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Detection of disease specific sialoglycoconjugate specific antibodies in bronchoalveolar lavage fluid of non-small cell lung cancer patients

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Abstract In this study, we report the presence of significantly higher level of GM3 specific IgG antibodies (IgG_{TL}) in the bronchoalveolar lavage fluid obtained from tumor bearing lung of non-small cell lung cancer (NSCLC) patients as compared to other non-neoplastic controls. The antibodies were isolated using DEAE-cellulose anion exchange chromatography and molecular weight of the subunits of IgG_{TL} was confirmed in SDS-PAGE. IgG_{TL} revealed high specificity to GM3 and the IgG distribution was confined to IgG1. Furthermore, IgG_{TL} showed strong reactivity with NSCLC cell lines as well as the tissue biopsies and cells obtained from fine needle aspirations of NSCLC patients. A 66 kDa membrane glycoprotein of NSCLC cell lines was found to interact specifically with IgG_{TL}, the intensity of which was drastically reduced in presence of GM3. Further, binding of *Maackia amurensis* agglutinin [specific for NeuAcα(2→3)Gal unit , the same disaccharide unit also known to be present in GM3] to the 66 kDa band confirmed it to be a sialoglycoprotein in nature. IgG_{TL} could not show any reactivity to alkaline borohydrate treated or periodate oxidised membrane fractions, suggesting the probable involvement of the carbohydrate moiety of the 66 kDa glycoprotein in the interaction with IgG_{TL}. Thus, the 66 kDa sialoglycoprotein seems to be the NSCLC specific sialoglycoconjugate. Taken together, IgG_{TL} antibodies may have the potential to serve as a unique probe for detail investigation of NSCLC specific cell surface sialoglycoconjugate. Further, due to high specificity of IgG_{TL} to GM3, it may be possible to develop a simple alternative diagnostic approach (GM3-ELISA) for NSCLC.

Keywords Non small cell lung cancer · Bronchoalveolar lavage fluid · Immunoglobulins · Sialoglycoconjugates

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Abbreviations

BALF	Bronchoalveolar Lavage Fluid
SDS-PAGE	Sodium Dodecyl sulphate polyacrylamide gel electrophoresis
NSCLC	Non Small Cell Lung Cancer
FNA	Fine Needle Aspiration
MAA	*Maackia amurensis* agglutinin
SCLC	Small cell lung cancer
ELISA	Enzyme Linked Immunosorbent Assay
OPD	Ortho Phenylenediamine
PBS	Phosphate Buffer Saline

ILD	Interstitial Lung Disease
BCA	Bicinchoninic Acid
DAB	Diaminobenzidine
PVDF	Polyvinylidene fluoride
HRP	Horseradish Peroxidase
PAS	Periodic Acid Schiff Staining
NCP	Nitrocellulose Paper
PI	Propidium Iodide
ANOVA	Analysis of Variance
SM-PBS	Skim Milk-Phosphate Buffer Saline

Introduction

Lung cancer is the most common cancer in the world today with a mortality rate of more than 90% [1]. It is the major cause of cancer related deaths in developed countries and is also rising at alarming rate in developing countries including India [2]. Despite major advances in cancer treatment in the past decade, the prognosis of patients with lung cancer has improved only minimally. Lung cancer has been classified into two major histological types: SCLC and NSCLC. SCLC is fast growing, highly metastatic and a rarely curable disease. The sub-type NSCLC accounts for approximately 80% of all human lung carcinoma cases, which represents a heterogeneous sub-group in terms of both behavior and therapeutic response and is usually associated with poor prognosis [3].

Lung cancer is characterized by a complex array of biomolecular alterations that drive uncontrolled growth and metastatic spread [4]. Studies have indicated that lung carcinoma is not a result of sudden transformational event occurring within the bronchial epithelium; rather the normal bronchial epithelium gradually acquires several cellular and genetic alterations, which ultimately result in the formation of invasive tumors [5]. Change in glycosylation pattern in the cellular glycoconjugates is a prominent biomolecular alteration in cancer cells [6]. Among many types of carbohydrate residues, sialic acids are extremely important, which are generally found as terminal residues in the vertebrate oligosaccharides. During oncogenesis, wide variation has been found in its quantity, derivatization and type of linkage to the sub-terminal sugar residue. Moreover, aberrant sialylation is thought to be associated with invasiveness and metastatic potential of cancer cells [7].

The organizational profiles of cancer associated cell surface carbohydrates are so different that they can be recognized by immunological methods. Several carbohydrate specific monoclonal antibodies of IgM and IgG type were developed, which could discriminate among various tumors and normal tissues [8]. Lung adenocarcinoma is known to be one of the abundant sources of Lewis-x and the related antigens and many murine monoclonal antibodies directed to lung cancer cells were shown to recognize these antigens [9, 10]. Furthermore, the presence of antibodies against the disease specific glycoconjugates has also been detected in the sera of cancer patients [11–13].

Application of immunological techniques in the study of lung cancer has raised hopes of finding newer methods for its easy diagnosis. For this purpose, the relatively safe and simple procedure of bronchoalveolar lavage has provided an access to both the cellular and humoral milieu. An increased level of immunoglobulin in bronchial washings was considered to be specifically related to lung cancer [14, 15]. Aberrant sialylation of glycoconjugates in sera of patients with lung adenocarcinoma was reported earlier [10]. The present study reports the detection of disease specific sialoglycoconjugate specific antibodies in the BALF of NSCLC patients.

Material and methods

Patient samples

This study was approved by the Institutional Review Board and all ethical guidelines were followed. A formal written informed consent was taken from all the patients enrolled for this study. The samples for the present study consisted of BALF, bronchial biopsies, transbronchial lung biopsies and FNA. BALF was obtained from the tumor bearing lung of NSCLC patients (n=36), contra-lateral normal lung of NSCLC patients (n=15), patients with sarcoidosis (n=7) and ILD (n=6) during the routine bronchoscopy after instillation of 20 ml of normal saline. BALF from contra-lateral normal lung of NSCLC patients, lung of patients with ILD and sarcoidosis served as non-neoplastic controls in this study. The collected BALF (10 ml) of each patient was centrifuged, the protein content of the supernatant was estimated by BCA assay [16]. In addition to BALF, bronchoscopic biopsies of lung cancer patients [NSCLC (n=10), SCLC (n=5)], trans-bronchial lung biopsies of the sarcoidosis patients (n=7) and one normal lung biopsy were collected and fixed immediately in 10% formalin. Biopsies were dehydrated through a graded series of ethanol solution (70%, 80%, 90% and 100%) and embedded in paraffin wax. Tissue sections (5 μm) were cut with a fine razor attached to microtome and taken on the poly-L-lysine (Sigma) coated slides for immunohistochemical analysis. Cells obtained from the FNA samples (NSCLC, n=7) were used for cytopathological examination and immunocytochemical analysis. The patients with the history of chemotherapy and radiotherapy were not included in this study.

Cell lines

Normal human lung fibroblast cell line (WI-38) and two NSCLC cell lines [NCI-H460 (large cell carcinoma) & NCI-H520 (squamous cell carcinoma)] were obtained from National Centre for Cell Science (Pune, India) and maintained in RPMI-1640 containing 10% FCS.

Detection of immunoglobulin level in BALF

The level of IgM, IgG and IgA in the supernatant obtained from BALF of each patient was determined by ELISA. Briefly, the samples were coated in the 96 well ELISA plates at a concentration of 10 μg/ml and incubated overnight at 4°C. After washing with 20 mM phosphate buffer (pH 7.2) containing 150 mM NaCl (PBS), non-specific sites of the wells were blocked with 5% skim milk in PBS (SM-PBS) for 2 h at 37°C. This was followed by washing and incubation with HRP-conjugated goat anti human IgG/IgM/IgA (diluted to 1:1,500; Santa Cruz Biotech, CA) for 1 h at 37°C. Finally, the color was developed using 0.05% OPD in 0.1 M phosphate/citrate buffer (pH 5.0) containing H_2O_2. The reaction was terminated with 3 M H_2SO_4 and the plates were read at 492 nm. Sialoglycoconjugate specificity of the IgG antibodies present in the supernatant obtained from BALF of each patient was assessed by ELISA using fetuin [10 μg/ml (ICN, USA)] / gangliosides [GM1/GM2/GM3/GD1a/GD3/GT1b (10 μg/ml), Sigma-Aldrich] as coating antigens. After washing, the non-specific sites of the wells were blocked with SM-PBS. Subsequently, samples (50 μg/ml) were added to the wells, incubated at 37°C for 2 h and its binding to fetuin / ganglioside was measured colorimetrically using HRP-labeled secondary antibody and OPD/H_2O_2. The isotyping of sialoglycoconjugate specific IgG_{TL} was done by ELISA using fetuin/GM3 as coating antigen (10 μg/ml) and HRP-labeled sheep anti-human IgG1/IgG2/IgG3/IgG4 (Serotec, UK) as secondary antibodies). Appropriate controls in each set of experiments were run in parallel.

Isolation and characterization of IgG from BALF

IgG was isolated from the pooled BALF of each group of patients using DEAE-cellulose anion exchange chromatography [17]. The unbound fractions containing IgG antibodies were collected and designated as IgG_{TL}, IgG_{NL}, IgG_{SL} and IgG_{IL} for IgG isolated from the BALF of the tumour bearing lung of NSCLC patients(n=10), contralateral normal lung of NSCLC patients(n=10), lung of sarcoidosis patients(n=7) and lung of ILD patients(n=6), respectively. Total protein content of each type of isolated IgG was measured by BCA assay and the molecular weight of the subunits was confirmed by 10% SDS PAGE [18]. The sialoglycoconjugate specificity of IgG_{TL}, IgG_{NL}, IgG_{SL} and IgG_{IL} was assessed in ELISA using fetuin / GM3 as the coating antigens. Isotyping of fetuin / GM3 specific IgG_{TL} was done by ELISA using HRP-labeled sheep anti-human IgG1 / IgG2 / IgG3 / IgG4 as the secondary antibodies.

Immunocytochemical staining of acetone-fixed cells

The cytospins of the NSCLC cell lines, normal lung fibroblast cell line as well as the cells obtained from the patient samples (FNA) were blocked for 30 min with 0.3% H_2O_2 in absolute methanol to remove the endogenous peroxidase. After washing with PBS, the cells were incubated with IgG_{TL}/ IgG_{NL}/ IgG_{SL} / IgG_{IL} (2.5 μg/ml) for 1 h at 37°C in a humidified incubator. The cells were washed with PBS, followed by incubation with HRP-labeled rabbit anti-human IgG (diluted to 1:250 in PBS; Bangalore Genei, India) for 1 h at 37°C. After washing, cytospins were stained with 0.05% DAB tetrahydrochloride in PBS containing H_2O_2 (1 μl/ml) for 10 min, counterstained with haematoxylin and analysed under light microscope.

Immunohistochemical staining of paraffin section

Paraffin-embedded sections of lung biopsies were deparaffinized. The sections were treated with 0.3% H_2O_2 in absolute methanol for 30 min at room temperature and stained with IgG_{TL}/ IgG_{NL}/ IgG_{SL} / IgG_{IL} as described for immunocytochemical staining.

Western blotting

The membrane proteins from the NSCLC cell lines (NCI-H520 and NCI-H460) were isolated [19]. Following 10% SDS-PAGE the membrane proteins were transblotted onto the PVDF membranes [20]. After blocking with 5% SM-PBS containing 0.1% Tween-20, the blots were incubated with IgG_{TL}/ IgG_{NL}/ IgG_{SL} / IgG_{IL} (2.5 μg/ml) for 1 h at 37°C. The membrane protein(s) reacting with four different types of IgG were detected using HRP conjugated rabbit anti-human IgG (diluted to 1:1,000) and DAB tetrahydrochloride/H_2O_2. In a separate set of experiments, the transblots containing the membrane proteins were treated with IgG_{TL}, preincubated in presence of GM3 (0.5 μg/ml) for 1 h at 37°C, followed by detection as described before. In another set of experiments, the PVDF membrane strips containing the transblotted membrane proteins, preincubated in presence or in absence of IgG_{TL}, were treated with MAA for 2 h at 37°C followed by IgG_{MAA} [IgG raised against MAA in rabbits by the method of Shant *et al.* (2002) [21];

(1:500) for 1 h at 37°C. After extensive washing, the immune complexes were detected with HRP-conjugated goat anti rabbit IgG (Santra Cruz Biotech, CA, 1:1,500) and developed with DAB/ H_2O_2.

PAS staining

The NCP containing the transblotted membrane proteins were subjected to PAS staining. Briefly, after washing, the NCP was incubated with 1% (v/v) periodic acid in 3% acetic acid for 30 min. Subsequently, the NCP was washed, treated with 0.1% (w/v) sodium metabisulphite in 0.01 M HCl for 10 min, incubated in Schiff's reagent for 15 min in the dark, washed and air-dried.

Chemical modification of the membrane fractions

The membrane fractions (300 μg/ml) of NCI-H460 and NCI-H520 cells were treated with 0.05 M NaOH and 2 M $NaBH_4$ for 18 h at 45°C [23]. The excess borohydride was removed by addition of acetic acid at 4°C and the pH was adjusted to 7.2. In a separate set of experiments, periodate oxidation was performed by treating the membrane preparation (300 μg/ml) with 0.1 M sodium metaperiodate in 0.02 M sodium acetate buffer (pH 4.5) for 24 h at 4°C in the dark [24]. The modified membrane fractions were subjected to SDS-PAGE and transblotted onto the PVDF membrane. After blocking, the strips were incubated with IgG_{TL} and developed as described above.

Apoptosis of NSCLC cells

NCI-H460 cells / NCI-H520 cells ($5x10^4$/well) were cultured for 24 h with different doses (0.005–1.0 μg) of IgG_{TL}. Cells were washed with PBS and fixed with 1% paraformaldehyde in PBS for 30 min at 4°C. After washing, the cells were permeabilized with the hypotonic buffer (0.1% sodium citrate/ 0.1% Triton X-100 in PBS) containing RNase H (10 μg/ml) for 15 min. Finally, PI, (ICN, USA) was added at a final concentration of 10 μg/ml. Analysis was done on a flow cytometer (Becton Dickinson, Mountain View, CA) using the Cell Quest software programme. The results were expressed in view of the mean fluorescence intensity of the labeled cells in the hypodiploid region, which was directly correlated to the percentage of apoptotic cells. Three independent sets of each experiment were run in duplicate.

Statistical analysis

The independent 't' test and ANOVA test were performed on the data sets as per requirement, 'p' value<0.05 was considered significant.

Results

Immunoglobulin level in BALF

The level of IgG was found to be significantly higher ($p<0.001$) than IgM and IgA in the BALF obtained from the tumor bearing lung of NSCLC patients. Further, the IgG level was more ($p<0.001$) in case of NSCLC patients than the non-neoplastic controls (Fig. 1a). To assess the sialoglycoconjugate specificity of the IgG, fetuin was taken as the broad spectrum sialoglycoconjugate and a significantly ($p<0.001$) high level of the fetuin specific IgG was detected in the BALF of the NSCLC patients (Fig. 1b). Since gangliosides are sialylated glycoconjugates, various gangliosides were also used to assess the sialoglycoconjugate specificity of the IgG in the BALF and the level of GM3 specific IgG was found to be significantly higher ($p<0.001$) than other ganglioside specific IgG in case of NSCLC patients. Further, the level of GM3 specific IgG in BALF obtained from the tumour-bearing lung of NSCLC patients was more ($p<0.001$) as compared to that in BALF obtained from the contra-lateral normal lung of the same patients (Fig. 1c). However, no such difference in the level of any ganglioside specific IgG was found in case of patients with ILD or sarcoidosis.

Characterisation of IgG

The yield of the four types of IgG i.e. IgG_{TL}, IgG_{NL}, IgG_{SL} and IgG_{IL} in the unbound fraction of DEAE-cellulose anion exchange chromatography of pooled BALF obtained from the tumour bearing lung of NSCLC patients, contra-lateral normal lung of NSCLC patients as well as patients having sarcoidosis or ILD were found to be 360 μg/ml, 200 μg/ml, 300 μg/ml and 50 μg/ml, respectively. The molecular weight of the subunits of the isolated IgG antibodies was confirmed by SDS-PAGE, in which two bands of apparent molecular weight 50 kDa and 25 kDa corresponding to the heavy and light chains of IgG, were obtained. The IgG_{TL} showed specificity for fetuin and GM3 (Fig. 2a) and IgG1 was found to be the most predominant fetuin and GM3 specific isotype in total IgG_{TL} (Fig. 2b). The interaction of BALF and IgG_{TL} with fetuin/GM3 could be significantly reduced when preincubated with GM3/ fetuin respectively (Fig. 2c).

Reactivity pattern of IgG

The Fig. 3 reveals the representative micrographs of the reactivity pattern of four different types of IgG with cells and tissue samples of different origin. IgG_{TL} showed strong reactivity with the cells of NSCLC origin *i.e.* NSCLC cell lines [NCI-H520 (panel A) & NCI-H460 (panel B)], FNA samples (panel D) and tissue biopsies of NSCLC patients

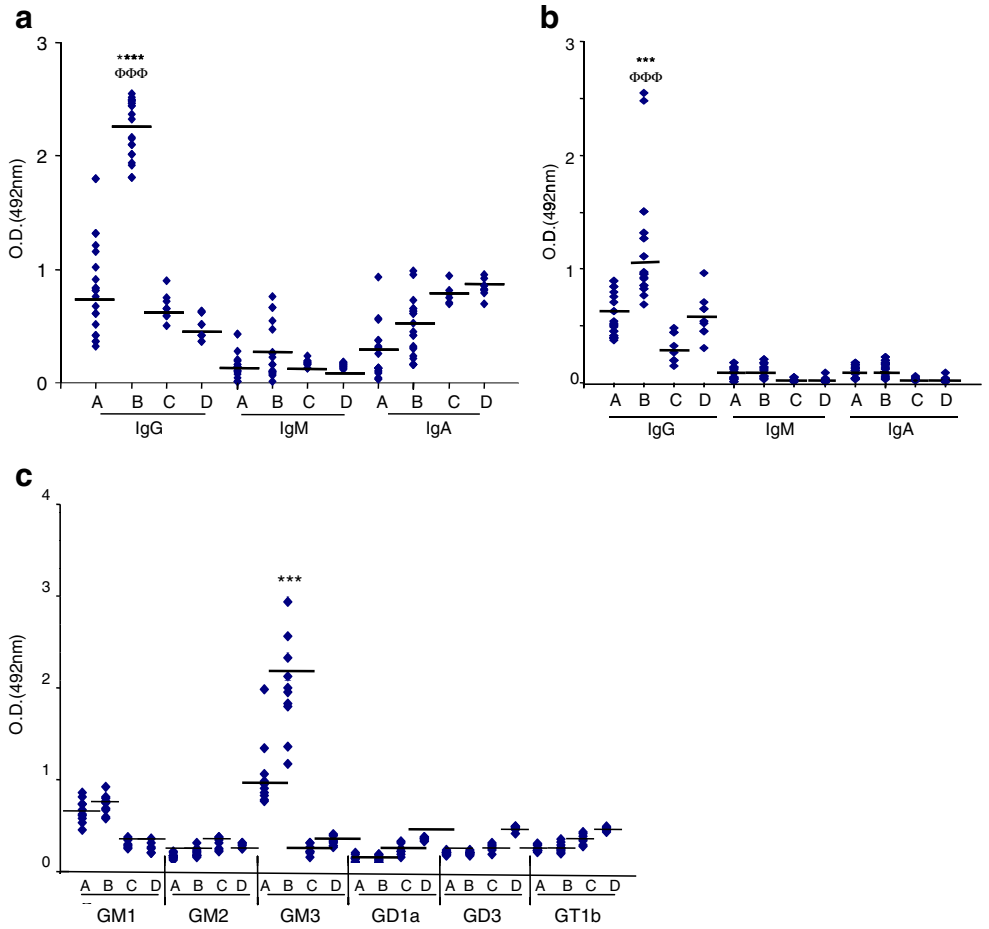

Fig. 1 **a** Level of total immunoglobulins in BALF obtained from normal lung of NSCLC patients (A, n=15), tumour bearing lung of NSCLC patients (B, n=36), patients with sarcoidosis (C, n=7) and patients with ILD (D, n=6); **b** Level of Fetuin specific immunoglobulins in BALF obtained from normal lung of NSCLC patients (A, n=15), tumour bearing lung of NSCLC patients (B, n=36), patients with sarcoidosis (C, n=7) and patients with ILD (D, n=6). ***p<0.001 (IgG vs. IgM; IgG vs. IgA); $^{\Phi\Phi\Phi}p$<0.001 (B IgG vs. A IgG; B IgG vs. C IgG; B IgG vs. D IgG), ANOVA followed by LSD; **c** Level of Ganglioside specific IgG in BALF obtained from normal lung of NSCLC patients (A, n=10), tumour bearing lung of NSCLC patients (B, n=10), patients with sarcoidosis (C, n=7) and patients with ILD (D, n=6). ***p<0.001 (GM3 vs. other gangliosides; B GM3 vs. A GM3, C GM3, D GM3), ANOVA followed by LSD

(representative micrograph, panel E). But it could not interact with normal lung fibroblast cell line (WI-38; panel C), the normal lung biopsy (panel H), tissue biopsies of SCLC patients (representative micrograph, panel F) as well as tissue biopsies of the sarcoidosis patients (representative micrograph, panel G). IgG_{NL} and IgG_{SL} did not show any reactivity to the cells of any origin. IgG_{IL} revealed weak cytoplasmic positivity with one FNA sample and two tissue biopsies of NSCLC patients (Table 1). Further, in Western immunoblot (Fig. 4a), IgG_{TL} was found to interact with a band of molecular weight ~66 kDa in the membrane fractions of NCI-H460 cells (lane2) and NCI-H520 cells (lane7) and such interaction was found to be inhibited when the transblotted membrane proteins were treated with IgG_{TL}, preincubated in presence of GM3 (lanes 6&11, respectively). The other three types of IgGs (IgG_{NL}, $IgG_{SL\&}$ IgG_{IL}) could not show any reactivity to any membrane protein (lanes 3–5&8–10). Periodic acid Schiff staining revealed the 66 kDa band to be a glycoprotein (Fig. 4b, lanes 1&2). Further, the sialoglycoprotein nature of the 66 kDa band was ascertained in Western blot (Fig. 5) by studying the interaction of membrane fractions of NCI-H460 cells (lane 2) and NCI-H520 cells (lane 8) with MAA, the intensity of which was reduced in presence of IgG_{TL} (lanes 3&9 respectively). IgG_{TL} could not detect any band in the membrane fractions of both the cell lines (NCI-H460 & NCI-H520) modified with alkaline borohydride treatment (lanes 4&6 respectively) and periodate oxidation (lanes 5 &7, respectively) separately.

Evaluation of IgG_{TL} induced apoptosis in NSCLC cell lines

The Fig. 6 shows the result of the flow cytometric analysis of IgG_{TL} treated NCI-H460 and NCI-H520 cells, respectively.

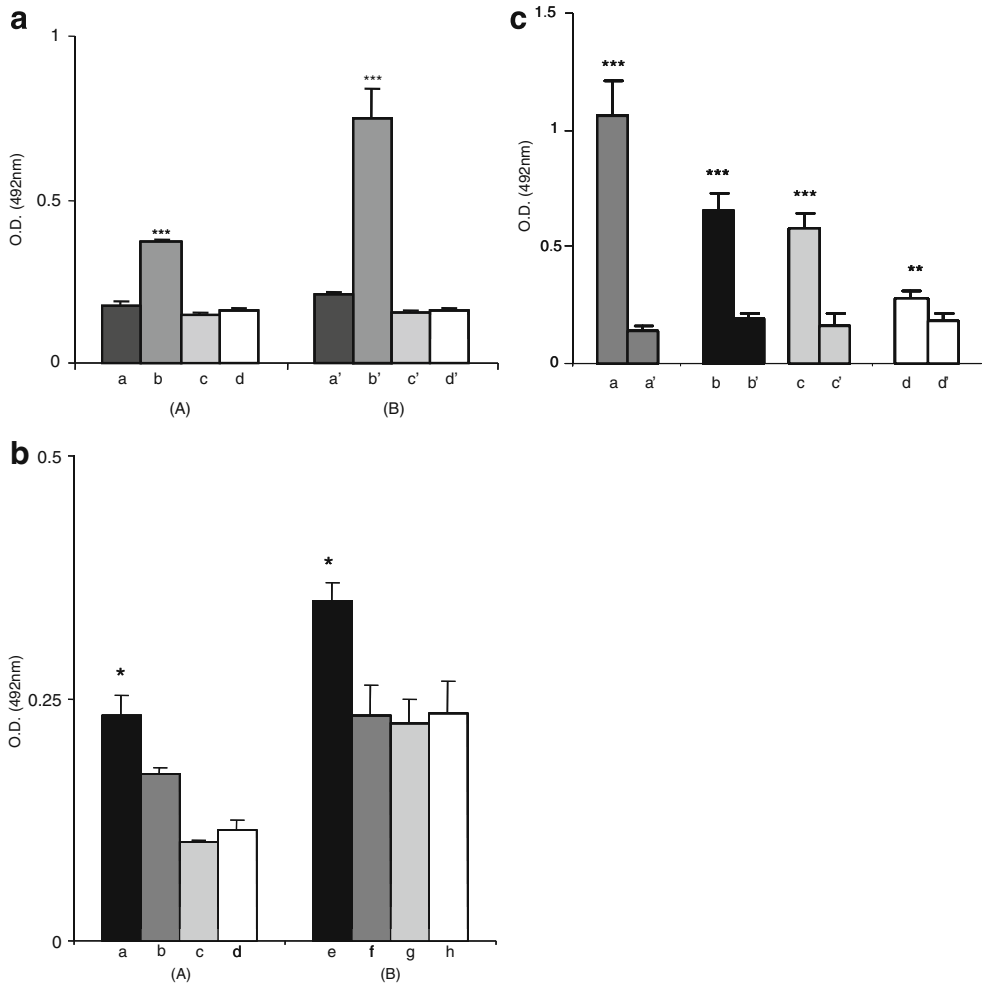

Fig. 2 **a** Fetuin (panel A) and GM3 (panel B) specificity of IgG antibodies isolated from pooled BALF of the various categories. IgG_{NL} (a, a'), IgG_{TL} (b, b'), IgG_{IL} (c, c') and IgG_{SL} (d, d'). ***$p<0.001$, independent 't' test [b vs. a, c, d; b' vs. a' c' d']. Each point represents the mean ± S.D. of the values obtained from three independent experiments run in duplicate; **b** Isotyping of Fetuin specific IgG (A) and GM3 specific IgG (B) of NSCLC patients. IgG1 (a, e), IgG2 (b, f), IgG3 (c, g) and IgG4 (d, h). *$p<0.05$ (a vs. b, c, d; e vs. f, g, h), ANOVA followed by independent 't' test. Each point represents the mean ± S.D. of the values obtained from three independent experiments performed in duplicate; **c** Interaction of BALF with GM3 and fetuin in absence and presence of fetuin (a, a') and in absence and presence of GM3 (c.c'), respectively. Interaction of IgG_{TL} with GM3 and fetuin in absence and presence of fetuin (b.b') and in absence and presence of GM3 (d,d') respectively. ***$p<0.005$ (a vs a', b vs b', d vs d'); **$p<0.05$ (c vs c') applying Student's paired 't' test

The number of apoptotic cells was found to be increased to 13-14% in presence of 1 μg of IgG_{TL} as compared to that of untreated cells.

Discussion

Recent developments in glycobiology have revealed the importance of sugar chains of glycoproteins in cellular recognition, which is essential for maintenance of ordered social behavior in the cells in multicellular organisms. Therefore, altered glycosylation in cellular glycoconjugates can be expected to be the basis of various abnormalities in tumor cells [6]. The presence of disease specific glycoconjugate specific antibodies in the sera of cancer patients has been reported earlier. Wantanabe *et al*. reported the presence of antibody against GD2 like ganglioside in the serum of patients with melanoma [25]. IgM against mucin (MUC-1) was found in the sera of patients with breast cancer, pancreatic cancer and colon cancer [26]. Smorodin *et al.* showed the presence of Thomsen-Friedenreich (TF) hapten (Galβ1→3GalNAcα) specific IgM in the sera of gastric cancer patients and breast cancer patients [27]. Pal *et al.* (2000) reported that serum IgG of children having acute lymhoblastic leukemia (ALL) showed specificity towards 9-*O*-acetylated sialoglycoconjugates [28]. However, there is no report regarding the presence of disease specific glycoconjugate specific IgG in BALF of NSCLC patients although an elevated level of IgG was reported in BALF and bronchial washings of NSCLC patients [14, 15]. In the

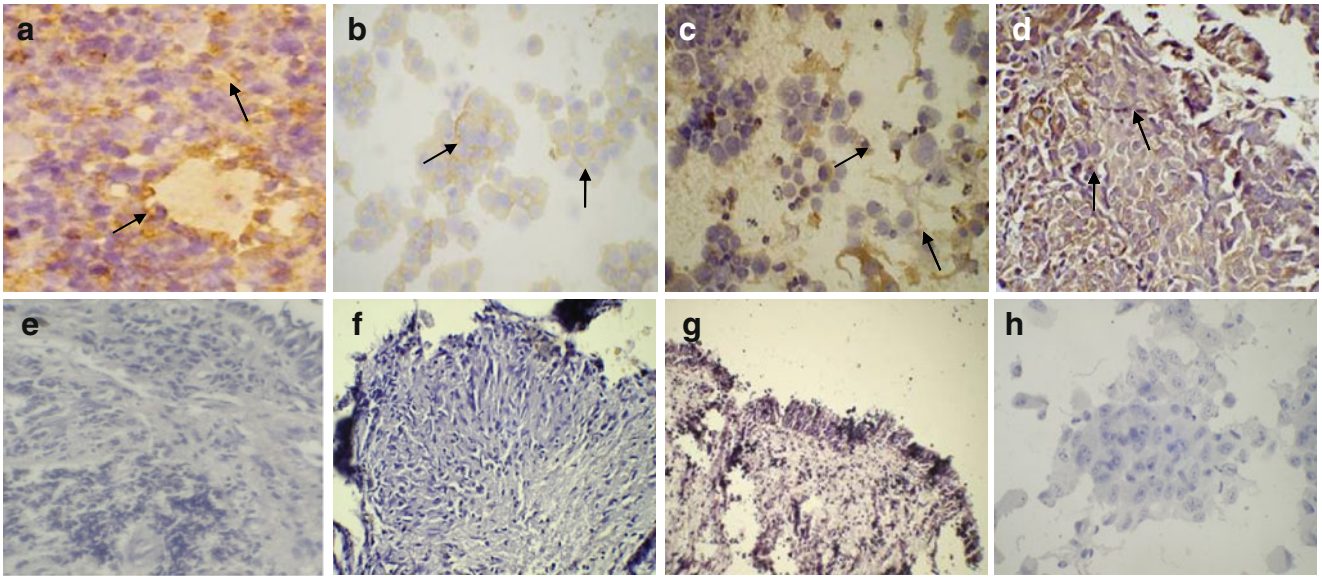

Fig. 3 Immunostaining of NCI-H460 (panel **a**), NCI-H520 (panel **b**), FNA of NSCLC patient (panel **c**), NSCLC biopsy (panel **d**), biopsy of SCLC patient (panel **e**), biopsy of sarcoidosis patient (panel **f**), normal lung biopsy (panel G) & WI-38 (panel H) with IgG_{TL}

present study, we have also found a significantly higher level of IgG in the BALF obtained from the tumor bearing lungs of NSCLC patients as compared to IgM and IgA.

The sialoglycoconjugate specificity of the antibodies present in the BALF of the NSCLC patients was assessed taking fetuin [a sialoglycoprotein having NeuAcα(2→3)/(2→6)Gal unit and NeuAcα(2→3)Gal, NeuAcα(2→6)GalNAc unit in its N-linked and O-linked oligosaccharide chains, respectively] /different gangliosides [glycolipids having NeuAcα(2→3)Gal terminal sugar moiety in their oligosaccharide portion] as the coating antigen in ELISA.

Table 1 Interaction of various IgGs with cells of different origin

	IgG_{TL}	IgG_{NL}	IgG_{IL}	IgG_{SL}
Cell lines				
NCI-H520	+	–	–	–
NCI-H460	+	–	–	–
WI-38	–	–	–	–
Biopsy samples of NSCLC patients (10)				
	10/10	0/10	2/10	0/10
FNA samples of NSCLC patients (7)				
	7/7	0/7	1/7	0/7
Biopsy samples of SCLC patients (5)				
	0/5	0/5	0/5	0/5
Biopsy samples of Sarcoidosis patients (7)				
	0/7	0/7	0/7	0/7
Normal lung biopsy (1)				
	0/1	0/1	0/1	0/1

Our results have revealed the presence of high level of fetuin specific IgG antibodies (IgG_{TL}) in BALF obtained from the tumor bearing lung of NSCLC patients and we have observed that the IgG_{TL} revealed maximum specificity for the monosialoganglioside GM3. Thus, probably the minimum required carbohydrate sequence for binding of the antigenic epitope of the sialoglycoconjugates to IgG_{TL} might be NeuAcα(2→3)Gal, present in both fetuin and GM3. Due to the presence of other sugar residue(s) as in case of other gangliosides, the required binding site might not be available owing to steric hindrance imparted by these residues.

In the present study, the isotype predominantly present in GM3 specific IgG_{TL} was IgG1. Pal *et al*. have also reported the presence of enhanced level of IgG1 and IgG2 type 9-*O*-AcSG specific antibodies in serum samples of children having ALL [28]. Moreover, various groups have observed that immunization of cancer patients with ganglioside based vaccines resulted in the production of antibodies mainly of isotype IgG1 and IgG3 [29]. IgG_{TL} selectively interacted with the NSCLC cell lines as well as FNA samples and biopsy samples of NSCLC patients. However, no such interaction of IgG_{TL} was observed with non-transformed human fibroblasts, normal lung biopsy and biopsy of sarcoidosis patients. Our finding regarding the reactivity of IgG_{TL} with NSCLC cells was further substantiated with the identification of a glycoprotein of apparent M_r 66 kDa in the membrane fractions of both the NSCLC cell lines. Moreover, the alkaline borohydride modified and periodate oxidised membrane fractions could not show any interaction with IgG_{TL} . Alkaline borohydride treatment removes the O-glycosidically linked carbohydrate chains of the

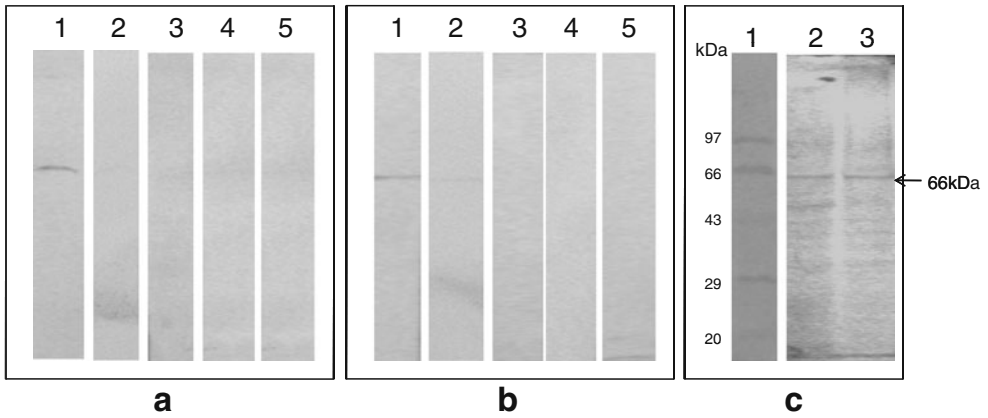

Fig. 4 Interaction of membrane fractions of NCI-H460 cells (**a**) and NCI-H520 cells (**b**) with IgG_{TL} in absence (lanes 1) and in presence of GM3 (lanes 2).The IgG_{NL} (lanes 3), IgG_{SL} (lanes 4), IgG_{IL} (lanes 5) could not show any reactivity to any membrane protein of both the cell lines. (**c**) Lane 1: Amido Black stained molecular weight markers; PAS stained membrane proteins of NCI-H460 cells (lane 2) and NCI-H520 cells (lane 3)

glycoproteins [30] and periodate oxidation modifies the vicinal diols of glycosyl residues of the glycoproteins to dialdehydes [31]. It is possible that in the 66 kDa glycoprotein, the O-glycosidically linked oligosaccharide chain containing the required carbohydrate epitope was removed by alkaline borohydride treatment and during periodate oxidation, the required carbohydrate epitope might have been modified. Thus, the modified glycoprotein could not reveal any interaction with the IgG_{TL}. Moreover, interaction of the 66 kDa band with *Maackia amurensis* agglutinin, a lectin specific for NeuAcα(2→3)Gal, confirmed it to be a sialoglycoprotein, probably having NeuAcα(2→3)Gal unit in its oligosaccharide portion. Further, such interaction was found to be inhibited in presence of IgG_{TL} thereby indicating that MAA and IgG_{TL} interacted with the same 66 kDa membrane glycoprotein. Specifically, we have used MAA, since we have already observed that IgG_{TL} could not interact with the 66 kDa glycoprotein in presence of GM3 and GM3 is known to contain the same unit [NeuAcα(2→3)Gal] in its oligosaccharide portion, to which MAA is highly specific. Thus, the minimum required carbohydrate sequence involved in the binding of the antigenic epitope of this 66 kDa sialoglycoprotein to IgG_{TL} might be NeuAc α(2→3) Gal and IgG_{TL} isolated from the BALF of NSCLC patients is the NSCLC specific sialoglycoconjugate specific antibody.

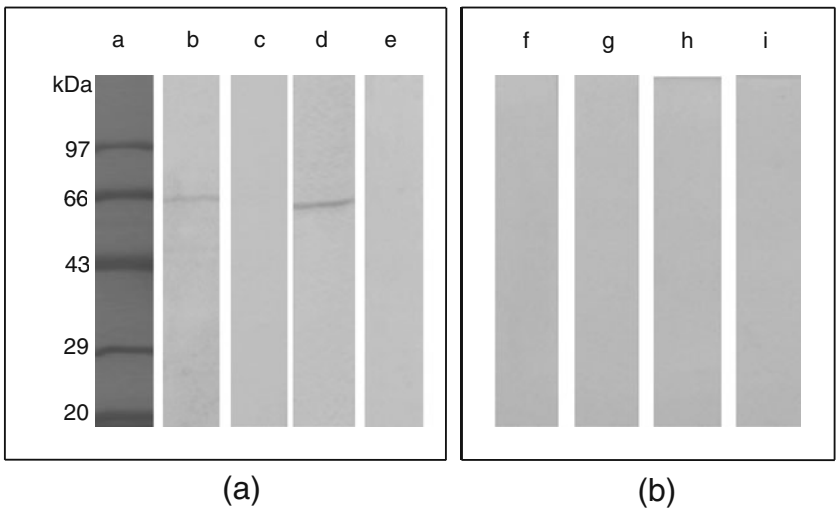

Fig. 5 **a** Interaction of membrane fraction of NCI-H460 cells and NCI-H520 cells with MAA in absence (lanes b & d, respectively) and in presence of IgG_{TL} (lanes c & e, respectively); Lane a: Amido black stained molecular weight markers. **b** IgG_{TL} could not detect any band in the membrane fractions of NCI-H460 cells and NCI-H520 cells, modified with alkaline borohydride treatment (lanes f & h, respectively) and periodate oxidation (lanes g & i, respectively)

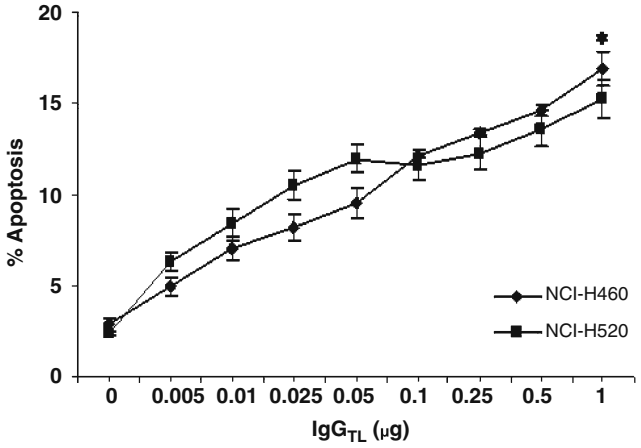

Fig. 6 Flow cytometric analysis of apotosis in IgG_{TL} treated NCI-H460 cells & NCI-H520 cells as evaluated as by propidium iodide staining. Cells cultured for 24h in absence of IgG_{TL}, in presence of IgG_{TL} at concentrations 0.005 μg, 0.01 μg, 0.025 μg, 0.05 μg, 0.1 μg, 0.25 μg, 0.5 μg & 1.0 μg. Each value represents mean ± SD of three independent experiments performed in duplicate. *$p<0.05$ (control vs. 1.0 μg IgG_{TL}) applying student's paired 't' test

It has been reported that human IgM antibodies isolated from different cancer patients could induce apoptosis in different cancer cell lines and also primary tumor cells isolated from cancer patients by binding to tumor specific receptors [19, 32]. Such apoptotic effect has not only been demonstrated in vitro [33], but also in animals as well as in patients when used in clinical trials of stomach cancer [19]. In this study, we have observed that IgG_{TL} could induce 13-14% apoptosis in both the NSCLC cell lines. Although the disease specific sialoglycoconjugate(s) on NSCLC cells elicited IgG1 type antibody response *in vivo*, it may be possible that such antibody was unable to induce effector immune function to kill the cancer cells due to low concentration [34]. Further, the antigenicity and immunogenicity might have been affected due to the aberrant glycosylation as well as crypticity of such antigens [35]. Moreover, since the tumor antigens are embryonic antigens, it is possible that these can be perceived as self by the immune system and thus tolerance is induced to these antigens [36].

Taken together, the findings of the present study suggest that IgG_{TL} antibodies have the potential to serve as a unique probe for detailed investigation of disease specific sialoglycoconjugate present on the surface of NSCLC cells. To the best of our knowledge, this is the first study which reports the identification of the disease specific sialoglycoconjugate specific IgG1 antibodies in the BALF of NSCLC patients. Further, due to high specificity of IgG_{TL} to GM3, it may be possible to develop a simple alternative diagnostic approach (GM3-ELISA) for NSCLC.

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