CHAPTER 5

SUMMARY AND CONCLUSION

5.1 Summary & Conclusion

Breast carcinomas in female mammary are reported as most notorious malignancy around the globe with highest incidence rate. Morphologically, Human breast having different parts include duct which is small tubes like structure which carry milk from the lobules to the nipple in lactating women, lobules which function as milk producing glands, blood vessels and lymph vessels, fatty tissue and connective tissue. Most of the cancer in breast is reported in duct cells. Some population of man also reported for breast carcinoma with low percentage. Different type of cancer of breast tissue is categorized on the bases of localization of cancer or tissue involved, some of which are very rare. These include ductal carcinoma in situ in which the malignant cells start in the cells coated to duct wall and it is generally not invading the tissue over a time. Invasive ductal carcinoma which is reported as most frequent type of carcinoma of breast tissue which invades the wall of ducts and disturbs the women breast tissue and it can be spread to other part include lymph nodes. Invasive lobular carcinoma i.e. ILC is also known as infiltrating lobular carcinoma which is caused in the lobules (milk glands) cells followed by spreading to other part of breast and body parts in human. Inflammatory breast cancer i.e. IBC is considered to be a infrequent type of carcinoma in breast in human, having multiple tumors and cause redness, itchy and larger of breast.

There are several risk factor for breast cancer, includes life style habits as smoking, drinking and junk food, gender, age, genetic risk includes mutations in some genes like BRCA1 and BRCA2, TP53 etc., family history, race, personal history of person, Non-uniform menstrual periods etc. Both BRCA genes are human genes that implicated in the production tumor suppressor proteins by different ways includes repair of damaged DNA hence responsible for integrity of genetic material in human. Mutation in these gene responsible for the production of nonfunctional protein or there is not expression of protein so the cells are more probable to develop supplementary genetic alterations that can lead to develop tumors. Sometime specific inherited mutations in both BRCA1/2 genes increases risk potential of carcinoma in breast and ovarian in human. TP53 is tumor suppressor protein code by TP53 gene in human. Optimal unaltered expression of TP53 is required for the
integrity of cell. Thus TP53 performed a significant role in suppression of various cancers. Cancer suppressor activity of p53 is reported by cells growth arrest, DNA repair, senescence, differentiation or by programmed cell death.

It was reported that till the age of forty five years carcinomas in breast tissue was more frequent in American/ African black females than white women but beside skin cancer, breast cancer diagnosis is reported as second largest reported clinical conditions (carcinoma) in America. It is reported that 85 % of women will develop breast cancer that had no genetic background for same. Only fifteen present of women’s have reported for genetic background for breast cancer and most of the mutation (at genetic level) is associated with BRCA1 and BRCA2 genes. It was projected that the total case of cancer in breast in 2016 will be over 2.8 million women with past history.

Mammography is still the backbone platform for the screening of cancer in breast along with imaging through magnetic resonance and ultrasound has become useful diagnostic assistants in select patients. Mammography a mechanical devide generated picture which is a precise type of breast imaging procedure uses x-rays at low-dose to detect cancer at early stage even before women experience symptoms. Same as X-rays, mammography uses doses of ionizing radiation to create images of breast followed by analysis for any abnormal findings.

Apart from mammogram and ultrasound, there are various diagnostic biomarkers (protein/ glycoprotein etc.) available for the diagnostic used of breast cancer includes Galectin-3, Her2, ST3-Gal1, Carcinoembryonic antigen (CEA), CA 27.29, tissue polypeptide antigen (TPA), and CA15-3 etc. Galectin 3 is a type of lectins having 14 different galectins, encoded by the LGALS3 gene. It is approximately 30 kDa protein and has been found in various developments, including angiogenesis, embryogenesis, and differentiation of cells, growth, inflammatory responses, cell progression and metastasis. Although Galectin-3 was majorly identified as cardiac markers but different experimental data proof the direct correlation of Galectin-3 concentration at great level and progression of tumor in breast carcinomas, liver, thyroid, colon and gastric, making Galectin-3 an emerging cancer marker.

Carcinoembryonic antigen (CEA) is glycoproteins present in human and shown involvement in adhesion of cells. The serum levels of CEA are become high in several clinical conditions (e.g. cancers) included breast carcinomas but expression
levels of CEA can also be high in smokers. CEA are also reported in several other cancer conditions.

CA27.29 is reported as marker of tumor growth and that is most often used for the diagnosis of this cancer other than cancer antigen 15-3. At early stage, the concentration of CA27.29 is not increased but in some cases with disease progression, the concentration goes high. TPA is come under the family of serine proteases which involved in the intravascular breakdown of blood clots which convert plasminogen to plasmin. This protein contains 562 amino acid with molecular mass approximately 180 kDa. It is reported as non-specific biomarker elevated in rapid cellular proliferation. It is composed of different cytokeratin units, i.e. of cytokeratin 18, cytokeratin 19, and cytokeratin 8 with proportion 36 %, 44 %, and 30 % respectively. Its normal concentration is 90 U/ml in serum and goes higher than 120 U/ml, regardless of type or localization of tissue. Unlike in other markers, the serum concentration of TPA is not proportional to the tumor burden in human.

ST3Gal-I is a sialyltransferase involved in in sialylation of T antigen to Sialyl T antigen in different cancer and its expression is increased in breast carcinogenesis with increased tumor progression stages. A study reported that the antibody against recombinant purified ST3Gal-I is specifically detects breast cancer tissue but as a promising marker, ST3Gal-I is still need to validate.

Human Her2 also known as CD340 is a protein found on the cell surface of normal breast. Several studies demonstrated that some breast cancer cells have a very high number of HER2 receptors and when the concentration of Her2 protein is high in breast cancer, it is called HER2 positive. Higher expression of this oncogene associated with significant function in disease development/ progression of certain destructive types of breast carcinoma. Recently Her2 oncoprotein has evaluated as significant bio-marker and target of therapy by immunological method, for approximately 25-30 % of patients having breast carcinoma.

As reported cancer antigen 15-3 is a carbohydrate product of mucin 1 oncogene originally identified by different mAbs 115D8 and DF3. Antibody DF3 specifically recognizes the amino acids of tandem repeats (i.e. DTRPAPGS amino acid) and 115D8 antibodies specifically recognizes glycosylation occurred on these tandem repeats amino acids as mentioned in introduction part of this report. The main application of cancer antigen 15-3 includes the follow up of mammary carcinoma
with continuous monitoring of various therapies in advancement of clinical disease conditions.

The incidence of carcinoma in breast in India is reported high and the most affected age of women is 40 to 60+ and trend was shifted from 20-40 to 40 to 60 and 60+ years in last few decades. 40 to 60 years age women are more affected may be because of post-menopausal stage. It was observed that in last 25 years, trend of incidence is shifter to older age women. In case of breast cancer the survival rate for 5 year is much better than other cancer conditions.

CA 15-3 is a carbohydrate product of mucin 1 oncogene originally identified by different mAbs 115D8 and DF3. It was reported that 115D8 mAb raised against membrane of milk fat globule and DF3 mAb developed against a membrane portion of metastatic liver. DF3 antibody (mAb) specifically recognizes the amino acids of tandem repeats (i.e. DTRPAPGS amino acid) and 115D8 antibodies specifically recognize glycosylation occurred on these tandems repeats amino acids.

The main application of cancer antigen 15-3 includes the follow up of mammary carcinoma patients with continuous monitoring of various therapies in advancement of clinical disease conditions. It was observed that Mucin 1 is expressed at high amount in breast carcinoma (aberrant O- glycosylation herewith) and this feature is exploited for the development of diagnostic assays as well as immunotherapy.

It was reported that cancer is associated with alteration in glycosylation pattern and also reported in breast cancer. In breast cancer, there are deviations in the structure of glycans (O linked). In this glycosylation type, GalNAc-transferases (belongs to polypeptide family) used to put a moiety of sugar i.e. N-acetyl galactosamine to threonine or serine amino acid in the core structure of protein, followed by extension of glycan chain by sugars in the Golgi pathway. Termination of chains was usually occurred by addition of specific sugars considering sialic acid molecule or fucose molecule.

In the condition of carcinoma of breast, few unique glycoproteins produced with unique antigenic sites because of early termination of glycoproteins. In case of mucins glycoproteins, the immunogenicity of protein is drastically changes because of availability of number of TR having of number of threonine (thr) and serine (ser) amino acid.

As demonstrated, MUC1 is composed of signal sequence, transmembrane domain, tandem repeats, and cytoplasmic tail. PDTR region of MUC1 tandem repeats are main
feature of MUC1 protein and immune response against PDTR region of MUC1 tandem repeats was well reported in different cancer conditions includes breast cancer, pancreatic and colon cancer.

The first antibody of immune response i.e. immunoglobulin IgM against mucin 1 (MUC1) was detected which were synthetic MUC1 native like protein. It was well reported that progression of carcinoma of mammary tissue includes increasing in tumor node metastases stages, increasing histological grade and lymph node (positive) status are extensively linked with the existence of Tn protein antigen and directly linked with MUC1.

In various studies, MUC1/Y protein is found as a part of MUC1 gene empty of tandem repeats array and its flanking amino acid sequences was found in different malignancy or carcinoma associated with breast. Several research studies suggested that MUC1/Y could be a good target for therapy potential. In the present study recombinant MUC1/Y protein was expressed in BL21 strain and purified by metal affinity column. For cloning of MUC1/Y gene into bacterial expression vector, pYBL bacterial expression vector was designed that contains T7 Promoter cassette, N terminus 7 x Histidine tag with multiple cloning site (MCS having EcoRI at N’ and HindIII at C’ cloning sites), C terminus 6 x Histidine tag and ampicillin resistance gene for clone selection.

Codon optimized cDNA of MUC1/Y (NCBI accession: AAP97018) was chemically synthesized from GenScript, USA in pUC57 cloning vector (ampicillin selection) with blunt end EcoRV site. Codon optimization is based on Wobble hypothesis which states that multiple codons can translate into same amino acid residue. This is because of third base of codon. Different species have different codon for same amino acid. So on the bases of several study, codon was biased for bacterial expression system so that it will produce high amount of protein and increase the solubility of protein. Cloning sites EcoRI at N’ and HindIII at C’ was inserted by PCR reaction into MUC1/Y cDNA insert. PCR primers were designed using online software. MUC1/Y_forw was forward primer containing BamHI and EcoRI site along with methionine and remaining DNA sequence of MUC1/Y. The size of forward primer was 26 bases and the both end contains guanine and cysteine residues for better binding to template DNA. The Tm value was calculated by on line software which was 68.29 with 10 mM salt concentration and GC content was 65 %. MUC1/Y_Rev was reverse primer containing HindIII site along with carboxy terminal DNA sequence of MUC1/Y. The
size of this primer was 33 bases and the both end contains guanine residues for better binding to template DNA. The Tm value was calculated by on line software which was 64.28 with 10 mM salt concentration and GC content was 48 %. Both the primer was not formed palindromes.

After insertion of cloning sites into insert (MUC1/Y), cloning was done using EcoRI and HindIII sites in *E.coli* DH5α competent cells. For cloning T4 ligase enzyme was used and different molecular ration of vector and insert was used. Ligation samples were incubated for overnight at 16 °C. Ligated samples were transformed in DH5α competent cells which were prepared by chemical method and the transformation was done with heat shock method. Final construct of pYBL-MUC1/Y was characterized by EcoRI and HindIII enzyme sites. Two clones (1 & 2) of pYBL-MUC1/Y were found positive on analytical Agarose gel stained within ethidium bromide solution.

For expression of MUC1/Y protein pYBL-MUC1/Y DNA clone 1 was transformed into BL21 (DE3) cells and expression was done under the influence of T7 promoter with induction with 1 mM IPTG. BL21 (DE3) E. coli strain having additional T7 RNA polymerase gene which help for higher expression of desired protein. It support the La promoter system hence cell only be induced by IPTG concentration. IPTG concentration may varied for higher production of different protein. After expression and localization study, it was found that MUC1/Y protein was coming as insoluble protein i.e. inclusion bodies (IBs). The desired expression band of MUC1/Y was confirmed by SDS-PAGE and western blot analysis. For purification of MUC1/Y pilot scale culture of 1 liter was prepared with same expression protocol. IBs were prepared from pilot scale culture. Pellet (4 grams) from pilot scale culture was harvested and was suspended in lysis buffer containing 150 mM NaCl, 100 mM Tris, 5% glycerol, 0.1 mg per ml lysozyme (Sigma) pH-8.0 ± 0.2. After freeze-thaw treatment for 3 cycle using Liquid N2 and 37°C water bath, again suspension was incubated on ice for 45 minutes with intermittent mixing. Sample was sonicated for 3 cycles with 50 amplitudes, 30 seconds. Between two sanitation cycle, samples was incubated on ice for 1 minute. Sonicated sample was spun at 10000 rcf for 30 minutes at 2-8 degree Celsius. Supernatant fraction of previous step was discarded and pellet was dissolved in 100 ml buffer contains 20 mM Tris, 150 mM NaCl, 0.1% Triton X 100, pH 8. Dissolved pellet was spun at 10000 rcf for 30 minutes at 2-8 degree Celsius using Kubota centrifuge rotor. This step was repeated once. Triton X 100 is usually dissolved the membrane protein present in IB’s preparation. Pellet was
washed two times with 100 ml of different composition of buffer containing only 150 mM sodium chloride, 20 mM tris, pH 8.0±0.2 and dissolved pellet was again spun at 10000 g for at 2-8 °C for thirty minutes. Purification of MUC1/Y from inclusion bodies was started with Ni-IDA purification because expressed MUC1/Y has 7 X Histidine tag at N terminal and has affinity for Ni ion in Ni-IDA gel. In the first step nickel sulphate was passed through column so that Ni ion was bind to the resin and had fee arm as an affinity for histidine motif. His tagged protein was bind to it and after washing elution of protein was done with higher concentration imidazole which has higher binding than his tag so that protein was eluted. Nickel from the column was removed by EDTA which chelates the Ni-ion from resin. After Ni-IDA purification protein profile was checked by SDS-PAGE and western blot analysis using anti his antibody and found that protein was not pure upto the immunization level. Further purification was done with anion exchanger (DEAE).

Theoretical isoelectric point (pI) of recombinant MUC1/Y was 6.86, calculated by ProtParam online software. For further purification, elution fractions of MUC1/Y from Ni-IDA purification were pooled and dialyzed using 10 kDa cutoff dialysis bag in 8 M urea, 20 mM tris acetate, pH 5.0 ± 0.2, 5 % glycerol. The dialyzed sample was filtered by using 0.2 µm (Pall Corporation) filter using filter apparatus (Nalgene, Rochester, NY). Single 3 ml DEAE cellulose (GE Healthcare, India) column was prepared in 10 ml plastic syringe column. Column was washed with DM water for at least 20 CV. Column was equilibrated with 5 CV of buffer containing 8 M urea, 20 mM tris acetate, pH 5.0±0.2, 5 % glycerol. Sample was loaded on column using peristaltic pump with the flow rate 1 ml/ minutes throughout the run. Protein post load was collected as the protein sample was gone through the columns in fresh collection tube. Since protein was coming in post load hence it is called negative chromatography. At pH 5 protein occupied positive charge hence coming is post load whereas contamination bands was bind to the column.

Refolding of protein is required to get native structure of protein and in the presence of urea protein will be linear hence conformational epitope is not form. So for refolding collected flow through from DEAE column was serially dialyzed in 12 kDa cutoff dialysis bag (Sigma) against buffer ‘A’ contains 20 mM tris, pH 8.0 ± 0.2 for three hours. Again dialyzed the sample in buffer B containing 4 M urea, 150 mM NaCl, 5 % glycerol for 3 hours at room temperature. Third time sample was dialyzed in buffer C contained 1 M urea, 20 mM tris, pH 8.0 ± 0.2, 150 mM NaCl, 5 %
glycerol for three hour at room temperature. Final dialysis was done with buffer D, contains 150 mM NaCl, 20 mM tris, pH 8.0 ± 0.2, 50 % glycerol at 25-30°C for three hours and final sample was taken off from dialysis bag and stored at -20°C deep freezer. Hence by serial dialyzed urea was removed and protein was not precipitated. Final dialyzed material was filtered through 0.22 µ - PES sterile syringe filter (Pall Corporation) in sterile Nalgene bottle under aseptic conditions. Final sample was analyzed on SDS-PAGE. Protein estimation was done by Bradford method and found 1.3 mg/ml as final concentration. Before immunization, cross immunoelectrophoresis (CIE) was done to check for any contamination of E.coli protein using E.coli antibody (Cat# B0357) from DAKO, Denmark. Crossed immunoelectrophoresis (CIE) is also known as two-dimensional (2D) quantitative immunoelectrophoresis. In CIE procedure, the proteins samples are first separated during the 1D electrophoresis in an electric field. Then the proteins are electrophoresed into 2D direction of gel containing specific antibody. As electrophoresis proceeds at 2D direction, Immunoprecipitation will form and it will form bell like shape. Each bell shape will represent one protein molecule and the height of bell is directly proportional to the concentration of that protein in solution. After analysis, it was found that protein having no contamination in preparation.

Purified MUC1/Y antigen was immunized in mice (BALB/c female mice) with Freunds adjuvant followed by several booster doses and fusion of Splenocytes with Myeloma cells Sp2 IL6. For fusion, splenocytes was prepared by collection of fresh splenocytes a day prior to fusion from designated mice. For the removal of spleen aseptically, mice was sacrifice by CO₂ euthanization followed by soaking in 70 % ethanol for decontamination. Spleen was removed by opening the abdomen in biosafety cabinet and splenocytes were prepared. For myeloma cells confluent T-75 flasks were taken of SP2 IL6 cells for next day use. For preparation of SP2 IL6, cells were disintegrated from the surface of the flask using a bent pipette and confirmed under the microscope followed by washing several times with media. After fusion experimentation, final hybrid clone 9A842G6 was immunized intra-peritoneal and after generation of intra-peritoneal fluid (ascetic fluid), fluid was carefully withdrawn with 16-gauge needle attached to a 5ml syringe. Purification of monoclonal antibody of MUC1/Y was done by using Protein G column and final concentration of antibody was measured by A280 nm (ε= 1.395) and it was 2.1 mg per ml. The result was able to demonstrate that antibodies generated against
rMUC1/Y are mostly within the 1-10 nM kDa affinity range, sufficiently high for diagnostic purposes.

To check the potential of mAb of MUC1/Y, human breast carcinoma and fibroid adenoma (fixed by formalin and paraffin embedded) blocks of four micrometer thick sections were treated by Citra in steamer for 30 minutes. Sections were probed with rMUC1/Y mAb and stained. The imaged with the Olympus phase contrast inverted microscope, CKX 41SF. Strong signal was detected in positive control tissue i.e. clinically diagnosed breast carcinoma tissues, which was strongly distinguishable. Although a small level of expected background staining in negative control was observed (fibroid adenoma tissue, one of the most common benign breast lesions). This result suggested that recombinant rMUC1/Y antigen and rMUC1/Y antibody can be used as secondary biomarker along with cancer antigen 15-3 assay for the detection of malignant breast cancer.

As mentioned CA15-3 is secreted product of mucin 1 oncogene and most exploited promising biomarker for cancer arising at breast tissue. MUC1 protein contains 1255 amino acid with variable degree of glycosylation but the amino acid sequence of CA 15-3 is not yet reported. In the present scenario, CA15-3 is purified from ascites of cancer patients and cross reactivity with other antigen is always a concerned for diagnosis and collection of ascites of cancer patients is again a difficult task. The exact sequence of CA15-3 is not reported yet, hence responsible for batch to batch variation. CA15-3 from native biomedical fluid having contamination of several antigens includes CA19-9, CA125, CA 72-4, AFP, Ferritin, CEA and several viral marker includes HBsAg, Anti- HCV, Anti-HIV I&II etc.

To utilized the advantage of specificity of recombinant protein, 10 tandem repeats (MUC1RS) and 20 tandem repeats (MUC20TR) of MUC1 protein were expressed in mammalian cells to mimic CA15-3 native antigen using CHO-K1 and AD293 cells to ensure post translational modifications. Mammalian system have some features which make this system more favorable for recombinant protein expression include post-translational modifications and protein folding which is essentially required for biological activity of protein.

As compared to the bacterial expression system, mammalian expression system is best for glycosylated protein. It was reported that the therapeutic protein produces in this system already proved their importance in human health sector. These therapeutics are more cost effective and high pure as compared to native proteins. The stable cell lines
produced for therapeutics are more durable and efficiently produce the protein after several years with same biological activity. Mammalian expression system was applied for the development of several recombinant diagnostic markers, viral vaccines, prognostic markers, therapeutics etc.

Although mammalian expression system have several benefits over other expression system in terms of biological activity, refolding and post translation modification of protein but this system also have some limitation including expression of protein with high glycosylation as compared to baculovirus system and highly glycosylation is interfere with crystallization of protein. Although this problem can be overcome by using selective cell line and expression system. Some key modification is now available and well known by which this system would be more productive at every scale. Gene can be codon optimized as per cell lines so that it will favor the transcription and translation process hence high yield. By selection of strong promoter system and signal system will enhance the production and hence production cost will be low. Recombinant protein can be expressed in intra cellular but the yield would be very limited but different signal sequence is available by which protein enables to come out into the media so that with time of culture we can recover more protein and yield would be increased with the combination of strong promoter system and suitable cell line.

Although protein expressed in serum containing media is high and after optimization of protein purification we can get good yield but purification from serum containing media is difficult and contaminating protein will strongly bind to desired band. If affinity of protein because of any tag or specificity is available, this feature can be used for purification upto some extant. This problem can be overcome by optimizing cells into serum free media. There are variety of serum free medium are available commercially for CHO, HEK93 cells and for myeloma cells.

‘Gluc’ protein (Gaussia luciferase) sequence was incorporated along with gene of MUC1 variants for secretion of protein outside the cells. cDNA of 10 tandem repeats of MUC1 gene (MUC1RS) and cDNA of 20 tandem repeats (MUC20TR) was cloned in mammalian expression vector. cDNA of MUC1RS was synthesized from Genscript, USA, which was came with in pUC57 cloning vector. MUC1RS name was given to specific selected gene contains cDNA of 61-320 followed by AA 941-1020 (Uniprot accession: P15941) of MUC1 protein. Codon optimized cDNA sequence of
MUC1RS was cloned into pcDNA3.1Zeo (+) vector using NheI/XhoI sites to make pcDNA3.1 Zeo_MUC1RS construct.

pcDNA3.1Zeo (+) vector is CMV promoter based vector and expressed the protein intracellular. Gluc protein (Gaussia luciferase) sequence was used to secrete MUC1RS protein into the media. cDNA of Gluc protein was inserted into the pcDNA3.1Zeo+MUC1RS, just before the cDNA of MUC1RS by site directional mutagenesis (SDM). Final constructs pcDNA3.1Zeo+MUC1RS (for intra cellular expression) and pcDNA3.1 Zeo+MUC1RS_GALU (for extra cellular expression) were characterized by restriction digestion with NcoI restriction endonuclease.

cDNA of MUC20TR was also synthesized from Genscript, USA, which was came with in pUC57 cloning vector. MUC20TR name was given to specific selected gene contains cDNA of targeted amino acids 61-120AA+ 20 TR (20 x 20aa) followed by AA 941-1020 (Uniprot accession: P15941) of MUC1 protein. Codon optimized cDNA sequence of MUC20TR contains cDNA of Gluc signal sequence, was cloned into pcDNA3.1Zeo (+) vector (Invitrogen co.) using NheI/XhoI sites to make pcDNA3.1 Zeo_MUC20TR construct. Final clone of pcDNA3.1Zeo (+)_MUC20TR was characterized by restriction digestion with XhoI and NheI enzymes.

Protein was characterized by western blot using cancer antigen 15-3 antibody as well as established for the occurrence of epitopes comparable to the ones, as recognized by 115D8 and DF3 on Siemens CLIA based platform. In case of MUC1RS (MUC1 protein contains 10 tandem repeats only), the total yield of protein was quantified in international units which were 106 kU/ Liter of media by ELISA (Calbiotech) and 3.7 kilo unit per liter by Advia Centaur Siemens platform. The size of protein was observed by reducing PAGE at ~130 kDa which was not the characteristic of native CA15-3 protein.

In case of MUC20TR (MUC1 protein contains 20 tandem repeats only), it was found that MUC20TR was highly expressed by AD293 cells (MUC20TR-AD293 clone) with in serum free medium. Purified sample was analyzed by 7 % reducing PAGE and immuno blot by using specific mAb of CA15-3_DF3 conjugate antibody. Because CA15-3 is highly glycosylated in nature with molecular size > 300 kDa, it was not observed in SDS-PAGE. Purified MUC20TR was also showed the same profile on SDS-PAGE as same as CA15-3 i.e. the band of MUC20TR is not stained by Coomassie staining. It was reported that silver staining is also not working for
CA15-3 antigen. Molecular weight (> 300 kDa) of MUC20TR and native CA15-3 was determined by western blot utilizing specific monoclonal antibody.

Western blot analysis of purified MUC20TR has shown the same profile as CA15-3 antigen, when probed by mAb of CA15-3_DF3 conjugate antibody. Total yield of protein in international units was 675 kU per liter of media by ELISA (Calbiotech) and 511 KU/ Liter by Siemens platform (Advia Centaur Immunoassay System). So the purified protein MUC20TR is active as CA15-3 native antigen. Thus this study may aid in the development of more specific and accurate diagnostic tools for detection of breast cancer and other carcinoma conditions in addition to existing primary cancer markers and lead to increase in total specificity of diagnostic tests. This study also helpful to understand the glycosylation pattern of MUC1 oncoprotein in breast cancer tissue. The CA15-3 like antigen (MUC20TR containing 20 tandem repeats with degenerated tandem repeats) also provide the alternative source of native CA15-3 which is currently purified from ascetic fluid of breast cancer patients.
5.2 Recommendation

While doing this study, several points were noticed and on the bases of analysis and result, several recommendations have come as mentioned.

1. For antibody development, used high pure antigen which increase the chance of getting specific clones. Some contaminants at very low concentration have high immunogenicity hence generate non specific clones. CIE with anti *E.coli* antibody confirmed the absence of any *E.coli* contaminating protein in purified protein preparation.

2. MUC1/Y antibody from 9A842G6 clone was specifically detected the clinically diagnosed breast carcinoma tissues (positive control tissue), which was strongly differentiate with negative control; although a small level of expected background staining is present in negative control (fibroid adenoma tissue, one of the most common benign breast lesions). This feature of 9A842G6 mAb can be used to understand the structure-functional relation of MUC1 gene product and O glycosylation pattern in mammary carcinoma tissue.

3. It was found that the final yield of MUC20TR was increased in serum free adaptation and serum free adaptation provided ease of purification because serum in media may interfere with purification.

4. While preparation of “Control”, it was observed that MUC20TR was more stable in artificial matrix 1 (AM1) so that this protein within AM1 can be used for the development of CA15-3 ELISA kit and would be utilized for diagnosis of breast cancer.
5.3 Future scope

In this study, monoclonal antibody against recombinant MUC1/Y was developed and characterized. The origin of this antibody is mouse hence it has mouse heavy and light constant region. This antibody was characterized by several procedures include western blot, ELISA, Biacore analysis. This antibody has shown good reactivity to primary breast cancer tissue against control tissue which revealed that this monoclonal antibody would be very much used for diagnostic purpose of breast cancer. But this antibody also can be utilized for therapeutic purpose but required humanization of this antibody. Because of mouse origin, human body will produce immune response, if immunized.

Recombinant MUC20TR expressed in AD293 cells are mimicking the native CA15-3 antigen in terms of SDS-PAGE profile and antigenic activity by Siemens closed system. So that this purified antigen can be utilized to develop the diagnostic kit for quantitation of CA15-3 in patients of breast carcinoma.

Both MUC1RS and MUC20TR were secretory expressed into the media using Gluc’ protein (Gaussia luciferase) sequence at N terminal of protein and the yield reported was high and hence it is cost effective. Adoption of MUC20TR AD293 cells in serum free media provides highest purity which minimized the purification and downstream processing. This strategy also can be used for the secretory expression of other glycoprotein in mammalian cells.
5.4 Limitations of research work

In the present study, MUC1/Y protein was developed from *E.coli* strain and developed the specific monoclonal antibody (9A842G6) which specifically detects the clinically diagnosed breast cancer tissue. But MUC1/Y generally not reported in serum of breast cancer tissue so that generated antibody can not be utilized to detect the breast cancer using clinical serum sample.

MUC1/Y monoclonal antibody (9A842G6) would be very convenient for the diagnosis of mammary cancer in patient tissues. This antibody was developed from mouse source so that this antibody can not be used for therapeutic purpose. Humanization of this antibody may provide the strong tool for therapeutic potential.

For the development of immuno assay with control preparation of MUC20TR required a set of specific antibody as same as available in the marker.