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DEVELOPMENT OF MONOCLONAL ANTIBODIES AGAINST MUC1/Y RECOMBINANT PROTEIN EXPRESSED IN *E.COLI*.

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ABSTRACT

Cancer antigen 15-3 (CA 15-3) is a MUC1 peptide fragment widely used for the diagnosis of breast cancer. However, because of a lack of sensitivity and specificity, especially during early stages, CA 15-3 alone has not proved to be a reliable marker for early diagnosis of breast cancer. High preoperative concentrations of CA 15-3 are, however, associated with adverse patient outcome. Therefore, an emergent need still exists to find out secondary markers to enhance specificity of serum based detection of breast cancer. MUC1/Y a trans-membrane protein (Non-polymorphic), and a part of MUC1 gene devoid of tandem repeats array and its immediate flanking sequences has also been reported to be expressed *in vivo* by tumor cells and suggested to be a potential target both for epithelial tumor diagnosis and immunotherapy. In this study, we have expressed and purified recombinant human MUC1/Y from *Escherichia coli* BL21 (DE3) strain. We could develop a repertoire of monoclonal antibodies, which bound to our recombinant protein. However, most of the antibodies exhibited cross reactivity against normal human serum or His Tag. However, only 9A842G6 specifically reacted to recombinant MUC1/Y protein. 9A842G6 monoclonal antibodies exhibited fluorescence specifically with established breast cancer tissue sections and did not bind to fibroid adenoma sections. Specific reactivity of 9A842G6 mAbs towards MUC1/Y was also confirmed by immunoblotting. Therefore, our observations suggest possible use of MUC1/Y as a potential secondary marker for Breast cancer diagnosis along with CA15-3.

KEY WORDS: **Human, MUC1, Breast cancer, MUC1/Y isoform, Escherichia *coli* expression, CA15-3**

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1. INTRODUCTION

Breast cancer is one of the most predominant cancers among women in the world and the breast cancer susceptibility genes *BRCA1* and *BRCA2* play important role in the development of breast carcinoma condition[1]. The protein product of MUC1 gene is the most widely used tumor marker for monitoring the disease status of all breast cancer patients and its expression level is intensely increased in breast cancer cells[2-9]. Mucins were originally described as large, heavily glycosylated proteins found mainly in the mucus secreted into the respiratory, gastro-intestinal and genitourinary tracts[10]. The best characterized of these molecules is a carcinoma-associated mucin, originally designated as polymorphic epithelial mucin (PEM)[11], episialin[12], epithelial membrane antigen (EMA)[13], according to the monoclonal antibody (mAb) detecting the mucin[14]. The most targeted *MUC1* gene product, MUC1/REP is a polymorphic, type 1 transmembrane mucin-like protein that contains a large extracellular domain, primarily consisting of a 20-amino acid repeat motif as well as a transmembrane domain and a 72-amino acid cytoplasmic tail[15–17]. Expression of the MUC1/TM, MUC1/X, MUC1/Y and MUC1/Z is also associated with the presence of malignancy, whereas expression of MUC1/SEC is observed mostly in non-malignant tissues[18]. The MUC1 gene product showed a high degree of length polymorphism due to the presence of different numbers (30–100) of tandem repeats[19-21]. Recently a supplementary and unique MUC1 isoform, MUC1/Y[22-24], was identified, which is generated by differential splicing. Even though MUC1/Y is a trans-membrane protein, it shares identical trans-membrane and cytoplasmic domains with the MUC1/REP protein, it contains neither the repeat array domain nor its flanking region and, henceforth, it is non-polymorphic and devoid of the hallmark mucin-like features. MUC1/Y protein is reported to be expressed by various human secretory epithelial tumors, but not detected in the adjacent normal tissues[22-26]. The MUC1/Y isoform has a cytokine receptor-like domain and has been suggested to play a role in signal transduction Furthermore, the involvement of the MUC1/Y protein in the oncogenic process was established by demonstrating its potential to enhance tumor initiation and progression *in vivo*[26]. The aim of the current study was to develop repertoire of monoclonal antibodies (mAbs) against human recombinant MUC1/Y purified protein expressed in *E. coli* in order to keep all amino acids exposed (without post translational modifications). We developed and characterized specific monoclonal antibodies against MUC1/Y. Our preliminary studies suggest that 9A842G6 monoclonal antibodies specifically recognize breast cancer tissues. However, more extensive clinical validation studies need to be carried out in order to establish the utilization of MUC1/Y as secondary marker along with CA 15-3 to improve the sensitivity and specificity of breast cancer diagnosis.

2. MATERIALS AND METHODS

2.1 Ethics Statement

Maintenance of mice and experimental procedures were approved by Institutional Animal Ethics Committees (IAEC), constituted by and following the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and forests, India.

2.2 Expression of recombinant MUC1/Y (rMUC1/Y)

Codon optimized nucleotide residues for amino acid 1-264 of *Homo sapiens* MUC1/Y (NCBI accession: AAP97018) was cloned using EcoRI/HindIII sites into the in-house developed pYBL plasmid vector which contains T7 promoter system, 7x His tag at N-terminus and ampicillin resistance gene for selection (data not published). *E. coli* strain, BL21 (DE3) (New England Biolabs Inc.) was used for recombinant protein expression. For expression, pYBL-MUC1/Y plasmid was transformed in BL21 (DE3) cells .The cells were cultivated at 37 °C in Luria Broth (LB) media containing ampicillin (100 µg/ ml) as a 5 ml culture overnight. Small scale expression (10 ml) was done at 37 °C for 3 hours with induction concentrations of IPTG, 1 mM at culture A600 nm 0.9. Cells were then

harvested by centrifugation at 4 °C and cell pellet was kept at -80°C for further processing.

2.3 Analysis of rMUC1/Y expression

To analyze the expression of recombinant protein (rMUC1/Y) containing N- terminal 7 x His tag, 10 ml small scale culture was centrifuged at 12,000 rpm for 10 min at 4°C in Kubota centrifuge (model No. 7780). Un-induced and induced cell pellet were suspended in buffer composed of 0.125 M Tris-HCl, 10 % 2 β-mercaptoethanol, 20 % glycerol, 0.004 % bromophenol blue, pH of approximately 6.8 and analyzed by SDS–PAGE. SDS–PAGE was done according to the method of Laemmli[27]. Protein bands were visualized by Coomassie blue staining. After localization study, we found that protein is expressed as inclusion bodies.

2.4 Purification of recombinant MUC1/Y

A total of 4 g wet weight of frozen cells from a 1 L BL21 (DE3) culture expressed at 37 ^{0}C was thawed on ice and resuspended in 100 ml of lysis buffer containing 20 mM Tris, pH 8.0, 150 mM NaCl, 5% glycerol, 0.1 mg/ml lysozyme. After sonication on ice for 5 min the lysate was clarified by centrifugation at 10,000 rpm in Kubota rotor for 30 min. The pellet was washed twice with washing buffer containing 20 mM Tris, pH 8.0, 150 mM NaCl, 0.1 % Triton X-100, followed by washing twice with 20 mM Tris, pH 8.0, 150 mM NaCl. Final pellet (inclusion bodies) was dissolved in 30 ml of equilibration buffer containing 20 mM Trsi-Cl, pH 8.0, 8 M urea, 150 mM NaCl, 5 % glycerol. The dissolved sample was filtered through a 0.2 µm pore size filter apparatus (Nalgene, Rochester, NY). Single 10 ml Chelating Sepharose (GE Healthcare, India) IMAC column, charged with 0.1 M nickel sulfate was pre-equilibrated with 20 mM Tris-Cl, pH 8.0, 8 M urea, 150 mM NaCl, 5 % glycerol (Equilibration buffer) prior to loading the filtered sample. Flow rate was set at 5 ml/min throughout the run. Protein flow through was collected as the sample passed through the columns. After loading was completed, columns were washed with equilibration buffer for at least ten column volumes (CV) until UV 280 nm absorbance (A280 nm) became stable at baseline. Washing was done with 5 CV of equilibration buffer (20 mM Tris-Cl, pH 8.0, 8 M urea, 150 mM NaCl, 5 % glycerol) containing 50 mM imidazole. For the elution of protein, 5 CV of elution buffer (20 mM Tris-Cl, pH 8.0, 8 M urea, 150 mM NaCl, 5 % glycerol, 300 mM imidazole) was applied. Protein fractions were collected at 10 ml each, and analyzed by SDS–PAGE using 15% gel. rMUC1/Y containing fractions were pooled and dialyzed against 20 mM Tris acetate, pH 5.0, 8 M urea, 5 % glycerol. The dialyzed sample was filtered through a 0.2 µm pore size filter apparatus (Nalgene, Rochester, NY). Single 3 ml DEAE cellulose (GE Healthcare, India) column was pre-equilibrated with 20 mM Tris acetate, pH 5.0, 8 M urea, 5 % glycerol prior to loading the filtered sample. Flow rate was set at 1 ml/min throughout the run. Protein flow through was collected as the sample passed through the columns. Collected flow through was serially dialyzed against buffer 1 (20 mM Tris, pH 8.0, 150 mM NaCl, 4 M urea, 5 % glycerol), buffer 2 (20 mM Tris, pH 8.0, 150 mM NaCl, 1 M urea, 5 % glycerol) and buffer 3 (20 mM Tris, pH 8.0, 150 mM NaCl, 50 % glycerol). Total protein concentration was quantified by Bradford assay using Bradford Assay Kit (Bio-Rad) according to the manufacturer's recommendations.

2.5 Generation of Monoclonal Antibodies

Five cohorts of three 6–8 weeks old female BALB/c mice each, were immunized by a subcutaneous injection of 10-50 µg of recombinant MUC1/Y. Hybridomas were generated as described by Kohler and Milstein[28]. For an initial immunization, the antigen was emulsified in complete Freund adjuvant (Sigma). Subsequent immunizations at days 21, 42 and 63 were performed with the antigen emulsified in Freund's incomplete adjuvant. Antisera were collected two weeks after each injection and tested for the presence of MUC1/Y-specific antibodies by an indirect ELISA. The mouse with the highest antibody titer was boosted intraperitoneally with 15 µg of MUC1/Y dissolved in PBS twice (24 hours interval between two boosters), 2 days before the cell fusion. Mouse splenocytes were fused with Sp2/0-Ag 14 mouse myeloma cells using Hybri-Max™ polyethylene glycol solution (50% W/v) (Sigma-Aldrich-P7181). Hybrid cells were selected in growth medium supplemented with HAT (hypoxanthine, aminopterin, and

thymidine) (50x HAT media supplement, Sigma-Aldrich-H0262). Culture supernatants from wells with viable clones were screened by an indirect ELISA using recombinant MUC1/Y protein. Stable hybridoma clones secreting MUC1/Y-specific antibodies were obtained after two cloning cycles by a limiting dilution assay. Hybridoma cells were grown in complete Dulbecco's modified Eagle's medium (DMEM, SIGMA) supplemented with 15% fetal bovine serum (FBS, Gibco), supplemented with Glutamax (Gibco) and anti-bacterial anti-mycotic (Gibco). The resulting hybridomas were screened for MUC1/Y protein specific antibodies by indirect antibody capture ELISA using Fc specific HRP conjugated anti-mouse IgG to select clones secreting immunoglobulin's of only IgG isotype. Clones secreting antibodies which bound to another His-tagged recombinant protein were neglected[29]. The isotype of generated mAbs was determined with a mouse monoclonal iso-typing kit (SIGMA) according to the manufacturer's instructions.

2.6 *Immunoblotting*

MUC1/Y purified antigen, Native human CA 15-3 antigen purified from cancer fluid[30], lysates of stable cell line transfected with MUC1 antigen contains only tandem repeats (NP_001191215.1) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. Blots were probed with 10 ml of monoclonal antibodies (100 ng/ml) purified from ascitic fluid raised *in-vivo* from *Balb/c* mice. Bound Abs were detected with the Fc specific HRP-conjugated goat anti-mouse immunoglobulin of IgG Isotype (Sigma), followed by a chemiluminescence reaction with TMB substrate (Sigma). Protein loading and transfer efficiency were monitored by Coomassie blue and Ponceau S staining, respectively as described elsewhere[31].

2.7 Biacore analysis

Determination of kinetic parameters and affinity constants using SPR analysis was carried out on GE healthcare T200 Biacore. The MUC1/Y molecule was immobilized to the Biacore sensor chip CM5 using conventional protocol for amine coupling. A total immobilization of 265 RUs was achieved. Binding was carried out at constant flow rates of 100 or 50 µl/min of mAb at various dilutions in Hepes complete buffer (10 mM Hepes, 3.4 mM ethylenediamine tetra-acetic acid (EDTA), 0.15 M NaCl, 0.05% Biacore surfactant P20, pH 7.4). Dissociations were carried out by passing buffer for 10 min for each cycle. Regeneration surface was carried out by injecting 10mM of NaOH for 1 min at a flow rate of 10 µl/min. No loss of binding capacity was observed under these conditions. The kinetic parameters and the affinity constants were calculated using BIACORE T200 EVALUATION software version 1.0.

2.8 Immunofluorescence

Human breast carcinoma and fibroid adenoma (formalin fixed paraffin embedded) blocks were received from Pathology Department, All India Institute of Medical Sciences, New Delhi. The 4 µM thick sections were treated by Citra in steamer for 30 min. Sections were blocked in 1 X TBS / 10% normal goat serum for 20 min at RT. Sections were incubated with primary antibody diluted 1:50 in 1xTBS overnight at 4°C. Sections were rinsed three times in TBS for 5 min each at RT. Sections were incubated with secondary antibody diluted 1:1000 in 1xTBS for 60 min at RT in dark. Sections were rinsed three times in TBS for 5 min each at RT. Coverslips were mounted on slides with DAPI (Invitrogen, Prolong Gold antifade reagent with DAPI). Stained sections were imaged with the Olympus Phase contrast inverted microscope, CKX 41SF.

3. RESULTS

3.1 Analysis of rMUC1/Y

The DNA construct of MUC1/Y (pYBL-MUC1/Y) was characterized with EcoRI/HindIII enzyme (fig. 1). The expression of rMUC1/Y proteins was performed using *E. coli* strain, BL21 (DE3), at induction temperatures, 37 ^{0}C at 1 mM IPTG concentrations for 3 hours (Fig. 2). The rMUC1/Y expressed in BL21 (DE3) at 1 mM IPTG and 37 ^{0}C was initially insoluble. We used IMAC column to purify rMUC1/Y based on its property of nickel-binding affinity eluting at 300 mM imidazole. The histidine-tagged rMUC1/Y showed 70-80 % purified protein on reducing SDS–PAGE.

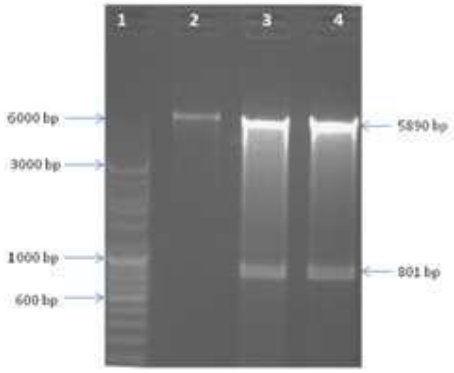


Figure 1

Characterization of pYBL-MUC1/Y clone: (lane 1: 1 Kb DNA ladder, Merck), (lane 2: Control DNA digested with EcoRI/ HindIII enzymes and the expected band size is 6020 bp), (lane 3 and 4: pYBL-MUC1/Y clone 1 and 2, digested with EcoRI/HindIII enzymes and the expected band sizes are 801 bp and 5890 bp).

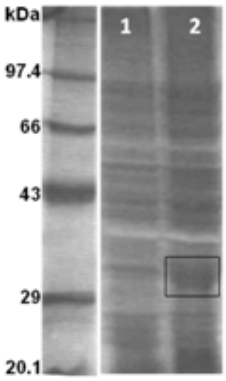


Figure 2

Coomassie blue stained SDS-PAGE analysis of recombinant MUC1/Y in E. coli strain BL21 (DE3): (lane 1: Un-inducedrMUC1/y culture), (lane 2- Induced rMUC1/Y culture). Protein Molecular weight marker from Merck was used. Rectangle showed induced recombinant protein.

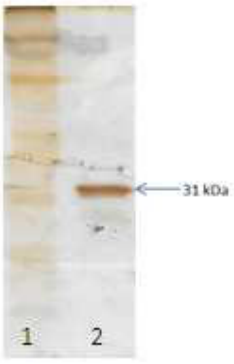


Figure 3

Silver stained SDS-PAGE analysis of purified recombinant rMUC1/Y. Lane 1: NEB Protein Molecular weight marker, Lane 2: purified recombinant Muc-1/y protein (2µg).

3.2 Purification of rMUC1/Y

rMUC1/Y purification was carried out by affinity purification using Chelating Sepharose IMAC column. The protein was further purified using DEAE, a weak anion exchanger and finally dialyzed into the buffer with 50% glycerol as stabilizing agent. The molecular weight of purified protein was observed to be approximately 31 kDa (Fig.3). We could achieve 95% purity as per the Bio-Rad GS900 Densitometer. We could purify almost 12 mg of rMUC1/Y antigen from 1L of bacterial culture.

3.3 Monoclonal antibody development

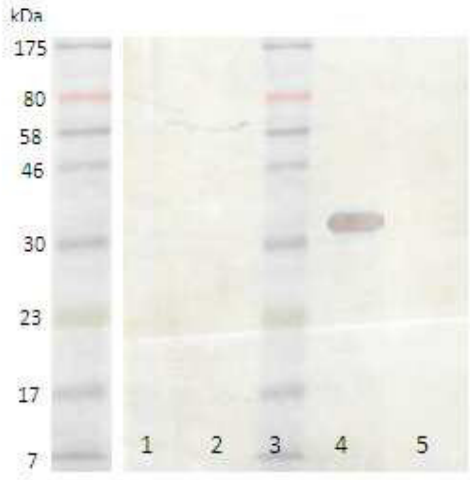
A repertoire of monoclonal antibodies was developed against the injected MUC1/Y (with only non-repeated sequences) and sub cloned twice by limiting dilution method. We could identify 7 monoclonal antibodies against recombinant protein, which did not recognize His tag as described elsewhere[29]. However, only 9A842G6 monoclonal antibodies exhibited MUC1/Y protein specific response as determined by ELISA. All other monoclonal antibodies reacted equally to Normal Human serum.

3.4 Western blot analysis

The specificity of the monoclonal antibodies was also analyzed by SDS-PAGE followed by Western blot. 9A842G6 monoclonal antibodies specifically recognized 31 kDa MUC1/Y protein. 9A842G6 antibodies did not exhibit any reactivity to purified CA 15-3 antigen or crude extract composed of a mixture of proteins represented by the different bands on the gel (data not shown) from mammalian CHO S cell lines transfected with Tandem repeated sequences of MUC-1. (Fig. 4).

Figure 4

Western blot analysis of rMUC1/Y with mAb of CA15-3: Lane 1: Crude Cho K1 cell extract after stable transfection with MUC1 (tandem repeat sequences). Lane 2: Crude Cho K1 cell extract (negative control without transfection), Lane 3: NEB Protein Molecular weight marker, Cat# P7709S, Lane 4: Purified recombinant Muc-1/y protein (3µg). Lane 5: Purified CA 15-3 protein 5KU.



3.5 Biacore Analysis

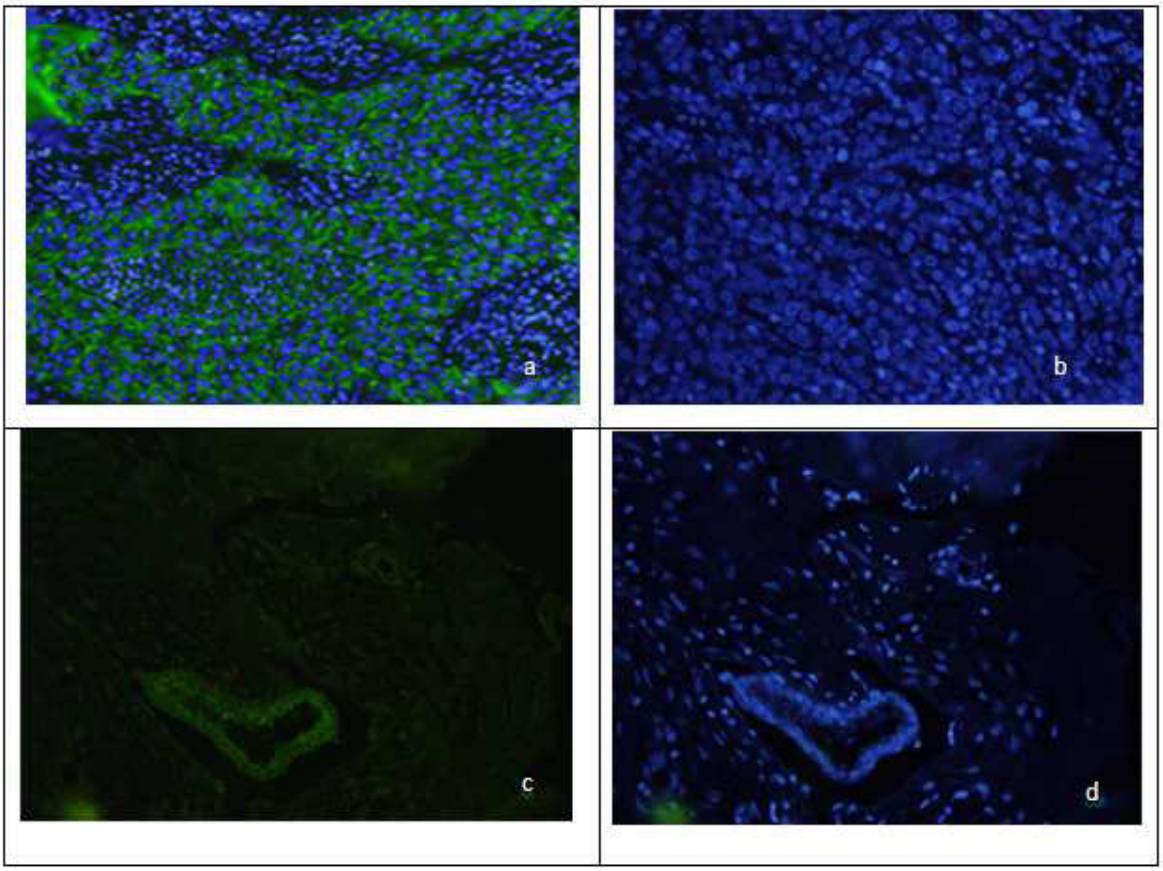
The kinetic binding parameters as well as the affinity constants for the binding between MUC1/Y and the 9A842G6 mAbs were determined by SPR based analysis at Biacore T200. Biacore sensorgrams indicated that 9A842G6 had association (*k*on) of 3.26X 10^6 and dissociation rates (*k*off), of 2.1 X 10^{-2}. Correspondingly, the *KD* values, calculated as *k*off/*k*on, were determined to be 6.72 × 10^{-9} M for 9A842G6 mAbs, suggesting very high affinity of 9A842G6 antibodies against MUC1/Y antigen.

3.6 Immunofluorescence

Strong signal was detected in positive control tissue i.e. clinically diagnosed breast carcinoma tissues, which was strongly distinguishable despite a small level of expected background staining in negative control (fibroid adenoma tissue, one of the most common benign breast lesions) as shown in Figure: 5.

Figure 5

a) Human breast carcinoma tissue stained with 9A842G6 monoclonal antibodies (green) and counterstained with DAPI (blue)
b) Human breast carcinoma tissue stained with isotype control antibody (green) and counterstained with DAPI (blue).
c) Human fibroid adenoma tissue stained with 9A842G6 monoclonal antibodies (green) and counterstained with DAPI (blue)
d) Human fibroid adenoma tissue stained with isotype control antibody (green) and counterstained with DAPI (blue).



4. DISCUSSION

Cancer Antigen 15-3 (CA 15-3) is a tumor-associated antigen used as a serum marker for breast cancer surveillance in patients and for monitoring the response to treatment[32, 33]. CA 15-3 is a mucinous carbohydrate antigen product of the MUC1 gene, originally identified by two monoclonal antibodies: DF3 raised against a membrane fraction of breast liver metastases and 115D8 raised against milk fat globule membrane[34, 35]. MUC1 protein is a large trans-membrane glycosylated molecule containing three main domains, a large extracellular region, a membrane-spanning sequence, and a cytoplasmic domain[1, 2]. Increased expression levels of MUC1 in primary tumor suggest that this protein acts as anti-adhesive molecule and facilitates detachment of malignant cells, both from adjacent cells and extracellular matrix (ECM) in primary cancer[36]. It is reported that MUC1 molecules devoid of the tandem repeat region (MUC-1/Y) are non-polymorphic[37], detected preferentially in carcinoma cells and are associated with their progression. Akagi J. *et al*. demonstrated that CA19-9 epitope is produced only on MUC-1/Y core protein, suggesting that CA19-9 epitope may be a

specific marker for MUC-1/Y protein[38]. To our knowledge, our study is the first report of simple expression system BL21 (DE3) capable of producing rMUC1/Y at significantly high-level than as reported earlier. This is the first report of recombinant His-tagged expression of MUC1/Y in *E. coli* and single step purification from inclusion bodies. Monoclonal antibodies were generated against this recombinant MUC1/Y which is not glycosylated, so as to target the epitopes which would be hidden, because of post translation modifications, in mammalian cell lines. At the same time our intention was to generate monoclonal antibodies against conformational specific epitopes of MUC1/Y protein which could not be mimicked in previous studies where in antibodies were generated against synthetic peptides. We were able to demonstrate that antibodies generated against r MUC1/Y are mostly within the 1-10 nM Kd affinity range, sufficiently high for diagnostic purposes. At the end we can conclude that these monoclonal antibodies against MUC1/Y will be used to further demonstrate structure and functional relationship of different cancer markers as CA15-3, CA19-9 or other epithelial markers in our upcoming studies. More studies have to be carried to validate recombinant MUC1/Y and mAb against MUC1/Y for diagnostic exploitation. Thus this study may aid in the development of more specific and accurate diagnostic tools for detection of breast cancer, ovarian cancer and other carcinoma conditions in addition to existing primary cancer markers and lead to increase in total specificity of diagnostic tests[38].

5. CONCLUSION

Monoclonal antibodies against *E. coli* expressed recombinant MUC1/Y are generated and characterized. Further research studies are warranted to establish the supportive role of MUC1/Y as secondary marker along with CA 15-3 to improve the sensitivity and specificity of breast cancer diagnosis.

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ABBREVIATIONS USED

LB, Luria broth; IPTG, isopropyl thio-β-D-galectosidase; PAGE, polyacrylamide gel electrophoresis; CV, column volumes; IMAC, immobilized metal affinity chromatography; mAb, monoclonal antibody; DEAE, Diethylaminoethyl; EMA, Epithelial membrane antigen; H23Ag, Epithelial tumor antigen recognized by H23 monoclonal antibodies; MCA, Mucin-like carcinoma-associated antigen; PEM, Polymorphic epithelial mucin.

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