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SECRETORY EXPRESSION OF CA15-3 LIKE RECOMBINANT ANTIGEN IN MAMMALIAN CELLS

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ABSTRACT

Breast cancer is most common life-threatening malignant lesion in women all over the world. Cancer antigen 15-3 (CA 15-3) is a widely used prognostic marker for breast cancer. CA 15-3 is a glycoprotein that is recognized with a match pair of DF3 and 115D8 monoclonal antibodies. DF3 antibody detects 8 amino acids sequence (DTRPAPGS) of tandem repeats in MUC1 protein, serves as a detection antibody in a sandwich assay while 115D8 monoclonal antibody binds to peptide- carbohydrates epitope on same repeat acts as the capture antibody in sandwich assay. In this study, we have developed a protein sequence containing 10 Tandem repeats with few approximate repeats out of MUC1 protein expressed in CHO-K1 cells to ensure post translational modifications. We have incorporated 'Gluc' protein (*Gaussia luciferase*) sequence along with gene of interest for secretion of protein outside the cells. Protein was characterized by western blot using CA15-3 antibody as well as confirmed for the presence of epitopes similar to the ones, as recognized by 115D8 and DF3 on Siemens CLIA based platform. Further validation studies are needed to demonstrate equivalence of the expressed protein with native purified CA15-3 protein to be used to develop diagnostic/prognostic tests.

KEYWORDS: **Human, MUC1, Breast cancer, CHO-K1 expression, CA15-3, DF3, 115D8 Mab, Gluc protein, Secretory expression.**

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1. INTRODUCTION

Breast cancer has been reported as one of the most common cause of death in women [1]. More than 1 million women are diagnosed with breast cancer every year [2]. Cancer antigen CA15-3 is one of the most promising markers for the diagnosis of breast cancer [3]. The main application of CA 15-3 includes the surveillance of patients diagnosed with breast cancer monitoring therapy in advance disease conditions [4]. Daniele *et.al.* found that the serum concentration of CA 15-3 levels in patients before surgery is significantly higher compared with those of CA 15-3 after surgery and demonstrated that in most of the cases CA 15-3 in patients with metastatic breast cancer and concentration of antigen is correlated with the clinical status of breast cancer [5].CA 15-3, a secreted product of MUC1 gene [6] is a mucinous carbohydrate product of MUC1 gene originally identified by two monoclonal antibodies: DF3 and 115D8 [7, 8]. The best characterized MUC1 gene product are MUC1/REP, MUC1/SEC and MUC1/Y. MUC1/REP contains the large extracellular domain, primarily consists of 20 amino acid tandem repeat, transmembrane domain and cytosolic tail of 72 amino acid [9-12]. MUC1/SEC has an extracellular domain identical to MUC1/REP but it is devoid of hydrophobic region [13]. MUC1/Y is a transmembrane protein that contains transmembrane and cytoplasmic domain as identical to MUC1/REP but devoid of tandem repeat and its flanking region [14-16]. MUC1/Y can be used as a secondary marker with CA15-3 antigen for the diagnosis of breast cancer [17]. Several studies have demonstrated that MUC1 is normally expressed on the apical surface of epithelial cells. In case of breast adenocarcinoma and other epithelial carcinoma, MUC1 is unregulated with unusual expression over the entire cell surface. These characteristics suggested MUC1 proteins can also be evaluated as marker or can complement CA15-3 in order to resolve the specificity issue associated with CA15-3 for diagnostic purposes [12, 18-20]. The full sequence of MUC1 (reference Uniprot ID # P15941) contains 1255 amino acids with theoretical molecular mass 122,102 Dalton. This 1255 amino acid composed of signal peptide (1-23 aa), approximate repeats of 20 aa (61-80, 81-100, 961-980, 981-1000 and 1001-1020 aa), tandem repeat of 20 aa (101 -960), transmembrane domain (1159-1181aa) and Topological domain (1182-1255 aa). MUC1 gene product MUC1/REP contains the large extracellular domain, is primarily constituted of 20 amino acid tandem repeat, transmembrane domain and cytosolic tail of 72 amino acids. The present study was undertaken to develop and characterize a hypothetical protein constituted of 10 tandem repeats and approximate repeats, with necessary Co/post translational modifications. The construct was prepared in pcDNA 3.1 Zeo (+) vector as a base vector and the protein was expressed in mammalian CHO K1 cells at an intermediate scale. The protein was characterized using SDS-PAGE, Western blot, and other commercially available platforms for the presence of required epitopes. However, more extensive clinical validation studies need to be carried out before the expressed MUC RS protein can be utilized as an antigen to detect anti-Muc1 antibodies or as calibrator in CA 15-3 antigen detection assays.

2. MATERIALS AND METHODS

2.1 Construction of vector of MUC1RS

For the expression of CA15-3 like antigen we have targeted specific regions of MUC1 protein. Codon optimized nucleotide residues for amino acid 61-320 followed by AA 941-1020 (Uniprot accession: P15941) was cloned using NheI/XhoI sites in the pcDNA 3.1 Zeo (+) vector (Invitrogen co.) to make pcDNA3.1 Zeo_MUC1RS construct. pcDNA 3.1 Zeo (+) vector contains CMV promoter cassette, and ampicillin resistance gene for selection in bacteria and Zeocin resistance gene for stable cell line generation. We used Gluc protein (*Gaussia luciferase*) sequence for secretion of desired protein from cells as reported elsewhere [21, 22]. We constructed pcDNA 3.1 Zeo+MUC1RS_Gluc by inserting nucleotide sequence of Gluc protein at N-term followed by MUC1RS cDNA sequence by

site directional mutagenesis process using primer set as follows:

Forward primer

5'CAAGCTGGCTAGCATGGGAGTGAAAGTTCT TTTTGCCCTTATTTGTATTGCT GTGGCCGAGG CCCA CCACCACCATCATC 3'

Reverse primer

5'GATGATGGTGGTGGTGGGCCTCGGCCACA GCAATACAAATAAGGGCAAA AAGAACTTTCACTCCCATGCTAGCCAGCTTG 3'

2.2 Cell culture, transfection, selection and cloning

The origin and culture of hamster CHO-K1 cells have been described elsewhere [7, 20]. Transfection of plasmid DNA was performed using the Effectene transfection reagent (Qiagen) according to the manufacturer's recommended protocol. The day before transfection, 0.3 million cells per well were seeded in 6 well plate with 2 ml of DMEM media containing 10% FBS (growth media). The cells were incubated under normal growth conditions i.e. 37ºC and 5% CO_2. On the day of transfection one microgram of DNA plasmid was mixed with transfection reagent and the transfection complex (DNA: Lipid complex) was incubated with the cells at 37°C, 5% CO_2. Transfection complex was removed after 16 hours; cells were dislodged and expanded in T 75 culture flask containing 15 ml of growth media. After 8-10 hr, of adherence of cell, Zeocin (250 µg/ml) was added as a selection antibiotic. Cells and control flask was kept under antibiotic selection for 15-20 days till colony formation. Single cell cloning of parent clone 4C9 (pool of mix clones) was done in 96 well plate (1 cells per 2 well) to develop sub clone 4C9-2E12. Further, to ensure the phenotypic stability of clone, second single cell cloning was done; MCB and WCB of clone 4C9-2E12-2e12 with highest expression of MUC1RS was cryopreserved in freezing mix (10% DMSO and 90% FBS).

2.3 Adaptation to Serum Free Media

Direct Adaptation of Clone to Serum-free Media (Excell CD-CHO)

The screened clone was adapted to serum free medium by direct adaptation. On revival, first 2-3 passages were done in basal media containing 10 % FBS, later seeded directly into pre-warmed serum free medium at a density 0.5 million cells/ml. Cells were sub cultured after every 3-4 days and were considered adapted after cell densities reached 2 million cells/ml with viability >94%.

Sequential Adaptation of Clone and Scale-up

During the sequential adaptation, initially cells were grown in basal media with 10 % of FBS and serum concentration from original medium was gradually (step-wise) reduced upto 2.5% of FBS before seeding cells in serum free media. Viability as well as cell morphology was monitored at each step during adaptation.

2.4 Purification of MUC1RS

For scale-up, cryopreserved clone thawed and suspended in DMEM containing 2.5% FBS and incubated at 37°C, 5% CO_2. Multiple passages were done before seeding the cells in large vessels for scale-up. 0.5 Million cells were seeded in 200 ml of DMEM media containing 2.5% FBS in Roller bottle (surface area~ 1700cm^2). Harvesting was done post stationary phase i.e. 3-4 days after cells reached 80% confluency. Supernatant was harvested from the expressed culture and dialyzed in equilibration buffer containing 20 mM sodium phosphate, 5% Glycerol pH- 8.0 at 4°C. The dialyzed sample was filtered through a 2 µm pore size filter apparatus (Nalgene, Rochester, NY). Single 10 ml UNO sphere Q gel (Biorad, India) column was equilibrated with 5 column volume (CV) of 20 mM sodium phosphate, 5% Glycerol pH- 8.0 (equilibration buffer). Sample was loaded and protein flow through was collected as the sample passed through the columns. Flow rate was set at 2-3 ml/min throughout the run. 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. First washing was done with 5 CV of equilibration buffer (20 mM sodium phosphate, 5% Glycerol pH- 8.0) containing 50 mM sodium chloride. Second washing was done with 5 CV of equilibration buffer (20 mM sodium phosphate, 5% Glycerol pH- 8.0) containing 100 mM sodium chloride. For the elution of protein, 5 CV of elution buffer (20 mM sodium phosphate, 250 mM sodium chloride, 5% Glycerol pH- 8.0) was applied. Protein fractions were collected at 10 ml

each, and analyzed by SDS–PAGE using 15% gel. rMUCRS containing fractions were pooled and filtered through a 0.2 µm pore size filter apparatus (Nalgene, Rochester, NY). Total protein concentration was quantified by Bradford assay using Bradford Assay Kit (Bio-Rad) according to the manufacturer's recommendations.

2.5 Analysis of MUCRS protein Immunoblotting

The culture supernatant containing secreted MUC1RS antigen was separated by SDS-PAGE and transferred onto Nitrocellulose membrane. Blots were probed with CA-15- 3_DF3 conjugated antibody from Calbiotech kit and commercial available Anti-CA15-3 (M002204) antibody by Fitzgerald. The section incubated with Anti-CA15-3 antibody was further incubated with Fc specific HRP-conjugated Goat anti-mouse immunoglobulin of IgG isotype (Sigma), followed by chemiluminescence reaction with TMB substrate (Sigma). Protein loading and transfer efficiency were monitored by Coomassie blue and Ponceau S staining respectively.

Chemiluminescence Immuno Assay (Siemens-Advia Centaur)

The samples of MUC1RS were analysed by Chemiluminescence Immuno Assay on Siemens-Advia Centaur platform. In brief, MUC1RS antigen, biotinylated monoclonal CA 15-3-specific antibody and monoclonal CA 15-3-specific antibody labeled with a ruthenium complex form a sandwich complex. After addition of streptavidin labeled micro-particles, the complex produced is bound to the solid phase via biotin-streptavidin interaction. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier. Results are determined via a calibration curve. This curve is instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

Enzyme linked Immunosorbent Assay (ELISA)

The culture supernatant of MUC1RS was analyzed by CA15-3 ELISA test kit (Calbiotech). It is based on the principle of a solid phase enzyme-linked immunosorbent assay. The assay system utilizes a monoclonal antibody directed against a distinct antigenic determinant on the intact CA15-3 molecule is used for solid phase immobilization (on the microtiter wells). A rabbit anti-CA15-3_DF3 antibody conjugated to horseradish peroxidase (HRP) is in the antibody-enzyme conjugate solution. The sample allowed reacting sequentially with the two antibodies, resulting in the MUC1RS molecules being sandwiched between the solid phase and enzyme-linked antibodies. After two separate 1-hour incubation steps at 37°C, the wells are washed with wash buffer to remove unbound labeled antibodies. A solution of TMB reagent is added and incubated for 20 minutes, resulting in the development of a blue color. The color development is stopped with the addition of stop solution changing the color to yellow. The concentration of MUC1RS is directly proportional to the color intensity. Absorbance is measured spectrophotometrically at 450 nm.

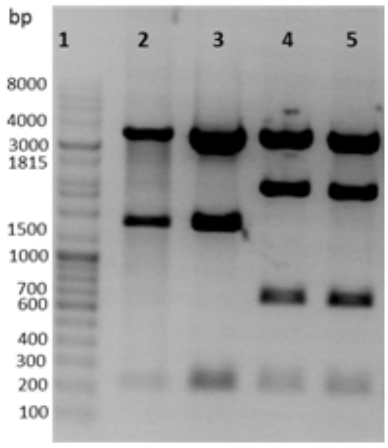
3. RESULTS

3.1 *Construction of vector of MUC1RS*

We cloned the cDNA of MUC1RS in to pcDNA 3.1 Zeo (+) vector using NheI and XhoI cloning site. Final DNA construct (pcDNA3.1 Zeo_MUC1RS) was characterized by NcoI restriction endonuclease (figure 1). The restriction digestion resulted in fragmentation of clone 1 and 2 and the characteristic bands at 220, 656, 1884 and 3443 were observed as per the expected band pattern.

Figure 1

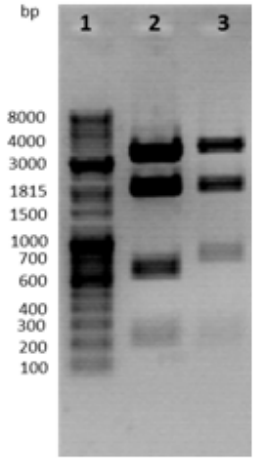
Characterization of pcDNA3.1 Zeo_MUC1RS clone: (lane 1: 1 Kb DNA ladder, Merck), (lane 2 and 3: Control Vector pcDNA3.1 Zeo+ digested with NcoI enzymes and the expected band sizes are 220, 1352 and 3443 bp), (lane 4 and 5: pcDNA3.1 Zeo_MUC1RS, clone 1 and 2 respectively, digested with NcoI enzymes and the expected band sizes are 220, 656, 1884 and 3443 bp)



For secretory expression, we inserted cDNA of Gluc protein in previous construct pcDNA 3.1 Zeo+MUC1RS at N terminus of MUC1RS DNA sequence by site directional mutagenesis process and made pcDNA 3.1 Zeo+MUC1RS_Gluc. Final DNA construct i.e. pcDNA 3.1 Zeo+MUC1RS_Gluc was once again characterized by restriction digestion with NcoI restriction endonuclease (figure 2) and we got the expected band sizes i.e. 220, 706, 1884 and 3443 bp of clone 1 and 2. In other words, addition of Gluc sequence was reflected by increment of second band from 656 to706bp. Insertion of Gluc sequence by SDM was also confirmed by DNA sequencing using universal T7 promoter primer set.

Figure 2

Characterization of pcDNA3.1 Zeo_MUC1RS_GALU clone: (lane 1: 1 Kb DNA ladder, Merck), (lane 2: Control Vector pcDNA3.1 Zeo_MUC1RS digested with NcoI enzymes and the expected band sizes are 220, 656, 1884 and 3443 bp), (lane 3: pcDNA3.1 Zeo_MUC1RS_Gluc clone 1 digested with NcoI enzymes and the expected band sizes are 220, 706, 1884 and 3443 bp)



3.2 Cell culture, transfection, selections and cloning

Parent clone; 4C9 was selected on the bases of resistance to zeocin, morphology and MUC1RS expression levels. Further, three sub-clone of 4C9 i.e. 4C9-2E12-1A3, 4C9-2E12-2E12 and 4C9-2E12-1C3 exhibited maximum expression level. CA 15-3 DF3 conjugate antibody recognized our MUC1RS protein at 130kDa (figure 3), further confirmed by immune-reactivity using Calbiotech kit (figure 4). 4C9-2E12-2E12 subclone exhibited maximum expression level of MUCRS protein (i.e. 122 U/ml), as determined by ELISA (Calbiotech kit).

Figure 3

Western blot analysis of MUC1RS with Mab of CA15-3_DF3 conjugate antibody: Lane 1: NEB Protein Molecular weight marker, Cat# P7709S, Lane 2: MUC1RS clone 4C9-2E12-2E12. Lane 3: MUC1RS clone 4C9-2E12-1C3, Lane 4: MUC1RS clone 4C92E121A3.

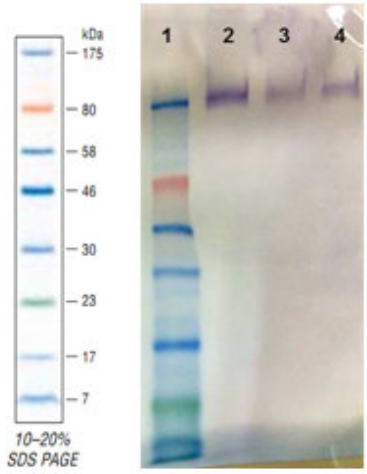
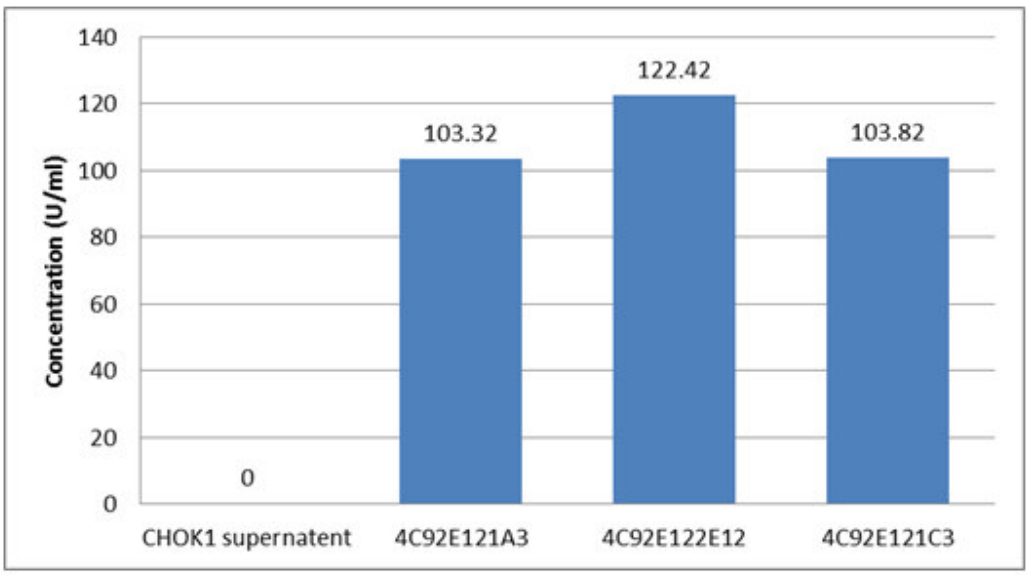


Figure 4

ELISA; Diagrammatic representation of expression of different sub-clones with corresponding graph. Among subclones, 4C92E122E12 showed maximum expression of MUC1RS : 122.42 KU/Lit.

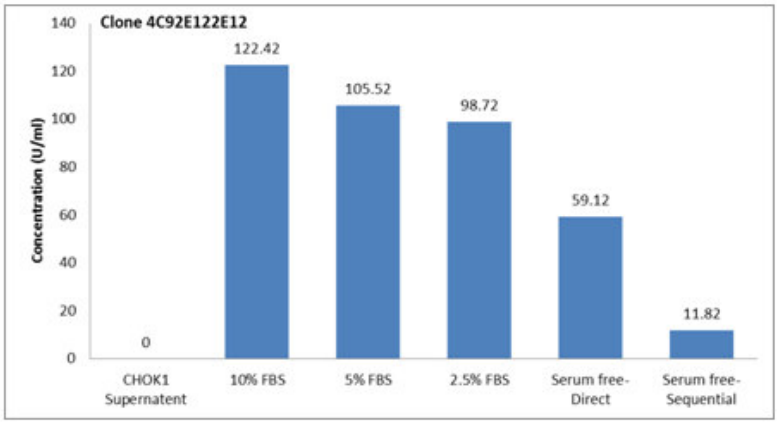


3.3 Adaptation of Clone to Serum Free Medium

Direct adaptation of clone to serum free medium did not exhibit favorable response as viability started decreasing significantly after 3 days of log phase. During sequential adaptation, the maximum cell density of 3M cells/ml was achieved with basal media containing 2.5% FBS. Furthermore, the qualitative profile of clone adapted sequentially showed no significant difference in expression of MUC1RS levels; from 5% to 2.5% FBS, as confirmed by immuno-reactivity by ELISA (figure 5). In order to facilitate downstream processing, clone adapted to grow in DMEM with 2.5% FBS was scaled up.

Figure 5

ELISA; Diagrammatic representation of expression levels of 4C9E122E12 clone during its sequential adaptation to serum free media.



3.4 Purification of MUC1RS

MUC1RS protein was purified using ion exchange chromatography using strong anion exchanger UNOsphere Q matrix. The final sample was eluted in 20 mM sodium phosphate, 250 mM sodium chloride, 5% Glycerol pH- 8.0.

3.5 Analysis of Purified MUC1RS

We pooled the eluted fractions of UNOsphere Q purification and analyzed by 7% reducing SDS-PAGE (figure 6a) and western blot (figure 6b) by using commercial available Anti-CA15-3 (M002204) antibody from Fitzgerald. We have quantitated the total protein by Bradford method and found the recovery of 30 mg per liter of media. We have quantitated the total yield of protein in international units which is 106 KU/ Liter of media by ELISA (Calbiotech) and 3.7 KU/ Liter by Siemens platform (Advia Centaur Immunoassay System).

Figure 6

Figure 6a and 6b: Analysis of purified sample by SDS-PAGE (Figure 6a) and western blot (Figure 6b) against commercial available Anti-CA15-3 (M002204) antibody from Fitzgerald.

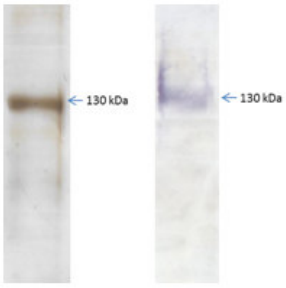


Figure 6a **Figure 6b**

4. DISCUSSION

Tumor Markers comprise a wide spectrum of bio-macromolecules synthesized in excess concentration by a wide variety of neoplastic cells. The appearance of tumor marker and their concentration have shown excellent clinical relevance in monitoring disease course, determining prognosis, and facilitating treatment planning, in a minimally invasive and cost effective fashion. The knowledge of marker used and its performance *in vivo*, however, remains critically important for accurate interpretation of the information provided. The application of individual markers varies frequently depending upon the criteria as per published recommendations as well as the outcome from the large scale studies to improve consistency in their use and interpretation. 13 Mucins, which are divided into seven structurally identifiable families (MUC1 to MUC7), are large glycoproteins normally found in a variety of epithelial cell types, including breast [23, 24]. The MUC1 gene product is a polymorphic transmembrane glycoprotein, frequently over expressed on malignant glandular cell surfaces, and is shed into the blood of some patients with carcinomas, thus resulting in increased levels [25]. Both CA15-3 and CA 27.29 are monoclonal antibody-defined markers. Monoclonal antibodies designated DF3 and 115D8 bind CA15-3, while the CA27.29 antigen is detected in the blood of a patient using a monoclonal antibody that recognizes MUC1. Because of the low sensitivity of the CA27.29 tumor marker, the test is used, only to follow a patient for breast cancer recurrence [25-27]. CA15-3 and CA27.29, both are used in conjunction with other assessments to monitor treatment response in patients with metastatic breast cancer as well as those who have been previously treated for stage II or stage III disease. The MUC-1 N-terminal ectodomain (MUC1-N) contains variable numbers of 20 amino acid tandem repeats, and is shed into circulating blood. The repeat units contain potential O glycosylation sites represented by serine and threonine residues, which act as a scaffold for the attachment of O-glycans, resulting in the formation of a highly glycosylated extended repetitive structure [28].Croce *et al*., reported that anti- MUC1 antibodies can bind MUC1 and form MUC1 circulating immune complexes (MUC1- CIC) in blood circulation [28]. MUC1 circulating immune complexes (MUC1-CIC) and free antibodies against MUC1 (IgG and IgM-MUC1) have been evaluated in order to compliment tumor MUC1 expression, secreted MUC1 levels. However, free and compound autoantibodies against MUC1 can be detected both in patients with malignant tumors and in healthy people [29-31]. Studies have demonstrated that circulating anti-MUC1 antibodies may be used as a favorable prognostic factor for patients with early breast cancer and pancreatic cancer [32-33]. In addition, previous studies have shown that the antibodies might contribute to limit tumor outgrowth and dissemination by antibody-dependent cellular cytotoxicity [34-36]. The patients with stage IV of breast cancer present low MUC1-CIC, although more common anti-MUC1 antibodies and MUC1 exist in their sera [37, 38]. A contradictory result indicated that anti-MUC1 antibodies in stage IV of breast cancer could not bind or neutralize MUC1 antigen, and they were of low affinity [37]. Thus far, there is no commercial enzyme- linked immunosorbent assay (ELISA) kit for detecting the anti-MUC1 antibodies in human serum. Mostly, synthetic MUC1 VNTR peptides along with recombinant peptides expressed in Escherichia coli have been tried as coating antigens in ELISA for detecting anti-MUC1 antibodies in human sera [34, 39]. However, as peptide expressed in *Escherichia coli* cannot be glycosylated as in eukaryotic cells, it has been demonstrated to be less efficient in detecting all anti-MUC1 antibodies. Looking at the ever increasing breast cancer cases, thus the demand for such antigens to be used to detect Anti-MUC1 antibodies, we have constructed recombinant MUC1 VNTR containing peptide with post translational modifications. The expressed protein contains ten tandem repeats, including a few approximate repeats, the characteristic of MUC1 in mammalian cells, with desired glycosylated Ser/thr residues. The protein's SDS-PAGE mobility around 130 kDa, which is approximately

180% higher than as expected (by calculating molecular weight of proteins, contributed by amino acids), and immunoreactivity profile as per Siemens CLIA based assay platform, ELISA based CA 15-3 detection kits from Cal-biotech, confirms all necessary co/post translational modifications, and presence of epitopes to be recognized by both DF3 and 115D8 antibodies. Therefore, Recombinant MUC1RS can also be used as calibrator in quantitative antigen detection tests for prognosis of Breast Cancer without any cross-reactivity of other cancer antigen antibody. However, further studies are warranted to validate and compare the generated protein with native CA 15-3 protein purified out of native sources, presently being used as calibrator control in an antigen detection assay.

ABBREVIATIONS

bp, Base pair; CHO, Chinese hamster ovary; CA, Cancer antigen; ELISA, Enzyme-linked immunosorbent assay; FBS, Fetal Bovine Serum; Mab, monoclonal antibody; MUC1, Mucin 1.

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