peptide sequence (STPS) that account for the heavy O-glycosylation of this subunit, as a surrogate antigen instead of α-galactoside groups (Geetha *et al.*, 2014).

Anti-Gal is a natural anti-carbohydrate antibody in humans, constituting ~1% of immunoglobulins. It consists predominantly of IgG, with traces of IgM and IgA isotypes (Galili *et al.*, 1984). It recognizes terminal α-1,3 linked as well as α-1,6 linked galactose moieties in glycoconjugates. Anti-Gal may owe its affinity for Lp(a) to the fact that Lp(a) occurs only in advanced mammals like man and apes, so

that apo(a) is evolutionarily too young to be considered as a self antigen. Incidentally anti-Gal also occurs only in the same animals that synthesize Lp(a) and both are of comparable evolutionary age from available evidence (Galili *et al.*, 1987, Lawn *et al.*, 1997). Analysis of different plasma samples has revealed that there is a strong inverse relationship between the size of Lp(a) synthesized and specific activity of anti-Gal in circulation, suggesting that the structure of apo(a) in Lp(a) affects the specific activity of anti-Gal. Sporadic antigenic stimuli generally produce IgM type antibodies whereas continued presence of antigen in the system is known to affect the affinity maturation of antibodies and direct its class-switching to IgG-domination (Li *et al.*, 2004). It appears that although anti-Gal synthesis is proposed to be triggered by α-galactoside-containing antigens synthesized by gut microbes as happens in the case of most other anti-carbohydrate antibodies, its affinity maturation seems to be affected by Lp(a) structure. Indeed unlike most other carbohydrate-specific circulating antibodies anti-Gal is predominantly IgG with IgA and IgM in traces (Galili *et al.*, 1984).

It has been reported that Lp(a) in circulation attaches additional molecules of LDL non-covalently to form adducts. Recent investigations in our laboratory has revealed that most Lp(a) molecules in circulation are in adduct form with LDL and that STPS-containing region of apo(a) is involved in adduct formation with LDL. Larger Lp(a) molecules with longer STPS-containing regions attach more LDL molecules and the increase in number of LDL molecules is out proportion with the increase in size of STPS-containing region of apo(a) (Kalaivani and Appukuttan, 2014). These results suggested that the reason for larger Lp(a) individuals possessing relatively less sharp (low affinity) anti-Gal may be that larger Lp(a) molecule, though

carrying longer STPS-containing regions are relatively more prone to have this region occupied by extra LDL molecules so that the net availability of STPS epitopes to effect affinity maturation of anti-Gal may be less. In addition generally smaller Lp(a) phenotype is accompanied by higher plasma circulation of Lp(a) (Utermann *et al.*, 1987).

The 'protection' offered by high circulating Lp(a) concentration to individuals against cancer may be explained by an interplay between Lp(a) and anti-Gal referred above. In view of our recent observation that high circulating Lp(a) which generally occurs in smaller Lp(a) phenotypes is accompanied by high specific activity anti-Gal, the above observation tends to suggest that increased anti-Gal reactivity protects high Lp(a) individuals from cancer. In support to this assumption is the report that cancer cells possess high surface distribution of hypoglycosylated MUC1 peptides (tumor-associated MUC1) with exposed serine and threonine rich peptide sequences which are also ligands for anti-Gal (Sandrin *et al.*, 1997).

Normal MUC1 is a heavily O-glycosylated transmembrane protein in which 50-90% of its molecular mass is due to carbohydrates. It is normally expressed on the apical surfaces of glandular and ductal epithelial cells. MUC1 is highly over expressed, under glycosylated and redistributed over the cell surface in majority of adenocarcinomas (Taylor-Papadimitriou *et al.*, 1999). Hypoglycosylation of tumor-associated MUC1 leads to exposure of immunogenic core peptides. Naturally occurring anti-Gal antibodies found in all human serum react with hypoglycosylated MUC1 peptides which are expressed in large amounts on the surface of tumor cells but not on normal cells. In individuals with high specific activity anti-Gal the antibody may be effective in binding to and destroying circulating metastatic tumor

cells. Various studies have reported the protection offered by high Lp(a) concentration to individuals against cancer. But studies relating the effect of Lp(a) size and concentration on anti-Gal synthesis and affinity maturation and also the binding of anti-Gal antibodies of differing specific activity to tumor cells are not yet conducted. So the present study aims to elucidate mechanisms of protection offered by high circulating Lp(a) concentration to individuals against cancer by the interplay between Lp(a) and anti-Gal. Verification of the hypothesis may offer better cancer care by increasing anti-Gal reactivity using vaccines.

Objectives

1. To find out the association between lipoprotein(a) size and concentration on one hand and anti-Gal antibody specific activity and affinity maturation on the other, using plasma samples from healthy individuals.
2. To find out the association between incidence of cancer and specific activity of anti-Gal antibody and lipoprotein(a) concentration of the individual.
3. To compare anti-Gal antibody binding to MUC1 from different grades of tumor.
4. To compare the reactivity of anti-Gal antibody samples with differing specific activity towards tumor-associated MUC1.

2. REVIEW OF LITERATURE

2.1 Glycoproteins

Glycoproteins are proteins carrying one or more glycans covalently attached to polypeptide backbone. They are present in all living organisms in soluble and insoluble forms with different functions and biological properties (Shylaja and Seshadri, 1989). In eukaryotes most of the secreted proteins contain long chains of covalently attached glycans. About 50% of all proteins and more than 80% of membrane proteins are glycosylated. Proteoglycans are proteins that are heavily glycosylated by glycosaminoglycans covalently attached to proteins. In glycoproteins carbohydrate content ranges from less than 1% to over 80% of the molecule. Glycosylation is the most important and complex co- or post translational modification of proteins catalyzed by glycosyl transferases and occurs in the endoplasmic reticulum or Golgi apparatus. It accounts for more than 50% of all post translational modifications and significantly increases proteome diversity and governs protein folding, stability, conformation, transport, and activity. However glycosylation is not a template driven process i.e. the order in which glycans are added is not encoded in the genome. Glycan structures are determined by a number of factors such as concentration of nucleotide sugar donors, presence of appropriate amino acid in the protein chain, expression and intracellular levels of glycosyltransferases, accessibility of glycosylation sites in the protein, and subcellular microenvironment (Varki and Sharon, 2009). Glycosyl transferases constitute a very large family of enzymes representing 1-2% of the genome and their expression varies widely among different cell types and changes significantly during development or stimulation of a cell, resulting in temporal and spatial variation of glycans.

Glycosylation

Glycosylation is the enzymatic process that attaches saccharides to other saccharides, proteins and lipids. Unicellular and multicellular organisms produce monomeric and multimeric glycan linkages through the process of glycosylation (Marth and Grewal, 2008). There are five distinct types of glycosylation in eukaryotes namely N-glycosylation, O-glycosylation, glypiation, C-glycosylation and phosphoglycosylation. In N-glycosylation glycan binds to the amino group in asparagine in the endoplasmic reticulum. In O-glycosylation glycan is attached to the hydroxyl group of serine or threonine in the endoplasmic reticulum, Golgi, cytosol/or nucleus. In glypiation glycan core links a phospholipid and a protein. In C-glycosylation α-mannose binds to the indole ring of tryptophan by carbon-carbon bond. In phosphoglycosylation glycan binds to the hydroxyl group of serine via a phosphodiester bond (Spiro, 2002).

N-Glycosylation

It is the most common type of glycosylation and it occurs co-translationally. About 90% of glycoproteins are N-glycosylated. Five different types of N-glycan linkages have been reported of which the most common type involves the formation of a β-glycosylamine linkage between glucosamine (GlcNAc) and amide nitrogen of asparagine. N-glycosylation occurs at the consensus sequence Asn-X-Ser/Thr and X represents any amino acid except proline. All N-glycans contain a common pentasaccharide core sequence, Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAcβ1-Asn-X-Ser/Thr. Based on the composition of oligosaccharides attached to α1-3 and α1-6 mannose branch points in the core, N-glycans are classified into 3 types; high-mannose, complex and hybrid type (Stanley *et al.*, 2009). In high-

mannose type only mannose residues are attached to core. In complex type N-acetyl lactosamine [LacNAc; Galβ1-3GlcNAc (Type I) or Galβ1-4GlcNAc (Type II)] is linked to α1-3 and α1-6 Man residues. In hybrid type, mannose residues are added to Man α1-6 arm while LacNAc residues are added in β1-4 linkage to α1-3 Man arm. Complex type of N-glycans exhibit enormous diversity and vary in the number of side chains attached to mannose residue resulting in the formation of bi-, tri-, tetra- and penta- antennary complex types that constitute the most of the cell surface and secreted N-glycans. Complex N-glycans commonly terminate with sialic acid residues. Additional modifications include addition of a GlcNAc at the mannosyl core which increases branching and/or the addition of a fucosyl residue to the core of N-glycans (Kornfeld and Kornfeld, 1985). Although the presence of the Asn-X-Ser/Thr sequon is necessary for N-glycosylation, transfer of N-glycan to this sequon does not always occur, due to conformational or other constraints during glycoprotein folding. Also the presence of acidic amino acids in the position of "X" may reduce the efficiency of glycosylation.

O-glycosylation

In this type of glycosylation, glycans are attached to the peptide chain through the hydroxyl group of serine and threonine and it occurs post-translationally in various compartments of Golgi apparatus. Peptide sites for O-linked glycosylation can also be tyrosine, hydroxylysine, or hydroxyproline. O-glycans are mainly of two types, mucin and non-mucin type. In mucin type of O-glycans, N-acetylgalactosamine (GalNAc) moiety is α-linked to the hydroxyl group of serine or threonine whereas in non-mucin type of O-glycans α-linked fucose, β-linked xylose, α-linked mannose, β-

linked GlcNAc (N-acetylglucosamine), α- or β-linked galactose, and α- or β-linked glucose are attached to the peptide site (Brockhausen *et al.*, 2009).

Mucin type O-glycans

Mucin type O-glycans are characterized by the presence of variable number tandem repeats at the peptide site which is rich in serine and threonine residues and have several O-GalNAc groups attached to the acceptor sites. These repeats are abundant in proline residues which facilitate O-glycosylation. Mucin type O-glycans begins with the addition of α-linked N-acetylgalactosamine to the serine or threonine followed by the addition of various sugars including galactose, N-acetylglucosamine, fucose, or sialic acid. There are four major types of O-GalNAc glycan core structures, designated as core 1 to 4 (Brockhausen *et al.*, 2009).

Table 1. Mucin type O-glycan core structures

O-glycan	**Structure**
Core	
Tn antigen	GalNAcαSer/Thr
Core 1 or T antigen	Galβ1-3GalNAcαSer/Thr
Core 2	GlcNAcβ1-6(Galβ1-3)GalNAcαSer/Thr
Core 3	GlcNAcβ1-3GalNAcαSer/Thr
Core 4	GlcNAcβ1-6(GlcNAcβ1-3)GalNAcαSer/Thr

Core 1 O-glycans contain Galβ1-3GalNAc group which is α-linked to serine or threonine and it forms the core of many longer and complex O-glycan structures and

known as T antigen. Subsequent branching of N-acetylglucosamine which is attached to core 1give rise to core 2, 3, and 4 structures of which core 2 structures are most common and found in many glycoproteins and mucins from variety of cells and tissues. Linear core 3 and branched core 4 structures are found in secreted mucins and all these core structures can be sialylated also.

Biological roles of glycans

Glycan structures have specific structural and modulatory properties and help in recognition by specific glycan binding molecules. Glycan mediated recognition can be either intrinsic (recognition of glycans from the same organism), or extrinsic (recognition of glycans from a different organism). Glycan structures on the cell surface (glycocalyx) act as a physical barrier. Glycans on extracellular matrix proteins help in maintenance of tissue structure, porosity and integrity. Glycans also provide protection to underlying polypeptide chains from proteolysis, helps in proper folding of proteins in the endoplasmic reticulum, provides extended conformation and aids in maintenance of solubility of proteins (Lis and Sharon, 1993).

Glycan structures also mediate cell–cell recognition, adhesion and cell–matrix interactions. An example for this is the interaction between selectin family of adhesion molecules and glycan structures on their ligands mediating interactions between blood cells and vascular cells in both in normal and pathological situations. Glycan structures also mediate clearance of proteins and intracellular trafficking of lysosomal enzymes. For example the removal of residues of Neu5Ac (a sialic acid) from ceruloplasmin facilitates its uptake by asialoglycoprotein receptors of hepatocytes enabling its destruction and replacement. Mannose-6-phosphate

residues of newly synthesized lysosomal proteins play an important role in their trafficking from ER and Golgi compartment to their final destination in lysosomes. Certain glycan structures also acts as specific binding sites for a variety of viruses, bacteria, and parasites. For example, the specific binding of hemagglutinins of many viruses (eg. influenza virus) specifically with the host sialic acid or its modifications (Varki and Lowe, 2009). HS1 and HS2 molecules on the surface of herpes simplex viruses bind specifically to heparan sulfate on the cell surface as a first step in their infection cycle. Some microbial pathogens have lectins that mediate bacterial adhesion to host cells or toxin entry into cells. Glycan structures on secreted mucins acts as decoys for microorganisms and parasites. The cognate oligosaccharide ligands attached to secreted mucins are recognized by pathogens trying to enter into host and they can be easily removed by the ciliary action of cells of mucosal membrane.

Impaired glycosylation leads to several human disorders such as I-cell disease, leukocyte adhesion deficiency, congenital dyserythropoietic anemia, rheumatoid arthritis, IgA nephropathy, Tn polyagglutinability syndrome and muscular dystrophies (Freeze and Schachter, 2009). Alteration in glycosylation is a universal feature of cancer cells and these altered glycan structures are well known tumor-associated antigens. Glycosylation can be altered in various ways in malignancy which includes loss of expression or excessive expression of certain structures, persistence of incomplete or truncated structures, accumulation of precursors or appearance of novel structures. Changes in carbohydrate structures in cancer cells leads to avoidance of immunological destruction, invasiveness and increased metastatic potential also (Varki *et al.*, 2009).

2.2 Lipoprotein(a) [Lp(a)]

Lipoprotein(a) [Lp(a)] is a unique lipoprotein which is present only in humans, apes and old world monkeys (Lawn *et al.*, 1997). It is similar to low density lipoprotein (LDL) in structure and it is unique in having an additional apolipoprotein, apo(a). It consists of a heavily glycosylated polymorphic apo(a) linked to apoB100 of LDL through a disulphide bond to form mature Lp(a).

Discovery of lipoprotein(a)

Lp(a) was discovered in 1963 by a Norwegian scientist Kare Berg at the University of Oslo (Berg, 1963). For several years Lp(a) was considered as a genetic variant of low density lipoprotein (LDL). During his experiments in rabbits to analyze antigenic differences in human beta-lipoproteins by immunizing with human LDL fraction, he noted that in addition to producing antibodies against LDL, the rabbits also produced an antibody against a variant form of LDL that was later named as lipoprotein(a) [Lp(a)]. It was found out that Lp(a) contained more carbohydrate than LDL and had pre-β electrophoretic mobility. It was shown that antigenic determinant of Lp(a) reside in a distinct high molecular weight glycoprotein apolipoprotein(a), designated as apo(a) (Karmansky and Gruener, 1994). Later numerous studies found that Lp(a) is an immunochemically and physicochemically distinct lipoprotein.

Structure of lipoprotein(a)

Lp(a) has two components, a large hydrophilic glycoprotein apolipoprotein(a) [apo(a)] attached to a particle of LDL. Like all lipoproteins, Lp(a) consists of a core of cholesteryl ester and triglyceride molecules and a surface layer made up of phospholipids and unesterified or free cholesterol molecules all enwrapped with a

single molecule of apoB100. A plasminogen like glycoprotein apo(a) is attached to apoB100 of LDL via a covalent disulfide bond. Thus Lp(a) contains apo(a) and apoB100 in 1:1 molar basis (Koschinsky and Marcovina, 2004).

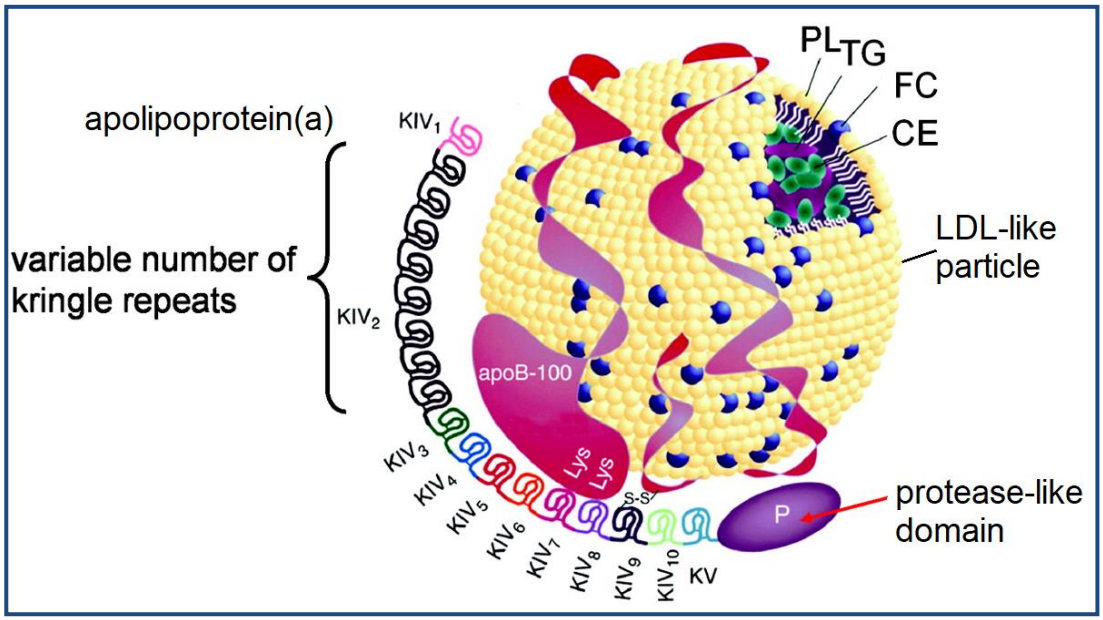


Figure 1. Schematic representation of structure of lipoprotein(a) (Albers *et al.*, 2007)

Structure of apolipoprotein(a) [apo(a)]

The glycoprotein apo(a) confers the unique structural and functional properties on Lp(a). DNA sequence comparisons and phylogenetic analysis indicate that the human apo(a) gene was evolved from plasminogen gene about 40 million years ago during primate evolution. Apo(a) gene is a member of the plasminogen (*PLG*) gene family, and it is located on human chromosome 6q26-27 in close proximity to the plasminogen gene (Frank *et al.*, 1988). The major structural feature of apo(a) is the presence of multiple loop like structures called kringles with structural homology to many proteins in the fibrinolytic system particularly plasminogen. Human apo(a)

gene shares a high sequence homology (78 to 100%) to human plasminogen gene in both the un-translated and coding regions. Apo(a) contains variable number of kringle domains (KIV type 1-10), one KV domain and an inactive protease like domain. KIV types 1 and 3-10 are present as single copies whereas kringle IV type 2 domain (KIV2) is present in variable numbers ranging from 2-50 copies. This repeat polymorphism is responsible for the isoform size heterogeneity of apo(a) among individuals (Karmansky and Gruener, 1994). The adjacent kringles are connected by interkringle regions called as linkers with 26-36 amino acid residues which are highly glycosylated by N-and O-linked sugars.

Kringles domains are non-catalytic cysteine rich internally looped structures stabilized by three internal disulphide bridges. Each kringle consists of approximately of 114 amino acids and containing six cysteines involved in three disulfide bond formation. Kringle domains facilitate the binding to substrates. Certain kringles of apo(a) function as lysine binding sites (LBS). The LBS consists of a trough lined by three aromatic residues flanked on one end by two anionic residues and at the other end by two cationic residues. Of which the KIV type 10 contributes to most of the lysine binding activity of apo(a), but weak LBS are also present in KIV types 5-8. KIV 10 LBS mediates the interaction of Lp(a) with fibrin whereas the KIV5-8 LBS mediate the initial non-covalent interaction between apo(a) and apoB-100 which is necessary for assembly of Lp(a) from apo(a) and LDL (Boonmark and Lawn, 1997).

Glycosylation of apo(a)

Glycans contribute upto 25-28% by mass of apo(a) and contains mannose, galactose, galactosamine, glucosamine, and sialic acid in the ratio 3:7:5:4:7 (Fless *et al.*, 1986).

Apo(a) contained two major N-glycans (~17% of total apo(a) glycans) of complex biantennary type in mono- or disialylated state whereas four to six O-glycans (~80% of total apo(a) glycans) of monosialylated core 1 type are seen at the inter kringle regions which are abundant in serine and threonine-rich peptide sequences (Garner *et al.*, 2001). This O-glycosylation that varies in density widely among individuals is unique to Lp(a) among lipoproteins. O-glycans play an important role in protecting the apo(a) from proteolytic degradation by serine protease. Apo(a) glycans have also role in intracellular processing, maintaining the tertiary structure of apo(a), and preventing aggregation. The hydrophilic apo(a) glycans ensures that the bulk of the apo(a) is extended out into the aqueous phase (Garner *et al.*, 2001).

Apo(a) size heterogeneity

Apo(a) proteins exhibit size polymorphism caused by variable number of kringle IV type 2 repeats in the apo(a) gene. These variable apo(a) sizes are known as apo(a) isoforms. SDS polyacrylamide and agarose gel electrophoresis have been used for the study of apo(a) isoforms and identified 6 apo(a) isoforms and classified them according to their mobility relative to apoB100 and designated as phenotypes F (smaller than apoB100), B (equal in size to apoB100), S1, S2, S3 and S4 (which are larger than apoB100 in different degrees) (Utermann *et al.*, 1987). So far about 34 apo(a) isoforms are identified (Marcovina *et al.*, 1993). The smallest apo(a) isoform contains two copies of KIV 2 whereas the largest apo(a) described so far have 43 KIV 2 repeats (Siekmeier *et al.*, 2010). Therefore the molecular weight of apo(a) ranges from 300 to 800 kDa. Either one or two apo(a) isoforms occur in a single individual. Null alleles with no production of apo(a) was also detected in individuals (Schmidt *et al.*, 2016).

Metabolism of Lp(a)

Assembly of Lp(a) involves a 1:1 stoichiometric association between apo(a) and apoB100 moiety of LDL to form covalent Lp(a) particles. Apo(a) particle is synthesized as a smaller-sized precursor in endoplasmic reticulum and after glycosylation mature form of apo(a) is secreted from liver. Small amounts of apo(a) mRNA have also been detected in testes and brain of rhesus monkeys (Tomlinson *et al.*, 1989). Various studies suggest that assembly of apo(a) with LDL occurs extracellularly on the hepatocyte surface or in plasma (Hoover-Plow and Huang, 2013). Formation of Lp(a) requires a high-affinity non-covalent interaction between apo(a) and apoB-100, where lysine binding sites in apo(a) KIV types 7 and 8 interact with the Lys-680 and 690 in the N-terminal region of apoB-100 (Becker *et al.*, 2001). This initial step can be inhibited by lysine and lysine analogues, such as epsilon-aminocaproic acid (ε-ACA). In the second step of Lp(a) assembly, a disulfide bond is formed between the unpaired Cys-4057 on KIV 9 of apo(a) with the Cys-4326 on carboxy terminus on apoB100 (Scanu and Edelstein, 1997). Majority of plasma apo(a) is covalently linked to LDL, only a very little free apo(a) [3-5%] is found in human plasma (Gries *et al.*, 1987). Binding of apo(a) to LDL occurs in close vicinity of the LDL receptor binding domain thereby yielding a particle with low LDL-R binding.

Lp(a) is not metabolized to other lipoproteins. The catabolic fate of Lp(a), including the precise sites and mechanisms of its degradation remains largely unknown. Turn over studies in rats, rabbits, mice suggested that liver is the major organ involved in Lp(a) degradation, with 50% of the Lp(a) taken up by the liver followed by kidney, spleen and muscles (Kostner and Kostner, 2017). Various reports suggested that

LDL receptor does not significantly involved in Lp(a) catabolism due to the possible masking of apoB recognition sites by apo(a). Other receptors, which can be involved in Lp(a) uptake are VLDL receptor, asialoglycoprotein receptor, megalin receptor, and macrophage scavenger receptor (Albers *et al.*, 2007). Studies reporting that patients with renal failure have increased plasma Lp(a) concentrations suggested a role for the kidney in Lp(a) catabolism (Hopewell *et al.*, 2018). Presence of apo(a) fragments in human urine was also detected. They are of 50-160 kDa in size, glycosylated and contain N-terminal region with KIV-1,-2, and -3 and are not complexed to apoB (Kostner *et al.*, 1996). Possible sites of fragmentation are skeletal muscle, liver and spleen. Neutrophil elastase and MMP-12 can cleave apo(a) between KIV-4 and -5, but at a different positions. Clearance studies in mice suggested that urinary apo(a) fragments are formed extrarenally and are then excreted by the kidney. Fraction of Lp(a) cleared by the kidney accounts only for a maximum of 1% of total plasma apo(a) catabolism.

Plasma Lp(a) concentration

In healthy subjects Lp(a) levels are mainly dependent on the apo(a) isoform type, with those with low molecular weight apo(a) isoforms having high plasma Lp(a) levels and those with high molecular weight isoforms having lower levels of Lp(a). The variation in the apo(a) gene accounts for 70–90% of the variability in plasma Lp(a) concentration in populations, with the kringle IV type 2 repeat polymorphism contributing to approximately half of this effect (Boerwinkle *et al.*, 1992). Thus there exists an inverse relationship between apo(a) size and plasma Lp(a) concentration (Utermann *et al.*, 1987). This is due to differences in the hepatic secretion of apo(a), with subjects carrying small apo(a) isoforms having increased

rate of apo(a) secretion from the liver. Rate of degradation of apo(a) in the endoplasmic reticulum, Golgi apparatus or in the proteasome was lower for apo(a) isoforms with low molecular weight compared to high molecular weight apo(a) isoforms which are strongly bound to hepatocytes (Siekmeier *et al.*, 2010). In vivo studies suggested that rate of Lp(a) production rather than catabolism is the major determinant of plasma Lp(a)concentration.

Plasma Lp(a) concentrations vary over 1000 fold among individuals. Plasma levels are usually between 0.1 and 300 mg/dl and are highly heritable and remarkably stable in any given individual. Lp(a) levels are relatively low at birth and gradually increase to adult levels over the first few months of life (Van Biervliet *et al.*, 1991). Levels are unaffected by age, sex, diet or by the levels of other lipoproteins (Jenner *et al.*, 1993). There is a large interindividual and interpopulation differences in average Lp(a) levels. In Caucasians and Chinese, the distribution of plasma Lp(a) is highly skewed towards lower levels. However, in Africans and African Americans, the distribution curve of plasma Lp(a) levels has a more Gaussian shape. African Americans have 2-3-fold higher plasma concentrations of Lp(a) than either Caucasians or Chinese (Kraft *et al.*, 1996).

Functions of Lp(a)

Despite of intensive research the physiological function of Lp(a) or apo(a) is still unknown. Individuals without Lp(a) or with very low Lp(a) levels seem to be healthy. Thus plasma Lp(a) may be certainly not vital at least under normal environmental conditions. There are several different human proteins and peptides whose biological functions are yet not clear, however it doesn't necessarily means that they are useless. This might be particularly true in the case of Lp(a). Some

studies reported beneficial effects of Lp(a) which may depend on external circumstances.

Several lines of biological evidence suggested a role for Lp(a) in promoting healing of wounds and repair of tissue injuries (Lippi and Guidi, 2000). During the acute phase response to a vascular injury, several mediators including interleukin-6 (IL-6) are released which enhance the hepatic synthesis of apo(a) as apo(a) gene contains several IL-6 responsive elements, which leads to accumulation of newly formed Lp(a) at the site of vascular injury (Ramharack *et al.*, 1998). Apo(a) of Lp(a) can be recognized by several receptors on the surface of macrophages, fibroblasts and platelets. Lysine binding sites of apo(a) mediates the binding of Lp(a) to vascular wall (Hughes *et al.*, 1997) and endothelial matrix components and apo(a) bound to the fibrin surface inhibits lysis of the clot. Lp(a) displays growth-factor-like properties, promoting the growth of human umbilical vein endothelial cells (hUVECs) and enhancing the human vascular smooth cells (hVSMCs) proliferation in culture by inhibiting the activation of transforming growth factor-β (Grainger *et al.*, 1994). Growth factor like properties of Lp(a) promotes tissue repair and cell regeneration is ensured by the tissue release of large amount of cholesterol carried by Lp(a).

According to the classic unified theory, Lp(a) is regarded as a surrogate for ascorbate (Vitamin C) as in human occlusive CVD which is a degenerative condition induced by chronic vitamin C deficiency, extracellular deposition of Lp(a) represents a biological defensive mechanism (Rath and Pauling, 1990). Various studies in animal models suggested that adequate amount of vitamin C prevented the accumulation of Lp(a) in the arterial wall, Lp(a) could contribute to the

strengthening of extracellular matrix during vitamin C deficiency and elevated Lp(a) level could be decreased by dietary supplementation with vitamin C, but there were no other reliable clinical or biological evidence regarding the biological relationship between Lp(a) ad vitamin C and this hypothesis demands further investigation (Lippi and Guidi, 2000).

Recently numerous evidences regarding the anti-tumor properties of Lp(a) are emerging. It was reported that individuals with high circulating Lp(a)/smaller Lp(a) phenotype are relatively less prone to cancer than their large Lp(a) counterparts (Sawabe *et al.*, 2012). Kringles produced by apo(a) degradation showed anti-angiogenesis and anti-tumor properties in animal studies and in in vitro studies (Lippi *et al.*, 2007). But these experimental studies supporting the anti-neoplastic effect of apo(a) are challenged by the growth factor-like functions of Lp(a) on endothelial and smooth muscle cells. Moreover systemic administration of intact kringle-containing proteins does not inhibit the growth of primary tumors and neo vascularization (Cao *et al.*, 1997). Hence the possible relationships between anti-angiogenic effect of Lp(a) and protection against tumor require further experimental investigation.

Several studies suggested an association between Lp(a) and longevity. These studies reported the presence of higher Lp(a) levels among centenarians (Thillet *et al.*, 1998, Panza *et al.*, 2007). A sheer in blood flow produced by frequent physical activity results in extended open type conformation of apo(a) from the Lp(a) particle. This may facilitates the enzyme degradation of apo(a) protein to generate kringle-containing degradation products with anti-cancer properties which might contribute to longevity (Hsieh Wu, 2011).

Pathophysiological aspects of Lp(a)

Large number of epidemiological studies, meta-analyses and genetic studies suggested that elevated Lp(a) concentration which is associated with low molecular weight apo(a) phenotype is an independent risk factor for cardiovascular events including coronary artery disease, atherosclerosis, myocardial infarction, stroke and peripheral vascular diseases (Berglund and Ramakrishnan, 2004, Malaguarnera *et al.*, 2013). Though Lp(a) concentration in circulation is nearly one twentieth of that of LDL, atherosclerotic plaques have been found to contain more Lp(a) than LDL (Pepin *et al.*, 1991). However the molecular mechanisms of Lp(a) sequestration into vascular tissue remains unclear. Many studies have suggested that serum level of Lp(a) >30 mg/dL is associated with CVD risk. Individuals with both elevated level of Lp(a) plus LDL cholesterol were at 10-fold or higher risk of MI. Elevated Lp(a) was proved to be an important CHD risk factor ranking 5th behind LDL-cholesterol, family history of myocardial infarction, fibrinogen, and HDL-cholesterol (Cremer *et al.*, 1994).

Various studies proposed a proatherogenic/thrombotic role for Lp(a). Lipoprotein(a) is believed to promote atherosclerosis by a number of separate but related mechanisms. Lp(a) induces the expression of adhesion molecules such as intercellular cell adhesion molecule (ICAM)-1, E-selectin, and vascular cell adhesion molecule (VCAM) which play a key role in leukocyte adhesion and migration. Lp(a) is reported to promote IL-8 and plasmin mediated inflammation, extracellular matrix degradation and rupture, foam cell formation and smooth muscle proliferation and inhibit nitric oxide synthesis leading to endothelial dysfunction (Deb and Caplice, 2004).

Lipoprotein(a) may also promote a thrombotic state by inhibition of the fibrinolytic system and enhancement of the tissue factor-mediated pathway of thrombosis (Edelberg and Pizzo, 1995). Because of the structural homology between apo(a) and plasminogen, Lp(a) was shown to inhibit competitively the binding of plasminogen to its endothelial receptor as well as to fibrinogen or fibrin, which might reduce the surface-dependent activation of plasminogen leading to impaired fibrinolysis (Miles and Plow, 1990). Small apo(a) isoforms are found to be more thrombogenic as small size apo(a) isoforms display high affinity binding to fibrin (Anglés-Cano *et al.*, 2001).

Lp(a) acts as main scavenger for proinflammatory and proatherogenic oxidized phospholipids (OxPLs) in human circulation. Both in vitro and in vivo studies demonstrated that majority of OxPLs travel on Lp(a) by binding to the KV domain of apo(a) (Bergmark *et al.*, 2008). Modification of apo(a) by OxPLs is responsible for Lp(a) mediated IL-8 expression by macrophages. Further Lp(a) and OxPLs induce macrophage apoptosis leading to plaque inflammation and instability (Berglund and Ramakrishnan, 2004).

Apart from its established role as a cardiovascular risk factor, studies have shown that elevated Lp(a) concentration is an independent risk factor for Alzheimer's disease (Solfrizzi *et al.*, 2002). Abnormally elevated plasma concentration of Lp(a) was observed as secondary to various diseases. Increased plasma Lp(a) concentration was observed in patients with nephrotic syndrome and in patients with end-stage renal disease (Siekmeier *et al.*, 2010). There are conflicting reports on the association between Lp(a) levels and diabetes mellitus and it is mainly due to insufficient sample size used in studies. Only a small change in plasma Lp(a)

concentration was observed in patients with type 1 diabetes mellitus compared to healthy individuals. Large studies and those including apo(a) phenotype analysis reported that in type 2 diabetes mellitus Lp(a) levels are not or only moderately elevated in patients compared to controls (Kronenberg *et al.*, 1996). A decreased plasma Lp(a) concentration was observed in patients with liver cirrhosis, liver failure and with different acute or chronic viral liver infections. But there are conflicting reports for patients with hepatocellular carcinoma who showed decreased as well as increased plasma concentrations of this lipoprotein (Siekmeier *et al.*, 2010).

Interaction of Lp(a) with LDL to form Lp(a)-LDL complexes

Lp(a) undergoes insoluble complex formation in the presence of physiological Ca^{2+} concentration by forming cross-bridges between different Lp(a) particles which is mediated by sialic acids on apo(a) which have high binding affinity for Ca^{2+}. Even though sialic acid is present on other lipoproteins they did not form insoluble complexes with Ca^{2+} which could be due to the higher sialic acid content of Lp(a) compared with other lipoproteins and due to the unique flexible extended open type conformation of apo(a) (Yashiro *et al.*, 1993).

Lp(a) was reported to form non-covalent complexes with LDL. Trieu *et al* first reported that Lp(a) can form complexes with LDL which is mediated by the interaction between kringle IV domains of apo(a) and proline residues on apoB in LDL (Trieu *et al.*, 1991). Later it was reported that Lp(a) can form insoluble immune complexes with LDL at physiological Ca^{2+} concentration. The interaction between Lp(a) and LDL results from the formation of Ca^{2+} cross-bridges between sialic acid on separate molecules, which appeared to be ionic in nature since the

increase in positive charge on LDL by desialylation increased this interaction. Due to the strong surface negative charge acetyl LDL interact more avidly with Lp(a) than native LDL at physiologic concentrations of Ca^{2+} (Yashiro *et al.*, 1993). This suggested that at physiologic Ca^{2+} concentration the interaction between Lp(a) and LDL especially its chemically modified forms such as oxidized LDL could contribute to the formation of insoluble complex of Lp(a) with LDL in atherosclerotic lesions leading to its preferential accumulation in the arterial wall. Several in vitro studies reported the presence of Lp(a) which is non-covalently bound to triglyceride-rich lipoproteins (Lp(a)-TRL complex) in hypertriglyceridemic subjects (Marcoux *et al.*, 1997) and Lp(a) with higher molecular weight apo(a) isoforms (i.e. > 500 kDa) appear to be preferentially form Lp(a)-TRL complexes both in native and reconstituted forms (Gaubatz *et al.*, 2001).

Recently it was reported from our laboratory that Lp(a) molecules in circulation attach additional molecules of LDL non-covalently to form adducts. Results revealed that most Lp(a) molecules in circulation are in adduct form and serine- and threonine-rich peptide sequences (STPS) in the O-glycan rich region of apo(a) in Lp(a) is involved in its adduct formation with LDL. Larger Lp(a) molecules with longer STPS-containing regions attach more LDL molecules than small Lp(a) molecules and the increase in number of LDL molecules is out of proportion with the increase in size of STPS-containing region of apo(a) (Kalaivani and Appukuttan, 2014).

2.3 Natural anti-carbohydrate antibodies

Natural antibodies (NAbs) are usually defined as antibodies circulating in normal individuals and are produced in the absence of stimulation by any exogenous antigen (Shoenfeld and Isenberg, 1989). Carbohydrates in the form of complex oligo and polysaccharides are predominant cell surface components in all three domains of life viz. archaea, bacteria and eukaryotes. Carbohydrate antigens enter the human body through diet, environment and microbes and offer antigenic epitopes thereby giving rise to anti-carbohydrate antibodies which react with polysaccharide antigens (Huflejt *et al.*, 2009). The identification of carbohydrate antigens by antibodies was evident from the studies on pneumococci which when grown in fluid media was precipitated specifically with anti-sera against pneumococcus (Dochez and Avery, 1917). Further it was identified that a polysaccharide and not a protein derived from bacterial surface caused precipitation (Heidelberger and Avery, 1924). The presence of anti-carbohydrate antibodies was also evident from the presence of antibodies against ABO blood group antigens which are also carbohydrates. A well equipped repertoire of anti-carbohydrate antibodies forms the first line of defense against invading pathogens, blood group antigens and xenoantigens (Cheng, 1998). They form a part of the house keeping functions by facilitating the removal of dysfunctional or malignant cells and cellular debris and a role for anti-carbohydrate antibodies in anti-tumor surveillance was also reported (Vollmers and Brändlein, 2007, Schwartz-Albiez, 2012). Since these antibodies are produced without any antigenic challenge they belong to the class of natural antibodies and form a part of the innate immune system. Some of them are found to be autoreactive because of

their ability to bind to self structures (Boes, 2000). These immunoglobulins belong to IgG, IgM, IgA, and IgD subtypes.

Examples of natural anti-carbohydrate antibodies include antibodies directed against blood group antigens A and B, anti-Gal antibodies directed against Galα1-3Galβ1-4GlcNac epitope, lactose-binding immunoglobulins (LIg), dextran-binding immunoglobulins (DIg), anti-β-glucan (ABG) antibodies, antibodies to tumor associated antigens such as Galβ1-3GalNAcα (TF, Thomson-Friedenreich antigen) and GalNAcα1-OSer/Thr (Tn antigen) (Huflejt *et al.*, 2009).

Anti-carbohydrate antibodies are classified into three groups conservative, allo and plastic based on the serum content, ligand specificity and binding properties (Bovin, 2013). Conservative antibodies remain the same in all healthy individuals with respect to epitope specificity and serum levels. Allo-antibodies occur naturally against foreign antigen from a person of the same species e.g. anti-blood group antibodies. Plastic antibodies vary among individuals depending on the disease states and they serve as potential markers for disease conditions e.g. anti-T antibody.

Origin of anti-carbohydrate antibodies

There is both a bacterial paradigm theory (Springer and Horton, 1969) and germline theory (Coutinho *et al.*, 1995) explaining the occurrence of natural antibodies. Natural anti-carbohydrate antibodies including those against blood group antigens are not found during first weeks of life. It was shown that anti-blood group A and B antibodies could be produced by immunological stimulus using appropriate antigens. When E.coli O strains were fed to individuals with blood group O and A there was

significant increase in anti-B antibodies. Based on these observations bacterial paradigm hypothesis proposed that anti-carbohydrate antibodies appear in response to stimulation of the immune system by bacterial O-antigens and lipopolysaccharides of gastrointestinal bacteria.

Natural antibodies are encoded by their genes in germline configuration and are not subjected to somatic hypermutation and affinity maturation (Siminovitch *et al.*, 1989). Germ line theory proposed that since natural antibodies are present in normal individuals, cord blood and antigen-free mice anti-carbohydrate antibodies derive from the pool of natural antibodies produced during fetal life, without stimulus by any exogenous antigen.

B-cell response to polysaccharide antigens

Immune response against carbohydrate antigens are characterized by lack of T-lymphocyte memory, isotype restriction and delayed ontogeny (Weintraub, 2003). Production of antibodies against polysaccharide antigens and not to protein antigens in athymic mice and T-cell defective mouse models suggested that polysaccharide antigens are thymus independent (Bos *et al.*, 1989). These antibodies are produced by splenic marginal zone B2-lymphocytes and B1 type of the peritoneal and pleural cavities which are characterized by the presence of CD5 (Hoffman *et al.*, 2016) These cells form only a minor fraction in spleen and lymph nodes but form the majority of B-cells in peritoneum and pleural cavity. B1-cells are further classified into B1a and B1b-cells of which B1a-cells produce natural antibodies which are stimulated by the danger signals as part of the innate immune system, B1b-cells and splenic marginal zone B2-lymphocytes are mainly responsible for the production of

anti-carbohydrate antibodies. Even though plasma cells are mainly produced by the marginal zone B-cells, peritoneal B1b-lymphocytes are also capable of producing plasma cells and memory cells contributing to the long lasting production of anti-carbohydrate antibodies (Foote and Kearney, 2009). Affinity maturation occurs mainly in B2-cells, but peritoneal B1-cells mainly of IgA and much less of IgM antibodies of B1b-cells are also reported to undergo somatic hypermutations (Roy *et al.*, 2009). Although anti-carbohydrate antibodies are predominantly of IgM type (Avrameas, 1991), presence of IgG type of anti-carbohydrate antibodies with somatic hypermutation in V gene suggests that they can also be generated by an antigen-driven process.

Properties of anti-carbohydrate antibodies

Most anti-carbohydrate antibodies are polyreactive but with distinct fine specificities which is required for the rapid and immediate recognition and protection against invading pathogens and monospecific antibodies are also present (Notkins, 2004). These polyreactive antibodies have low affinity as compared to monospecific antibodies which is compensated by their multivalent nature. Ligand binding pocket of these antibodies is more flexible which are characterized by the presence of several arginine and lysine residues in their complementarity determining regions which facilitates formation of salt bridges between antibody and negatively charged groups on the surface of antigens (Avrameas, 1991). This salt bridge formation followed by conformational changes lead to a more intimate contact between the antigen and the antibody. Levels of these antibodies remain practically unchanged during life time and their repertoire is conservative.

Anti-β-glucoside antibody (ABG)

β-Glucans are long chain polymers of glucose in β(1→3) and β(1→6) linkages. They are known for long as plant constituents and as major components of the cell walls of plants, fungi, and some pathogenic bacteria including Streptococcus mutans and are absent in animals including humans. Human exposure to β-glucan occurs through breathing, fungal infections and systemic administration of β-glucan containing pharmaceuticals. These microbial or dietary antigens can trigger synthesis of anti-β-glucoside antibody. ABG recognizes β-linked glucose moieties and binds to β1→4 linked (cellulose), β1→3 linked and β1→6 linked glucans (yeast and fungal glucans) (Masuzawa *et al.*, 2003).

ABG was isolated from human plasma by affinity chromatography on cellulose and were found to contain three times higher IgA content compared to IgG and substantially higher polymeric IgA (mostly IgA1) than total serum immunoglobulins. ABG which is mainly of IgA type bind to commonly encountered microbial and dietary antigens and form immune complexes which have inflammatory potential (Geetha *et al.*, 2007). ABG in normal, healthy Italian adults consists of mostly IgG2 (Chiani *et al.*, 2009). The titre of ABG differs significantly in the sera from healthy human volunteers and was significantly decreased in patients with deep mycosis (Ishibashi *et al.*, 2005) and ABG forms immune complexes with fungal and yeast cell wall β-glucan. Plasma β-glucan concentration in normal individual is less than10 pg/ml and was found to increase to 1 ng/ml in fungal infections (Obayashi *et al.*, 1995). ABG plays a role in host defense against pathogenic fungi.

Anti-dextran antibody (DIg)

Dextrans are polymers of D-glucose units with α(1→6) linkages in main chains and α(1→2), α(1→3) and α(1→4) linkages in branches and are present as contaminants in sugar, as bacterial extracellular polysaccharides, as a component of bacterial biofilms. Dextrans are also synthesized from sucrose by Lactobacillus and Leuconostoc and are present in dental plaques and dental caries also. These persistent sources of antigenic epitopes cause synthesis and maturation of DIg (Kabat and Berg, 1953). Anti-dextran antibodies with high affinity for α-linked glucose and dextran are present in healthy donors in substantial titres (Chacko and Appukuttan, 2003). DIg isolated from human plasma consists mostly of IgM and IgG and enriched in polymeric IgA. DIg is multispecific and recognize α(1→6) and β(1→3) linked glucose and α(1→3) linked galactose in natural glycoconjugates (Paul *et al.*, 2009).

Presence of DIg containing immune complexes has been reported in several pathological conditions. DIg of IgG and IgA type were found to be increased in IgA nephropathy patients (Kennel *et al.*, 1995). IgG and to a lesser extent IgM DIg was detected in liver cirrhosis and chronic hepatitis. High level of DIg of IgG type in patients with gastric and duodenal ulcers, ulcerative colitis, Crohn's disease, colorectal carcinomas and rheumatoid arthritis was reported (Palosuo and Milgrom, 1981). Normal serum glucose concentrations (4-5 mM) can hardly inhibit antigen binding by DIg whereas in diabetic serum glucose concentration reaches upto 20 mM, which competitively inhibits DIg activity. This can make the individual susceptible to infections (Chacko and Appukuttan, 2003).

Lactose-binding immunoglobulin (LIg)

Antibodies specific to lactose were produced upon injection of lactose (Gal β1-4Glc) bound to carrier protein, into mice (Tsukasa *et al.*, 1987). Terminal N-acetyl lactosamine (Galβ1-4GlcNAc or LacNAc) is the ligand for LIg in glycoconjugates (Gupta *et al.*, 1996). LIg exhibits extended specificity towards terminal α- and β-galactoside groups. LIg isolated from plasma by affinity chromatography consisted of mainly IgG followed by IgA and IgM. Biological role of LIg remains unexplored. LIg levels were reported to be elevated in patients of lung carcinoma (Dong *et al.*, 1997). LIg has been shown to form LacNAc-dependent immune complexes (ICs) with desialylated human lipoprotein(a) (Sabarinath *et al.*, 2014). Tissue deposition of these ICs can be a possible mechanism for Lp(a) deposition on vessel walls in CHD patients.

Anti-T antibody

Antibodies against T antigen i.e. desialylated core 1 O-glycans (Galβ1-3GalNAc) are present in the sera of all individuals (Springer, 1984). These antibodies are absent in cord blood and they develop only during the first year of life and levels remain unaltered in healthy individuals throughout life. Anti-T antibodies consist mainly of IgM followed by IgA and IgG and its synthesis was elicited by bacteria of the intestinal flora. In normal tissues T-antigen is masked by sialic acid and altered glycosylation leads to exposure of these antigens in about 90% of carcinomas (Springer, 1997) and anti-T titre in serum decreased with tumor proliferation and was restored rapidly following tumor removal so that its titre is an indicative of tumor burden (Desai *et al.*, 1995).

2.4 Anti-α-galactoside antibody (anti-Gal)

Anti-α-galactoside antibody (anti-Gal) is the naturally occurring anti-carbohydrate antibody in humans constituting ~1% of circulating IgG. It interacts specifically with the terminal α-galactosyl epitope, Galα1-3Galβ1-4GlcNAc-R present on the carbohydrate chains of glycoproteins and glycolipids (Galili *et al.,* 1984). It recognizes terminal α-1,3 and α-1,6 linked galactose moieties in glycoconjugates. Approximately 1% of human B-cells produce anti-Gal, a small proportion of B-cells in the lymphoid tissues along the gastrointestinal tract produce this antibody continuously against antigenic stimulation by bacteria of normal flora (Galili *et al.,* 1988). Most of the anti-Gal B-cells in spleen and lymph nodes are quiescent. Anti-Gal is predominantly of IgG subtype, with traces of IgM and IgA in contrast to IgM domination in the case of most other carbohydrate-specific circulating antibodies. Anti-Gal heavy chain in humans is encoded by several closely related genes indicating the polyclonality of anti-Gal (Wang *et al.*, 1995). Even though it is polyclonal, anti-Gal specifically interacts with α-gal epitopes on glycoconjugates. Presence of somatic hypermutations in human VH genes encoding anti-Gal antibodies suggests that these antibodies could be synthesized by B-cells in a T-cell dependent mechanism (Wang *et al.*, 1995, Cretin *et al.*, 2002)

Anti-Gal reactive epitopes are present in humans on normal senescent red blood cells, thalassemic and sickle cell RBCs (Galili, 2013). The presence of anti-Gal reactive epitopes in human brain grey matter was also reported (Jaison *et al.*, 1993). Later the presence of antibodies reactive against terminal α-gal epitope was reported in atherosclerotic plaques in humans (Mosedale *et al.*, 2006). Our

laboratory demonstrated that anti-Gal antibody binds to serine- and threonine-rich peptide sequences (STPS) present in the heavily O-glycosylated regions of apolipoprotein(a) of human lipoprotein(a) forming circulating immune complexes (Geetha *et al.*, 2014).

Evolutionary appearance of anti-Gal antibody

Anti-Gal is an evolutionary milestone as it occurs only in advanced mammals such as man, apes and old world monkeys which lack α-gal epitopes (Galili and Andrews, 1995). Development of anti-Gal in the above animals occurred along with suppression of synthesis of the enzyme α1,3-galactosyl transferase (α1,3GT) that is needed for the synthesis of anti-Gal reactive epitope Galα1-3Galβ1-4GlcNAc-R. A reciprocal situation exists in non-primate animals, promisians and new world monkeys, characterized by lack of anti-Gal antibody but possess anti-Gal reactive terminal α-galactosyl epitope. Inactivation of α1,3GT gene in above animals occurred approximately 20-30 million years ago followed by production of anti-Gal antibodies reactive against the α-gal epitope (Galili and Swanson, 1991).

A possible reason for the inactivation of α1,3GT gene in ancestral Old World primates could have been associated with epidemics of an infectious agent that expressed α-gal epitopes in the Old World. Such an evolutionary scenario could have induced the inactivation of α1,3 GT gene and producing anti-Gal antibody as a means of defense. It is reported that anti-Gal could provide immune protection against several pathogens, including enveloped viruses, bacteria and protozoa as they were found to express α-gal epitopes and so can be destroyed by anti-Gal (Galili, 2013). An alternative scenario for the inactivation of α1,3GT gene could be

an infection by bacteria or protozoa that used α-gal epitopes in these primates as docking receptors. Ultimately, any of these evolutionary processes result in extinction of Old World primates synthesizing α-gal epitopes and they were replaced by offspring populations lacking α-gal epitopes and producing the anti-Gal antibody. New World monkeys and lemurs were protected from pathogens of the Old World by oceanic barriers, thus they continue to synthesize α-gal epitopes and lack the ability to produce the anti-Gal antibody (Galili, 2005).

Pathophysiology of anti-Gal antibody

The physiological role of anti-Gal antibody is not clear yet. It was postulated that anti-Gal antibody may participate in the removal of normal and pathologically senescent red cells by binding to the cryptic α-galactosyl epitopes leading to the destruction of senescent red cells by reticuloendothelial system (Galili, 2013). Anti-Gal is a major immunological barrier against xenotransplantation of organs into humans and monkeys. In vivo binding of anti-Gal to α-gal epitopes on xenograft cells induces rapid rejection of such grafts as a result of complement activation and antibody dependent cell mediated cytotoxicity (Galili, 2005).

Anti-Gal also contributes to several immunological pathogeneses. Occasionally anti-Gal of IgE type was produced in individuals due to isotype switching, which cause allergies by binding to α-gal epitopes. In cancer patients treated with monoclonal antibody cetuximab carrying α-gal epitopes on its Fab, anti-Gal IgE binding to this epitope induces a systemic allergic reaction (Chung *et al.*, 2008). Anti-Gal IgE produced in some individuals also causes allergies to red meat presenting α-gal

epitopes (Commins *et al.,* 2009). Aberrant expression of α-gal epitope has been found on human cells and the interaction of anti-Gal with such epitopes may result in autoimmune reactions. In patients with Graves' disease anti-Gal IgG titre is elevated which may be due to aberrant expression of α-gal epitopes on Graves' disease thyrocytes and binding of this antibody to the aberrantly expressed epitopes results in excessive stimulation of these thyrocytes (Galili, 2013). An increase in anti-Gal titre was also reported in autoimmune diseases such as Henoch-Schonlein purpura and in Crohn's disease (Davin *et al.,* 1987, D'Alessandro *et al.,* 2002)

In Chagas' disease and American cutaneous leishmaniasis, caused by *Trypanosoma cruzi* and *Leishmania mexicana* respectively, the serum anti-Gal titre was found to be markedly increased. Anti-Gal antibody interacts with the α-gal epitopes on glycoinositolphospholipids and lipophosphoglycans produced by *Trypanosoma cruz*i and induces complement-mediated lysis of this pathogen. However the intracellular parasites escape recognition by anti-Gal and continue to produce α-gal epitopes which interact with anti-Gal and induce autoimmune like inflammatory reactions in Chagas' disease (Avila, 1999, Galili, 2013).

Because of its abundance in humans, anti-Gal antibody may be exploited for various clinical uses. Anti-Gal antibody can be used as an endogenous adjuvant for the effective targeting of vaccines to antigen presenting cells (APCs) by exploiting its interaction with the ligand α-gal epitope. Anti-Gal mediated enhancement in the immunogenicity of viral and microbial vaccines expressing α-gal epitopes by effective targeting to APCs was well established with studies in α1,3GT knockout mice (mice that lack α-gal epitopes and produce anti-Gal antibody similar to

humans). α1,3GT knockout mouse was immunized with influenza virus vaccine or gp120 vaccine of HIV engineered to express α-gal epitopes. Anti-Gal forms immune complexes with the α-gal epitopes on vaccines and induces effective uptake by APC via Fc/Fcγ receptor interaction (Abdel-Motal *et al.*, 2009).

Healing of wounds and burns may be accelerated by applying α-gal liposomes or α-gal nanoparticles to the injured tissue (Hurwitz *et al.*, 2012). The binding of anti-Gal antibody to α-gal epitope activate complement, and recruit and activate macrophages that induce tissue regeneration. Preliminary studies in mice with chemically induced diabetes impaired wound healing suggest that α-gal nanoparticles can induce healing of wound (Galili *et al.*, 2010). This therapy has further significance in regeneration of ischaemic myocardium and of injured nerves.

Use of anti-Gal-α-gal epitope interaction in cancer immunotherapy

Currently available treatment strategies fail to completely eliminate all tumor cells in many cancer patients, and this raises the need for an active immunotherapy treatment to achieve complete remission. Since for most types of cancer the identity of the multiple autologous tumor associated antigens (TAA) is not known, the tumor itself serve as a source for vaccinating TAAs and for a successful vaccination, it should be targeted to APCs for effective uptake, processing and presentation of antigen.

Autologous tumor cells or cell membranes in cancer patients can be engineered to express terminal α-galactosyl epitopes and can be effectively targeted to APCs by exploiting the binding of anti-Gal to these epitopes on vaccinating tumor cells

(Galili, 2005). This can be achieved by mimicking in vitro the natural synthesis of α-gal epitopes by α1,3GT enzyme. Tumor cells for vaccine preparation can be obtained easily from patients having hematological malignancies and in those with solid tumors, the tumor obtained from the surgery should be homogenized, washed and subjected them to the enzymatic reaction (LaTemple *et al.,* 1999). Such a synthesis of α-gal epitopes was achieved on cell membranes of mammary, colon, and ovarian carcinomas in humans. Adenovirus mediated transfection with α1,3GT gene can also lead to expression of α-gal epitope on tumor cells (Deriy *et al.,* 2005). Anti-Gal IgG opsonizes the processed autologous tumor vaccines expressing the α-gal epitopes. Then the interaction of Fc portion of anti-Gal with Fcγ receptors of APCs leads to its effective internalization and also induces dendritic cell maturation. APCs then process and present the antigens for the activation of tumor antigen specific T-cells to produce a systemic anti-tumor immune response.

Efficiency of autologous tumor vaccines with α-gal epitopes was studied in the animal model of α1,3GT knockout mouse. For this mouse was challenged with the highly immunogenic mouse melanoma cell line BL16 engineered to express α-gal epitopes. Vaccination with these cells elicited an immune response that prevented tumor growth after challenge with live cells lacking this epitope (LaTemple *et al.,* 1999). Similar protection was achieved in mice with pre-existing melanoma after immunization with tumor cells expressing α-gal epitopes (Rossi *et al.,* 2005). Similar efficacy was also observed in α1,3GT knockout mouse bearing pancreatic adenocarcinoma, vaccinated with tumor cells expressing α-gal epitopes (Deguchi *et al.,* 2010).

A phase III clinical study is designed to conduct in pancreatic cancer patients after surgery (NCT01072981) to assess survival after treatment with a regimen of Gemcitabine alone or with 5-fluorouracil with immunotherapy using Hyper-Acute Pancreas (Algenpantucel-L), containing two allogeneic cancer cell lines expressing α-gal epitopes after in vitro processing (Tanemura *et al.*, 2013).

Another approach is intratumoral injection of α-gal glycolipids into tumors and subsequent binding to anti-Gal antibodies leads to destruction of tumor lesions and converting them to endogenous vaccines. Pre-clinical studies indicated that when injected into tumors, α-gal glycolipids get inserted into the membranes of tumor cells and resulted in the expression of α-gal epitopes on tumor cells. This epitopes then interacts with anti-Gal and target tumor cells to APCs. Efficacy of intratumoral injection of α-gal glycolipids was studied in α1,3GT knockout mice having B16 melanoma which is devoid of α-gal epitopes. Injection of α-gal glycolipids into the tumor prevented growth at the challenged sites and also at different sites challenged with B16 cells (Galili *et al.*, 2007). Intratumoral injection of α-gal glycolipids as an immunotherapy treatment for patients with advanced stages of malignant tumors was studied in a Phase I safety assessment trial (IND12946). Increased survival rate was found in patients with tumors such as renal and pancreatic carcinomas whereas in others no significant change in survival was observed (Whalen *et al.*, 2012).

2.5 Affinity maturation of antibodies

The functionality of an antibody can be best described by two properties that define antibody-antigen associations: affinity and avidity. Affinity is the specific strength of binding between an antigen and antibody, while avidity gives a measure of strength of total antigen- antibody complex. Low affinity antibodies are involved in the initial immune response (Eisen and Siskind, 1964) but the affinity increases as the response progresses, particularly after secondary challenge (Steiner and Eisen, 1967). Affinity maturation is basically a Darwinian selection process by which B-cells increase their affinity for a particular antigen. This fine tuning of B-cell specificity involves repeated process of somatic hypermutation of immunoglobulin genes in B-cells and subsequent clonal selection which results in B-cells encoding high affinity antibodies necessary for an efficient and effective immune response, and is a remarkable property of adaptive immunity and B-cell biology (Nossal, 1992).

Affinity maturation occurs within the germinal center where positive selection for B-cells with the highest antigen affinity and apoptosis of B-cells with lower affinity or autoreactivity occurs. Finally, the germinal center reaction produces two types of cells, memory B-cells and antibody secreting plasma cells.

Process of affinity maturation in the germinal center

During the first week of primary immune response antigen specific antibodies are produced by the foci of antibody forming cells in the T-cell areas along the periarteriolar lymphoid sheath which first secrete IgM type (Smith *et al.*, 1997).

Subsequently isotype switching leads to the production of IgG type (Nossal and Riedel, 1989). Low affinity antibodies which are secreted by these B-cells are encoded by V gene segments that are not subjected to somatic hypermutation (McHeyzer-Williams *et al.*, 1993).

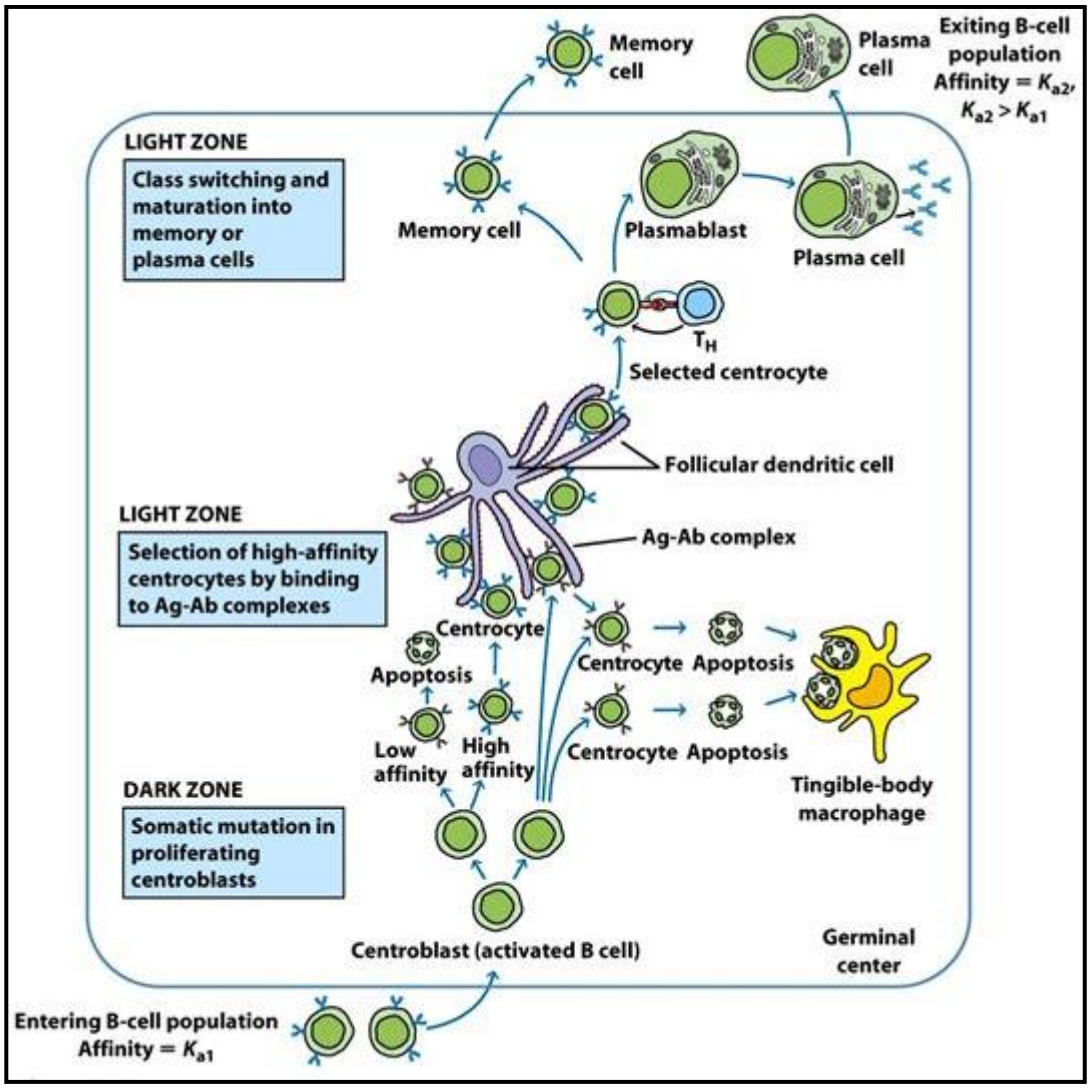


Figure 2. Cellular events within germinal centers (Kindt *et al.*, 2007)

Germinal centers develop in the B-cell follicles of secondary lymphoid tissues during T-cell dependent (TD) antibody response. Upon encounter with an immunogen, B-cells with receptors or membrane bound antibodies that recognize

the immunogen become activated. The activated B-cells, associate with follicular dendritic cells (FDCs) and antigen specific T helper cells (Th cells) and form a germinal center (GC) inside the B-cell follicle. The GC is where the affinity maturation, proliferation, and plasma and memory cell differentiation takes place (MacLennan, 1994).

The GC is divided into two functionally distinct regions: the dark zone (DZ) and light zone (LZ). B-cells undergo several rounds of proliferation, generally 5 or 6 cycles, in the dark zone , while selection of the most active B-cell clones occurs in the light zone which is characterized by the presence of follicular dendritc cells with bound antigen (Victora and Nussenzweig, 2012). During affinity maturation, B-cells traffic between the two zones. Alternating phases of division and mutation occurs in the dark zone while affinity-dependent selection by antigen takes place in the light zone, for optimization of antibodies (Kepler and Perelson, 1993).

In the dark zone, as B-cells are replicating somatic hypermutation is initiated to increase diversity of B-cell receptors (potentially the binding affinity) through the introduction of point mutations in the variable region genes. Following the B-cell expansion, the B-cells migrate into the light zone where Th cells and the FDCs reside and provide presentation of antigen (FDCs) and survival signals (Th cells) to the highest affinity B-cell clones. On encounter with FDCs which have antigen trapped, generally in immune complexes on their cell surface, capture antigen from the FDC surface depending on the B-cell receptor affinity for the antigen. B-cells carrying the receptors with highest affinity for the antigen are able to capture it more efficiently. B-cells then process the antigen and present it through MHCII to

follicular T helper cells (Meyer-Hermann *et al.*, 2012). B-cells that can most efficiently capture, process, and present the antigen will receive the survival signals from T helper cells for the perpetuation of that B-cell clone (Schwickert *et al.*, 2011). Class switch recombination (CSR) results in isotype switching and takes place in the light zone based on cytokines produced by the Th cells. The B-cells that fail to compete for the antigen or fail to receive survival signals from T helper cells undergo apoptosis, leaving the highest affinity B-cell clones to move forward. Approximately 90% of selected B-cells return to the dark zone and repeat the cycle, while the remaining 10% escape from the GC and differentiate into either memory B-cells or plasma cells (Tarlinton and Smith, 2000). As a result of several rounds of the expansion, mutation, and selection cycle, high affinity B-cell clones and antibodies which are highly mutated from their naive precursors are generated.

Various selection mechanisms contributing to affinity maturation in GCs includes competition for FDC binding sites, time interval between two attempts to bind antigen (Meyer-Hermann *et al.*, 2012), and masking of FDC-bound antigen by soluble antibodies (Tarlinton and Smith, 2000) and survival signals from follicular helper T-cells (Victora *et al.*, 2010). These alternating processes of somatic hypermutation and selection in the germinal center continue with sustained or subsequent antigen exposures leading to the generation of antibody repertoire with highest the affinity or specific activity towards that antigen.

Affinity maturation of anti-carbohydrate antibodies

B-cells secrete antibodies that mediate protection against a great variety of invading pathogens. There are two classes of antigens for B-cells, T-cell dependent

(thymus-dependent, TD) and T-cell independent (TI) antigens, depending on whether T-cell help is needed to induce an antibody production. Thymus dependent antigens require the presence of T helper (Th) cells to trigger a B-cell response, whereas the T-independent antigens can elicit an antibody response in the absence of T helper cells. T-independent antigens are further classified into TI types 1 and 2. Classical example for a TI type 1 antigen is lipopolysaccharide (LPS) and it act as B-cell mitogens, which function by nonspecifically or polyclonally activating most B-cells. The TI type 2 antigens, such as polysaccharides, are large molecules with repeating epitopes and are able to activate complement pathway but lack the ability to induce MHC-dependent T-cell help. Immune response to TI antigens however influenced by T-cells, either directly or by cytokines produced by T-cells (Lesinski and Westerink, 2001). In the case of TD antigens such as protein antigens B-cells obtain help from T-cells in the antibody response by acting as antigen-specific antigen presenting cells and B-cells activated by antigen in a T-cell dependent manner undergoes isotype switching, affinity maturation and somatic hypermutation and accompanies the development of memory cells producing antibodies more of IgG type (Stein, 1992).

Carbohydrate antigens belong to the class of T-independent antigens and secondary immune responses to T-independent antigens are usually characterized by little or no affinity maturation, a phenomenon attributed to limited somatic hypermutation and these B-cells are characterized by little proclivity to isotype switching and differentiation to memory B-cells (Scott *et al.*, 1988). To overcome this restricted response, glycoconjugate antigens have been developed in which glycan antigens or

fragments coupled to proteins can be used and the protein moieties can then recruit T helper cells.

Even though pneumococcal capsular polysaccharides are classified as thymus independent antigens several studies reported the presence of T-cell dependent antibody response to these capsular polysaccharides (Jeurissen and Bossuyt, 2004). Most carbohydrate binding natural antibodies are T-cell independent for their synthesis and are IgM-dominated (Haji-Ghassemi *et al.*, 2015) exceptions being antibodies to lipid-associated antigens which are presented by MHC molecules and engage T-cells as well (Haji-Ghassemi *et al.*, 2015), (Schneider *et al.*, 2015). The characteristic feature of T-dependent antibodies is their domination of IgG (Schneider *et al.*, 2015) rather than IgM. It was also reported that human anti-carbohydrate antibody repertoire shows a broader spectrum of isotype-switched immunoglobulin G (IgG) molecules that are not restricted to the IgG2 subclass (von Gunten *et al.*, 2009). Unlike other anti-carbohydrate antibodies, anti-α-galactoside antibody is predominantly of IgG type, which indicates isotype switching. The presence of somatic hypermutations in the V_H genes encoding for anti-Gal antibodies suggested the possibility of an antigen driven affinity maturation process. However, the α-gal epitope itself cannot activate helper T-cells that are required for activation of anti-Gal B-cells and Th cells are activated by immunogenic xenoproteins and xenogenic peptides of the xenograft cells expressing α-gal epitopes. These elicited anti-Gal antibodies were of increased affinity because of affinity maturation process in which anti-Gal B-cells with high affinity B-cell receptor undergo preferential expansion (Yu *et al.*, 1999, Tanemura *et al.*, 2000).

Adjuvant induced enhancement in affinity maturation

Adjuvants are defined as components capable of augmenting immune response to antigen. Immunogenicity of purified protein antigen may be low compared to vaccines containing live attenuated or inactivated pathogens. Natural adjuvants such as particulate forms of proteins, lipids and oligonucleotides etc. are present in live attenuated or inactivated vaccines. Adjuvants are of different types such as aluminum salts (alum), oil-in-water emulsions, such as MF59, or monophosphoryl lipid A (MPL), microbial products, microparticles, and liposomes (Reed *et al.*, 2013).

Most widely used adjuvant for human use is the aluminum-containing adjuvants which provide a depot effect by increasing the persistence of antigen in addition to the effects on immune cells. Most of the adjuvants have effect on acute effector functions, such as induction of Th1, Th2, or Th17 T-cell responses while some of them are reported to promote affinity maturation of antibodies (Reed *et al.*, 2013). Various studies reported that novel adjuvants induce B-cell maturation and increase both antibody titre and affinity of antibodies (Kasturi *et al.*, 2011). Studies in mice and non-human primates reported increased immunogenicity of 3'aminomethylnicotine-DT-conjugate anti-nicotine vaccine with CpG (Toll-like receptor 9 agonist) and aluminum hydroxide adjuvants and both anti-nicotine antibody titre and affinity was increased in mice (McCluskie *et al.*, 2013). Cholera toxin A1 fusion protein CTA1-DD adjuvant was reported to interact with follicular dendritic cells and complement factors to enhance somatic hypermutation and affinity maturation of antibodies and to increase the size of germinal center

promoting persistence of long-term plasma cells in the bone marrow and memory B-cells in the spleen (Bemark *et al.*, 2011).

Lipid and lipophilic substances also act as adjuvants to enhance immune response. Classical example for a lipid adjuvant is monophosphoryl Lipid A (MPL) derived from Salmonella minnesota R595 and is safe and effective in inducing Th-1 type immune responses to protein antigen vaccines. It was reported that a lipid nanoparticle adjuvant enhanced both B-cell and T-cell immune response to viral antigens (Swaminathan *et al.*, 2016). Lipid adjuvants also augment the affinity maturation of antigen specific antibodies. Vaccine systems containing monophosphoryl lipid A as adjuvant produced strong and persistent B- and T-cell responses and induced the production of high affinity antibodies against hepatitis B surface antigen (Vandepapelière *et al.*, 2008). ISCOMATRIX™ is a lipid adjuvant contains cage-like structures, composed of phospholipid, saponin, and cholesterol components. An influenza virus vaccine H7N9 virus like particle with ISCOMATRIX™ adjuvant showed enhanced neutralizing antibody responses and promoted affinity maturation of antibodies to hemagglutinin antigen (Chung *et al.*, 2015). Vaccine with lipid enveloped PLGA [poly(lactic-co-glycolic acid] nanoparticles displaying the VMP001 malaria antigen produced antibodies against *Plasmodium vivax* sporozoites with enhanced avidity and affinity (Moon *et al.*, 2012). Even though various studies analyzed the use of adjuvants in promoting affinity maturation the role of adjuvants alone in providing necessary immunological stimuli for promoting affinity maturation remains unclear.

2.6 Cancer immunotherapy

Immunotherapy is the treatment approach aimed to boost or restore the ability of the immune system to fight cancer, infections, and other diseases which includes strategies to improve anti-tumor immune responses by either boosting components of the immune system that produce an effective immune response or by inhibiting components that suppress the immune response. There are several reasons for the failure of immune system to mount an immune response against tumor which include failure to activate specific T-cells, inadequate antigen processing and presentation, insufficient T-cell repertoire, ineffective T-cell differentiation into effector cells and lack of homing of primed T-cells to tumor sites.

Immune-mediated tumor destruction can be promoted through activation of cytolytic T-lymphocytes and through antibody-dependent cytotoxicity. Tumor antigens are presented by activated dendritic cells to CD4 T-cells, which in turn activate CD8 cytotoxic T-lymphocytes (CTL) and leads to antibody production by B-cells. The activated NK and NKT cells use perforin and granzymes to destroy the tumor cells. In cancer, failure can occur at various stages of this process. The interactions between cancer cells and host immune cells results in an immunosuppressive network in the tumor microenvironment which promotes tumor growth, protects the tumor from immune recognition. Immunosuppression in tumor microenvironment can be due to T-cell anergy resulting from overexpression of CTLA-4, insufficient B7 co-stimulation, direct inhibition through inhibitory ligands such as PD-L1 or through extrinsic suppression by T-regulatory cells (Gajewski, 2007). Various forms of immunotherapeutic approaches in cancer include use of Toll-like receptor

agonists, cancer vaccines, immune checkpoint inhibitors, cytokine therapy, adoptive T-cell therapy, monoclonal antibody therapy etc.

Lethal nature of a tumor is due to relapse which results from the presence of residual tumor cells in patients after completion of standard therapies in the form of metastases. Detectable ones can be removed by surgery. Usually they are invisible for imaging and can develop into lethal ones. Complete elimination of metastases can be achieved by immunotherapy which can stimulate the immune system to produce an anti-tumor response against multiple tumor-associated antigens (TAAs) on the tumor cells to provide a long term protection. Presence of T-cells in tumors suggests that immune system can mount a response against tumor-associated antigens. But in majority of cancers T-cells are not found because tumors escape from recognition by cells of immune system such as antigen presenting cells (APCs).

Most of the TAAs are unique to each patient and many of them are not identified or isolated. Hence tumor cells expressing TAAs can serve as target for active immunotherapy against metastatic cells. Immunotherapy with a single type of TAA molecule cannot elicit a protective immune response. This is due to genome instability resulting in appearance of tumor cells with low or no expression of the specific TAAs (Khong and Restifo, 2002). For a long term anti-tumor response tumor vaccines with multiple TAAs are required. Since the identity of most of the TAAs is unknown it is difficult to identify the TAAs on an individual basis for vaccine preparation for immunotherapy. Hence tumor itself can be a source for autologous TAAs for vaccination purpose in cancer patients.

Tumor-associated antigens (TAA)

Malignant transformation leads to the expression of antigens on tumor cells which are useful markers in identifying tumor cells and are potential candidates for use in cancer therapy. Based on their patterns of expression tumor antigens are of two categories. Tumor specific antigens are present only on tumor cells not on any normal cells while tumor-associated antigens which are preferentially expressed by tumor cells are often found in normal tissues. Expression of tumor-associated antigens differs in their degree of expression in tumor, alterations in their protein structure by their aberrant subcellular localization within tumor. Different classes of tumor antigens include oncofetal antigens, oncoviral antigens, over expressed self-antigens, cancer-testis antigens, lineage-restricted antigens, mutated self-antigens and altered glycolipids and glycoproteins for maintenance of cellular transformation. These tumor antigens provide survival or dissemination benefits to developing cancers, allows for dysregulated growth, promotes tumor survival, enhance the metastatic potential of tumor cells and inhibit immune response to tumors (Vigneron, 2015, Buonaguro *et al.*, 2011).

Tumor-associated antigen MUC1 (TA-MUC1)

Altered human mucin 1 (MUC1) is a well known tumor-associated antigen and target for cancer immunotherapy for over a decade. In healthy tissues, MUC1 is expressed at low levels on the apical surface of glandular and ductal epithelial cells as a heavily O-glycosylated transmembrane protein, whereas MUC1 is highly over expressed and under glycosylated in majority of adenocarcinomas (Horm and Schroeder, 2013).

MUC1 is heavily O-glycosylated transmembrane glycoprotein and are typically found on the apical surface of glandular or ductal epithelial cells of various organs. In healthy tissues, the sugar chains of MUC1 oligomerizes to form a mucinous gel which have a role of lubrication and protection to the underlying epithelia from changes in pH, osmolarity. High negative charge on the surface of MUC1 creates a physical barrier and prevents the colonization of pathogens.

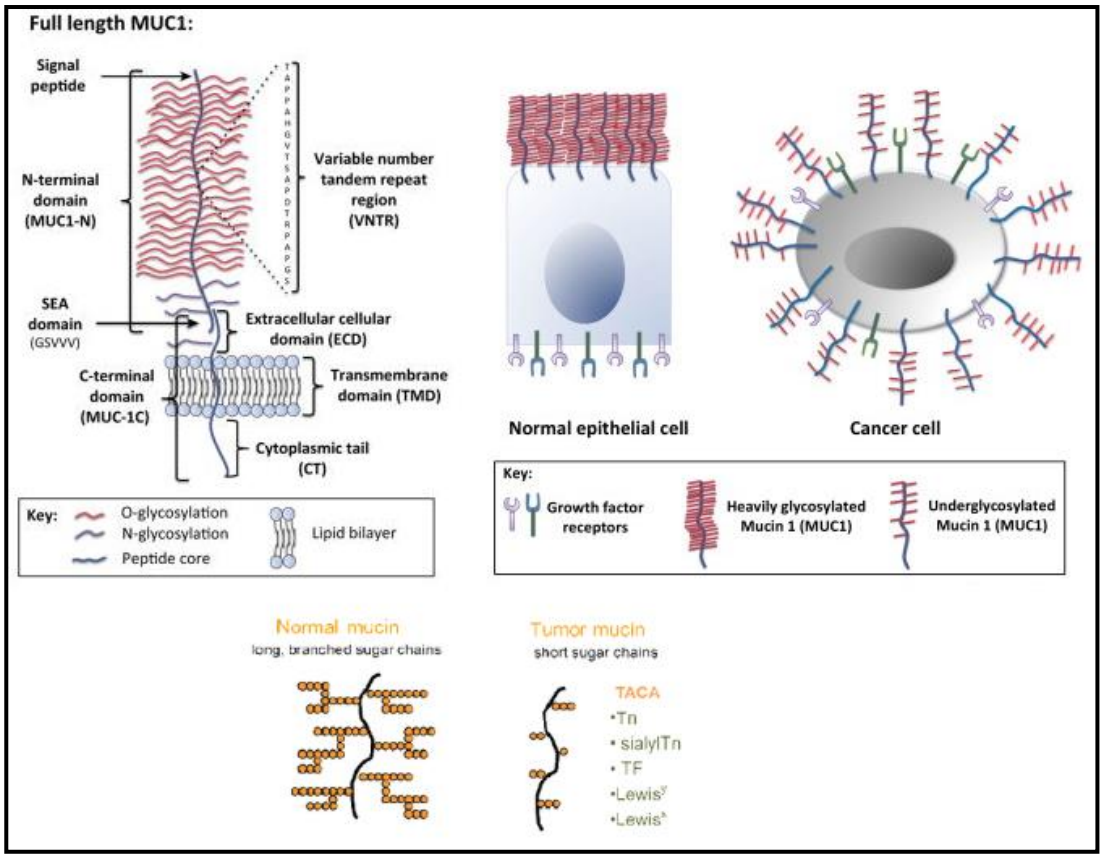


Figure 3. Schematic representation of the structure of normal MUC1 and its glycosylation in normal and tumor-associated MUC1 (Nath and Mukherjee, 2014**,** von Mensdorff-Pouilly *et al.*, 2011)

The mature MUC1 consists of two subunits; large N-terminal subunit (MUC1-N) and short C-terminal subunit (MUC1-C) associated around the SEA domain and form a stable heterodimeric complex. The smaller subunit MUC1-C contains a C-terminal cytoplasmic domain of 72 amino acids, followed by a transmembrane domain of 31 amino acids, and a short extracellular sequence which is non-covalently linked to the larger extracellular subunit MUC1-N. The N-terminal subunit contains SEA domain, variable number tandem repeat (VNTR) region and the signal peptide. Major portion of MUC1-N is the VNTR domain which is a highly polymorphic sequence motif marked by the presence of a PTS domain rich in proline/threonine/serine residues. Each VNTR domain of MUC1-N is composed of 20 amino acids that are extensively O-glycosylated at the serine and threonine residues and varies from 20 to 125 repeats. The heavily O-glycosylated extracellular region of MUC1 extends up to 200–500 nm from the cell surface.

MUC1 is an extensively O-glycosylated and moderately N-glycosylated protein. Glycosylation contributes to 50–90% of the total weight of MUC1 and based on the degree of glycosylation and number of tandem repeats molecular weight of MUC1 ranges from 300 to 600 kDa (Nath and Mukherjee, 2014). Each VNTR repeat possesses two serine and three threonine residues offering five O-glycosylation sites whereas N-glycosylation occurs at five sites four of which reside in the MUC1-N subunit and one in the extracellular domain (ECD) of the MUC1-C subunit. MUC1 exhibits a tissue specific glycosylation pattern also. O-glycosylation contributes to the biological properties of MUC1, whereas N-glycosylation is essential for proper protein folding, sorting, secretion, and its apical expression. In normal cells, MUC1

is heavily glycosylated, with the immunogenic peptide core regions masked by the sugar moieties which provide protection from proteolytic cleavage by environmental enzymes (Gendler, 2001).

Tumor-associated MUC1 differs in its structure, cellular distribution and function compared to normal MUC1. MUC1 is highly over expressed and under glycosylated in majority of human adenocarcinomas including those derived from breast, pancreas, ovary, lung, gastrointestinal tract, prostate, urinary bladder etc. MUC1 overexpression was also demonstrated in hematological malignancies, such as multiple myeloma, B- and T-cell lymphomas. In adenocarcinomas TA-MUC1 is redistributed over the cell surface and within the cytoplasm. Glycan chains of normal MUC1 contains extensively branched Core 2 O-glycans whereas in cancer cells the loss of Core 2 β1-6-GlcNAc-transferase, the enzyme responsible for initial chain elongation results in premature termination of chain elongation and exhibits Core 1 O-glycans with early addition of sialic acid. TA-MUC1 is highly sialylated due to increased expression of the α2,3- and α2,6- sialyl transferase in cancer cells (Taylor-Papadimitriou *et al.*, 1999).

Core peptides rich in serine and threonine residues in the VNTR domain of normal MUC1 are masked by the sugar moieties. Hypoglycosylation of TA-MUC1 leads to exposure of these immunogenic core peptides and the truncated sugar chains on TA-MUC1 shows the presence of tumor-associated carbohydrate antigens (TACA), such as Tn, sialyl-Tn, and the Thomsen-Friedenreich (TF) antigens that are not exposed on normal MUC1 molecule (Singh and Bandyopadhyay, 2007). Normally MUC1 exists on the plasma membrane as a heterodimeric complex, but following

stimulation with proinflammatory cytokines interferon-γ and tumor necrosis factor-α, matrix enzymes such as TNF-α converting enzyme and matrix metalloproteases causes dissociation of this complex resulting in release of MUC1-N from MUC1-C and also catalyze the cleavage of the extracellular domain of MUC1-C, thereby generating smaller peptide fragments (Nath and Mukherjee, 2014).

In tumors, MUC1 functions as an oncoprotein and is involved directly or indirectly in most of the hallmarks of cancer either through the extracellular MUC1-N subunit or through the transmembrane (MUC1-C) subunit. MUC1-N subunit has immunosuppressive properties, and MUC1 overexpression on tumor cells alters their adhesive properties favoring tumor progression and metastases through signaling pathways that induce transformation and promote growth and survival of tumors and activate genes involved in invasion, angiogenesis and metastases (von Mensdorff-Pouilly *et al.*, 2011).

TA-MUC1 as a target for cancer immunotherapy

MUC1-N subunit is secreted into the circulation in cancer patients, and is elevated in their serum. MUC1/CA 15-3 is used as a marker of breast cancer staging and to monitor response to treatment (Safi *et al.*, 1991). Overexpression and its aberrant membranous and cytoplasmic localization in tumor is associated with poor prognosis in cancer patients (Guddo *et al.*, 1998).

Presence of circulating antibodies directed against the extracellular subunit of MUC1 was reported in healthy individuals and in patients with benign and malignant diseases and are either in free form or in the form of complexes with

MUC1 (Croce *et al.*, 2003, Rughetti *et al.*, 1993). MUC1-immune complexes containing IgG and/or IgM are present in pregnant and lactating women and in patients with cancer (Croce *et al.*, 2001, Gourevitch *et al.*, 1995). Cytotoxic T-cell dependent immune response to immunogenic core peptides of tumor-associated MUC1 are also reported in cancer patients (Ioannides *et al.*, 1993, Jerome *et al.*, 1991). These reports suggested that immune system is capable of inducing immune response to MUC1. It was also reported that naturally occurring anti-Gal antibodies found in all human serum react with hypoglycosylated MUC1 tandem repeat peptides expressed in large amounts on the surface of tumor cells but not on normal cells (Sandrin *et al.*, 1997). Antibodies generated against TA-MUC1 in cancer patients have prognostic significance and associated with increased survival in patients (Blixt *et al.*, 2011, Hamanaka *et al.*, 2003).

Overexpression of aberrantly glycosylated MUC1, the truncation of the glycan side chains, and the loss of polarized expression which results in an antigenically distinct molecule and the identification of cellular and humoral responses to this antigen makes tumor-associated MUC1 an immunotherapeutic target. TA-MUC1 was ranked as the second best target out of 75 tumor-associated antigens for the development of cancer vaccines (Cheever *et al.*, 2009). Various in vitro studies demonstrated the anti-tumor effects of antibodies to MUC1-N subunit. These antibodies are capable of restoring cell-cell interactions, cell adhesion properties which are altered by tumor-associated MUC1 thereby preventing metastasis (Denda-Nagai and Irimura, 2000, Li *et al.*, 2010) and these antibodies are also reported to prevent the immunosuppressive properties of soluble mucin forms (Chan *et al.*,

1999). Anti-MUC1 antibodies are also implicated in tumor cell killing by antibody-dependent cell mediated cytotoxicity with natural killer cells as the major effector cells (von Mensdorff-Pouilly *et al.*, 2011, Snijdewint *et al.*, 2001).

Several MUC1-based immunotherapies are in various stages of development. Development of transgenic mice expressing human MUC1 in a tissue-specific manner has led to a better model system for developing immunotherapy protocols (Peat *et al.*, 1992, Taylor-Papadimitriou *et al.*, 2018). In addition, adenocarcinomas induced in MUC1 transgenic mice express the abnormal tumor-associated form similar to that of humans (Tinder *et al.*, 2008), (Mukherjee *et al.*, 2003). A variety of immune-based therapies targeting the tumor-associated antigen MUC1 including MUC1 vaccines, anti-MUC1 antibodies and adoptive T-cell transfer with MUC1-specific cytotoxic T-lymphocyte (CTL) are in clinical trials for testing toxicity and ability to elicit anti-tumor immune response in patients with cancer (Kimura and Finn, 2013). A synthetic aberrantly glycosylated MUC1 peptide vaccine which is covalently linked to a Toll-like receptor agonist, was shown to produce humoral and cellular immune responses in mice (Lakshminarayanan *et al.*, 2012). Immunotherapies to induce antibodies to MUC1 are mainly based on tandem repeat peptides or glycopeptides which is the most immunodominant portion of MUC1 (Tang *et al.*, 2008). Many of the oncogenic properties of MUC1 reside in the cytoplasmic tail region, making it an attractive target. A peptide inhibitor of MUC1-CT which prevents MUC1-CT mediated oncogenic signaling is in clinical trial (Yin and Kufe, 2011). Strategies to strengthen immune response against TA-MUC1 could benefit cancer patients especially by preventing metastatic dissemination.

3. MATERIALS AND METHODS

3.1 Materials

1-O-methyl α-D-galactopyranoside, galactose, cellulose, celite, melibiose, cellobiose, bovine serum albumin, soybean trypsin inhibitor, soluble guar gum, Tween-20, horse radish peroxidase (HRP) type II, sulpho-NHS-biotin, avidin-HRP, ortho-phenylenediamine (OPD), acrylamide, N,N'-methylene bisacrylamide, tetramethylene diamine (TEMED), Coomassie brilliant blue G-250 and R-250, tris, dithiothreitol (DTT), riboflavin, bromophenol blue, sodium cyanoborohydride, cyanogen bromide (CNBr), potassium borohydride, epichlorohydrin, potassium bromide (KBr), trisodium citrate, 1-fluoro-dinitrobenzene (FDNB), sodium periodate, dextran (molecular weight 400,000–500,000 kDa), phenylmethylsulfonyl fluoride (PMSF), dimethyl sulfoxide (DMSO), *Griffonia simplicifolia* IB4 (GS-IB4), fluorescein isothiocyanate (FITC), Triton X-100, poly-L-lysine were purchased from Sigma-Aldrich (India), Bangalore. Glycine, boric acid, ammonium persulfate, disodium salt of EDTA, hydrogen peroxide, solvents and buffer components were purchased from Merck, India. Amicon ultra centrifugal filter units (10,000 Da molecular weight cut-off), Sephadex G-200 and Sepharose 4B and 6B were from Pharmacia Fine Chemicals, Uppsala, Sweden. Bio Gel P-4 was purchased from Bio-Rad Laboratories, USA. Polystyrene 96-well flat bottom microplates (MAXISORB) were purchased from Nunc, Denmark. Antibodies to human IgA, human IgG, human IgM raised in goat and antibodies to apo(a) and apoB raised in rabbit were purchased from Dako, Denmark. 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 in solvents and buffers were of analytical

grade and obtained from local sources. The seeds of *Artocarpus integrifolia* (jack fruit) were obtained locally.

3.2 Methods

3.2.1 Collection of samples

Outdated plasma samples from healthy individuals of age 18-40 were collected from the Department of Blood Transfusion Services of this Institute with Institutional Ethics Committee approval (SCT/IEC/926). 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 as approved 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. Histologically confirmed (FNAC) cases of breast cancer patients who underwent surgery in the Department of Surgical Oncology, Regional Cancer Centre, Trivandrum, Kerala, India

ii. No history or documentary evidence of hepatic and renal dysfunction

iii. Age <70yrs

iv. No history of neoadjuvant chemotherapy

The controls were selected from healthy volunteers outside SCTIMST. The selection criteria for controls were as follows:

i. Age- and sex-matched to the cases

ii. No past history of malignancy or cancer treatment

iii. No history of any chronic diseases such as tuberculosis

iv. Normal hepatic and renal functions

Table 2. 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
Clinical Stage		I (5/46) II (32/46) III (9/46)
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) and stored at -20 °C and subsequently processed for the isolation of MUC1 glycoprotein.

3.2.2 Protein estimation by Bradford's method

Coomassie brilliant blue G-250 dye solution was prepared as a 0.06% solution in 3% perchloric acid. The reagent was filtered through Whatman No.1 filter paper before use. The dye and protein solutions were mixed in the ratio 1:1 and the absorbance was measured at 620 nm immediately (Bradford, 1976).

3.2.3 Protein estimation by bicinchoninic acid (BCA) assay

Protein was estimated in tissue homogenate samples by using Pierce® BCA Protein Assay Kit as per the experimental protocol given by Thermo Scientific, USA. Bovine serum albumin was used as the standard.

3.2.4 Preparation of cross-linked guar gum (CLGG)

Soluble guar galactomannan (from *Cyamopsis tetragonoloba* beans) was cross-linked to form an insoluble gel by a modification of the procedure described earlier (Appukuttan *et al.*, 1977). Guar gum powder 20 g was weighed and lumps were broken by gentle pressure. Powder was mixed thoroughly with a finely dispersed emulsion of 4 ml epichlorohydrin and 50 ml 3 N NaOH at room temperature until the mixture became a solid cake. It was then kept at 40 °C in a water bath for 24 h and mixed occasionally and then kept at 70 °C in a hot air oven for 10 h. The resulting gel was soaked in distilled water and repeatedly washed with water until washings became neutral. The gel was then equilibrated with PBS pH 7.4 and homogenized in a blender to obtain particles of about 300 µm in size. Fine particles were discarded by repeated decantation. The rest was kept at 4 °C in the presence of 0.02% sodium azide or packed into a column of required size for chromatography.

3.2.5 Isolation of jacalin (jack fruit seed agglutinin, JSA)

Jacalin was isolated from the seeds of *Artocarpus integrifolia* (jack fruit) by the procedure described earlier (Suresh Kumar G *et al.*, 1982). Thirty gram of jack fruit seeds were dehusked and soaked in PBS pH 6.5 for 12 h. The seeds were then cut into small pieces, homogenized in 300 ml of PBS pH 6.5 and stirred for 1 h at 4 °C. The supernatant of homogenate obtained by centrifugation at 12,000 g for 20 min was subjected to 65% saturated ammonium sulphate precipitation by stirring at 4 °C for 1 h. The precipitated proteins recovered by centrifugation at 12,000 g for 20 min were dissolved in PBS pH 6.5 and dialyzed against PBS pH 6.5. The retentate was centrifuged at 12,000 g for 20 min and the supernatant was loaded on to cross-linked guar galactomannan (CLGG) column. The column was washed with PBS pH 6.5

and eluted with 0.15 M galactose in PBS pH 6.5. Fractions containing proteins were pooled and concentrated using Amicon ultra centrifugal filter units and stored with 30% v/v glycerol at 4 °C. Lectin activity was assayed by haemagglutination with human RBC.

3.2.6 Preparation of Jacalin-Sepharose 4B matrix

Sepharose 4B was activated using cyanogen bromide as described earlier (Cuatrecasas and Anfinsen, 1971). Sepharose 4B was washed thoroughly with distilled water by suction over G2 sintered glass funnel and 20 g of this washed gel was added to 40 ml 2 M Na_2CO_3 followed by 20 ml of distilled water. The gel suspension was kept at 9 °C with constant stirring and 800 mg cyanogen bromide dissolved in 1-2 ml dimethylformamide was added and stirring was continued for 5 min. Activated gel thus obtained was immediately washed with 20 times gel volume of cold 0.1 M $NaHCO_3$ buffer pH 8.5. The protein sample in 0.1 M $NaHCO_3$ was added to the activated gel (2 mg protein per ml gel) and stirred gently overnight at 4 °C followed by incubating the gel with 0.1 M ethanolamine hydrochloride at 4 °C for 1 h to block the unconjugated activated groups on the gel. The uncoupled protein was removed by washing the gel with 20 times the gel volume using 0.1 M $NaHCO_3$ followed by distilled water, then with 50 mM acetate buffer containing 1 M NaCl pH 5**,** then with distilled water and finally washed and equilibrated in PBS pH 7.4. The jacalin-sepharose 4B affinity matrix was stored at 4 °C with 0.02% sodium azide or packed into a column of required size for chromatography.

3.2.7 Isolation of anti-α-galactoside antibody (anti-Gal)

Anti-Gal antibody was isolated human plasma samples. Since a portion of anti-Gal had been observed to form immune complex with lipoprotein(a) plasma anti-Gal

was prepared by affinity chromatography on cross-linked guar galactomannan gel after dissociating the lipoprotein using specific sugar (Geetha *et al.*, 2014). All steps were carried out at 4 °C. Plasma (70 ml) treated with 0.2 M galactose overnight at 4 °C was subjected to ultracentrifugation at 202,000 g in 1.24 g/ml density at 4 °C for 4 h in 4 ml tubes. Bottom 30% volume from all tubes were pooled, dialyzed against PBS pH 7.4 and loaded onto CLGG column. After washing with PBS to remove unbound proteins bound proteins were eluted with 150 mM galactose in the same buffer in 3 ml fractions. Protein containing fractions were pooled, concentrated by ultrafiltration, dialyzed against PBS to remove galactose and stored at 2-4 °C.

3.2.8 Isolation of anti-β-glucoside antibody (ABG)

ABG from human plasma was isolated as described earlier (Geetha *et al.*, 2007). All steps were carried at 4 °C. Human plasma sample from healthy donors was thawed, dialyzed thoroughly against PBS pH 7.4 and centrifuged at 12,000 g for 20 minutes. The supernatant was passed through a column (2 cm x 15 cm) consisting of a mixture of cellulose (microcrystalline) and celite (type 545, E. Merck, Germany) in 1:1 ratio (v/v). The column was washed with PBS pH 7.4 till effluent was protein free. Bound protein was eluted using 0.2 M dialyzable dextrose (obtained by thorough dialysis of 20 ml 1 M dextrose in PBS pH 7.4 against 80 ml PBS) into 3 ml fractions. Protein fractions were pooled, concentrated by ultrafiltration and dialyzed against PBS pH 7.4 to remove dextrose and stored at 2-4 °C.

3.2.9 Isolation of dextran-binding immunoglobulin (DIg)

Dextran-binding immunoglobulin was isolated by a method reported earlier (Paul *et al.*, 2009). All steps were carried out at 4 °C. Out-dated human plasma (50 ml) from healthy donors was dialyzed extensively against PBS pH 7.4 centrifuged at 12,000 g

for 20 min and passed through a 2 cm x 30 cm column of Sephadex G-200 equilibrated in the same buffer. After washing out unbound proteins using PBS pH 7.4, bound protein (DIg) was eluted using 0.25 M dextrose in PBS pH 7.4 as fractions. Protein containing fractions were pooled, concentrated by ultrafiltration, dialyzed against PBS pH 7.4 to remove dextrose and stored at 2-4 °C.

3.2.10 Conjugation of horse radish peroxidase (HRP) to lectins/antibodies

A. Periodate activation of HRP

HRP (2 mg) was dissolved in 0.2 ml of freshly prepared 0.3 M $NaHCO_3$ and 10 µl fluorodinitrobenzene (FDNB; 1% in absolute ethanol) was added, the contents were mixed and incubated for 1 h at room temperature (to prevent self-coupling). To create reactive aldehyde groups on HRP the solution was treated with 0.2 ml sodium metaperiodate (0.06 M in distilled water) for 30 min at room temperature, following which 0.2 ml of ethylene glycol (0.32 M in distilled water; 32 µl made up to 1.78 ml with distilled water) was added, mixed and incubated for 1 h at room temperature to remove excess periodic acid. Finally the solution was made up to 1 ml with 0.01 M carbonate buffer pH 9.5 (concentration is 2mg/ml) and dialyzed in 0.01 M carbonate buffer pH 9.5.

B. Conjugation of activated HRP to proteins

Conjugation of HRP to proteins was done according to the method described earlier (Heyderman *et al.,* 1986). Briefly, 1 mg of lectin or antibody in 1 ml of 10 mM sodium bicarbonate buffer, pH 9.5 was mixed with 0.67 mg periodate activated HRP in the same buffer and incubated at 25 °C for 2 h in dark. Then potassium borohydride (1%) solution in distilled water was added to a final concentration of 0.1% (to block unconjugated aldehyde groups). After 30 min incubation, the mixture

was dialyzed against PBS pH 7.4 and the labeled lectin or antibody was stored at 4 °C with 30% glycerol v/v.

3.2.11 Fluorescein isothiocyanate (FITC) labeling of anti-Gal

FITC was conjugated to antibodies as described earlier (Hudson and Hay, 1980). Antibody samples were concentrated to 1mg/ml and dialyzed in 0.25 M sodium carbonate/bicarbonate buffer pH 9. Prior to fluorescence labeling antibody samples were pre-incubated for 1 h with its specific sugar (25 mM melibiose) to block sugar binding sites. FITC-celite mixture was added to obtain FITC concentration of 150 µg per mg protein and mixture was incubated overnight at 4 °C for conjugation. The conjugated antibody was separated from the unreacted fluorochrome by gel filtration chromatography on Bio Gel P-4 column. The protein fractions were pooled and concentrated using Amicon ultracentrifugal filter units. FITC-conjugated antibodies were dialyzed extensively in dark (to remove sugar) and stored in amber colored bottles at 4 °C. Fluorescence was measured in BioTek fluorescence reader model FLx800TBI using excitation at 485 nm and emission at 520 nm.

3.2.12 Biotin labeling of *Griffonia simplicifolia* IB4 (GS-IB4) lectin

Griffonia simplicifolia IB4 (1 mg/ml) was dialyzed in 0.1 M Na_2HPO4 buffer pH 8. Lectin was pre-incubated with its specific sugar (melibiose 25 mM) for 1 h at 4 °C to prevent biotinylation at the carbohydrate binding site. Sulfo-NHS-biotin (0.002 M) was added and mixture incubated for 2 h at 4 °C. The biotinylated proteins were then dialyzed extensively in PBS pH 7.4 to remove the sugar and unconjugated Sulpho-NHS-biotin. The labeled lectin was stored at 4 °C with 30% glycerol v/v (Paul *et al.*, 2011).

3.2.13 Preparation of neoglycoconjugates

Cellobiose and melibiose were conjugated to protein soybean trypsin inhibitor by reductive amination using sodium cyanoborohydride. Soybean trypsin inhibitor, melibiose/cellobiose, sodium cyanoborohydride were taken in the ratio 1: 2: 4 by weight in 2 ml 0.2 M trisodium phosphate-phosphoric acid buffer pH 9.0. The contents were incubated at 25 °C for 14 days. The neoglycoconjugates thus formed were dialyzed in PBS 7.4 and stored at -20 °C (Baues and Gray, 1977).

3.2.14 Enzyme-linked immunosorbent assay (ELISA) and enzyme-linked lectin assay (ELLA)

Antibodies, lectins, glycoproteins or other proteins were coated on polystyrene microtitre plate wells by incubation of 200 µl of specified concentration of their solution in PBS pH 7.4 at 37 °C for 3 h. The wells were then washed thrice with PBS containing 0.05% Tween 20 (PBS-T) and blocked with PBS containing 0.5% Tween 20 for 30 min at 37 °C and finally washed thrice with PBS-T. Coated wells were then incubated with specified concentrations of solutions (200 µl) in PBS-T for 2 h at 4 °C followed by washing with PBS-T. Wells were then incubated at 4 °C with specified concentrations of HRP-labeled lectins or antibodies in 200 µl PBS-T for 2 h followed by washing with PBS-T. To assay the bound HRP, the washed wells were treated with 200 µl ortho-phenylene diamine (0.5 mg/ml) in 0.1 M citrate-phosphate buffer pH 5.0 containing 0.03% H_2O_2 for 15 min at 25 °C, followed by addition of 50 µl of 12.5% H_2SO_4 to stop the reaction. Bound HRP activity was measured as absorbance at 490 nm in a BioTek (USA) ELISA reader.

3.2.15 Lp(a) assay by jacalin-based enzyme immunoassay

Lp(a) in plasma and other lipoprotein samples was assayed by an apoB-independent jacalin-based enzyme immunoassay as described earlier (Sreekumar *et al.*, 2013). Briefly polystyrene wells were coated with jacalin (1 µg/well) and after blocking 500 times diluted plasma was added and wells were incubated for 2 h at 4 °C. The wells were then washed with ice-cold PBS-T and probed with 200 µl HRP-conjugated anti-human apo(a) [1.5 µg per ml in PBS-T]. The bound HRP was assayed using OPD as substrate as described above. Standard Lp(a) [99 mg/dL; International Reference SRM 2B Standardization, APTEC Diagnostics nv, Belgium] was assayed parallel to samples for quantitation of Lp(a) in samples.

3.2.16 Plasma anti-Gal assay

Polystyrene wells were coated with melibiose-conjugated soybean trypsin inhibitor (TIM, 1 µg/well) and after blocking 50 times diluted plasma was added and wells incubated for 2 h at 4 °C. After washing the wells, 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 of each antibody) for 2 h at 4 °C. The bound HRP was assayed using OPD as substrate. Unconjugated soybean trypsin inhibitor served as control for TIM.

3.2.17 Determination of specific activity of antibody

Specific activity of anti-Gal was defined as the ratio of ligand-binding activity to immunoglobulin content of the same amount of antibody (George *et al.*, 2015). These parameters were measured by ELISA. Binding to polystyrene well-coated TIM was measured as an indicator of activity of anti-Gal. Polystyrene wells were coated with TIM (1 µg/well) and after blocking, wells were incubated with 50 ng anti-Gal and bound antibody was assayed by probing with a mixture of HRP

conjugates of anti-human IgG, IgM and IgA (1.5 μg per ml of each antibody) and bound HRP activity 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 ELISAs was taken as specific activity of the antibody.

Specific activity of ABG was defined as the ratio of TIC (cellobiose-conjugated soybean trypsin inhibitor) binding activity to immunoglobulin content of the same amount of antibody. Specific activity of ABG was measured by binding the antibody (250 ng) to polystyrene well-coated TIC (1 μg/well) and measuring the bound antibody using HRP-labeled anti-immunoglobulins. Immunoglobulin content of the antibody sample (50 ng) was also assayed by direct coating on microplates as described above for anti-Gal.

Specific activity of DIg was defined as the ratio of dextran (molecular weight 400,000–500,000 kDa) binding activity to immunoglobulin content of the same amount of antibody. Specific activity of DIg was measured by binding the antibody (1 μg/well) to polystyrene well-coated dextran (1 μg/well) and measuring the bound antibody using HRP-labeled anti-immunoglobulins. Immunoglobulin content of the antibody sample (50 ng) was also assayed by direct coating on microplates as described above for anti-Gal

3.2.18 Isolation of Lp(a) from plasma

Lp(a) was prepared by affinity precipitation of plasma proteins with lectin jacalin followed by Tris-Borate-EDTA (TBE) electrophoresis followed by elution of lipoprotein bands as described below (Kalaivani and Appukuttan, 2014).

Isolation of jacalin-binding plasma lipoproteins

Jacalin-binding plasma proteins were precipitated by diluting plasma (2.5 ml) with 2.5 ml of 20 mM potassium phosphate buffer containing 150 mM NaCl, pH 7.4 (PBS) containing 0.8 mg/ml jacalin and incubated overnight at 4 °C. Precipitated proteins were collected by centrifugation at 64,000 g for 45 min at 4 °C. Pellet was redissolved in 1ml PBS containing 150 mM 1-O-methyl α-galactopyranoside by incubation at 4 °C for 2 h with occasional mixing to dissociate jacalin from proteins. The density of the solution was adjusted to 1.24 g/ml with potassium bromide and subjected to ultracentrifugation at 535,000 g for 4 h at 4 °C. The lipoproteins precipitated by jacalin from plasma (collectively termed as JL1) containing Lp(a) was sequestered in the top 20% volume and dialyzed in 10 times diluted TBE buffer pH 8.7.

Purification of Lp(a) from JL1 by Tris-Borate-EDTA (TBE) electrophoresis

Lp(a) and the associated LDL (adduct) were separated by subjecting JL1 to non-denaturing 3.75% polyacrylamide disc gel electrophoresis as described earlier with some modifications.

Reagents

Solution A: TBE buffer containing 0.05 M Tris, 0.025 M boric acid and 0.003 M disodium salt of EDTA pH 8.7 was prepared freshly in deionized distilled water.

Solution B: 1ml of solution A was mixed freshly with 2 ml of deionized distilled water containing 14 µl of TEMED.

Solution C: 15% acrylamide/bisacrylamide (19:1,w/w) 15% solution was prepared by dissolving 14.25 g acrylamide and 0.75 g bisacrylamide in 100 ml

deionized distilled water and filtered using Whatman No.1 filter paper and stored at 4 °C in amber colored bottle.

Solution D: 25% acrylamide/bisacrylamide (20:5, w/w) Acrylamide (20 g) and bisacrylamide (5 g) were dissolved in 100 ml deionized distilled water and filtered using Whatman No.1 filter paper and stored at 4 °C in amber colored bottle.

Solution E: Riboflavin (4 mg) riboflavin in 100 ml of deionized distilled water.

Solution F: Ammonium persulfate (0.2%) dissolved in deionized distilled water.

Solution G: Tracking dye: Bromophenol blue, 0.005% solution.

Solution H: Fixative: 12.5% trichloroacetic acid in distilled water.

Solution I: Tube gel stain: 0.12% Coomassie brilliant blue (CBB R-250) in a methanol: acetic acid: water mixture (11:3:11, v/v).

Solution J: Destaining solution: methanol: acetic acid: water mixture (1:1.5:17.5, v/v).

Gel preparation

The gels were cast in 5 mm internal diameter (ID), 9 cm long glass tubes (BROVIGA DISC electrophoresis apparatus).

A. Separating gel (3.75% acrylamide)

Solution A - 2 ml

Solution C - 2.5 ml

Solution F - 5.5 ml

TEMED -10 µl

Polymerization was achieved at room temperature.

B. Spacer Gel

Solution B - 1 ml

Solution D - 1 ml

Solution E - 1 ml

De ionized distilled water - 5 ml

Polymerization was achieved under fluorescent light and gels were kept at 4 °C in a wet condition for overnight.

Reservoir buffer

Pre-cooled (4 °C) TBE buffer, pH 8.7 (0.05 M Tris, 0.025 M boric acid, 0.003 M disodium salt of EDTA).

Sample loading and disc gel electrophoresis:

Dialyzed JL1 sample (50 μg) containing 10% glycerol and bromophenol blue (tracking dye) was loaded per tube and run was carried out at current of 3 mA per tube till dye came out and continued for 50% more time. The bands were located by staining one gel with Coomassie brilliant blue R-250 and destained. The stained gel served as reference to cut out the required protein bands from other tube gels which was kept at 4 °C after the run. The corresponding position in unstained gel was cut, minced well and passively eluted into minimum volume of PBS pH 7.4 by overnight incubation at 4 °C. Eluted samples were recovered by filtering through glass wool. Eluted fractions were coated on polystyrene wells and probed with HRP conjugates of anti-apo(a) and anti-apoB. Bands containing both apo(a) and apoB was identified as Lp(a) and bands containing only apoB was identified as LDL.

3.2.19 Molecular size index of Lp(a) isoforms

Appropriate dilution of eluted Lp(a) isoforms from different individuals were directly coated on microtitre 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. 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).

3.2.20 Preparation of apo(a)

Apo(a) was prepared by DTT reduction of plasma lipoproteins by a method described earlier (Geetha *et al.*, 2014). Plasma (1 ml) was subjected to ultracentrifugation at 535,000 g for 4 h at 4 °C after adjusting the density to 1.24 g/ml with potassium bromide and top 20% layer (L1) containing lipoproteins was collected and dialyzed against PBS pH 7.4 and the latter was subjected to reduction using 4 mM DTT at 37 °C for 15 minutes. After adjusting density of the solution to 1.24 g/ml with potassium bromide the solution was again subjected to ultracentrifugation at 535,000 g for 4 h at 4 °C. Bottom 20% layer containing apo(a) was collected and dialyzed against PBS pH 7.4.

3.2.21 Alkaline Polyacrylamide Gel electrophoresis (Alkaline-PAGE)

Alkaline PAGE was done as described by (Davis, 1964).

Reagents

Solution A: pH 8.8-9.0

1N HCl	24 ml
Tris	18.1 g
TEMED	0.12 ml

Made up to 100 ml with water

Solution B: pH 6.6-6.8

1N HCl	48 ml
Tris	5.98 g
TEMED	0.46 ml

Made up to 100 ml with water

Solution C:

Acrylamide	28 g
Bisacrylamide	0.735 g

Made up to 100 ml with water

Solution D:

Acrylamide	20 g
Bisacrylamide	5 g

Made up to 100 ml with water

Solution E: Riboflavin 4 mg dissolved in 100 ml water

Solution F: 14 mg ammonium persulfate dissolved in 10 ml distilled water

Solution G: Tracking dye: Bromophenol blue, 0.005% solution

Solution H: Fixative: 12.5% trichloroacetic acid in distilled water

Solution I: Tube gel stain: 0.12% Coomassie brilliant blue (CBB R-250) in a methanol: acetic acid: water mixture (11:3:11, v/v)

Solution J: Destaining solution-methanol: acetic acid: water mixture (1:1.5:17.5, v/v).

Gel Preparation

A. Separating Gel (7% acrylamide)

A	1 part
C	1 part

F 2 parts

B. Spacer Gel

B 1 part

D 1 part

E 1 part

Water 5 parts

Polymerization was achieved under fluorescent light.

Reservoir buffer: 0.05 M Tris /0.38 M glycine, pH adjusted to 8.3.

The gels were cast in 5 mm glass tubes (BROVIGA DISC electrophoresis apparatus) and electrophoresis run at 3 mA per tube till the bromophenol blue used as tracking dye had reached the bottom of the gel. The gels were fixed, stained and destained.

3.2.22 Preparation of O-glycan-rich glycoproteins other than apo(a) from plasma

After preparation of L1 from 2 ml plasma as described above the non-L1 plasma proteins (bottom 80% of ultracentrifuged plasma) was collected, dialyzed against PBS pH 7.4 and loaded to jacalin-Sepharose 4B affinity chromatography column (5 ml). Unbound protein was washed with PBS pH 7.4 and bound protein was eluted with 0.8 M galactose in PBS pH 7.4. The galactose solution was subjected to ultrafiltration using PM-10 membrane to filter out undialyzable materials present in it and the filtrate was used for elution. Eluted proteins were collected, concentrated and then subjected to 6% alkaline PAGE electrophoresis. After staining different bands of O-glycan-rich glycoproteins were passively eluted as described above and protein was estimated in each sample.

3.2.23 Plasma anti-Gal IgG and IgM levels

Polystyrene wells were coated with melibiose-conjugated soybean trypsin inhibitor (TIM, 1 µg/well), blocked as described, 50 times diluted plasma was added and wells incubated for 2 h at 4 °C. After washing wells were probed with 200 µl HRP-conjugated anti-human IgG and IgM separately (1.5 µg/ml in PBS-T) for 2 h at 4 °C. After washing bound HRP was assayed using OPD as substrate as described above.

3.2.24 Extraction and partial purification of MUC1 glycoprotein

The isolation and extraction procedures were carried out by a modification of the procedure described earlier (Paszkiewicz-Gadek *et al.*, 2008). All steps were carried out at 4 °C. Preliminary homogenate (10% w/v) was prepared in phosphate buffered saline, PBS pH 7.4 containing 1 mM EDTA and 1 mM phenyl methyl sulfonyl fluoride 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 16000 rpm and supernatant was collected.

In order to partly purify MUC1 glycoprotein, supernatant was passed through Sepharose 6B column (1.8 × 60.0 cm) which was equilibrated and eluted with PBS. Fractions were collected in 2 ml volume. Each protein fraction was examined with jacalin-HRP and anti-MUC1 antibody. For this each protein fraction was directly coated on microtitre plate wells and blocked and washed as described above. Wells 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 was quantitated as described above.

MUC1 positive fractions (which are jacalin reactive also) 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.

3.2.25 Assay to check the inhibition of binding of anti-Gal antibodies to its ligand TIM by tumor-associated MUC1

Polystyrene wells were coated with TIM (1 μg/well). Anti-Gal (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 to the wells, followed by incubation at 4 °C for 2 h. Washed wells were incubated with 200 μl of a mixture of HRP conjugates of anti-human IgG, IgA and IgM (1.5 μg per ml of each antibody) for 2 h at 4 °C. The bound HRP was assayed using OPD as substrate as described above. Untreated anti-Gal added to TIM coated wells served as control.

3.2.26 Assay to check the inhibition of binding of GS-IB4 lectin to its ligand TIM by tumor-associated MUC1

Biotinylated GS-IB4 lectin (75 ng in 200 μl PBS-T) pre-incubated at 4 °C for 18 h with 2 μg TA-MUC1 was added to TIM coated wells and incubated for 2 h at 4 °C. After washing, the bound lectin was assayed using HRP-labeled avidin (60 ng avidin per ml). Untreated biotinylated GS-IB4 added to TIM coated wells served as control.

3.2.27 Immunofluorescence analysis of anti-Gal antibody binding to tumor tissue

Immunofluorescence staining of the formalin fixed tissue sections using FITC-labeled anti-Gal were carried out by the following procedure.

Solutions and reagents

10% neutral-buffered formalin

Distilled water	900 ml
Di-sodium hydrogen phosphate, anhydrous (Na_2HPO_4)	6.5 g
Sodium di-hydrogen phosphate, monohydrate (NaH_2PO_4.H2O)	4.0 g
Formaldehyde, 37% solution	100 ml

Dissolved all the ingredients and mixed well and stored at room temperature.

Poly-L-lysine coating of slides

A rack containing clean slides was placed in diluted (0.01%) poly-L-lysine solution for 5 min at room temperature. Slides were then drained and dried in an oven at 60 °C for 1 h.

Antigen retrieval buffer-Sodium Citrate Buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0)

Tri-sodium citrate (dihydrate)	2.94 g
Distilled water	1000 ml

Mixed to dissolve and adjusted pH to 6.0 with 1 N HCl.

Added 0.5 ml of Tween 20 and mixed well and stored at 4 °C.

Wash buffer-TBS-T (Tris-Buffered Saline, 0.05% Tween 20)

Tris base	6.05 g

Sodium chloride 8.76 g

Dissolved in 900 ml of distilled water and adjusted pH to 7.6 with 12 N HCl. Added distilled water to a final volume of 1 L. Added 0.5 ml of Tween 20 and mixed well and stored at 4 °C.

Glycerol-PBS mounting medium

Phosphate-buffered saline (20 mM PBS, PH 7.4) 10 ml

Glycerol 90 ml

Mixed well and stored at 4 °C.

i. Tissue fixation and paraffin embedding

The surgically resected breast tumor tissue sections were fixed in 10% neutral buffered formalin for 24 h at room temperature. Dehydration of the tissue with graded alcohols and paraffin embedding at 58 °C was carried out with fully automated tissue processor (Leica ASP300 S).

ii. Tissue cutting and mounting the section

Five µm transverse sections were cut from the paraffin embedded tissue using a fully automated rotary microtome (Leica RM2255). Sections were spread on to poly-L-lysine coated slides and dried in an oven at 37 °C for 1 h followed by incubation in an oven for 1 h at 60 °C to melt the paraffin wax.

iii. Deparaffinization and rehydration of tissue sections

Slides were placed in a rack and performed the following washes

a. Immersed the slides in xylene (mixed isomers) 2 times for 10 minutes each.

b. Immersed the slides in 100% ethanol 2 times for 10 minutes each.

c. Immersed the slides in 95% ethanol for 5 minutes.

d. Immersed the slides in 70% ethanol for 5 minutes.

e. Immersed the slides in 50% ethanol for 5 minutes.

f. Rinsed the slides with deionized H_2O.

iv. Antigen retrieval

Most formalin-fixed tissue requires an antigen retrieval step before immunohistochemical staining can proceed. This is due to the formation of methylene bridges during fixation, which cross-link proteins and therefore mask antigenic sites. There are several methods of antigen retrieval and the most common is heat-induced epitope retrieval in citrate buffer.

Slides were kept in a coplin jar containing the antigen retrieval buffer. Pressure cooker without lid was placed on the hotplate with water. Coplin jar was placed inside the pressure cooker. Once boiling started, the pressure cooker lid was secured and as soon as the cooker reached full pressure, heating for 3 minutes was given. After 3 minutes, cooker was de-pressurized and the slides were then cooled down at room temperature and washed thrice with wash buffer.

v. Immunostaining

All incubations were carried out in a humidified chamber to avoid drying of the tissue. Drying at any stage will lead to non-specific binding and ultimately high background staining. Non-specific staining was blocked by incubating the tissue section with 1% human serum albumin (glycoprotein free) in TBS-T for 2 h at room temperature. Drained the slides for a few seconds and wiped around the sections with tissue paper. Sections were incubated overnight at 4 °C in dark with FITC-labeled anti-Gal (1 µg/ml) in TBS-T. Sections were washed thrice with wash buffer 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 analysed using QCapture Pro7.

3.2.28 Statistical analysis

All results were expressed as mean ± SEM. One-way ANOVA followed by Tukey's multiple comparison tests or unpaired Student's t-test was used to determine the significance of difference between the groups where appropriate. Pearson's correlation coefficient, r was used to measure the strength and the direction of a linear relationship between two parameters. Statistical significance of categorical variables was determined by Pearson chi-square test. Binary logistic regression analysis was used to estimate odds ratios 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).

4. RESULTS AND DISCUSSION

PART-I

Role of lipoprotein(a) [Lp(a)] as an antigenic stimulus for affinity maturation of anti-α-galactoside antibody

Introduction

Overexpression of serine- and threonine-rich peptide MUC1 with reduced glycosylation is a hallmark of cancer cells (Taylor-Papadimitriou *et al.*, 1999) so that vaccination for increased production of anti-MUC1 antibodies is among current therapeutic strategy (Deguchi *et al.*, 2010). Naturally occurring anti-α-galactoside antibody (anti-Gal) present in plasma of primates had been shown to recognize MUC1 as surrogate ligand by accepting the serine- and threonine-rich peptide sequence (STPS) of MUC1 at its binding site (Sandrin *et al.*, 1997). Role of anti-Gal in natural anti-tumor defense has however been hardy explored though variations in specific activity (ligand-binding activity per unit immunoglobulin protein) appear to be decisive in the anti-MUC1 defense of the individual, based on the above observations. Incidentally another circulating macromolecule, namely lipoprotein(a) [Lp(a)] which is also exclusive to primates has been found to form immune complex with anti-Gal (Geetha *et al.*, 2014). The ligand recognized by anti-Gal on Lp(a) is also the STPS in the O-glycosylated apo(a) subunit since de-O-glycosylation of apo(a) only increased anti-Gal binding (Geetha *et al.*, 2014). High plasma concentration of Lp(a) which is generally accompanied by smaller size of the lipoprotein (Utermann *et al.*, 1987), correlates positively with atherosclerosis, stroke and neurodegenerative disorders (Momiyama *et al.*, 2012). Curiously low plasma Lp(a) concentration is associated with increased incidence of cancer (Sawabe *et al.*, 2012) though no causative relationship has been established.

While synthesis of anti-Gal has been suggested to be triggered by gut microbial antigens (Galili *et al.*, 1988), persistent presence of systemic molecules possessing

antigenic ligands in the host circulation is known to effect affinity maturation of the antibody resulting in antibodies with greater complementarity and affinity to the systemic antigenic ligand (Tam *et al.*, 2016). Anti-Gal-Lp(a) immune complex in plasma underlined the potential of Lp(a) as an autologous antigenic effector of affinity maturation of this antibody. A major determinant of availability of STPS in Lp(a) for recognition by anti-Gal is likely to be the number of LDL molecules that non-covalently adhere to the circulating form of the lipoprotein in addition to its integral LDL molecule attached covalently to apo(a) subunit. Reason is that the adhering or adduct LDL molecules also utilize the sialic acid-rich and negatively charged O-glycosylated region of apo(a) subunit of Lp(a) for adhesion. Accordingly marked increase in the number of adduct LDL molecules with increasing Lp(a) size had been reported (Kalaivani and Appukuttan, 2014). The present study examines how individual variations in size and plasma concentration of Lp(a) could affect the specific activity of anti-Gal, a factor that may decide the individual's antibody-mediated resistance to cancer.

Results

(A). Non-covalently attached LDL blocks anti-Gal binding to Lp(a)

In general, plasma Lp(a) concentration is inversely proportional to Lp(a) size (Utermann *et al.*, 1987) the former parameter which is easily measurable was used as an indicator of Lp(a) size. Since Lp(a) concentration below 10 mg/dL and above 30 mg/dL are considered below and above normal respectively, plasma containing Lp(a) between these extremes were chosen for preparation of purified (free from

adduct LDL) Lp(a) of average size. Reactivity of Lp(a) sample as a ligand for anti-Gal was measured in terms of the percentage increase it brings about in the fluorescence of fluorescently labeled anti-Gal. This increase had been reported from our laboratory to be a measure of conformational shift in Fc part of the antibody which in turn reflects the strength of antigen binding at the binding site since low molecular weight ligands that produce insignificant conformational changes in Fc part do not appreciably enhance the fluorescence of the fluorescently labeled antibody (George *et al.*, 2015).

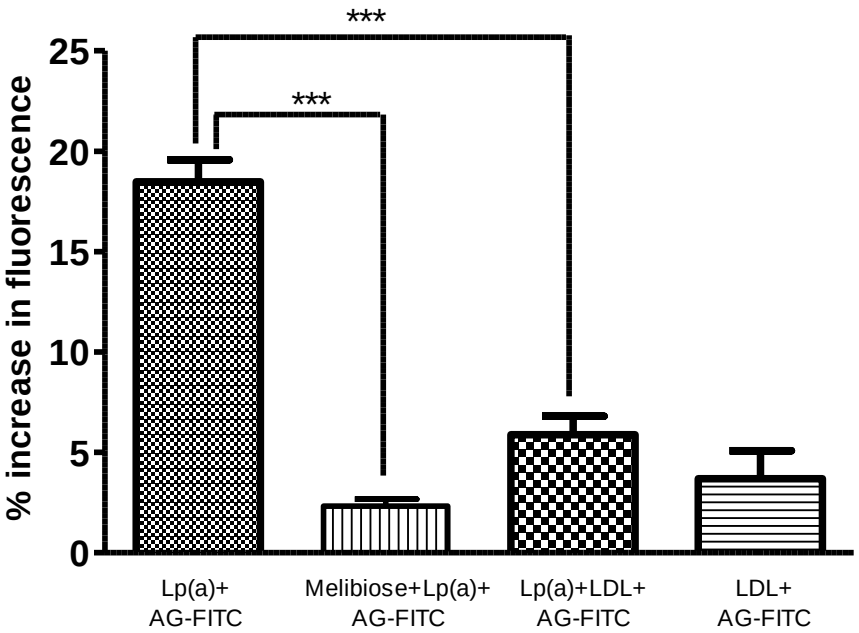


Figure 4. Inhibition of anti-Gal binding to Lp(a) by non-covalently attached LDL. *Purified Lp(a) (4 µg in 50 µl PBS) was incubated with LDL (2 µg in 25 µl PBS) or with 25 µl PBS only for 6 h at 4 °C. These samples or LDL (2 µg in 75 µl PBS) were further incubated with anti-Gal-FITC (3 µg in 25 µl PBS) for 18 h at 4 °C. Anti-Gal-FITC (3 µg in 25 µl PBS) pre-incubated with its specific sugar (25 mM melibiose) in PBS for 1 h at 4 °C was incubated with purified Lp(a) (4 µg in 75 µl PBS) for 18 h at 4 °C. Volume was made upto 300 µl with PBS just before fluorescence measurement*

*by excitation at 485 nm and emission at 520 nm. Fluorescence of 3 μg of FITC-anti-Gal alone in PBS was used as a benchmark to calculate the % increase in fluorescence on addition of other ligands. ***: p value <0.0001 for blocking of anti-Gal by its specific sugar melibiose and for blocking of Lp(a) by LDL. Mean ± SEM of 6 consecutive plasma samples as Lp(a) source.*

Result in Fig.4 shows that increase in the fluorescence of labeled anti-Gal brought about by pure Lp(a) was solely due to its attachment on the sugar-binding site of the antibody since blocking the binding site with excess of the disaccharide anti-Gal ligand melibiose fully prevented Lp(a)-mediated fluorescence increase in anti-Gal-FITC (*p* value <0.0001). Lp(a) pre-treated with LDL produced only a marginal rise in fluorescence of the antibody (*p* value <0.0001) showing that the latter lipoprotein also competed with anti-Gal for binding to Lp(a). Specificity of anti-Gal towards Lp(a) was also clear from the failure of LDL alone to enhance fluorescence. Result in Fig.4 suggested that though non-covalently attached adduct LDL in circulating Lp(a) molecules may not use the same binding domain on Lp(a) as anti-Gal does, being bulky molecules they are deterrents to recognition of Lp(a) by the antibody possibly by blocking the O-glycan-rich region and thereby the STPS in Lp(a).

(B). LDL-mediated blocking of anti-Gal binding is more, the larger the Lp(a) size

Purified samples of apo(a) subunits from high and low molecular weight Lp(a) samples (plasma Lp(a) concentrations <10 mg/dL and > 30 mg/dL respectively) and possessing equal antigenic apo(a) content (equal to 2 μg standard Lp(a), determined by apoB-independent assay (Sreekumar *et al.*, 2013) were compared for the effect of same amount of LDL on their capacity to bind anti-Gal. Result (Fig.5) showed that

binding of anti-Gal to lower molecular weight apo(a) was relatively much less blocked by LDL which indicated that in circulation low molecular weight Lp(a) are more available for recognition by anti-Gal antibody as compared to high molecular weight Lp(a).

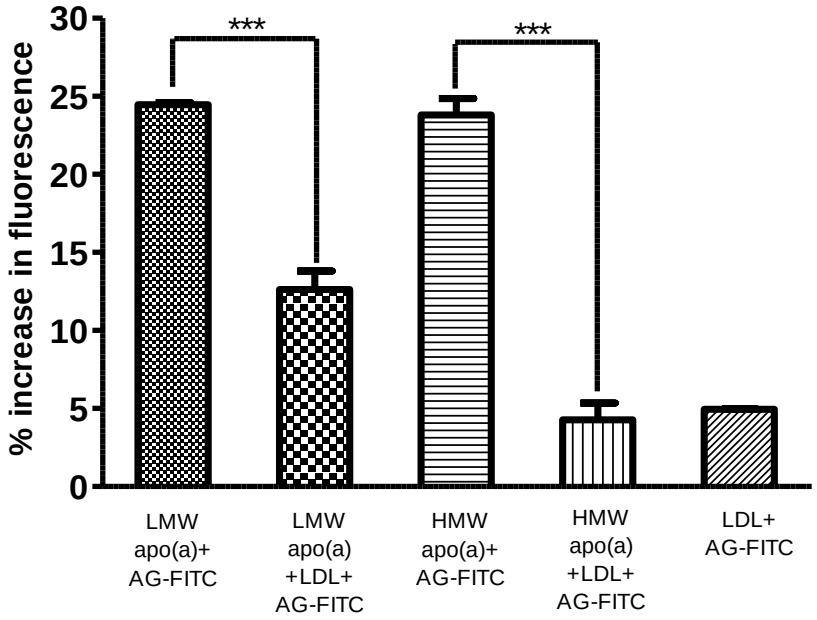


Figure 5. Inhibition of anti-Gal binding to low and high molecular weight apo(a) by LDL. *Purified low (LMW) and high (HMW) molecular weight apo(a) (50 ng in 200 µl PBS) was directly coated on microtitre plate wells, blocked and followed with anti-apo(a)-HRP (1.5 µg antibody per ml) and anti-apo(a) response was assayed.*

LMW apo(a) (2 µg) and an equivalent amount of HMW apo(a) based on anti-apo(a) response in 50 µl PBS were incubated with LDL (4 µg in 25 µl PBS) or with 25 µl PBS only for 6 h at 4 °C. These samples or LDL (4 µg in 75 µl PBS) were further incubated with anti-Gal-FITC (3 µg in 25 µl PBS) for 18 h at 4 °C. Volume was made upto 300 µl with PBS just before fluorescence measurement by excitation at 485 nm and emission at 520 nm. Fluorescence of 3 µg of FITC-anti-Gal alone in PBS was used as a benchmark to calculate the % increase in fluorescence on

*addition of other ligands. ***: p value =0.0006 for blocking of LMW apo(a) by LDL and p value =0.0002 for blocking of HMW apo(a) by LDL. Mean ± SEM of 6 consecutive plasma samples as apo(a) source.*

(C). Circulating forms of smaller Lp(a) bind to anti-Gal more strongly than those of larger Lp(a).

Circulating forms of Lp(a) precipitated by jacalin and redissolved on addition of jacalin specific sugar, was segregated from other proteins to top 20% layer following ultracentrifugation in 1.3 ml tubes (Methods 3.2.18). This sample (JL1), from plasma contains only Lp(a) with adduct LDL if any (Kalaivani and Appukuttan, 2015). JL1 samples from low and high molecular weight Lp(a) individuals (according to criteria described for Fig.5), all possessing the same apo(a) content, were treated with the same sample of FITC-labeled anti-Gal.

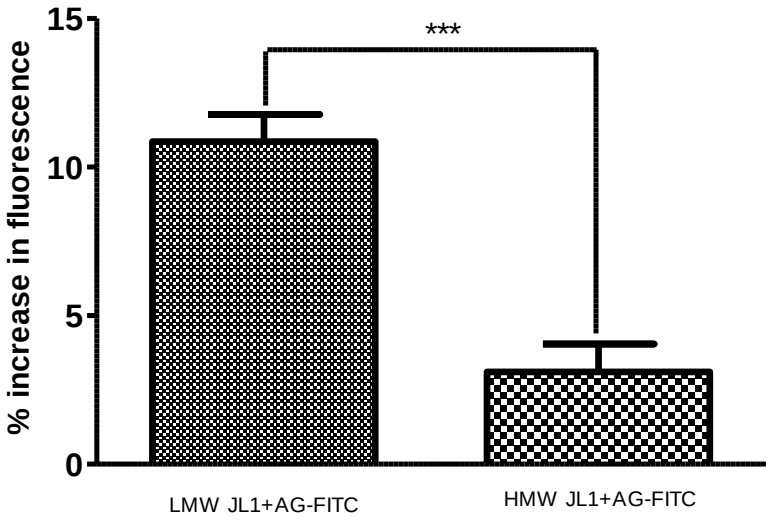


Figure 6. Recognition of circulating forms of low and high molecular weight Lp(a) samples (JL1) by anti-Gal antibody. *Polystyrene wells were coated with jacalin (1 µg/well) and after blocking diluted JL1sample was added and incubated*

for 2 h at 4 °C followed by probing with anti-apo(a)-HRP (1.5 μg antibody per ml) and anti-apo(a) response was assayed.

*Low molecular weight (LMW) JL1 (6 μg) and an equivalent amount of high molecular weight (HMW) JL1 based on anti-apo(a) response in 75 μl PBS were incubated with anti-Gal-FITC (3 μg in 25 μl PBS) for 18 h at 4 °C. Volume was made upto 300 μl with PBS just before fluorescence measurement. Fluorescence of 3 μg of FITC-anti-Gal alone in PBS was used as a benchmark to calculate the % increase in fluorescence on addition of other ligands. ***: p value <0.0001. Mean ± SEM of 18 consecutive plasma samples as source of JL1 samples.*

Fig.6 results show that increase in fluorescence produced in anti-Gal-FITC by JL1 of small Lp(a) individuals was significantly greater than that produced by JL1 of large Lp(a) individuals (*p* value <0.0001). Result suggested that despite large Lp(a) molecules possessing larger numbers of repeats of STPS-containing kringle IV type 2 repeats, the latter were not accessible in their circulating forms for anti-Gal to bind. Increasing number of LDL molecules in proportion to Lp(a) size, reported to be adhering to Lp(a) in circulation by non-covalent adhesion (Kalaivani and Appukuttan, 2014) could have blocked access to anti-Gal as evidenced by the result in Fig.4. Result in Fig.6 also established that a qualitative difference in Lp(a) structure in circulation, and not just the large number of Lp(a) molecules, is reason for the higher affinity of circulating low molecular weight Lp(a) for anti-Gal.

(D). Lp(a) size and plasma concentration decide the specific activity of anti-Gal

Macromolecules that contribute towards affinity maturation of an antibody should mandatorily offer their antigenic ligands in a format in which the latter are accessible to the antibody. Since Lp(a) in JL1 samples is the natural form in which these lipoproteins are available in circulation, but is occupied by varying numbers of

adduct LDL molecules depending on Lp(a) size, it followed that effect of Lp(a), if any, on affinity maturation of anti-Gal would depend on the accessibility of STPS of O-glycosylated region of Lp(a) to the antibody. Since the latter factor is decided by the number of adduct LDL, which in turn is dependent on Lp(a) size, we examined the correlation between Lp(a) size and specific activity of anti-Gal in 73 random samples of plasma from healthy individuals. Since apo(a) is attached by a disulfide bond to the apoB of LDL molecule during Lp(a) synthesis, apo(a) is peripherally available while apoB gets masked in proportion to the size of the apo(a) (Kalaivani and Appukuttan, 2015). Consequently anti-apoB response [B] to microplate well-coated purified Lp(a) [free from adduct LDL] varied depending on apo(a) size whereas anti-apo(a) response [a] was independent of apo(a) size, so that the ratio of responses [a] to [B] was an easily measurable index of Lp(a) size (Kalaivani and Appukuttan, 2015).

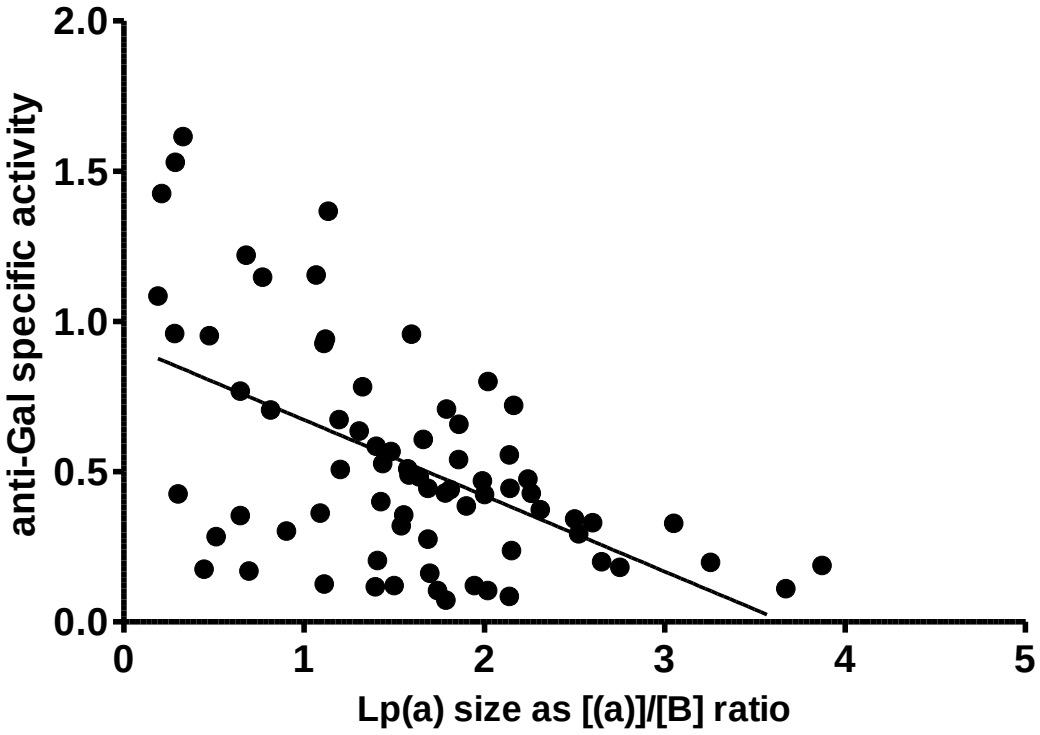


Figure 7. Correlation plot of Lp(a) size and anti-Gal specific activity. *As an index of molecular size of Lp(a) ratio of anti-apo(a) response to anti-apoB response (absorbance at 490 nm) of microplate well-coated Lp(a) was measured as described in Methods 3.2.19. Specific activity of anti-Gal antibody was determined as described in Methods 3.2.17.n=73, Pearson's correlation coefficient, r= -0.5443, p value <0.0001.*

Fig.7 shows that specific activity of anti-Gal in normal individuals varied inversely with their Lp(a) size with a Pearson's correlation coefficient of r= -0.5443 and *p* value <0.0001. An inverse relationship between size of Lp(a) synthesized and plasma concentration of the lipoprotein(a) had been reported earlier (Utermann *et al.*, 1987). To confirm the above effect of Lp(a) size on anti-Gal activity we measured the variation of specific activity of anti-Gal with plasma Lp(a) concentration. Since the assay used for plasma Lp(a) deciphered only the apo(a)

content regardless of actual size of Lp(a), the assay values reflected the number of Lp(a) molecules irrespective of their size.

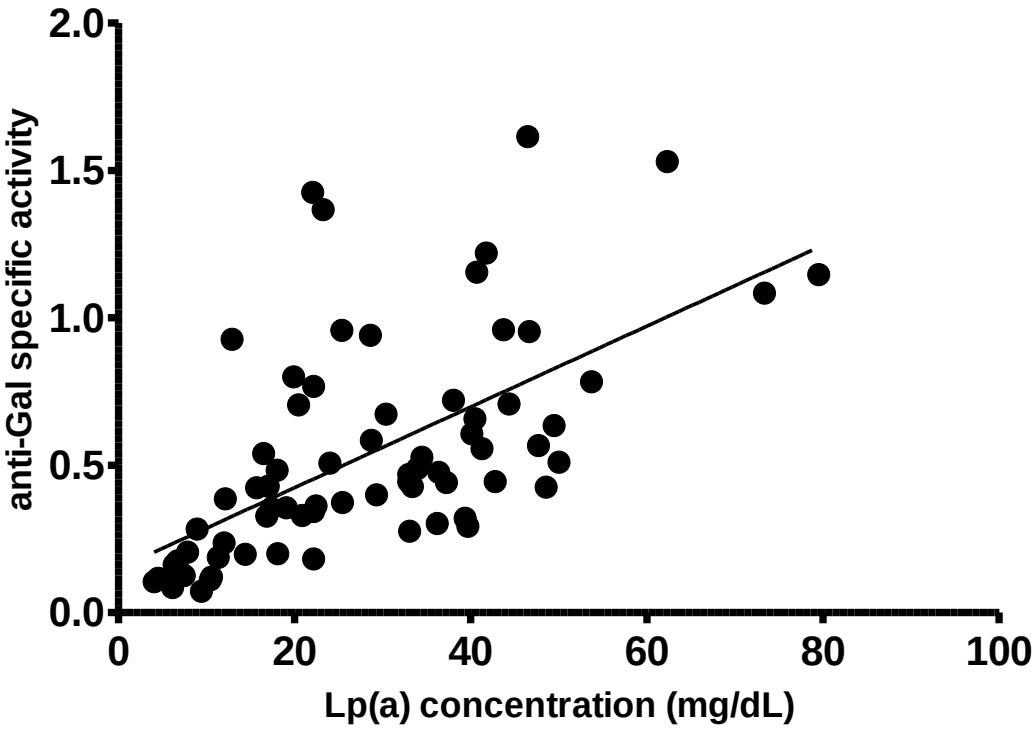


Figure 8. Correlation plot of Lp(a) concentration and anti-Gal specific activity. *Lp(a) in plasma samples was assayed by an apoB-independent jacalin-based enzyme immunoassay as described in Methods 3.2.15. Specific activity of anti-Gal antibody was determined as described in Methods 3.2.17.n=73, Pearson's correlation coefficient, r=0.6202, p value <0.0001.*

Data in Fig.8 shows that specific activity of anti-Gal in plasma increased with the concentration of Lp(a) with a Pearson's correlation coefficient of r=0.6202 and *p* value <0.0001.

(E). Apo(a) is superior to other plasma O-glycosylated proteins as ligand for anti-Gal

To ascertain the anti-Gal binding affinity of Lp(a) vis a vis those of other plasma O-glycoproteins the latter were isolated by affinity chromatography on immobilized jacalin and compared to apo(a) in anti-Gal binding activity measured in terms of percentage increase in fluorescence of anti-Gal-FITC.

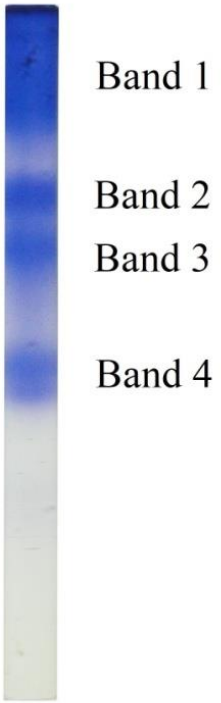


Figure 9. 6% Alkaline PAGE electrophoretic pattern of plasma O-glycan-rich glycoproteins other than apo(a). *Plasma O-glycan rich glycoproteins other than apo(a) were isolated from plasma as described in Methods 3.2.22.*

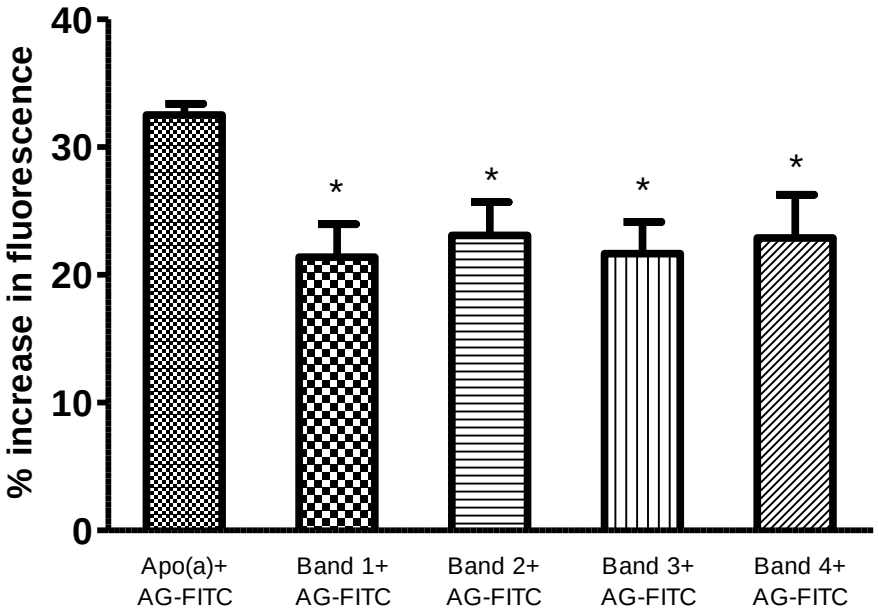


Figure 10. Comparison of apo(a) and other O-glycan rich glycoproteins as ligands for anti-Gal. *Apo(a) was prepared by DTT reduction of plasma lipoproteins as described in Methods 3.2.20. Purified apo(a) or other O-glycan rich glycoproteins isolated from different bands of gel (4 μg in 75 μl PBS) were incubated with anti-Gal-FITC (3 μg in 25 μl PBS) for 18 h at 4 °C. Volume was made upto 300 μl with PBS just before fluorescence measurement. Fluorescence of 3 μg of FITC-anti-Gal alone in PBS was used as a benchmark to calculate the % increase in fluorescence on addition of other ligands. *: p value =0.0176 vs Apo(a)+AG-FITC. Mean ± SEM of 6 plasma samples as source of apo(a) and other O-glycan rich glycoproteins.*

Fig.10 shows the significantly higher affinity of apo(a), than of the same mass of any other plasma O-glycosylated protein for anti-Gal indicating that Lp(a) is the prime candidate as affinity maturation ligand during anti-Gal synthesis.

(F). Anti-Gal is IgG dominated, more so in high Lp(a) individuals

While most carbohydrate binding natural antibodies are T-cell independent for their synthesis and are IgM-dominated (Haji-Ghassemi *et al.*, 2015) exceptions are

antibodies to lipid-associated antigens which engage T-cells as well (Haji-Ghassemi *et al.*, 2015, Schneider *et al.*, 2015). The characteristic feature of T-cell dependent antibodies is their domination of IgG rather than IgM in them (Schneider *et al.*, 2015). Responses of purified anti-Gal samples coated on microplates to anti-IgG and IgM were compared between low Lp(a) plasma group and high Lp(a) plasma group (16 each).

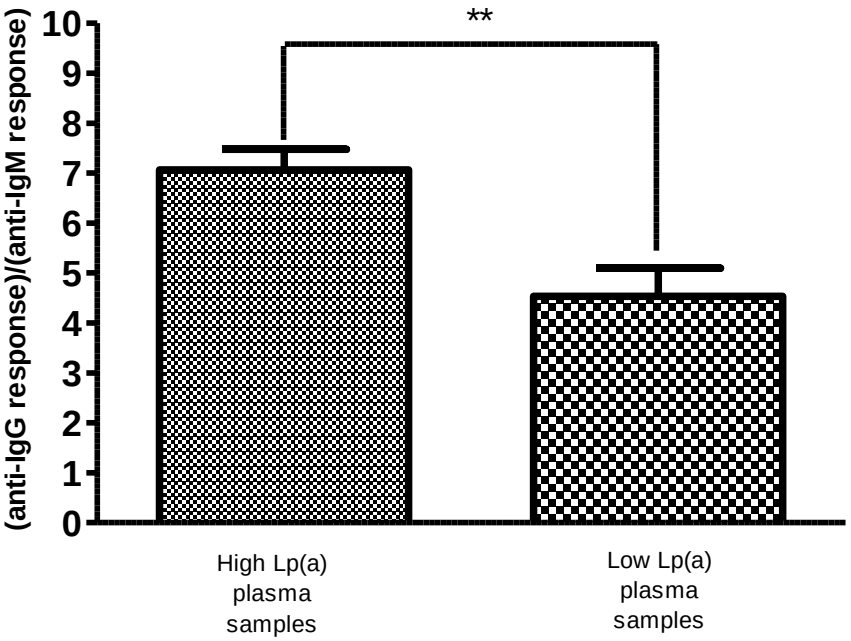


Figure 11. Anti-Gal IgG and IgM levels in low and high Lp(a) plasma samples. *Polystyrene wells were coated with TIM (1 µg/well), blocked and 50 times diluted plasma was added and incubated for 2 h at 4 °C followed by probing with 200 µl HRP-conjugated anti-human IgG and IgM separately (1.5 µg/ml in PBS-T) and bound HRP was assayed. **: p value =0.0014. Mean ± SEM of 16 plasma samples.*

Result in Fig.11 showed that while anti-Gal antibodies from both groups were dominated by IgG, IgG domination over IgM was significantly higher in high

plasma Lp(a) concentration/small Lp(a) group than in low plasma Lp(a) concentration/ larger Lp(a) group (*p* value =0.0014).

(G). Small Lp(a) phenotype did not increase specific activity of anti-β-glucoside antibody

Specific activity of a non-anti-Gal human plasma antibody, viz anti-β-glucoside antibody (ABG) was measured using cellobiose-conjugated trypsin inhibitor (TIC) in polystyrene well-coated form for antibody capture in the procedure described for specific activity assay (Methods 3.2.17).

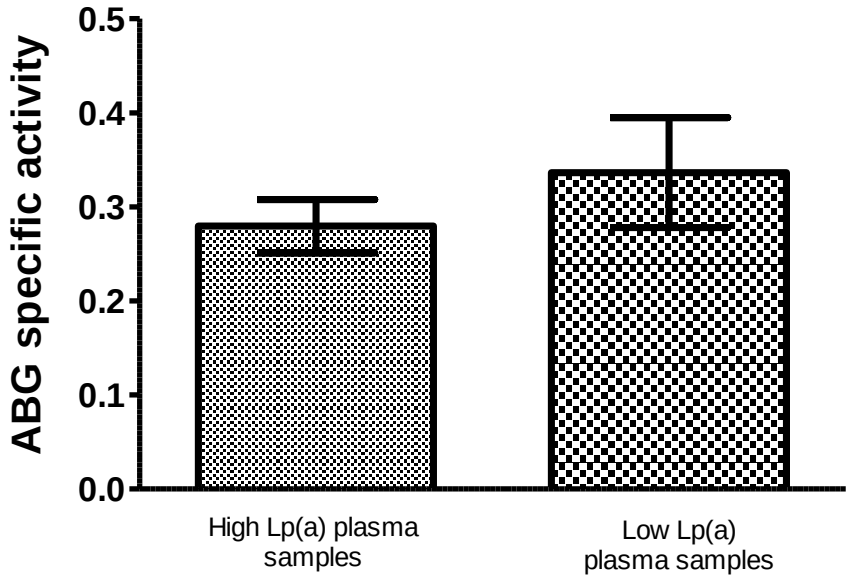


Figure 12. Specific activity of ABG in high and low Lp(a) plasma samples. *The specific activity of ABG antibody isolated from plasma samples with high and low Lp(a)concentration was compared. p value =0.402. Mean ± SEM of 6 plasma samples.*

Small Lp(a) phenotype in high plasma Lp(a) individuals did not increase the specific activity of ABG over that of large Lp(a) individuals. (n=6 in each group; *p* value

=0.402) asserting that specificity of anti-Gal for STPS in apo(a) subunit of Lp(a) was responsible for the Lp(a) size-dependent modulation of specific activity of anti-Gal antibody.

Discussion

Circulating Lp(a) had been found to carry non-covalently adhering LDL (adduct LDL) in addition to the lone LDL molecule to which the apo(a) subunit is attached covalently by a disulfide linkage (Marcovina *et al.*, 1993). The number of adduct LDL molecules attached to each Lp(a) molecule increased with the size of the apo(a) subunit of the individual so that Lp(a) free from adduct LDL was undetectably low in very large Lp(a) molecules (Kalaivani and Appukuttan, 2014). Increase in the number of adduct LDL molecules with Lp(a) size and sharp drop in non-covalent attachment of LDL to Lp(a) following desialylation of the latter (data not shown) indicated that the negative charge carried by the sialic acid moieties at the terminals of O-glycans mediated attachment of LDL through the positively charged amino acid residues of the apoB chain in the latter (Yashiro *et al.*, 1993) . Nevertheless this occupation by LDL is likely to obstruct the approach of anti-Gal, mostly IgG and much smaller than LDL to the O-glycan-rich region preparatory to accommodating the underlying STPS. In support of this assumption the O-glycan-specific lectin jacalin also blocked recognition of Lp(a) by anti-Gal (Geetha *et al.*, 2014). In further evidence LDL-mediated blocking of anti-Gal binding to apo(a) subunit, reflected in the drop in apo(a)-mediated increase of antibody-associated fluorescence, was significantly more pronounced in high molecular weight apo(a) subunit than in the same number of low molecular weight apo(a) subunits (Fig.5) apparently since a

large number of LDL molecules adhering to the former would more effectively block the approach of anti-Gal molecules to the O-glycan-rich region of apo(a).

The differential effect of LDL on anti-Gal binding to Lp(a) of varying size was confirmed using the circulating forms of Lp(a). The latter, prepared by jacalin-mediated precipitation and having adduct LDL still adhering to them (Kalaivani and Appukuttan, 2015) possess adduct indices (adduct LDL per Lp(a) molecule) strictly governed by their Lp(a) size since isolated pure Lp(a) captured exactly the same number of adduct LDL as in circulation (Kalaivani and Appukuttan, 2014). Consequently circulating large Lp(a) molecules, despite possessing much longer O-glycosylated STPS-rich regions were significantly less efficient in binding to anti-Gal than the same number of small Lp(a) molecules (Fig.5).

Negative correlation of Lp(a) size with its efficiency as an anti-Gal ligand, seen here implied that had Lp(a) been a homologous antigen that effected affinity maturation of anti-Gal, the specific activity of the antibody would be greater, the smaller the Lp(a) size. This was indeed found to be the case, with a convincing correlation coefficient and *p* value. In further confirmation of this effect plasma Lp(a) concentration showed an even more significant positive correlation with anti-Gal specific activity. These two results underlined the contribution of apo(a) size in Lp(a) towards determining the specific activity of anti-Gal and suggested that Lp(a) which differs widely among individuals in size and thereby its availability for anti-Gal binding, is an autologous antigen dictating affinity maturation of the antibody. Evidence here indicate that adduct LDL would attenuate this function of Lp(a).

Increasing adduct formation and a steep decline in adduct-free plasma Lp(a), as Lp(a) size increases (Kalaivani and Appukuttan, 2014) support this conclusion.

Besides being the most reactive among plasma O-glycoproteins towards anti-Gal, Lp(a) may owe its role as affinity maturation antigen, to its lipid component which could act as an adjuvant (Chung *et al.*, 2015). Lipoproteins had been shown to become less antigenic following removal of their lipid components (Patton *et al.*, 1982). Notably glycan-specific antibodies to glycolipid antigens were shown to be T-cell dependent (Haji-Ghassemi *et al.*, 2015). Several human anti-carbohydrate antibodies other than anti-Gal have been shown to be T-cell dependent and isotype-switched (Schneider *et al.*, 2015). Sustained presence of antigen had been shown to result in upto ten times more antibody production and affinity maturation (Chung *et al.*, 2015). Another reason for the pre-eminence of Lp(a) as an affinity maturation antigen for anti-Gal synthesis is the unique property of size variation of this lipoprotein among individuals, thus explaining the variations in specific activity of anti-Gal as well.

Confirmation of elevated expression and/or exposure of MUC1 as cancer phenotypes spurred efforts towards developing vaccines that generate antibodies targeting MUC1 (Deguchi *et al.*, 2010). Present result suggests a role for a natural antibody in the recognition of MUC1-rich cancer cells. Association of low plasma Lp(a) concentration which is accompanied by low specific activity anti-Gal in circulation, with increased incidence of cancer (Sawabe *et al.*, 2012) supports this conclusion. Also in agreement with this is the inverse association between incidence of cancer and Alzheimer's disease among population (Driver, 2014) since the latter

disease is marked by high plasma concentration/small size Lp(a) phenotype. In summary, results above suggest the possibility that variation in susceptibility to cancer depending on plasma concentration/size of Lp(a) operates through Lp(a)-dependent modulation of anti-Gal activity. Clinical research could reveal whether high specific activity anti-Gal protects from cancer.

PART II

Small lipoprotein(a) phenotype reduces breast cancer susceptibility by enhancing specific activity of MUC1-binding anti-Gal antibody through affinity maturation

Introduction

Though it appears quite mundane at present to implicate the host defense systems in destroying tumor cells, immunobiology was at its infancy when Paul Ehrlich suggested in 1909 that the body possesses mechanisms to counter and eliminate aberrant cells such as tumors (Ehrlich, 1909). Thereafter it took half a century to demonstrate development of specific immune responses in the host triggered by tumor-specific or neo-antigens in a manner similar to the homograft rejection process (Thomas, 1959, Burnet, 1957). Presently immunotherapy is the fastest advancing therapeutic option in cancer and is predicted to constitute a major part of anti-cancer therapy in a few years (Ledford, 2014). In this context the role of naturally occurring host antibodies that recognize cancer-specific antigens is increasingly being investigated (Maccalli *et al.*, 2017). Since MUC1 is a conspicuous tumor-associated antigen as mentioned in the introduction (Taylor-Papadimitriou *et al.*, 1999) and is recognized by anti-Gal, the contribution of anti-Gal antibody towards anti-tumor defense emerged as a major research question. Since anti-Gal circulates in all individuals, while only a few develop tumors, the factor(s) modulating anti-Gal activity could be suspected to determine the individual's susceptibility to cancer. Results in the previous chapter revealed that high circulating concentration of Lp(a) which is often accompanied by low molecular weights of these lipoproteins gave rise to anti-Gal molecules with relatively greater specific activity values. Since more active anti-Gal could be expected to bind faster to MUC1 bearing cancer cells in the process of antibody-mediated anti-tumor defense, this effect offered a possible route by which small

Lp(a) phenotype could be operative in anti-tumor defense, since Lp(a) per se could not be conceived to take part in this defense. The present chapter details our investigation on whether higher specific activity of anti-Gal brought about by small size/ high concentration Lp(a) phenotype offered protection from breast cancer in women subjects, employing a randomized control study.

Results

(A). Serum Lp(a) levels are lower in breast cancer patients than in controls

Enzyme-linked immunoassay used earlier for Lp(a) assay in sera were based on detecting the apoB content of Lp(a) captured on microplate-coated anti-apo(a). We had shown that apoB molecule was differentially available for anti-apoB binding since the apo(a) subunit which was much larger than apoB and was overlying the latter masked it to varying degrees depending on the size of the apo(a) subunit which varied widely among individuals (Kalaivani and Appukuttan, 2015). More importantly circulating Lp(a) molecules contained non-covalently attached LDL molecules in proportion to apo(a) size, in addition to the lone apoB molecule covalently attached to apo(a) as an integral part of Lp(a) (Kalaivani and Appukuttan, 2014). Since both the above factors interfere in apoB-dependent Lp(a) assay, we had developed an apoB-independent ELISA for Lp(a) using microplate-coated form of the lectin jacalin for Lp(a) capture and assay using anti apo(a)-HRP (Sreekumar *et al.*, 2013).

Reports on contribution of serum Lp(a) levels to incidence of cancer have been conflicting. A recent study reported that low serum Lp(a) was a significant risk

factor for all the cancers though this trait was protective against lung cancer (Mieno *et al.*, 2014). In contrast elevated serum Lp(a) was reported earlier in breast cancer. (Kökoğlu *et al.*,1994). A marginally significant protection from cancer death was reported for individuals with high plasma Lp(a) (Sawabe *et al.*, 2012). This uncertainty as well as the availability of a new apoB-independent Lp(a) assay procedure prompted a reassessment of the contribution of Lp(a) concentration towards cancer incidence. More importantly high plasma Lp(a) titer was shown by results in the previous chapter to cause synthesis of more reactive ant-Gal antibodies towards the most ubiquitous cancer-specific antigen tumor-associated MUC1.

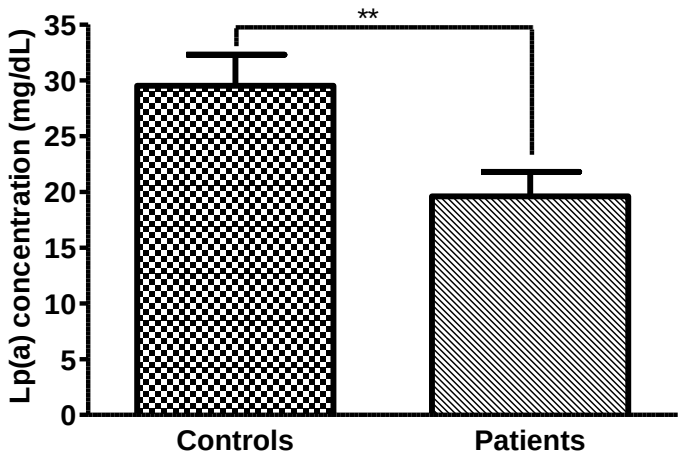


Figure 13. Lipoprotein(a) concentration of individuals. *Lp(a) concentration of controls and patients was assayed by an apoB-independent enzyme immunoassay as described in Methods 3.2.15. **: p value =0.0067; n=46 in each group.*

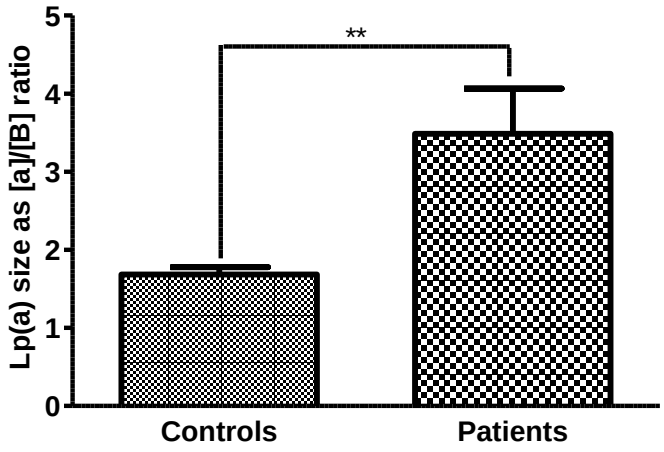


Figure 14. Molecular size of lipoprotein(a) in individuals. *Molecular size of Lp(a) as ([a]/[B] ratio was measured in controls and patients as described in Methods 3.2.19. **: p value =0.0023; n=46 in each group.*

Lp(a) concentration in serum were significantly reduced in patients compared to controls (Fig.13; *p* value =0.0067; n=46 in either group). This relationship was corroborated by the observation (Fig.14) that controls had significantly lower molecular weight Lp(a) than patients (*p* value =0.0023, n=46) since it had been shown in the previous chapter that high Lp(a) concentration was generally accompanied by small size Lp(a).

To test whether low Lp(a) levels were associated with an increased risk of cancer Pearson Chi-Square test was used. Chi-square test for trend was applied to test linear trend. 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 odds ratio and 95% confidence interval (CI).

Table 3. Binary logistic regression analysis of low Lp(a) level as a risk factor for cancer

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

Binary logistic regression analysis of low Lp(a) level as risk factor for cancer with odds ratio (OR) and confidence interval (CI). n: number of subjects with low and high Lp(a) concentration values.

Analysis revealed that low Lp(a) level is associated with a risk for cancer. The above odds ratio indicates that the chance of getting lower Lp(a) level (≤ 30 mg/dL) in cancer patients is 3.769 times greater as compared to controls. That is there is 3.769 times greater risk of getting cancer in low Lp(a) level group.

The risk of cancer as a function of serum Lp(a) level was also studied by categorizing Lp(a) levels into quartiles (serum Lp(a) level ≤ 10, 10.1-20, 20.1-30, >30 mg/dL) based on its distribution in the control population. Binary logistic regression analysis using group of participants (cases/controls) as the dependent variable and level of Lp(a) as independent variable was carried out.

Table 4. Binary logistic regression analysis expressing OR and 95% CIs for quartiles of Lp(a)

Quartiles of Lp(a) (range, mg/dL)[a]	>30	20.1-30	10.1-20	≤ 10
Controls (n) (%)	22 (47.8)	9 (19.6)	7 (15.2)	8 (17.4)
Patients (n) (%)	9 (19.6)	10 (21.7)	8 (17.4)	19 (41.3)
Unadjusted OR	1	2.716	2.794	5.806
95% CI		0.828-8.914	0.779-10.016	1.870-18.027
p value		0.099	0.115	0.002
p value for trend	0.002			

[a] *Quartiles were defined according to Lp(a) distribution in control population.*

Table 4 shows odds ratios of risk for cancer for lower quartiles of Lp(a) compared to the highest quartile taken as reference. In this model risk of cancer increased with decrease in concentration of Lp(a) (*p* value for trend 0.002) but the odds ratio was statistically significant only in the lowest quartile (OR 5.806, CI 1.870-18.027) where individuals with Lp(a) value below 10 mg/dL were compared to those with Lp(a) values above 30 mg/dL. Logistic regression revealed a higher odds ratio for lower levels of Lp(a) indicating it as an important risk factor for cancer in the population.

(B). Correlation of anti-Gal activity with incidence of cancer

It had been observed in the previous chapter that individuals with relatively smaller size and higher plasma titre Lp(a) synthesized higher specific activity anti-Gal molecules. Since both specific activity and antibody concentration could be expected to count in MUC1-dependent anti-tumor defense by anti-Gal, the combined effect of the two parameters, termed total activity per given volume or antibody level, and measured as optical density response in ELISA for anti-Gal was compared between patients and controls.

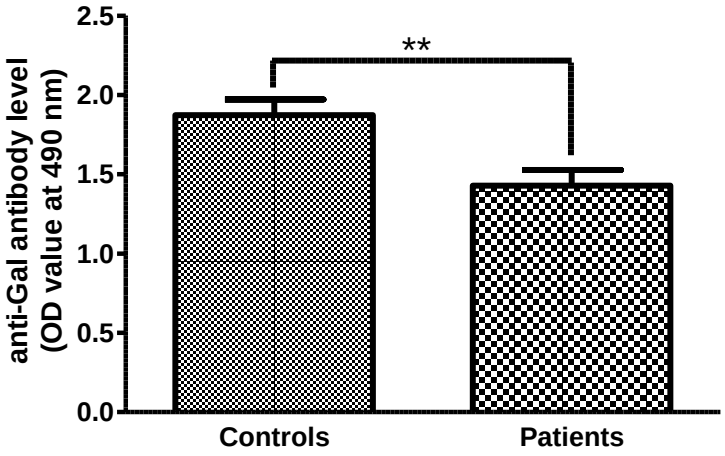


Figure 15. Total anti-Gal activity in given volume in patients and controls. *Serum anti-Gal activity was assayed in controls and patients as described in Methods 3.2.16. **: p value =0.0033; n=46 in each group.*

Patients had decidedly less total anti-Gal activity response than controls (Fig.15) indicating that small Lp(a)-mediated protection from cancer may be operating through greater total anti-Gal activity response (*p* value =0.0033; n=46 in either group). Anti-Gal assay response shown in Fig.15 was a combined effect of both the number and the specific activity of anti-Gal molecules. The latter factor is governed

by affinity maturation mediated by the widely varying size and concentration of the autologous antigen Lp(a) as revealed in the previous chapter.

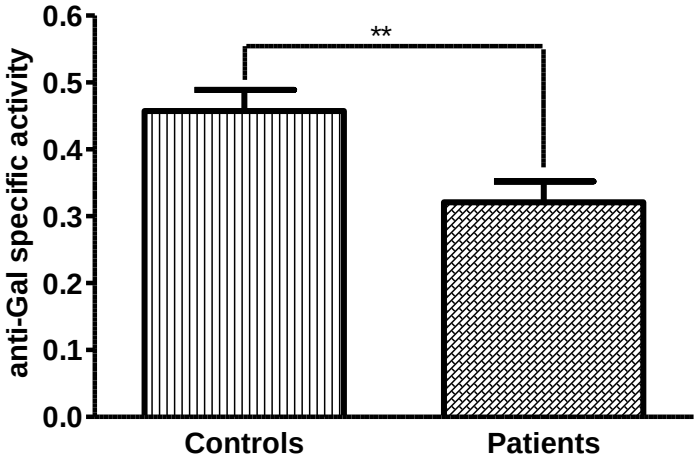


Figure 16. Specific activity of anti-Gal in individuals. *Specific activity of anti-Gal isolated from controls and patients was determined as described in Methods 3.2.17. **: p value =0.0033; n=46 in each group.*

Fig.16 results showed that cancer patients had anti-Gal with significantly lower specific activity (*p* value =0.0033; n=46 in either group) indicating that differences in specific activity, dictated by Lp(a) size, could alone account for the increased incidence of cancer in low titer/high molecular weight individuals. In comparison specific activity of another anti-carbohydrate antibody, dextran-binding immunoglobulin (DIg) which was non-reactive towards Lp(a) was nearly the same in both groups as shown in Fig.17 (n= 8 consecutive samples from either group).

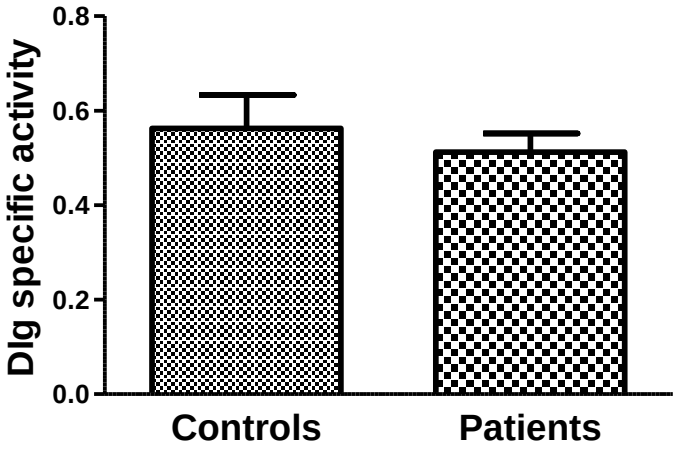


Figure 17. Specific activity of dextran-binding immunoglobulin (DIg) in individuals. *Specific activity of DIg isolated from controls and patients was determined as described in Methods 3.2.17. p value =0.5412; n=8 in each group.*

Correlation of anti-Gal specific activity 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 activity as independent variable, to estimate odds ratio (OR) and 95% confidence interval (CI).

Table 5. Binary logistic regression analysis of low anti-Gal specific activity as risk factor for cancer

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.

Analysis revealed that low specific activity anti-Gal was associated with a risk for cancer. The above odds ratio indicates that the chance of getting low specific active anti-Gal (≤ 0.4) in cancer patients was 3.215 times greater compared to controls.

(C). Lp(a) levels in patients at different stages of cancer

Lp(a) levels did not vary significantly among patients belonging to different stages of cancer determined by clinical assessment (Fig.18) or histological grading (Fig.19). This was not unexpected since cancer phenotype per se are not known to generate any condition that affects plasma Lp(a) concentration which is genetically determined.

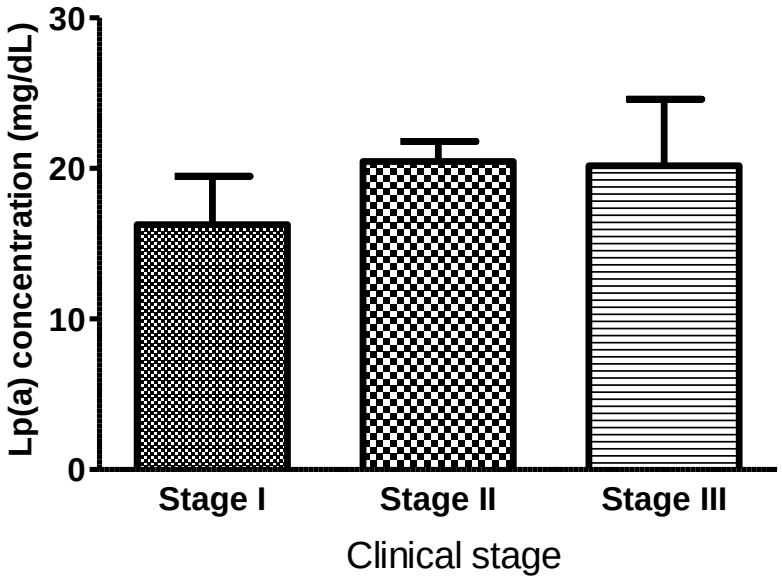


Figure 18. Variation of Lp(a) concentration with different clinical stages of breast cancer. *Lp(a) concentration in patients in different clinical stages was assayed by an apoB-independent jacalin based enzyme immunoassay as described in Methods 3.2.15. n=5 for stage I, n=32 for stage II, n=9 for stage III.*

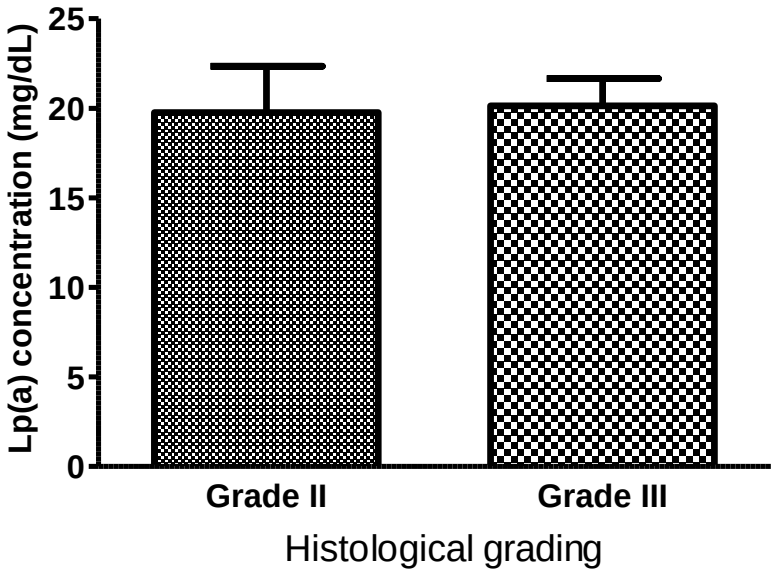


Figure 19. Variation of Lp(a) concentration in patients with different histological grades of tumor. *Lp(a) concentration in patients with different histological grades of tumor was assayed by an apoB-independent jacalin based enzyme immunoassay as described in Methods 3.2.15. n= 10 for grade II, n=36 for grade III.*

(D). Anti-Gal level in patients of different stages of cancer

In contrast to Lp(a) concentration total anti-Gal activity in serum increased significantly from stage I to stage III patients determined by clinical assessment (Fig.20). The increase from stage II to stage III was less significant. However there was significant increase in some anti-Gal from stage II to stage III determined by histological grading (Fig.21).

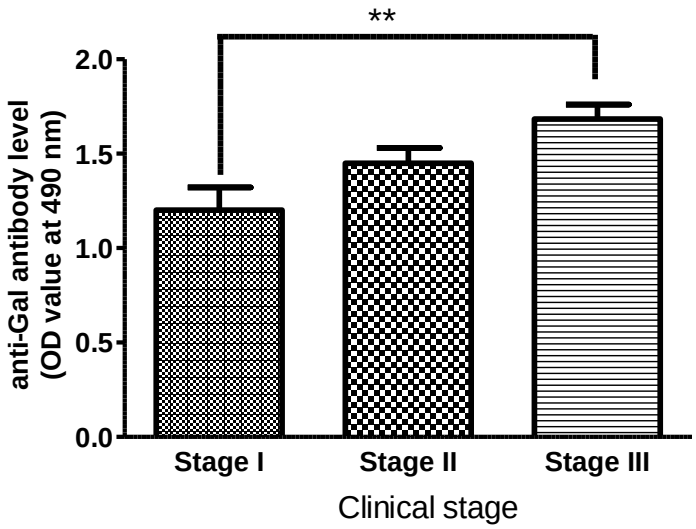


Figure 20. Variation of anti-Gal titre with different clinical stages of breast cancer. *Anti-Gal titre in patients in different clinical stages was assayed as described in Methods 3.2.16. **: p value = 0.004 for stage I vs stage III. n=5 for stage I, n=32 for stage II, n=9 for stage III.*

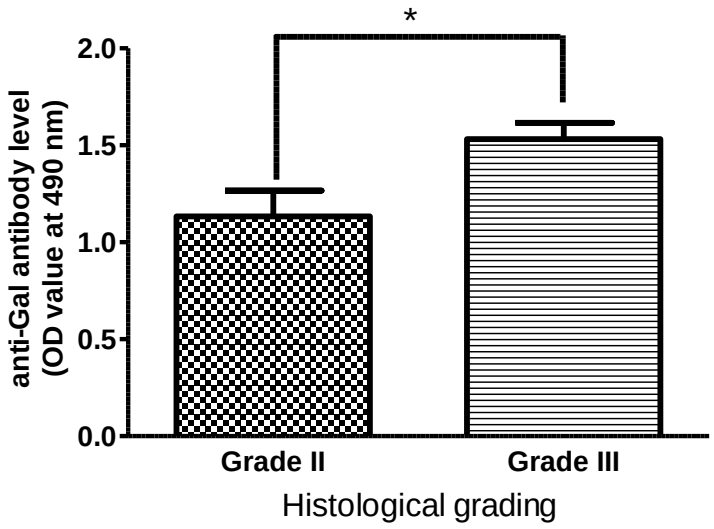


Figure 21. Variation of anti-Gal titre in patients with different histological grades of tumor. *Anti-Gal titre in patients with different histological grades of tumor was assayed as described in Methods 3.2.16. *: p value = 0.0258 for grade II vs grade III. n= 10 for grade II, n=36 for grade III.*

(E). Specific activity of anti-Gal in cancer patients; changes with advancing stages

Specific activity of anti-Gal increased significantly as the cancer progressed from stage I to stage II and from stage I to stage III, assessed clinically (Fig.22). Obviously the low sample numbers in stages I and III compared to stage II could have affected the *p* value since actual differences between stage I and II and between stage I and III were more marked than what the *p* values would indicate. The latter conclusion was supported by comparing specific activities of serum anti-Gal between histological grades II and III, which showed a *p* value of 0.0286 (Fig.23).

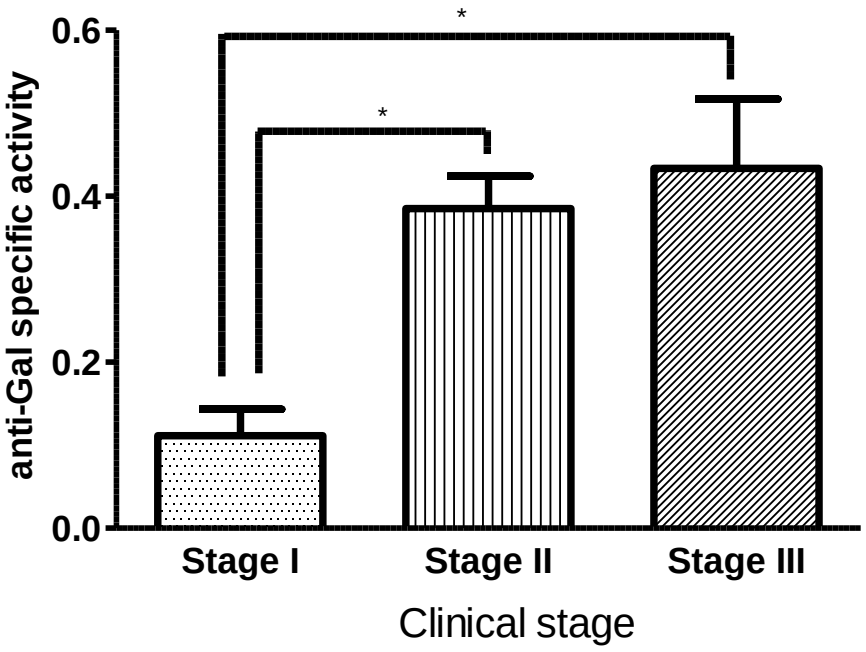


Figure 22. Variation of anti-Gal specific activity with different clinical stages of breast cancer. *Specific activity of anti-Gal antibody in patients in different clinical stages was determined as described in Methods 3.2.17. *: p value = 0.0413 for stage I vs stage II, p value =0.0497 for stage II vs stage III. n=5 for stage I, n=32 for stage II, n=9 for stage III.*

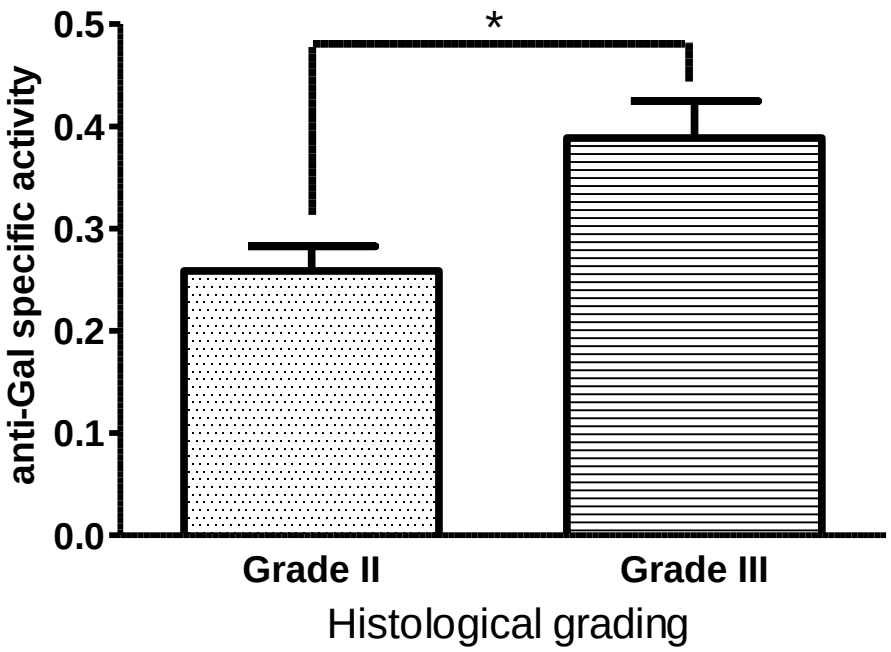


Figure 23. Variation of anti-Gal specific activity in patients with different histological grades of tumor. *Specific activity of anti-Gal antibody in patients with different histological grades of tumor was determined as described in Methods 3.2.17. *: p value = 0.0286 for grade II vs grade III. n= 10 for grade II, n=36 for grade III.*

(F). Confirmation of presence of MUC1 in samples of proteins isolated from tissues

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 these samples to inhibit binding of anti-Gal to its ligand, trypsin inhibitor melibiose (TIM) coated on microplate. Firstly, 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 (Fig.24).

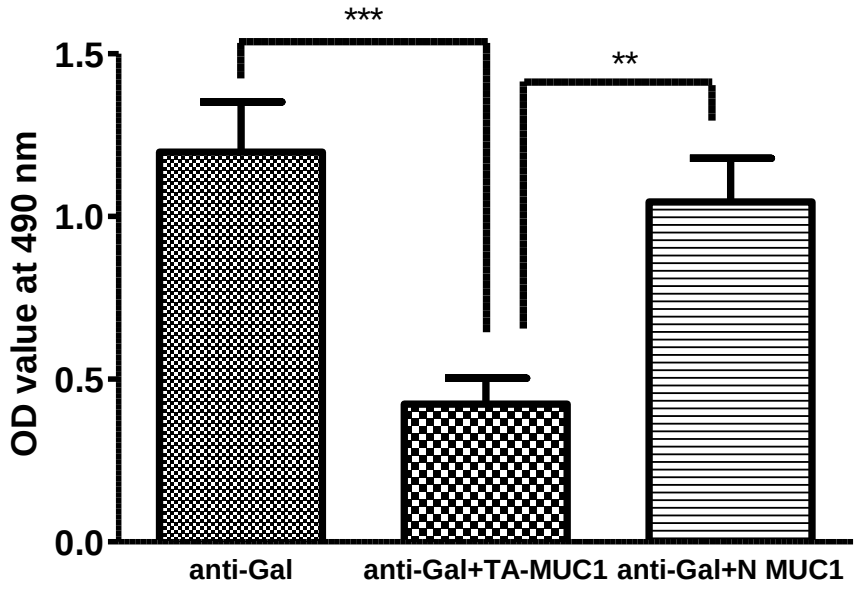


Figure 24. Recognition of tumor-associated MUC1 by anti-Gal antibody. *Polystyrene wells were coated with TIM (1 μg/well). Anti-Gal (75 ng in 200 μl) pre-incubated at 4 °C for 18 h with either 2 μg tumor-associated MUC1 (TA-MUC1) or 2 μg MUC1 derived from normal tissue (N MUC1), was added to the wells, followed by incubation at 4 °C for 2 h. After washing the wells 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 of each antibody) for 2 h at 4 °C. The bound HRP was assayed using OPD as substrate. Untreated anti-Gal added to TIM coated wells served as control. ***: p value =0.0003 for blocking of anti-Gal by TA-MUC1. **: p value =0.0011for difference in blocking of anti-Gal by TA-MUC1 and N MUC1. n= 12 in each group.*

GS-IB4 is a lectin from the plant *Griffonia simplicifolia* with nearly identical specificity as anti-Gal and binds to terminal α-galactoside groups as well as to MUC1 accommodating STPS of the latter in place of sugar at its binding site. Presence of MUC1 antigen in the protein samples isolated from tissues was further confirmed by the ability of these samples to inhibit the binding of GS-IB4 to its ligand, trypsin inhibitor melibiose (TIM) coated on microplate wells (Fig.25).

Binding of biotinylated GS-IB4 to TIM, its specific ligand was strongly inhibited by TA-MUC1 but poorly by normal MUC1 (N MUC1).

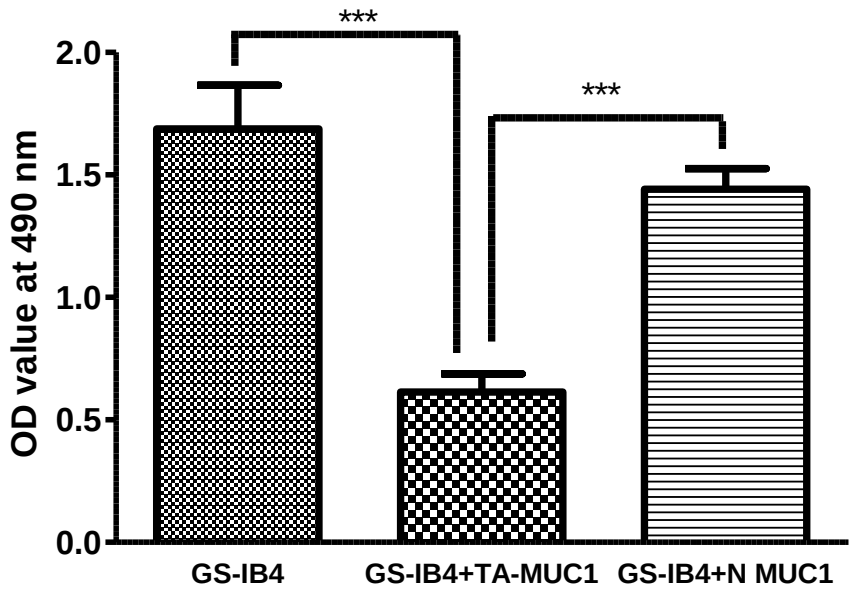


Figure 25. Recognition of tumor-associated MUC1 by GS-IB4 lectin. *Biotinylated GS-IB4 lectin (75 ng in 200 µl) pre-incubated at 4 °C for 18 h with either 2 µg tumor-associated MUC1 (TA-MUC1) or 2 µg MUC1 derived from normal tissue (N MUC1) was added to TIM (1 µg/well) coated wells and bound lectin was assayed using avidin-HRP. Untreated GS-IB4-biotin added to TIM coated wells served as control. ***: p value <0.0001 for blocking of GS-IB4 by TA-MUC1 and also for difference in blocking of GS-IB4 by TA-MUC1 and N MUC1. n= 12 in each group.*

Presence of MUC1 in tumor extracted proteins was further confirmed by 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 (Fig.26).

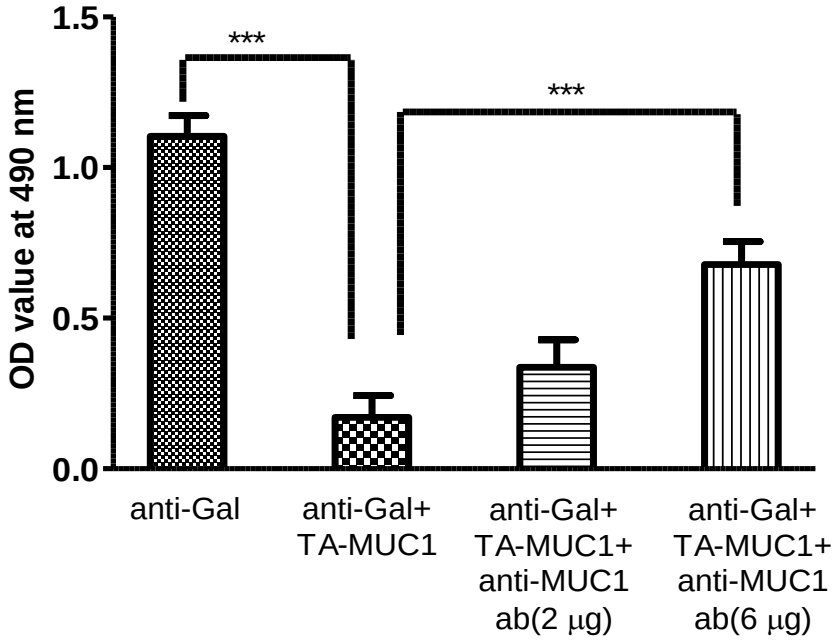


Figure 26. Confirmation of presence of MUC1 in tumor extracted proteins using anti-MUC1 antibody. *Polystyrene wells were coated with TIM (1 μg/well). Anti-Gal (75 ng in 200 μl) pre-treated at 4 °C for 18 h with either 2 μg MUC1 alone or 2 μg MUC1 previously incubated at 4 °C for 6 h with either 2 or 6 μg anti-MUC1 antibody (goat) was added to the wells, followed by incubation at 4 °C for 2 h. Washed wells were incubated with 200 μl of a mixture of HRP conjugates of anti-human IgG, IgA and IgM (1.5 μg/ml of each antibody) for 2 h at 4 °C. The bound HRP was assayed. Untreated anti-Gal added to TIM coated wells served as control. ***: p value <0.0001 for blocking of anti-Gal by TA-MUC1 and p value =0.0007 for the reversal of TA-MUC1 binding of anti-Gal antibody by anti-MUC1 antibody (6μg). n=6 in each group.*

(G). Effect of O-glycosylation status of TA-MUC1 on anti-Gal reactivity

Response of microplate-coated MUC1 to jacalin (O-glycan-specific lectin) in enzyme-linked lectin assay was significantly lower for TA-MUC1 compared to N MUC1 (Fig.27) indicating marked hypoglycosylation in TA-MUC1 and suggesting a possible reason for TA-MUC1 to be more accessible and reactive to anti-Gal.

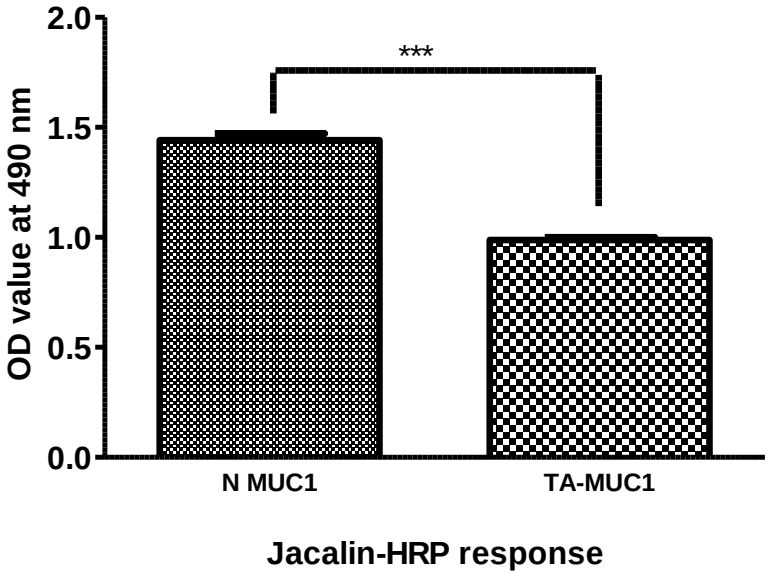


Figure 27. Comparison of O-glycosylation of MUC1 derived from normal and tumor tissues. *N MUC1 and TA-MUC1 (500 ng/well) were directly coated on microplate wells and blocked. After washing wells were probed with jacalin-HRP. Bound lectin was assayed. ***: p value <0.0001. n=12 in each group.*

To assess variation of anti-Gal reactivity with O-glycosylation of TA-MUC1, 24 samples of the latter were coated on polystyrene wells and binding response to HRP-conjugated form of the O-glycan-specific lectin jacalin to the TA-MUC1 samples measured as described earlier. Percentage of inhibition by TA-MUC1 samples of anti-Gal binding to TIM 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 (Fig.28) revealed a Pearson's correlation coefficient r= -0.7618 with *p* value <0.0001 and suggested that anti-Gal reactivity to MUC1 increased as O-glycosylation decreased. This conclusion was further confirmed by a nearly identical pattern with the above for inhibition of GS-IB4 binding to TIM (r= -0.7485, *p* value <0.0001) (Fig.29).

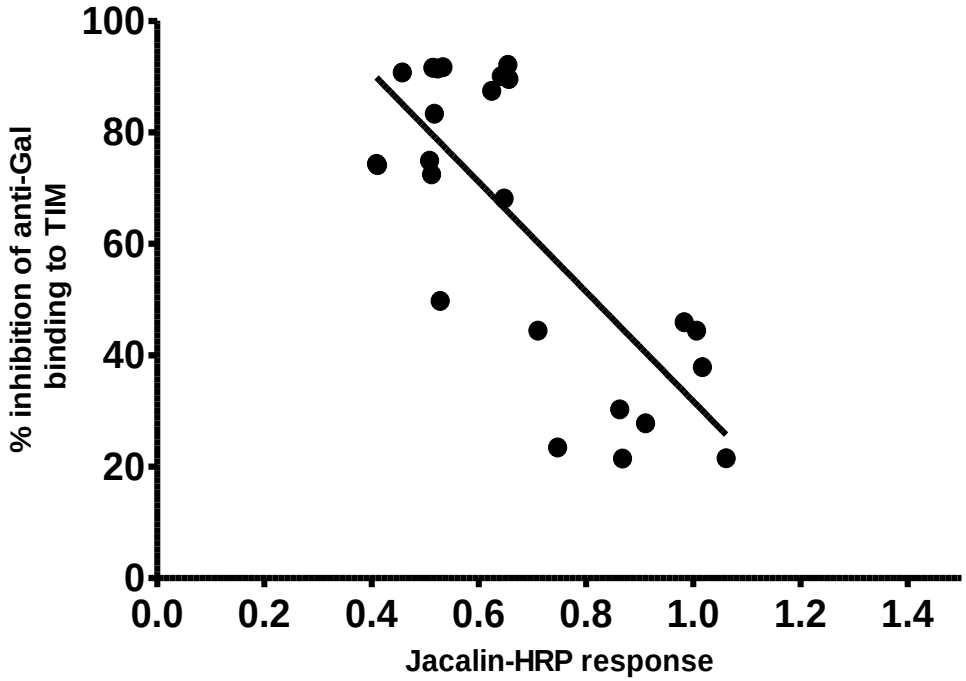


Figure 28. Correlation between O-glycosylation of tumor-associated MUC1 and percentage inhibition of anti-Gal binding to TIM. *In order to assess the O-glycosylation status of different tumor-associated MUC1 samples, 500 ng of TA-MUC1 was directly coated on microplate wells and incubated with HRP conjugate of jacalin for 2 h at 4 °C and bound HRP activity was assayed.*

Ability of tumor-associated MUC1 to inhibit binding of anti-Gal 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) for 18 h at 4 °C followed by addition to TIM (1 µg/well) coated wells and incubation at 4 °C for 2 h. Washed wells were probed with 200 µl of a mixture of HRP conjugates of anti-human IgG, IgA and IgM (1.5 µg/ml of each antibody) for 2 h at 4 °C. The bound HRP was assayed. HRP response for anti-Gal alone sample added to TIM coated wells was used as a benchmark to calculate the percentage inhibition of anti-Gal binding to TIM by TA-MUC1 samples. n=24, Pearson's correlation coefficient, r= -0.7618, p value <0.0001.

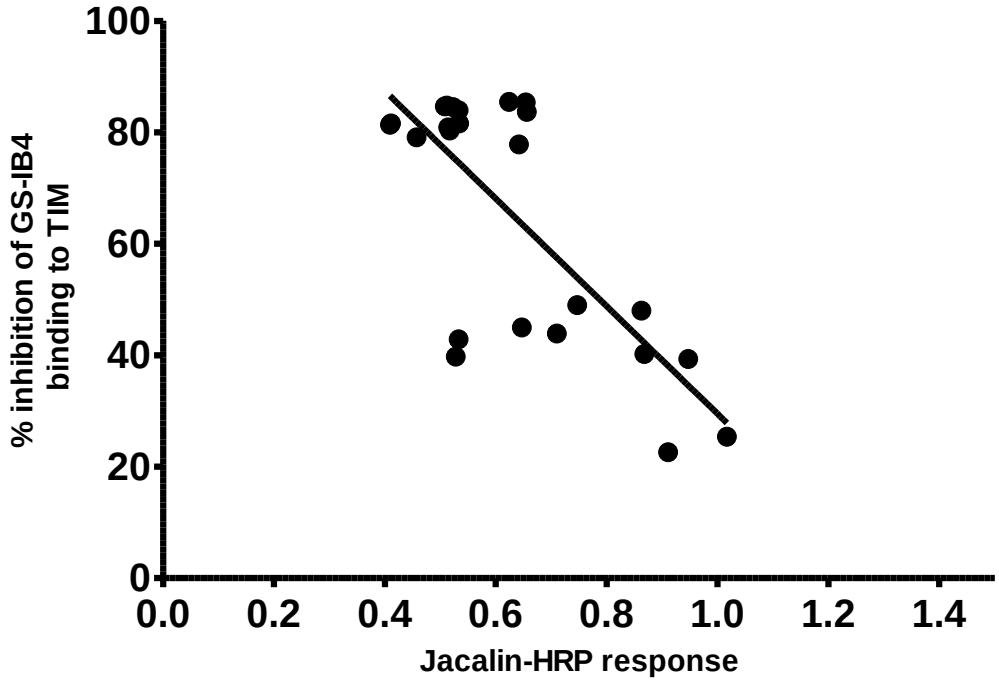


Figure 29. Correlation between O-glycosylation of tumor-associated MUC1 and percentage inhibition of GS-IB4 binding to TIM. *In order to assess the O-glycosylation status of different tumor-associated MUC1 samples, 500 ng of TA-MUC1 was directly coated on microplate wells and incubated with HRP conjugate of jacalin for 2 h at 4 °C and bound HRP activity was assayed.*

Ability of tumor-associated MUC1 to inhibit binding of GS-IB4 to its ligand, TIM was measured by incubating 2 µg tumor-associated MUC1 (TA-MUC1) from different tumor samples with biotinylated GS-IB4 (75 ng in 200 µl) for 18 h at 4 °C followed by addition to TIM (1 µg/well) coated wells and incubation at 4 °C for 2 h. Bound lectin was assayed using avidin-HRP. HRP response for GS-IB4 alone sample added to TIM coated wells was used as a benchmark to calculate percentage inhibition of GS-IB4 binding to TIM by TA-MUC1 samples. n=24, Pearson's correlation coefficient, r= -0.7485, p value <0.0001.

(H). Effect of tumor progression on anti-Gal reactivity of TA-MUC1

Anti-Gal reactivity of MUC1 from tumors at different stages was measured in terms of their inhibition of anti-Gal binding to its classical ligand TIM. Results (Fig.30) show 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.

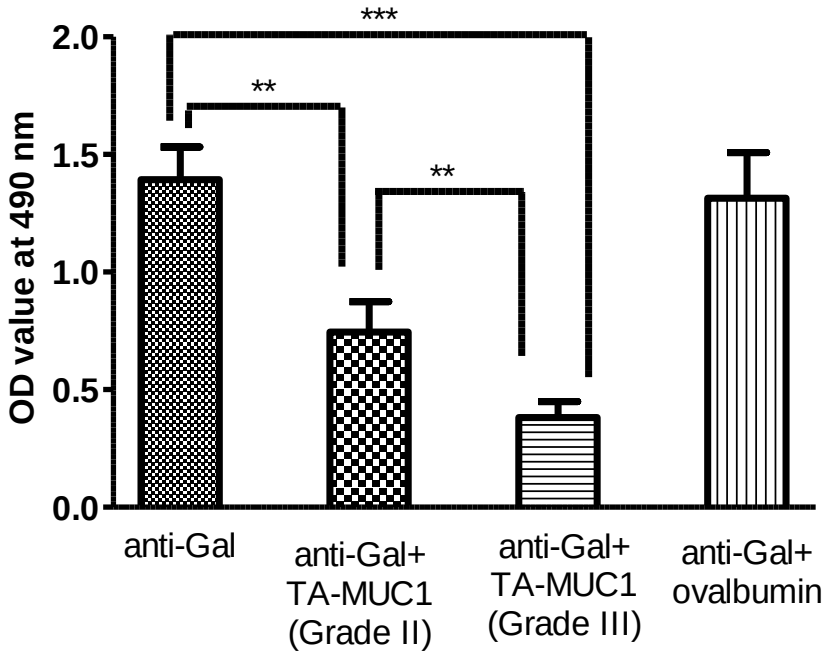


Figure 30. Inhibition of binding of anti-Gal antibodies to TIM by MUC1 from different grades of breast tumor. *Polystyrene wells were coated with TIM (1 µg/well). Anti-Gal (75 ng in 200 µl) pre-incubated at 4 °C for 18 h with 2 µg tumor associated MUC1 (TA-MUC1) from different grades of tumor or ovalbumin (2 µg) was added to the wells, followed by incubation at 4 °C for 2 h. Washed wells were probed with a mixture of HRP-conjugated goat antibodies against human immunoglobulins IgG, IgM and IgA. Bound antibody was assayed. Untreated anti-Gal 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*

*<0.0001 for blocking of anti-Gal by TA-MUC1 (grade III). **: p value =0.0034 for blocking of anti-Gal by TA-MUC1 (grade II) and p value =0.0099 for difference in blocking of anti-Gal by TA-MUC1 from grade II and grade III. n=8 in each group.*

Nearly identical pattern of inhibition was exhibited by the TA-MUC1 of grade II and III towards GS-IB4 lectin (Fig.31).

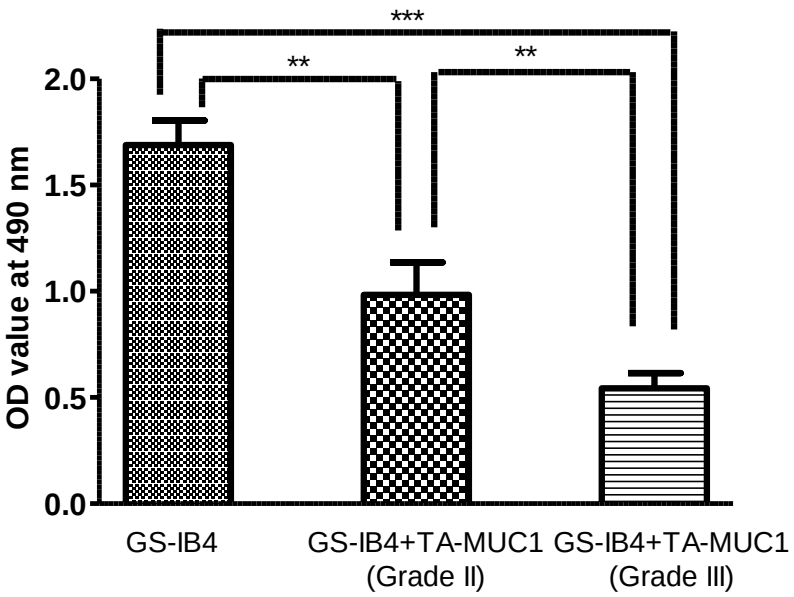


Figure 31. Inhibition of binding of GS-IB4 to TIM by MUC1 from different grades of breast tumor. *Biotinylated GS-IB4 lectin (75 ng in 200µl) pre-incubated at 4 °C for 18 h with 2 µg tumor associated MUC1 (TA-MUC1) from different grades of tumor was added to TIM (1 µg/well) coated wells and bound lectin was assayed using avidin-HRP. Untreated GS-IB4-biotin added to TIM coated wells served as control. ***: p value <0.0001 for blocking of GS-IB4 by TA-MUC1 (grade III). **: p value =0.0031 for blocking of GS-IB4 by TA-MUC1 (grade II) and p value =0.0056 for difference in blocking of GS-IB4 by TA-MUC1 from grade II and grade III. n=8 in each group.*

To further confirm the stage-dependent increase in anti-Gal reactivity of TA-MUC1 another parameter namely increase in fluorescence of fluorescently labeled anti-Gal

(anti-Gal-FITC) by these samples 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 (George *et al.*, 2015). Result (Fig.32) showed that increase in fluorescence of anti-Gal-FITC was twice as much by TA-MUC1 grade II and 4 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 as also the cancer stage-dependent increase in their affinity. The increase in fluorescence of anti-Gal was exclusively owing to occupation of binding site of the antibody by the MUC1 samples since presence of melibiose totally abolished enhancement of fluorescence.

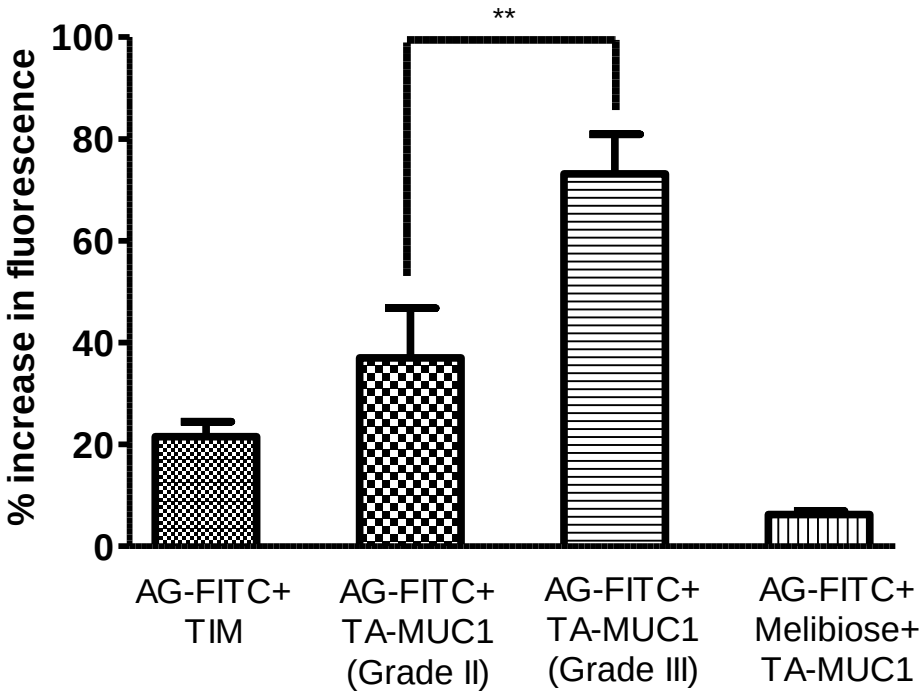


Figure 32. Binding of FITC-labeled anti-Gal to MUC1 from different grades of breast tumor. *MUC1 (10 µg) isolated from different grades of tumor (TA-MUC1) was incubated at 4 °C for 18 h with FITC-labeled anti-Gal (3 µg) pre-incubated*

*with or without 25 mM melibiose at 4 °C for 1 h. Volume was made upto 300 µl with PBS just before fluorescence measurement by excitation at 485 nm and emission at 520 nm. As 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. **: 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.*

(I). MUC1 binding increases with specific activity 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 activity) of the antibody. Three groups of purified anti-Gal samples, each consisting of 6 anti-Gal samples were selected so that average specific activity 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 group was noted.

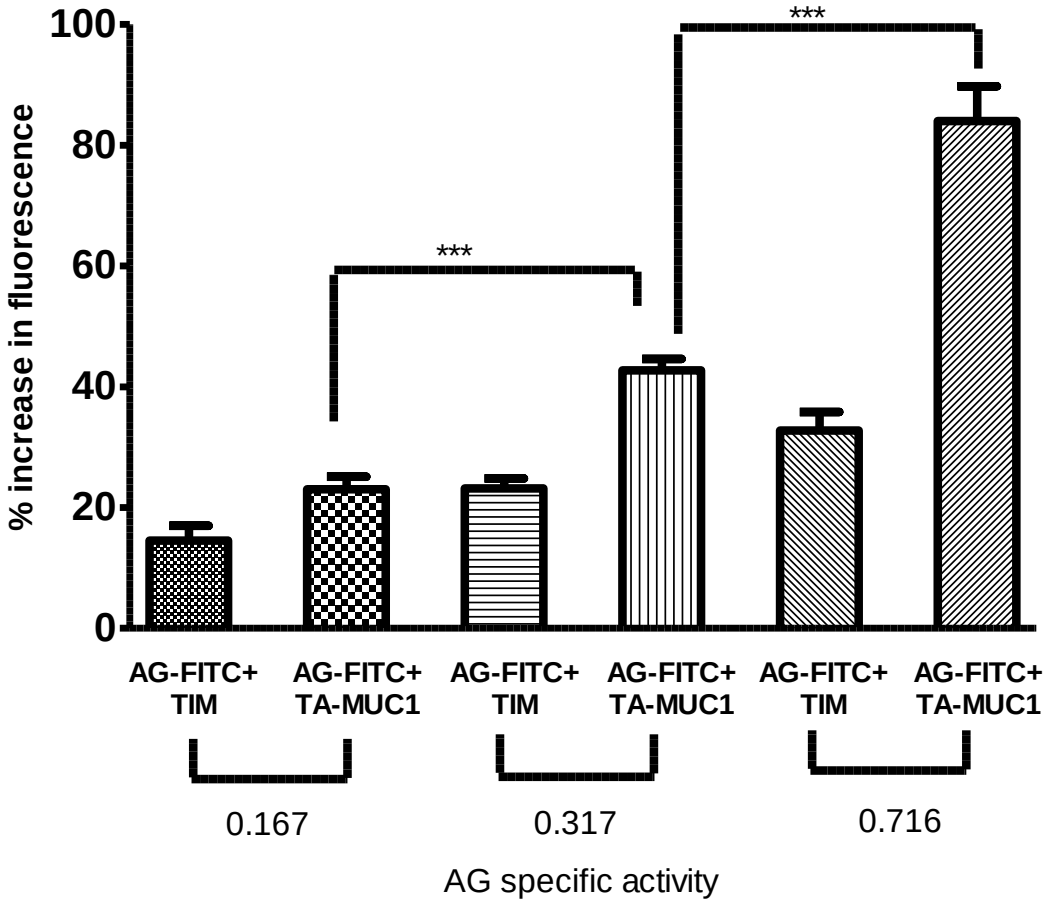


Figure 33. Comparison of binding of anti-Gal of varying specific activity to TA-MUC1. *Three groups (n=6 in each) of anti-Gal samples with average specific activity values 0.167, 0.317, 0.716 were used. Anti-Gal samples with varying specific activity were then labeled with FITC. Same amount of three randomly selected TA-MUC1 samples (10 µg) or the known ligand (TIM, 10 µg) was separately mixed with each of the FITC-labeled anti-Gal (3 µg) of varying specific activity at 4 °C for 18 h. Volume was made upto 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 <0.0001 for difference in fluorescence enhancement of different groups of FITC-anti-Gal produced by TA-MUC1 from grade II.*

Result (Fig.33) revealed that as anti-Gal specific activity doubled from group 1 to group 2, percentage of fluorescence increase which represents the binding affinity of the ligand towards the antibody also doubled. A similar increase of reactivity was also observed from group 2 to group 3 of antibodies. This result established that the reactivity of the same TA-MUC1 as antigen varied enormously depending on anti-Gal specific activity. In view of the demonstration in the previous chapter that lipoprotein(a) concentration/size, which varies among individuals, govern the specific activity, the results here allow us to suggest that anti-Gal with its specific activity decided largely by affinity maturation mediated by Lp(a), possibly acts as the major immunosurveillance and immunoprotective natural antibody against cancer.

The ability of tumor-associated MUC1 to inhibit binding of anti-Gal to its ligand, trypsin inhibitor melibiose (TIM) coated on microplate was compared with the ability of TA-MUC1 to inhibit binding of ABG to its ligand, trypsin inhibitor cellobiose (TIC) coated on microplate. Anti-β-glucan antibody is only weakly inhibited by TA-MUC1 (Fig.34).

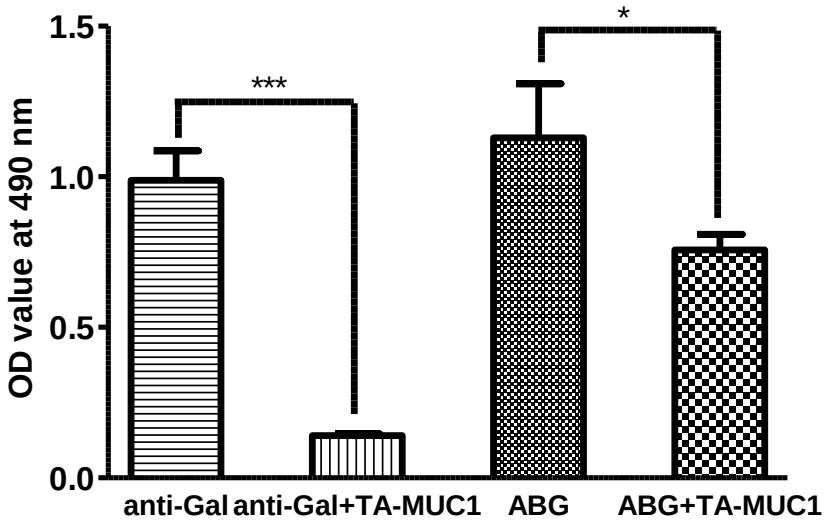
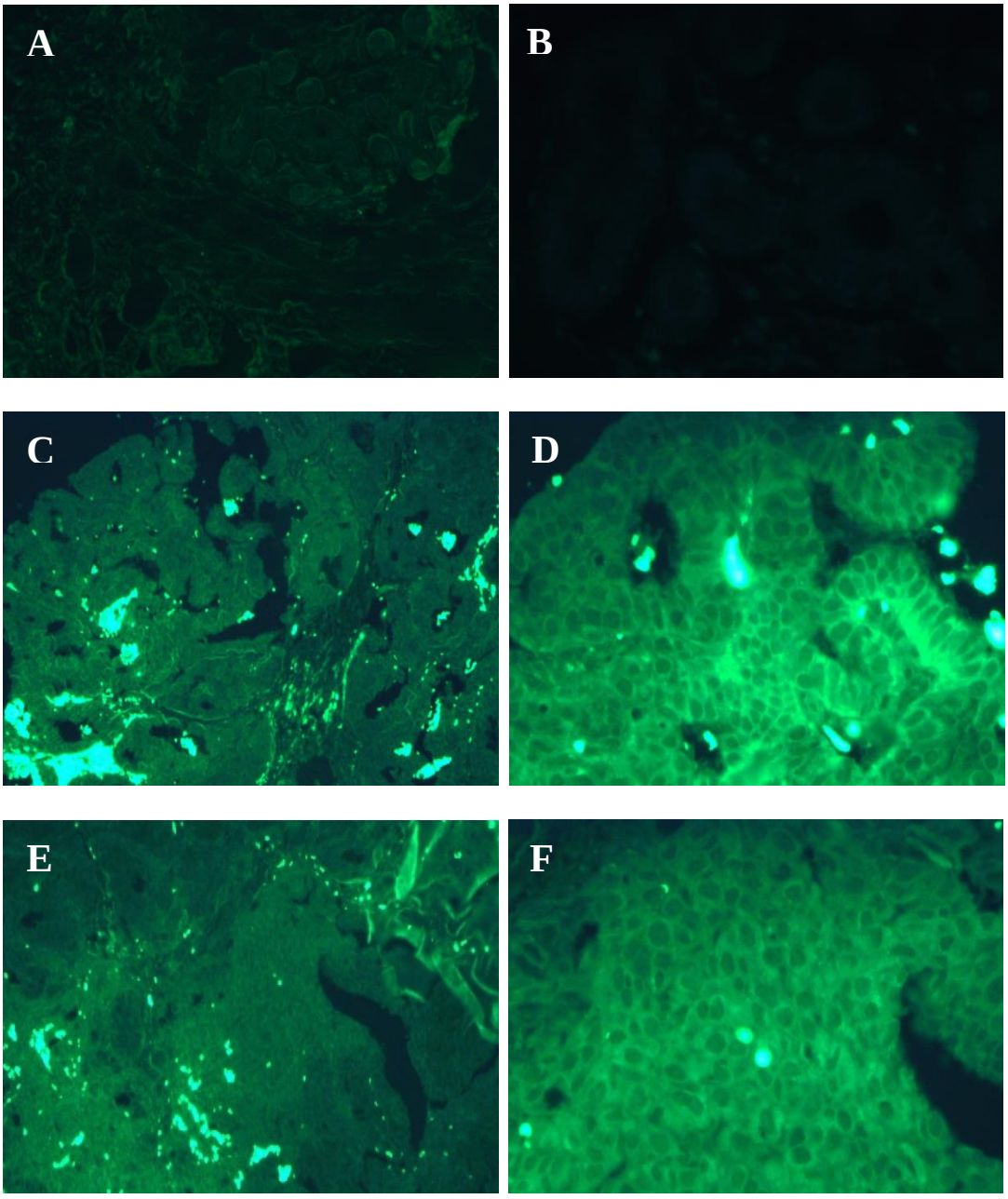


Figure 34. Comparison of anti-Gal and anti-β-glucoside antibody for TA-MUC1 binding. *Anti-Gal (100 ng in 200 µl) pre-incubated at 4 °C for 18 h with 2 µg tumor associated MUC1 (TA-MUC1) was added to TIM coated wells, followed by incubation at 4 °C for 2 h. Washed wells were probed with a mixture of HRP-conjugated goat antibodies against human immunoglobulins IgG, IgM and IgA. Bound antibody was assayed. Untreated anti-Gal added to TIM coated wells served as control. Anti-β-glucoside antibody (200 ng in 200 µl) pre-incubated at 4 °C for 18 h with 2 µg tumor associated MUC1 (TA-MUC1) was added to TIC coated wells, followed by incubation at 4 °C for 2 h. Washed wells were probed with a mixture of HRP-conjugated goat antibodies against human immunoglobulins IgG, IgM and IgA. Bound antibody was assayed. Untreated ABG added to TIC coated wells served as control. ***: p value <0.0001 for blocking of anti-Gal by TA-MUC1. *: p value =0.0319 for blocking of ABG by TA-MUC1. n=6 in either group.*

(J). Immunofluorescence analysis of anti-Gal antibody binding to tumor tissue

Recognition of MUC1 glycoprotein in breast tumor tissue by anti-Gal antibody was further confirmed by immunofluorescence staining of the formalin fixed tissue sections using FITC-labeled anti-Gal.

10X **40X**



10X **40X**

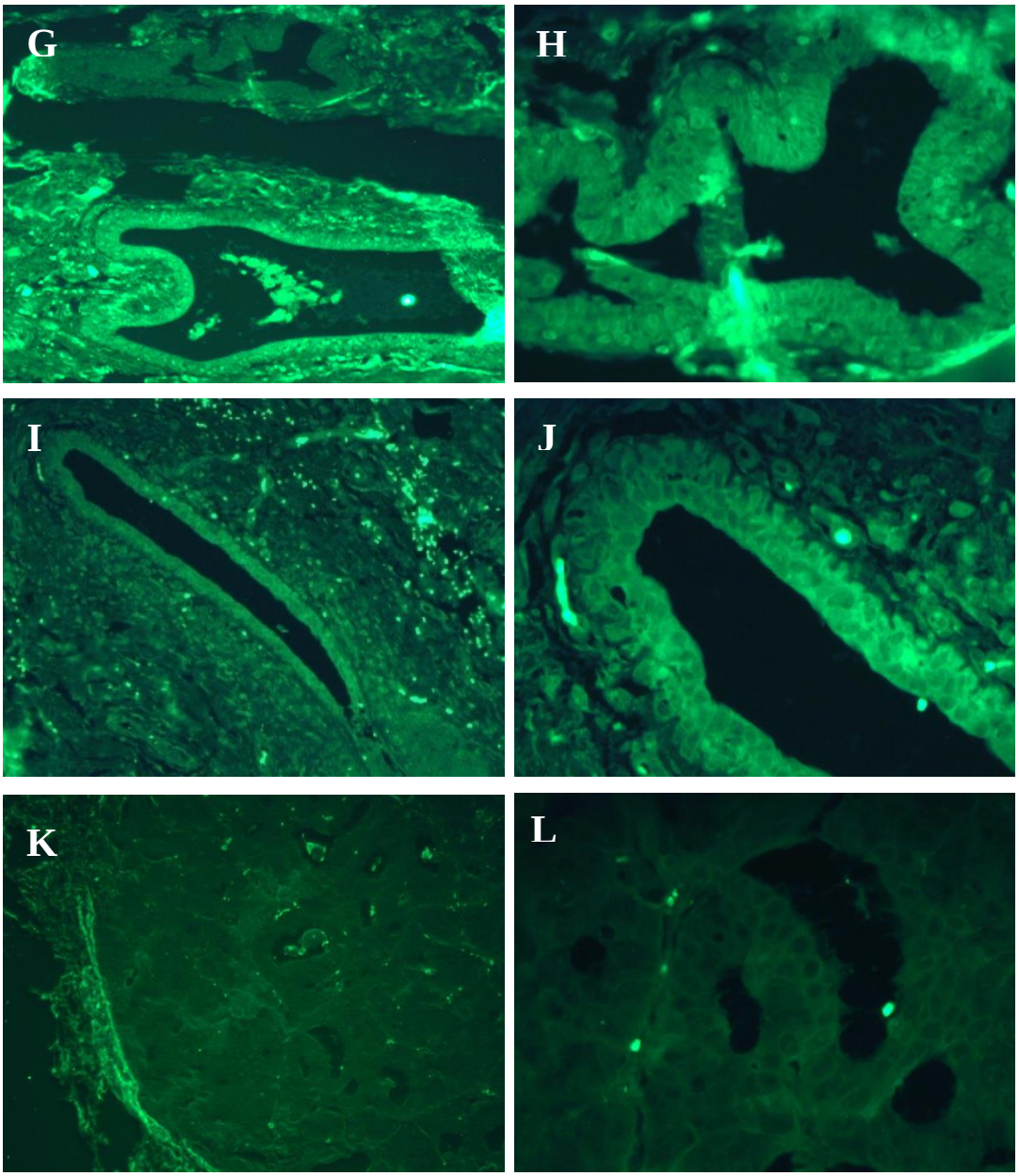


Figure 35. Immunofluorescence analysis of human breast tissue. *FITC-labeled anti-Gal was used on formalin fixed and paraffin embedded tissue. (A, B) Normal breast tissue showing acinar lobule with surrounding stromal tissue. (C, D, E, F) Moderately differentiated infiltrating breast carcinoma. (G, H, I, J) Poorly differentiated infiltrating breast carcinoma. (K, L) Moderately differentiated infiltrating breast carcinoma tissue treated with anti-Gal antibody which had been previously incubated with its specific sugar (25 mM melibiose).*

Representative figures of the immunofluorescence analysis of human normal/malignant tissues are shown in Fig.35. Anti-Gal antibody was not reactive with the formalin fixed normal breast tissue, in contrast reactivity was observed with infiltrating breast carcinoma tissue where both cytoplasmic staining and cell membrane staining can be seen. Intensity of fluorescence decreased when anti-Gal antibody which had been previously treated with its specific sugar melibiose (25 mM) was incubated with the tumor tissue sample confirming that the staining of tumor tissue was due to recognition of MUC1 in tumor tissue by anti-Gal using the latter's sugar-binding site.

MUC1 was expressed by most breast epithelial cells and expression was usually limited to the apical membrane of these cells, whereas breast carcinomas exhibited increased expression of MUC1 with different patterns, over the entire surface. Reduced glycosylation of the peptide backbone of MUC1, demonstrated in the previous chapter would have also facilitated increased exposure of cryptic MUC1peptides.

Discussion

Our hypothesis that low Lp(a) phenotype may be providing protection from tumors by modulating anti-Gal activity through affinity maturation of the latter rested mainly on two assumptions: i) lipoprotein(a) size-dependent alterations in specific activity of anti-Gal, and ii) specific activity dependent increase in binding of anti-Gal to tumor-associated antigen MUC1. These assumptions were verified by results in the previous and present chapter respectively. Specific activity-dependent increase in MUC1-induced change in anti-Gal conformation reflected in enhanced fluorescence of labeled antibody established that the specific activity of the antibody

assayed by ELISA based on binding avidity to the anti-α-galactoside ligand (TIM) hold good equally towards MUC1. Another notable conclusion from results above is that while anti-Gal specific activity and total activity were in general less in cancer patients compared to controls, advances in cancer stage resulted in significant increase in anti-Gal specific activity. This was in agreement with a marked decrease in O-glycosylation with tumor advancement since reduced O-glycosylation could increasingly expose the underlying MUC1 peptide sequence for recognition by the antibody. Remarkable increase in synthesis of anti-Gal-reactive MUC1 accompanying tumorigenesis and global distribution of these antigens on cell surface in contrast to the mostly apical distribution in normal cells would also have contributed to the increased rate of anti-Gal synthesis. These results suggest the likelihood of the role of antigenic stimulus for anti-Gal getting taken over by MUC1 after tumor differentiation. Results also tend to suggest that as tumor progresses, the altered MUC1 with increased accessibility to anti-Gal may itself act as affinity maturation antigens capable of influencing the specific activity of the antibody.

5. SUMMARY AND CONCLUSION

Affinity maturation involves the fine tuning of B-cell specificity through repeated processes of somatic hypermutation of immunoglobulin genes in B-cells and subsequent clonal selection which results in B-cells encoding high affinity antibodies necessary for an efficient and effective immune response which occurs in germinal center. These alternating processes of somatic hypermutation and selection continue with sustained or subsequent antigen exposures leading to the generation of antibody repertoire with higher affinity or specific activity towards that antigen. The present study examines the role of lipoprotein(a) as an affinity maturation agent for tumor antigen-reactive anti-Gal antibodies and its effect on tumor susceptibility.

Plasma concentrations of Lp(a) show remarkable variation among individuals and an inverse relation exists between plasma Lp(a) concentration and size. Most Lp(a) molecules in circulation attach additional molecules of LDL non-covalently to form adducts and STPS-containing region of Lp(a) is involved in Lp(a):LDL adduct formation. Larger Lp(a) molecules with longer STPS-containing regions attach more LDL molecules and smaller Lp(a) molecules attach nil or few LDL molecules as adducts. Lp(a) size correlated negatively with cancer incidence for unknown reasons. Anti-α-galactoside (anti-Gal) is a natural anti-carbohydrate antibody in humans and are mainly of IgG type and constitute ~1% of human IgG. This laboratory has reported that anti-Gal antibody that is present only in the animals that synthesize Lp(a) forms immune complex with Lp(a) by binding to serine- and threonine-rich peptide sequence (STPS) in the O-glycan rich region of apo(a) subunit of the lipoprotein. Notably STPS are also present at very high levels in tumor-associated MUC1 molecules in cancer cells. Further it is reasonable to expect

that hypoglycosylation that usually occurs in tumor cell glycoproteins would result in greater accessibility of the STPS to specific antibodies. Incidentally MUC1, especially its hypoglycosylated variant has been shown to be an anti-Gal ligand. Specific activity of anti-Gal antibody is the ratio of ligand binding activity to immunoglobulin content of the same amount of antibody and it differs among anti-Gal samples of different individuals.

Lp(a) molecules in circulation attach additional molecules of LDL non-covalently to form adducts. We show here that STPS, anti-Gal ligand akin to MUC1 in the apo(a) subunit of Lp(a), were decreasingly accessible to the antibody as Lp(a) size increased, due to occupation of the latter by LDL. LDL inhibited Lp(a) binding to anti-Gal probably due to ionic interaction between LDL and the negatively charged sialic acid moieties present on the STPS regions of apo(a), thereby blocking anti-Gal binding. Lower LDL-adduct index in low molecular weight Lp(a) samples suggested that they are more available for recognition by anti-Gal in circulation. Sporadic antigenic stimuli generally produce IgM type antibodies whereas continued presence of antigen in the system is known to affect the affinity maturation of antibodies and direct their class-switching to IgG-domination. In contrast to other carbohydrate-specific circulating antibodies anti-Gal is predominantly IgG with IgA and IgM in traces. Greater dominance of IgG type of anti-Gal antibodies in individuals with high Lp(a) plasma concentration suggested that lower molecular weight Lp(a) molecules that generally cause higher Lp(a) concentration are better antigenic stimuli for affinity maturation of anti-Gal than higher molecular weight Lp(a).

Reduced availability of STPS as plasma Lp(a) size increases, as reflected in reduced reactivity towards anti-Gal and caused by increased blocking of accessibility to the antibody by larger number of adduct LDL, would result in attenuated Lp(a)-mediated affinity maturation of anti-Gal causing negative correlation of Lp(a) size with anti-Gal specific activity in normal individuals. This was confirmed by the result that specific activity of anti-Gal in plasma increased with the concentration of Lp(a). These results suggested that although anti-Gal synthesis is proposed to be triggered by α-galactoside-containing antigens synthesized by gut microbes as happens in the case of most other anti-carbohydrate antibodies, its affinity maturation seems to be affected by Lp(a) structure. Apo(a) glycoprotein was found to be significantly superior to other plasma O-glycan rich glycoproteins as ligand for anti-Gal, suggesting Lp(a) as the major affinity maturation agent for anti-Gal antibody.

Lower Lp(a) concentrations and higher molecular weight Lp(a) were observed in patients compared to controls. Anti-Gal titre and its specific activity were significantly lower among breast cancer patients. No stage-wise difference in Lp(a) concentration was found in breast cancer patients whereas level and specific activity of anti-Gal antibody increased with stage of tumor which may be due to tumor-associated MUC1 acting as an affinity maturation agent for anti-Gal antibody in cancer patients. Binary logistic regression analysis revealed that low Lp(a) level and low specific activity anti-Gal were risk factors for cancer.

Underlining the anti-tumor activity of anti-Gal, tumor associated-MUC1 isolated by chromatography and verified by anti-MUC1 antibody was significantly more

reactive than normal MUC1 towards anti-Gal. This was confirmed by using in place of anti-Gal, a MUC1-specific lectin Griffonia simplicifolia IB4 (GS-IB4) which can also accommodate STPS in place of specific sugar at its binding site. Anti-MUC1 antibody dose-dependently inhibited MUC1 thereby blocking anti-Gal recognition of MUC1. This again confirmed the presence of MUC1 as anti-Gal-reacting component in tumor extracted proteins.

Decreased jacalin reactivity of tumor-associated MUC1 confirmed the reduced O-glycosylation of MUC1 in tumor cells and consequent increased exposure of STPS with which the anti-Gal antibody interacts. Reactivity of anti-Gal or GS-IB4 towards TA-MUC1 increased steadily with tumor stage and decreasing O-glycan content of the TA-MUC1. This result confirmed the reduced O-glycosylation and increased exposure of STPS in MUC1 as cancer stage advances. Presence of low molecular weight anti-Gal ligand abolished anti-Gal binding to TA-MUC1 establishing that MUC1 occupied the sugar-binding site of anti-Gal. Finally avidity of tumor-associated MUC1 towards anti-Gal increased proportional to the latter's specific activity. Recognition of MUC1 in tumor by anti-Gal antibody was further confirmed by immunofluorescence analysis of formalin fixed tissue sections using FITC-labeled anti-Gal antibody.

Taken together these results suggested that in circulation low molecular weight Lp(a) are more available for recognition by anti-Gal because of low LDL adduct index. Low molecular weight Lp(a) molecules offer better antigenic stimuli for affinity maturation and class-switching to IgG-domination for anti-Gal than high molecular weight Lp(a). This results in high specific activity anti-Gal in individuals

with high plasma Lp(a) concentration. Role of anti-Gal activity modulated by Lp(a) size in tumor susceptibility was established by these results. Besides identifying specific activity of anti-Gal as the factor through which Lp(a) size modulates cancer susceptibility, these results also offer prospects for natural immunotherapy. Immunotherapeutic interventions to augment anti-Gal specific activity seem to be a viable strategy in preventing and controlling tumorigenesis.

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ANNEXURE

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