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Review

Advances of MUC1 as a target for breast cancer immunotherapy

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Summary. MUC1 is a potential target in breast cancer immunotherapy as MUC1 is overexpressed in breast cancer, and is absent or expressed in low level in normal mammary gland. In addition, MUC1 is mostly aberrantly underglycosylated in cancer and the antigens on the cancer surface are different from normal cell. Therefore targeting MUC1 for cancer immunotherapy can exploit the difference between cancer and normal cells, and eliminating the cancerous cells while leaving the normal mammary cells unharmed. This review will focus on the recent advance of MUC1 breast cancer immunotherapy currently being investigated.

Key words: Mucin, MUC1, Breast cancer, Immunotherapy, Signaling transduction

Introduction

Breast cancer is the most frequent cancer in women. There is an estimated of 1.15 millions new cases worldwide that accounts for 23% of all cancers in women in 2002 (Parkin et al., 2005). The current method of breast cancer treatment often involves removal of the breast (part or whole), radiation therapy, chemotherapy and hormonal therapies. Many patients developed minimal residual diseases that become resistance to further chemotherapy and eventually lead to relapse and tumor progression. Therefore, there is a great demand of developing better treatment for breast cancer patients.

Currently, cancer vaccine has become a potential therapy in treating the minimal residue diseases and preventing cancer occurrence. There is a number of tumor oncoproteins currently under investigation as an appropriate target; however most of them are still in experimental stage and a few of them in the clinical trials. Mucin 1 (MUC1), a member of mucin family, is one of the tumor oncoproteins that has demonstrated to be a potential target and currently in the clinical trials.

Mucins are expressed by epithelial cells of lung, trachea, stomach, intestinal tract and secretory surface of specialized organs such as liver, kidney. Their environments are subjected to change in pH, ionic concentration, hydration and oxygenation. In general, mucins lubricate the cell surface, protect mucus membrane from harsh condition by maintaining the homeostasis and promoting cell survival. However, recently more evidences has shown that mucins, especially MUC1, play an important role in signal transduction. In responses to external stimuli, mucins might act as cell-surface receptors and sensors, and conduct signals that lead to coordinated cellular responses including proliferation, differentiation, apoptosis, metastasis and secretion of specialized cellular products (Hollingsworth and Swanson, 2004).

Mucins have a high content of O-linked oligosaccharides (50-90% of their molecular mass) and rich in serine, threonine and proline. The majority of mucins contain extracellular domain, a region with variable number of tandem repeats (VNTR), transmembrane and cytoplasmic region. The tandem repeats provide scaffold for building the oligosaccharide structure. Mucin family can be classified into membrane, gel-forming and small soluble mucin. The membrane mucins, including MUC1, MUC3 (Williams et al., 1999), MUC4 (Porchet et al., 1991), MUC12 (Williams et al., 1999), MUC13 (Williams et al., 2001), MUC15 (Pallesen et al., 2002), MUC16 (Yin and Lloyd, 2001), MUC17 (Gum et al., 2002), MUC18 (Lehmann et al., 1989) and MUC20 (Higuchi et al., 2004) are found as both membrane and soluble forms. The membrane mucins provide a barrier to the harsh environments. The gel-forming mucins, such as MUC2 (Gum et al., 1992), MUC5AC (Escande et al., 2001), MUC5B (Desseyn et al., 1997), MUC6 (Toribara et al., 1997), MUC8 (Shankar et al., 1994), MUC9 (Lapensee et al., 1997) and MUC19 (Chen et al., 2004) are produced and

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secreted from mucous cells of glandular tissue and goblet cells of luminal epithelia. MUC7 is a small soluble mucins found in saliva (Bobek et al., 1993). At present, MUC11 (Williams et al., 1999) is an unclassified mucin that is only identified by its short cDNA sequence of tandem repeat.

MUC1 is overexpressed in breast cancer patients, and also is upregulated on majority of adenocarcinomas including lung, colon, pancreas, stomach, prostate and ovary (Ho et al., 1993). Overexpression of MUC1 in breast cancer patients are associated with metastatic and poor survival (McGuckin et al., 1995). MUC1 is weakly expressed in normal epithelial cells; conversely MUC1 is overexpressed in breast cancer. In immunohistochemical staining, normal epithelial cells and benign breast cancer cells showed staining of MUC1 on the apical surface; however in metastatic breast cancer, MUC1 had both intracellular and cell surface staining. Therefore, the presence of MUC1 on only the apical surface of breast cancer is an indication of good prognosis (Rahn et al., 2001). However, MUC1 is not only a useful maker for the diagnosis and prognosis, but also showed to be a potential target for immunotherapy. Hence, this review will focus on the recent advances of MUC1 as a target for breast cancer immunotherapy, and also relevant structure and its function of MUC1.

Structure and biosynthesis of MUC1

MUC1 has a molecular weight of approximately 500kDa to more than 1000kDa (Baeckstrom et al., 1991), extends 200 to 500nm beyond the cell membrane above the plasma membrane (Bramwell et al., 1986). MUC1 is a large transmembrane mucin containing an extracellular region, a transmembrane region and a cytoplasmic tail. The N terminus extracellular region has 104 amino acids and 40 to 80 repeats (up to 125 repeats) of the same 20 amino acid sequence PDTRPAPGSTAPPAHGVTSA (VNTR) (Gendler et al., 1990). The C terminus consists of 228 amino acids including the extracellular motif, a transmembrane region of 28 amino acids and a phosphorylated cytoplasmic tail of 69 amino acids. There is a similar structure between murine and human MUC1 with significant protein homology: 59-62% in extracellular region, 34% in the repeat region and 87% in both transmembrane and cytoplasmic tail (Gendler and Spicer, 1995). As a result of a high percentage of MUC1 homologous in mouse and human, it is possible with caution to extrapolate the function of MUC1 in mice and predict its function in human. Therefore mouse is a reasonable model for investigating the function of MUC1 in pre-clinical setting before commencement of human clinical trial.

MUC1 biosynthesis involves many distinct steps. Full-length MUC1 is expressed as a type 1 transmembrane heterodimer following synthesis as a single polypeptide chain. Then several N-glycans attaches to the transmembrane region of the single polypeptide chain in the endoplasmic reticulum. MUC1 is proteolytic cleaved at FRPG/SVW site (located 65 amino acid upstream of the transmembrane domain) into two different size fragments while it is still in the endoplasmic reticulum (Parry et al., 2001). The smaller transmembrane fragment anchors to the larger fragment by non-covalent bond. After cleavage, MUC1 precursors move into Golgi where N-glycans become more intricate, and O-glycosylation begins on the VNTR region. The molecular weight of MUC1 increased considerably within 30 minutes of synthesis. Before leaving the Golgi as a premature form, MUC1 is partially sialylated on its O-linked oligosaccharides. The full mature form of MUC1 is generated by addition of sialic acid residues to the premature form during many rounds of internalization and recycling (Hilkens and Buijs, 1988). MUC1 is completely sialylated on the surface of normal cells, however MUC1 contains both completely and incompletely sialylated molecules on tumor cells. This phenomenon in tumor cells may be due to greater abundance of MUC1 and/or less efficient of sialylation process (Litvinov and Hilkens, 1993).

The larger MUC1 fragment is consisted of VNTR and rich in serine, threonine and proline. In addition, the smaller fragment (~20kDa) of MUC1 consists of a short extracellular region, a transmembrane region and a short intracellular tail. Extracellular region is N-linked glycosylated, and the transmembrane region contains cysteines that may assist in fatty acid acetylation to help anchor MUC1 on the cell membrane. Moreover, cytoplasmic tail has potential sites for phosphorylation and intracellular protein binding, and therefore may play a role in signal transduction (Hilkens and Buijs, 1988). A study utilized the nuclear magnetic resonance to demonstrate that the MUC1 structure became more ordered when the number of repeats increased from one to three (Fontenot et al., 1993). A further study showed that APDTR sequences within each tandem repeats are protruding knob-like structures connected by extended spacers on the MUC1 backbone (Fontenot et al., 1995).

Glycosylation occurs in the tandem repeat of MUC1. The tandem repeat provides a scaffold for building the oligosaccharide structures (O-glycans). Each tandem repeat has five potential sites for O-linked glycosylation. The actual glycosylated sites *in vivo* depend on the expression of particular GalNAc transferases by the mammary gland and the location of the enzymes within the Golgi apparatus (Muller et al., 1997).

In normal lactating mammary gland, 50% of the sites in the tandem repeat are glycosylated (Muller et al., 1997). Glycosylation involves the addition of galactose (Gal) to N-acetylglucoasmine (GalNAc) to form the core 1 structure (Galß1,3GalNAc-O-Ser/Thr). Then core 1 is acted as a substrate for the formation of branched and complex core 2 glycans (Gal,1-3[GalNAcß1-6]GalNAcα1-O-Ser/Thr) in the presence of ß1,6GlcNAc transferase (C2GnT) enzyme. Further addition of GalNAc to the existence GalNAc leads to the formation of polylactosamine side chain. This side chain continues

to extend until terminated by sialic acid or fucose (Hanisch et al., 1989, 1990).

Aberrant glycosylation of MUC1 in cancer

MUC1 in breast cancer is core 1 O-glycan instead of core 2. The level of α2,3 sialyltransferase, which generates the sialylates core 1, was increased in breast cancer patients, and was correlated with poor prognosis (Whitehouse et al., 1997; Burchell et al., 1999). A study shown that a 2,3 sialyltransferase was elevated several fold, but ß1,6 GlcNAc transferase (generate core 2) is absented or reduced by 50% in breast cancer cell lines (BT20, MCF7 and T47D) (Brockhausen et al., 1995). Therefore α2,3 sialyltransferase may compete with ß1,6 GlcNAc transferase to produce core 1 based structures found on cancer cells.

Soluble MUC1 is detectable in the supernatant of the cancer cell lines and in the sera of cancer patients. The possible mechanism involved in the production of soluble MUC1 including proteolytic cleavage, simple dissociation and cleavage by external proteases. Tumor necrosis factor α converting enzyme (TACE) may be a possible protease responsible for this cleavage (Thathiah et al., 2003). The external proteases are not likely to be involved in cleavage, as the addition of proteolytic inhibitors has no effect on the amount of soluble MUC1 detected (Julian and Carson, 2002). In addition, simple dissociation is not possible because MUC1 remains a stable heterodimer during repeated recycling for further glycosylation and sialylation (Litvinov and Hilkens, 1993). Moreover, even a mutated form of MUC1 lacking the cleavage site can still be released from the cell (Ligtenberg et al., 1992). Hence, only proteolytic cleavage is a feasible mechanism in releasing soluble MUC1 into the supernatant and sera.

In cancer cell, aberration of glycosylation leads to revelation of immunodominat peptide epitopes in tandem repeats, which are often hidden by glycosylation in normal tissues. Studies shown that MUC1 has shorter and less branches of O-linked oligosaccharides in breast cancer, resulting in unmasking of the peptide core and allowing antibodies (such as SM-3) to bind (Burchell et al., 1987; Girling et al., 1989). In addition, an immunohistochemical study demonstrates that epitopes recognized by SM-3 was absent or weakly expressed on normal tissues comparing to breast cancer tissues (Burchell et al., 1987). Therefore antibody against the MUC1 peptide core may only bind to MUC1 of the cancer and not bind to MUC1 of the normal cell, even though their sequences of the core protein are the same.

Moreover, the aberration of glycosylation in cancer also causes the formation of shortened carbohydrate side chains. Exposures of these antigens are normally masked in normal tissue by chain elongation. Tn antigen (GalNAcαl-O-Ser/Thr), a short carbohydrate structure, is O-linked to serine or threonine on the MUC1 backbone, and can be extended with a galactose residue to form the TF antigen (Galß1-3GalNAc·l-O-Ser/Thr) or substitutes with sialic acid to form sialyl-Tn (Neu5Acα2-6GalNAcα1-O-Ser/Thr).

MUC1 in cancer has shorter O-glycan chain and are more sialylated O-glycan (Lloyd et al., 1996). Sialyl-Tn antigen on the O-glycan has been shown to be overexpressed in breast cancer patients but not on normal mucosa (Thor et al., 1986), and is related to the resistance to chemotherapy (Miles et al., 1994). ST6GalNAc-1 sialyltransferase is responsible for the synthesis of sialyl-Tn in human cancer by catalysing the transfer of sialic acid to GalNAc (Marcos et al., 2004; Sewell et al., 2006), and its presence led to a reduction of the O-glycosylation sites occupancy in MUC1 from an average of 4.3 to 3.8 per tandem repeat (Sewell et al., 2006). In addition, the presence of sialic acid on the core GalNAcs may prevent the action of polypeptide-GalNAc transferases and therefore fewer GalNAc were added to the protein core. This led to a reduction of glycosylated sites leading to exposure of antigenic epitopes on the protein core and thus changed the expression of MUC1 glycoform antigens (Sewell et al., 2006). Moreover, inhibition of sialylation (Kijima-Suda et al., 1986) and enhancement of mucin glycosylation (Bresalier et al., 1991) reduces the metastatic potential of cancer cells in experimental mouse models.

MUC1 is a signaling molecule

The cytoplasmic tail of MUC1 is involved in signal transduction. There are seven tyrosines (Wreschner et al., 1990) available for phosphorylation in the cytoplasmic tail (Fig. 1). The MUC1 cytoplasmic domain can be phosphorylated on i) Ser^{44} by glycogen synthase kinase 3ß binding to $TDRS^{44}P$ sequence (Li et al., 1998), ii) Tyr^{46} by Src-family kinases (Li et al., 2001a,b) or epidermal growth factor receptor (EGRF) (Li et al., 2001a,b) binding to Y46EKV sequence, iii) Thr^{41} by protein kinase Cδ binding to $T^{41}DR$ (Ren et al., 2002) and iv) Tyr60 by Grb2 binding to Y60TNP (Pandey et al., 1995). In addition, the phosphorylation at Tyr^{46} (Li et al., 2001a,b) and Thr^{41} (Ren et al., 2002) enhances the binding of ß-catenin to SAGNGGSSLS

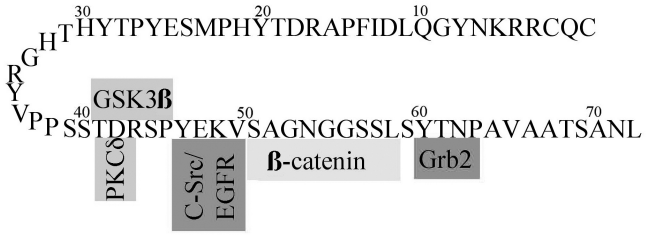

Fig. 1. MUC1 cytoplasmic tail signaling. MUC1 cytoplasmic tail can be phosphorylated on i) Ser^{44} by GSK3ß binding to TDRSP, ii) Tyr^{46} by c-Src or EGRF binding to YEKV, iii) Thr^{41} by PKCδ binding to TDR and iv) Tyr^{60} by Grb2 binding to YTNP. In addition, the phosphorylation at Tyr^{46} and Thr^{41} enhances the binding of ß-catenin to SAGNGGSSLS of MUC1 cytoplasmic region.

sequence of MUC1 cytoplasmic region, while phosphorylation of Ser^{44} (Li et al., 1998) decreases the binding interaction.

The phosphorylated YTNP sequence of MUC1 cytoplasmic domain provides a binding site for SH2 domain of the Grb2 (adaptor protein). This MUC1-Grb2 complex then interacts with the Sos (guanine nucleotide exchange protein) through its SH3 domain of Grb2, and associates with Ras at the plasma membrane of cancer cells (Pandey et al., 1995). Consequently, mitogen-activated protein kinase (MAPK) pathway is activated through Ras-MEK-ERK2 pathway. This pathway can be blocked by a dominant negative Ras mutant or a (MAPK) kinase (MEK) inhibitor (Meerzaman et al., 2001).

The cytoplasmic MUC1 has been shown to associate with c-Src tyrosine kinase and activated ERK1/2 *in vivo* (Schroeder et al., 2001) and indirectly activated ERK2 via Ras and MEK *in vitro* (Meerzaman et al., 2000). In addition, cytoplasmic domain of MUC1 bound directly to Catenin p120 and induced the nuclear localization of p120 (Heukamp et al., 2001). Moreover, cytoplasmic tail of MUC1 associated with all four erbB receptors by forming heterodimers (Schroeder et al., 2001).

Anti-adhesion of MUC1

Overexpression of MUC1 on cancer cells was shown to have a reduction in cell-cell (Kondo et al., 1998) and cell-matrix adhesion (Wesseling et al., 1995), and this was due to steric hindrance by the sialylated O-glycan on the mucin tandem repeat domain (Wesseling et al., 1996). In addition, the sialic acid on the O-glycan is strongly negative charge (Ligtenberg et al., 1992), and the abundance of them on MUC1 may contribute to the anti-adhesion effect by charge repulsion. Moreover, the high density of MUC1 may disrupt the interaction of cell surface proteins with the surrounding macromolecules on adjacent cell membranes (Ligtenberg et al., 1992). Besides inhibiting the cell-matrix adhesion by steric effect, MUC1 cytoplasmic domain binds to ß-catenin and prevents the interaction of ß-catenin with E-cadherin in the formation of cellular adherens junctions (Wesseling et al., 1995). The interaction of MUC1 cytoplasmic tail and glycogen synthase kinase 3 beta (GSK3ß) leading to decrease MUC1 binding to ß-catenin. This can be reversed if MUC1 is phosphorylated by c-Src SH2 domain, resulting in reduction of MUC1 and GSK3ß interaction and enhancement of MUC1 binding to ß-catenin (Li et al., 2001a,b). However, Huang's finding shown that overexpression of MUC1 in human breast cancer and other carcinomas, leading to decrease in ß-catenin phosphorylation by GSK3ß, resulting in stabilization of ß-catenin by preventing ubiquitination and degradation, and thereby increasing the level of ß-catenin (Huang et al., 2005). There are some discrepancies between Huang's and Li's finding about the function of GSK3, and MUC1. Nevertheless their findings directed to similar conclusion about the degree of anti-adhesion, which is determined by the amount of MUC1 and extent of phosphorylation of MUC1, as enhanced level of MUC1 phosphorylation will lead to increase MUC1 binding to ß-catenin and thereby inhibiting cell-matrix adhesion. Thus, tumor may use the anti-adhesion properties to escape from the primary site, and migrate to the secondary sites via lymphatic and blood circulation.

Furthermore the tandem repeat domain of MUC1 can function as a ligand for the ICAM-1 (intracellular adhesion molecule) (Hayashi et al., 2001). The adhesion properties of cancer can be inhibited by antibody against the ICAM-1 (Regimbald et al., 1996). Besides ICAM-1, VNTR of MUC1 can bind to E-selectin (an adhesion molecule) and mediate adhesion events (Zhang et al., 1996). Hence, MUC1 may play a potential role in the metastasis of epithelial breast cancer.

Invasion and metastasis

Interestingly, in a transfection study the upregulation of MUC1 with deletion of either the cytoplasmic tail or the tandem repeat, leading to increase tendency of S2-013 (human pancreatic tumor cells) to display invasive and metastastic phenotype compared to full length MUC1. Analysis by DNA microarray found that many genes were differentially expressed by cells overexpressing the full-length MUC1 compared with cells overexpressing MUC1 with deletion of tandem repeat or cytoplasmic tail (Kohlgraf et al., 2003). Furthermore an increase in expression of MUC1/Y isoforms (Zrihan-Licht et al., 1994) in mouse DA3 mammary epithelial cell, has shown to enhance the tumorigenic potential (Baruch et al., 1997). In addition, flow cytometry analyses shown that MUC1/Y was expressed on the cell surface of the malignant epithelial cell obtained from the effusions of the breast cancer patients (Hartman et al., 1999). This is further supported by the northern blot that demonstrated a significant level of MUC1/Y in primary breast cancer tissue sample (Zrihan-Licht et al., 1994). Hence, MUC1/Y is a possible malignant marker for the breast cancer.

Furthermore, an *in vitro* study shown that the increased expression level of ICAM-1 would lead to augmented migration of MCF7 cells (MUC1-bearing breast cancer cells). Inhibition of ICAM-1 binding to MUC1 by antibodies caused a reduction in the migration of the MCF7 cells. Transendothelial migration was greatly enhanced when MCF7 cells were co-cultured with human umbilical vein endothelial cell and fibroblasts in the presence of cytokines (TNFα and IL-ß1,) (Rahn et al., 2005). Therefore microenvironment surrounding the tumor is important in cancer metastasis.

MUC1 and apoptotic response

Since mucins act as a defensive physical barrier to harsh environment, MUC1 in carcinoma cells can exploit the normal physiological mechanisms of mucins, which protect the normal epithelial cells against apoptosis during adverse condition. This is supported by

a study demonstrated that overexpression of MUC1 in cancer cells can protect the cell from the oxidative stress-induced apoptosis (Yin et al., 2003). Therefore, the use of genotoxic anticancer drugs alone may not be efficient in killing the cancer with upregulation of MUC1, as cancer exploits the mucins response to external stress for evasion of the immune system. Hence MUC1 should be targeted and the combination of genotoxic anticancer drug with MUC1 immunotherapy may enhance the killing of cancer cells.

MUC1 induced immune response

The immune system badly recognizes tumor cells due to (i) tolerance to self-antigen, (ii) evasion of the immune recognition and (iii) suppression of the immune system by a variety of mechanism such as down-regulating MHC class I (Zheng et al., 1999), inducing immunosuppressive molecules (eg. Prostaglandin E) (Blobe et al., 2000), and secreting inhibitory cytokines (eg. TGFß) (Yang et al., 2003). MUC1 reduces the efficiency of antitumor immune response by suppressing the T cell function. Affinity purified MUC1 from cancer patients and synthetic tandem repeats peptides can suppress the proliferation response of human T-cell. This inhibition response can be reversed by exogenouse IL-2 or anti-CD28 monoclonal antibody (Agrawal et al., 1998). Furthermore, MUC1 can interfere with NK cell-mediated lysis. The purified secreted form of MUC1 could suppress the cancer cell lysis by NK cells in a dose dependent way (Zhang et al., 1997).

Early breast cancer patients, with the presence of naturally occurring anti-MUC1 antibodies in serum, have decreased the likelihood of breast cancer metastasis and have a better survival rate. Anti-MUC1 antibodies produced by the immune system of the patients may control the invasion of tumor by destroying the circulating MUC1 expressing tumor cells (von Mensdorff-Pouilly et al., 2000) by mediating complement-dependent cytotoxicity and/or antibody-dependent cell-mediated cytotoxicity, and thereby restrain the tumor to its primary site.

Multiparity can protect the women against breast cancer in future, as T cells can be primed during pregnancy. T cell has been shown to proliferate from biparous women but not nulliparous women in response to MUC1 core peptides (Agrawal et al., 1995). This may be because there are many changes anatomically and physiologically in uterus and breast during pregnancy and lactation, which cause an increase in MUC1 production that lead to priming of immune system. Serum levels of MUC1 and free anti-MUC1 IgG and IgM antibodies were evaluated in pregnant women compared with nonpregnant women. It was also demonstrated that MUC1 was dramatically increased during second trimester of pregnancy to puerperium; while the level of free anti-MUC1 IgG and IgM antibodies decreased and reached the lowest point in puerperium and gradually increased after delivery. In addition, lactating women have a higher titre of anti-MUC1 IgG compared with non-lactating women (Croce et al., 2001a,b). An epidemiological study (Kalache et al., 1993) suggested that there was a correlation between pregnancy and reduce risk of breast cancer. The incidence of breast cancer will continue to rise as the fertility rate decline. Furthermore, pregnancy can prevent the recurrence of breast cancer where MUC1 specific immune response may be triggered, as demonstrated by a pregnant long-term breast cancer survivor (Jerome et al., 1997), who became pregnant after five years of removing her breast cancer and developed fulminant lymphocytic mastitis in her breast. The lactating breast tissue expressed the same MUC1 tumor specific epitopes as the original cancer. She had a high titre of circulating anti-MUC1 IgM and IgG antibodies and a high frequency of MUC1 specific cytotoxic T lymphocytes in the peripheral blood. She remained free of tumor for five additional years of follow-up. The original breast tumor may have primed the patient's immune response against MUC1 epitopes and the re-expression of MUC1 epitopes may evoked a secondary immune response on the lactating breast (Jerome et al., 1997). It is possible that her anti-MUC1 immunity may have protected her from recurrent breast cancer.

Expression of MUC1 and T cells function

MUC1 is expressed on the surface of activated T cells but not resting T cells, as shown by immunohistochemistry (Delsol et al., 1984), flow cytometry (Agrawal et al., 1998; Correa et al., 2003), confocal microscopy (Correa et al., 2003) and RT-PCR (Agrawal et al., 1998; Correa et al., 2003). The function of MUC1 on activated T cells has not been fully elucidated, and it has been suggested that MUC1 may be involved in regulation of immunity (Agrawal et al., 1998) and modulation of cell-cell interaction (Correa et al., 2003). The activated T cells have an increased expression of ß1,6GlcNAc-transferase that lead to the synthesis of long, highly branched O-glycans on MUC1 backbone (Piller et al., 1988), and therefore do not have the same epitope as found on tumor cell. A study had demonstrated that B27.29, a monoclonal antibody against the MUC1 was raised against a mucin fraction from the ascites fluid of a cancer patient, inhibited the proliferation of activated T cells *in vitro*. The proliferation was restored by the addition of either IL2 or costimulatory anti-CD28 antibody (Agrawal and Longenecker, 2005). The function of MUC1 in activated T cells is unclear, further studies are required to elucidate their involvement in breast cancer.

MUC1 is a useful serum marker for detection of breast cancer

MUC1 is a serum marker useful for detecting recurrence or prognosis in breast cancer patients (Duffy et al., 2000) as serum level increased in breast cancer (Gourevitch et al., 1995). Studies shown that MUC1

serum level was increased in breast cancer patients with distant metastasis while it was not significantly elevated in benign breast cancer patients (Hayes et al., 1985; Kerin et al., 1989). This phenomenon may be due to circulating MUC1 expressing tumor cells that broke off from its primary site and traveling to its distant sites. In addition, MUC1 can provide indication of recurrence even before diagnosed by conventional clinical or radiological diagnosis (such as chest x-ray, liver ultrasonography and bone scan) in 41-54% of treated patients (Molina et al., 1995; Tomlinson et al., 1995). Therefore enabling earlier diagnosis and treatment decisions, and thereby resulting in cost saving of at least 50% when compared to the cost of diagnosis by expensive imaging techniques (Robertson et al., 1995). Hence MUC1 is useful as a marker to monitor patients for early detection of recurrence and metastasis following treatment of primary breast cancer.

CA15.3 (MUC1 mucin glycoproteins) is used only for the monitoring patients in advanced disease as it lacks sensitivity for early-stage disease. The concentrations of CA15.3 are elevated in ~10% of patients in stage I, 20% in stage II, 40% in stage III and 75% in stage IV. A 5-10 fold increased in CA15.3 indicated the presence of metastatic disease (Zhang et al., 1996). However, increased CA15.3 can be found in a small percentage of healthy people and in patients with benign diseases, such as chronic active hepatitis, liver cirrhosis, sarcoidosis (Duffy, 2006). Hence CA15.3 may not be suitable for early diagnosis and early prognosis of breast cancer.

The use of other serum markers with MUC1 greatly enhances the sensitivity in detection of patients with distant metastasis. CA15.3 and CEA (carcinoembryonic antigen) level are increased in 50-70% and 40-50% in advanced breast cancer patients respectively (Molina et al., 1995, 1996; Jager et al., 2000). The combination of CA15.3 and CEA serum marker allow early detection of metastasis in 60-80% patients (van Dalen, 1992; Soletormos et al., 1996). The detection sensitivity is likely to increase in the advanced stage of cancer patients by using a mixture of several serum markers.

In addition, MUC1 can be used as an immunohistochemistry marker in breast cancer diagnosis and prognosis. Immunohisotochemistry is used for the pathological diagnosis of breast cancers, such as differentiate between benign and metastatic adenocarcinomas. Since MUC1 is overexpressed in breast cancer and absent or weakly expressed in the apical surface of healthy mammary gland, anti-MUC1 antibodies are valuable immunohistochemical markers in diagnosis of breast cancer. There are a number of anti-MUC1 antibodies against different region of MUC1, such as VNTR and cytoplasmic tail. Studies demonstrated that anti-MUC1 cytoplasmic tail monoclonal antibodies (anti-MUC1 CT) are better than antibodies against the MUC1 VNTR (anti-MUC1 VNTR) for diagnosis of metastatic adenocarcinomas. CT33 and CT2 (anti-MUC1 CT) have higher percentage of positively stained in malignant carcinoma, 90% and 93% respectively, compared to C595 (anti-MUC1 VNTR), 73.5% (Croce et al., 2003, 2006). This observable fact may be due to MUC1 VNTR cleaved from the cell, and released into the serum. In addition, the anti-MUC1 VNTR may bind to multiple sites of VNTR within the same MUC1 molecule, and thereby amplifying the staining; whereas each anti-MUC1 CT only bind to one MUC1 molecule, and therefore giving a quatitative staining pattern. Moreover, MUC1 expression may have a prognostic value in predicting the patients' outcome, as shorter survival time is related to aberrantly located MUC1 in the tumor cell cytoplasm and nonapical membrane (Rakha et al., 2005).

Additionally, MUC1 antibodies may be able to differentiate different type of breast cancer by immunohistochemistry. A study has shown that MUC1 antibodies can differentiate between invasive micropapillary carcinoma (IMPC) and invasive ductal carcinoma (IDC) of the breast. IMPC (a subtype of IDC) is associated with lymphatic invasion and lymph node metastasis and poorer prognosis. IMPC showed a reversed apical membrane pattern of MUC1 expression in neoplastic cell cluster; whereas MUC1 expression in pseudo-IMPC was present in the whole cytoplasmic membrane and/or cytoplasm (Li et al., 2006). Hence MUC1 antibodies can greatly assist in the diagnosis of breast cancer and may be used in the prognosis of the patients' outcome.

MUC1 is a target in cancer immunotherapy

MUC1 is a potential target for immunotherapy, as it is aberrantly overexpressed in tumor and has distinct antigens due to truncation of the oligosaccharide side chain. There are two forms of immunotherapy: passive and active immunotherapy.

Passive immunotherapy

Passive immunotherapy involving the use of monoclonal antibodies alone, or monoclonal antibodies conjugates with radioisotopes or cytotoxic drug to treat cancer. The anti-MUC1 monoclonal antibody induces antitumor response by antibody-dependent cellular cytotoxicity and complement dependent cytotoxicity, or interfering with receptor-mediated signaling adhesion and metastasis. Since early 1980s, monoclonal antibodies against MUC1 have been developed and most of them have been used as carriers of radioisotopes, as it is more effective if conjugated with radioisotopes. A clinical trial had shown that antibody alone can inhibit metastasis in colorectal cancer (Riethmuller et al., 1994), but not as efficient than the radioisotopes conjugated monoclonal antibodies. 111In (indium-111) and 90Y (yttrium-90)-labeled BrE-3 against MUC1 has been used in breast cancer clinical trial. Unfortunately, the patients in this trial produced human anti-mouse antibody (HAMA) response to these antibodies (Kramer et al., 1994), and led to the rapid clearance of these antibodies and prevented further repeat dosing. Therefore

humanized monoclonal antibody (^{111}In and ^{90}Y-labelled BrE-3) was used in 11 patients to avoid the HAMA response, where there were four clinical improvement, three stable, two partial response, one too early to evaluate and one progressive disease (Cagnoni et al., 1998, 1999).

R-1549 (formerly known as pemtumomab), a murine monoclonal antibody radiolabeled with 90Y, is under development by Antisoma and Roche. The antibody was raised against the epithelium-specific components of the human milk fat globule (HMFG) membrane (Taylor-Papadimitriou et al., 1981) and specifically recognizes MUC1. It had showed promising results in improving the long-term survival rate in ovarian and gastric cancer patients in phase I/II clinical trial (Xing, 2003). Therefore, this antibody may have the potential to treat other epithelial carcinomas, such as breast cancer, as MUC1 is also aberrantly upregulated in breast cancer. Unfortunately, R-1549 has failed to meet the primary endpoint in the Phase III SMART trial in ovarian cancer (Verheijen et al., 2006). The antibodies may bind to soluble MUC1 shed from cancer, and thereby limiting the amount of unbound antibodies reaching the tumor (Peterson et al., 1995).

In addition, the size of the initial tumor may also play a role in the partial response. IgG antibody has been estimated to take 2 days to penetrate 1mm of the tumor and require 7-8 months to penetrate 1 cm of the tumor, due to disordered tumor vasculature and increased hydrostatic pressure within the tumor (Jain and Baxter, 1988). Therefore the bigger the size of the tumor, the harder and longer it took for the antibody to penetrate. Moreover, radioisotope labeled antibody against MUC1 may only kill the MUC1-bearing cancer and the immediate surrounding cancer. Unfortunately, since tumor contains a heterogeneous of antigens (Jain and Baxter, 1988) due to mutation, this MUC1 antibody may not eliminate some cancer cells that do not have MUC1 antigen on their surface or close to the MUC1-bearing cancer cell. In future, the radioisotope labeled multiple antibodies against more than one cancer antigen should be used, as cancer not killed by one antibody may be eliminated by the other.

MUC1 capping induced by anti-KL6 MUC1 monoclonal antibody was able to restore cell-cell interactions, and thereby inhibiting the tumor proliferation by means of increasing the accessibility of target molecules to effector cells. Capping of MUC1 may expose target epitopes on the tumor cell surface that are normally masked by the bulky and abundant MUC1, and increased the cytotoxicity of lymphokine-activated killer cells. In addition, the exposure of E-cadherin may re-establish the interaction of cell-cell adhesion (Doi et al., 2006). Consequently, other anti-MUC1 monoclonal antibodies may have similar function in preventing the proliferation of MUC1 bearing tumor cells.

Active immunotherapy

Active immunotherapy utilizes the patient's own immune system to eradicate the tumor cells. It has many theoretical advantages over other traditional therapies, such as low toxicity, specificity, and continued antitumor effect attributable to immunologic memory. There are currently many active immunotherapies under investigation, including carbohydrate vaccines (George et al., 2001), peptide vaccines (Xing et al., 1995; Goydos et al., 1996), fusion proteins (Karanikas et al., 1997) (Acres et al., 2000) and dendritic cells-based immunizatioms (Gong et al., 1998; Koido et al., 2000; Loveland et al., 2006).

Peptide vaccine

Peptide vaccines have the advantage of readily available, but the precise epitope recognized by T or B cells must be identified. Many of the tumor-associated antigens (TAA) peptide used in the peptide vaccine are presented in association with MHC class I molecules, and recognized by tumor-specific $CD8^+$ cytotoxic T cells, thereby elicited cellular response. Whereas only a small number of TAA epitopes are discovered that can present in association with MHC class II molecules and are recognized by $CD4^+$ helper T cells. $CD8^+$ T cells recognize 8-10mer peptides bound to MHC class I molecules on antigen presenting cells (APCs) (Zinkernagel and Doherty, 1979); while $CD4^+$ T cells recongnize 12-20 mer peptides bound to MHC class II molecules on APCs.

Our laboratory performed the first phase I clinical trial using synthetic MUC1 peptides in 13 breast cancer patients. The patients were immunized with diphtheria toxoid conjugated MUC1 VNTR peptide, which induced antibody response but showed no anti-tumor effect, nevertheless was appeared to be safe (Xing et al., 1995). This pioneered MUC1 peptide vaccine lead to further development of other MUC1 vaccines and clinical trial of MUC1 in cancer patients. The MUC1 peptide vaccine containing five MUC1 tandem repeats, which was linked to both GST fusion protein and oxidized mannan, was shown to have high cellular immune response in mice. The oxidized mannan MUC1-GST fusion proteins stimulated antigen presentation in the MHC class I pathway and cross-presented to cytotoxic T lymphocytes, and thereby elicited cellular immune response. Whereas reduced mannan MUC1-GST fusion proteins encouraged antigen presentation in the MHC class II pathway and produced humoral immune response (Apostolopoulos et al., 1995). However in a clinical trial (Karanikas et al., 1997), 25 patients (advanced metastatic carcinoma of breast, colon, stomach, or rectum) immunized with oxidized mannan-MUC1 fusion protein produced strong antibody responses and moderate cellular cytotoxic responses. A pilot phase III trial using oxidized mannan-MUC1 in early-stage breast cancer patients. Patients undertaking this trial had a primary lesion; their lymph nodes surgically removed and had no evidence of disease at the commencement of the trial. No recurrence occurred in all 16 patients receiving oxidized mannan-MUC1 fusion

protein, whereas four out of 15 patients receiving placebo had recurrent disease after 7 years and 10 months. All treated patients have immunity to MUC1 VNTR, and none in placebo patients (Apostolopoulos et al., 2006). Hence immunotherapy may benefit the patients in early stage than the advanced disease stage, as the advanced stage patients have poor immune response and significant tumor size.

Goydos (Goydos et al., 1996) underwent a clinical trial using a 105 amino acid synthetic MUC1 peptide containing five VNTR mixed with bacillus Calmette-Guérin (BCG). BCG is an adjuvant of the attenuated form of *Mycobacterium bovis*. In that study, 100 μg of the synthetic MUC1 peptides were administered three times at three-week intervals to 63 patients with adenocarcinoma of breast, colon or pancreas. Skin biopsies at the injection sites showed that 37 patients had intense T cell infiltration and seven patients had lesser infiltration. Seven of 22 patients had two-to four fold increases in MUC1-specific CTL precursor frequency.

Besides targeting the VNTR region, peptide vaccine can also contain non-VNTR (outside VNTR region) or cytoplasmic tail epitopes. Peptides of non-VNTR region of MUC1 had induced effective anti-tumor immunity and HLA-restricted anti-tumor CTL response in transgenic mice. Effector T cells are able to discriminate between MUC1 overexpressing tumor and normal MUC1 tissue as epitopes from the non-VNTR sequences are far less abundant than those from VNTR. Therefore T cell immune system is less prone to develop toleration for these epitopes (Heukamp et al., 2001). In addition, MUC1 cytoplasmic tail peptide vaccine had shown to increase the survival of MUC1 transgenic mice challenge with murine melanoma cell line B16. No detectable autoimmune response was observed in the vaccinated mice. The tumors arose late in the vaccinated mice had low or undetectable MUC1 level as demonstrated by immunohistochemical studies (Kohlgraf et al., 2004). Therefore the results from this experiment may be extrapolated for the study of breast cancer.

Carbohydrate vaccine

Furthermore, carbohydrate antigens (Tn, TF and STn) of mucin can also be a target for vaccine, as they are exposed in tumor cell due to aberrant glycoslyation. These antigens are exclusively expressed in adenocarcinomas and have restricted distribution on normal adult tissues (Springer, 1984). A breast cancer trial of STn-KLH (Theratope) with Detox in patients pretreated with low-dose of cyclophosphamide shown to induce high titre of IgM and IgG antibodies against synthetic STn. In contrast, patients not pretreated with cyclophosphamide had a two to four fold lower titre of antibodies (MacLean et al., 1993). In Phase III clinical trial, Theratope failed to meet the endpoints of time-to-disease progression and overall survival in metastatic breast cancer patients. Nevertheless, the combination of Theratope and hormone therapy had improved the survival rates compared with hormone therapy alone, 8.3 months and 5.8 months respectively (Holmberg and Sandmaier, 2004).

Recently, glycopeptides vaccines (Tn and STn) with complete O-glycan occupancy (five sites per repeat) has shown to produce the strongest humoral response against MUC1 expressed in breast cancer cell lines in both BALB/c and MUC1 transgenic mice (Sorensen et al., 2006). Glycopeptide vaccine consists of cancer associated carbohydrate epitopes on the natural mucin peptide backbone. This type of vaccine is better than just carbohydrate antigens because (i) antibodies to the peptides have higher affinity than antibodies to carbohydrate antigens; (ii) the carbohydrate antigens were present in a specific clustering pattern on the natural mucin backbone that resemble the immune target on the cancer cell (Sorensen et al., 2006); (iii) the natural anti-MUC1 antibodies in cancer patients shown to bind strongly to MUC1 glycopeptides than unglycosylated peptides (von Mensdorff-Pouilly et al., 2000); (iv) the antibodies produced by the glycopeptide vaccine may bind to both the carbohydrate and the peptide backbone, or the conformational peptide epitopes that are dependent on glycosylation, thereby eliciting the immune response specifically target the MUC1 expressing tumor (Schuman et al., 2003).

However the glycosylation may cause steric hindrance by the bulky carbohydrate chains and prevent the access of the backbone by the protease in antigen presenting cells (APC). In addition, the binding of the glycopeptide fragments to the MHC proteins may be disturbed by the glycans (Werdelin et al., 2002). Therefore the soluble glycosylated human MUC1 from patient's serum does not cause anti-tumor immune response, as they are not processed by antigen presenting cells and does not prime helper T cell responses *in vitro* (Hiltbold et al., 1999). Nevertheless, a study had shown that the steric hindrance is dependent on the position of glycans on the peptide sequence, where the glycosylation at GSTA was able to be processed, but not at VTSA or PDTR in the VNTR (Vlad et al., 2002). This is further supported by *in vitro* studies that had demonstrated that O-glycans at either threonine or serine in the VTSA motif (proteolytic cleavage site) of MUC1 sterically hinder the access of cathepsin L (protease), and therefore resistant to proteolysis (Hanisch et al., 2003; Hanisch, 2005). Hence, when designing the glycopeptide vaccine, the steric hindrance of glycans at particular motif should be taken into consideration.

Additionally, multivalent vaccine is also used in preclinical studies, which may be better than monovalent because most cancers express heterogeneous of antigens. A unimolecular multivalent vaccine is formed by joining multiple antigens in a single molecule. The immune system will be able to generate a multifaceted immune response where antibodies are produced against each antigen. This kind of vaccine has a higher chance of targeting a greater number of cancer cells than the

single-antigen containing vaccine, thereby increasing the likelihood of eliminated all cancer cells (Slovin et al., 2005). A trivalent vaccine containing globo-H, Lewis^y and Tn has been examined in mice and shown that antibodies were generated with specificity for each antigens (Ragupathi et al., 2002). A potential problem with polyvalent vaccine may be dominant response of one particular antigen and eliminate the response against other antigens due to steric hindrances. In future, multivalent vaccine may be used in breast cancer therapy.

Peptide vaccine may have some disadvantage that need to be addressed, such as (i) the peptide vaccine is restricted by the human histocompatibility leukocyte antigen (HLA) of characterized tumor antigen epitope, and the peptide vaccine need to match the patients' HLA, therefore limited number of patients will benefit from this vaccine. (ii) MUC1 is a self-antigen, which may induce immune tolerance. New strategy for developing more effective cancer vaccines may involve modifying peptides that may enhance the antitumor T cell response. The idea behind this process is that high avidity T cell specific for the most dominant MUC1 epitopes are deleted in the thymus due to self-protein, while the subdominant epitopes may not be deleted. However, this subdominant epitopes may have suboptimal immunogenicity because of suboptimal affinity for the MHC molecule. To overcome this problem, the sequence of the subdominant epitopes can be modified in the residues interacting with the MHC molecules, thereby increasing the affinity for the MHC molecules and not affecting the binding of the T cell receptor to the peptide-MHC complex (Berzofsky et al., 2005). Hence, there are many improvements required in peptide vaccine development.

Tumor eradication induced by MUC1 DNA vaccine

DNA vaccine is simple, stable and inexpensive. It consists of plasmids with strong promoter, an intron, a multiple cloning site for inserting gene of interest and an appropriate transcription terminator. DNA vaccines can be delivered by intramuscular (i.m.), intradermal (i.d.), subcutaneous (s.c.), oral, pulmonary (aerosols) administrations. After injecting the vaccine i.m., gene expression can still be detected for up to 19 months in the mouse skeletal muscle (Wolff et al., 1992). DNA vaccine can activated both cellular and humoral immune response as the encoded antigen is processed through both endogenous and exogenous pathways and its peptide epitopes generated by proteolysis in antigen processing cell (APC) are presented by major histocompatibility complexes (MHC) class I and II. The antigen are taken up into APC by both direct transfection and uptake of antigen released from other transfected cells (lysis or secretion) (Corr et al., 1999). Furthermore, the vaccine delivers the antigen gene of interest can be coupled with a number of genes that modify the immune response. Unlike peptide-based vaccine that usually has a limited number of epitopes, DNA vaccine is able to involve multiple different antigenic epitopes and a wider range of MHC restriction.

DNA vaccination of MUC1 cDNA to C57/BL6 wild type mice showed rejection of human MUC1-expressing tumor cell line MC38 (Johnen et al., 2001). Tumor growth inhibition was observed in 85% of immunized mice with both humoral and cellular mediated immune response detected. The validation of this study is being questioned, because the wild type mice would see the human MUC1-bearing tumor cell line as "foreign" and develop strong antihuman MUC1 immune response. Hence future study will need to demonstrate the effectiveness of DNA vaccine in human MUC1 transgenic mice. Human MUC1-transgenic mice provide a suitable model for examining the human immunogenicity, as they have a better reflection on the immuopathology of human tumors bearing MUC1 where MUC1 is a self-antigen subjected to tolerance. Currently, there are a number of human MUC1-transgenic mice being generated on the C57BL/6 (Rowse et al., 1998), BALB/c (Acres et al., 2000) and DBA (Carr-Brendel et al., 2000) strains. The transgenic mice challenged with MUC1-bearing synegeneic tumors were unable to develop effective antitumor response and tumors were able to grow, whereas wild-type mice eliminated all MUC1 tumor (Rowse et al., 1998). Therefore MUC1 transgenic mouse is a better model than the wild type mouse in developing strategies for breaking the tolerance and eliciting effective antitumor immune response.

Human MUC1 transgenic mice were used in MUC1/IL 18 DNA (MUC1 plasmid containing murine interleukin 18 DNA) vaccine study and demonstrated a significant tumor protection and survival after tumor challenge. The MUC1 DNA vaccine alone was not sufficient to provoke protection against tumor challenge. IL18 was chosen as the adjuvant to induce NK cell activation, leading to tumor cells destruction and subsequent stimulation of tumor-specific immune response (Snyder et al., 2006).

MUC1 DNA vaccination may not be effective, as MUC1 produced by *in vivo* transfected muscle cells or APC would be normal rather than the tumor forms. The normal heavily glycosylated MUC1 may not be processed by adjacent DC due to defective intracellular trafficking (Hiltbold et al., 2000). In addition, persistent of normal MUC1 expression may lead to autoimmunity. Hence, DNA vaccination of MUC1 may not be useful as a cancer vaccine unless the vaccine can deliver MUC1 cancer aberrant forms rather than the normal form. However, DNA vaccination of gene involved in modifying immune response can be used in conjunction with other cancer vaccines to enhance the antitumor immune response.

Dendritic cell vaccine

Dendritic cells (DC) are the most potent antigen-

presenting cells for priming T cell. Mature DC express high level of HLA class I and II molecules, costimulatory molecules and adhesion molecules; mature DC also produces some chemokines and cytokines for T cells proliferation and activation. DC-based immunizations are reported to induce an effective immune response to MUC1.

Cancer can be eliminated by DC activation. Immature DC (iDC) leave the bone marrow and have high level of MHC class II mRNA and protein in the cytoplasm but their expression was low at the cell surface. iDC have low or absence expression of co-stimulatory molecules (such as CD40, CD80 and CD86) at the cell surface, and IL12 production is low or absence (required for T cell proliferation). In the tissue, iDC can engulf apoptotic or necrotic tumor cells, which have tumor-associated antigens on their cell surface (Gabrilovich, 2004). Necrotic tumor cells are potent activator for maturation of iDC (Sauter et al., 2000), which results in increase cell surface expression of MHC class I molecules and co-stimulatory molecules on DC. Once DC are activated, they migrated to the draining lymph node, where they interact with $CD4^+$ and $CD8^+$ T cells. $CD4^+$ T cells activate DC to produce IL12 and supplies cytokine for clonal outgrowth of antigen-specific $CD8^+$ cytotoxic T cells that are directly induced by activated DCs. Subsequently cytotoxic T cells can recognize and eradicate the specific tumor cells (Gabrilovich, 2004).

Unfortunately, the level of DC decreases dramatically in cancer patients. Cancer patients in their early stages had more than twofold lower level of DC in their peripheral blood than healthy donors (less than 0.5% of the peripheral blood mononuclear cells (Markowicz and Engleman, 1990)); while in advance stage cancer patients DC decreased to fourfold lower than control healthy individuals. After surgical removal of tumor, the level of DC increased in the peripheral blood of breast or prostate cancer patients (Almand et al., 2000). The significant reduction of DC was only observed in the myeloid population of DC (iMC), and the lymphoid DC was not affected (Della Bella et al., 2003). In addition, iMC have reduced expression of the co-stimulatory molecules CD80 and CD86 found in the peripheral blood of the breast cancer patients (Gabrilovich et al., 1997).

The defect in differentiation of iMC was mediated by soluble factors produced by tumor cells. Studies shown that conditional medium from tumor cells cultured *in vitro* can suppress the differentiation of DC from the bone marrow of control mice *in vitro*. In addition, the isolation of DC precursors from tumor-bearing mice cultured *in vitro* in the absence of tumor cell conditioned medium, the differentiation of DC was normal (Gabrilovich et al., 1996). Moreover, tumor releases vascular endothelial growth factor (VEGF) (Gabrilovich et al., 1998), interleukin10 (IL10) (Allavena et al., 1998) , IL-6 (Menetrier-Caux et al., 1998), macrophage colony-stimulating factor (M-CSF) (Menetrier-Caux et al., 1998), and/or gangliosides (Birkle et al., 2003) and thereby inhibit DC differentiation and function *in vitro* and *in vivo*. Hence cancer patients may not be able to eliminate the tumor by differentiating and activating their DC *in vivo*.

MUC1 aberrantly expressed on tumor cells may be a potent chemoattractant for immature dendritic cells and able to induce maturation but unable to promote Th1 type immunity. A study had shown that aberrantly glycosylated form of MUC1 on tumor cells could attract iDC through its polypeptide core, where the DC took up the MUC1 antigens for the presentation to T cells in the lymph nodes. The short sialylated carbohydrates of the MUC1 were able to bind to and internalize by DC and induce DC activation and maturation. MUC1 was able to induce an increased expression of CD40, CD80, CD86 and CD83 on DC, similar to that induced by LPS. However, DC induced by MUC1 failed to induce a type 1 response, which is important for tumor rejection (Carlos et al., 2005). Another study demonstrated that tumor MUC1 impaired the differentiation and function of DC by increasing the expression of CD1a and CD206, which are associated with immature DC phenotype. Moreover, in the presence of tumor MUC1, DC secreted higher level of IL10, but incapable to produce IL12 even after LPS stimulation. This altered balance of IL12/IL10 production led to impaired ability of APC to produce allogeneic and autologous immune response (Rughetti et al., 2005). Hence MUC1 bearing tumor can escape immunosurveillance by impairing the differentiation and maturation of DC.

DC vaccines are able to bypass the defect in DC differentiation and function in cancer patients. DC vaccine is generated by *ex vivo* expansion of DC from monocytes of the peripheral blood mononuclear cell cultured *in vitro* with IL-4 and granulocyte macrophage colony-stimulating factor (GM-CSF) for 3-5 days to differentiate into iDC (Sallusto and Lanzavecchia, 1994). These iDC have high level of endocytotic activity, but low T-cell stimulatory capacity, and are often referred to as the antigen capturing cells. iDC take up the required soluble molecules (such as peptide) by fluid phase pinocytosis or by receptor-mediated internalisation. Then the iDC is differentiated into mDC by incubating with inflammatory stimuli, such as tumor necrosis factor α (TNFα) (Sallusto and Lanzavecchia, 1994), CD40 ligation (Sallusto and Lanzavecchia, 1994), IL-1, LPS. The mDC are often termed as the antigen presenting cells.

Animal studies demonstrated that DC injected subcutaneously preferentially migrated to the draining lymph nodes and induced greater antitumor effect (Bonifaz et al., 2002) than intravenously injected DC that migrated to the spleen (Eggert et al., 1999). Whilst in clinical trials, patients developed antigen specific T cell immune response was independent of the route of administration (i.v., i.d. and i.l. (intralymphatic)). However, induction of Th1 type immunity was seen only in i.d. and i.l. routes of administration (Fong et al.,

2001).

DC-tumor cell hybrids are fusions of DC with MUC1 expressing cancer cells. These fused cells expressed both MHC class I and II and the costimulatory molecules that are normally found on DC. They also express MUC1 and possibly other tumor-specific antigens on their surface (Gong et al., 1997). After injected subcutaneously in the MUC1 transgenic mice, the fused cells migrated to the draining lymph node and distributed to the T cell area in a similar manner to unfused DC (Koido et al., 2002). These fused cells were able to defeat T cell tolerance to MUC1 antigen in MUC1 transgenic mice and triggered cytotoxic T lymphocytes to eliminate MUC1 expressing cancer (Gong et al., 1998).

Dendritic cell vaccine are made by fusing DC with either a MUC1-expressing carcinoma cells or transfected with MUC1 RNA or pulsed with a MUC1 tandem repeat peptide (Hiltbold et al., 1999). A clinical trial (Kontani et al., 2003) demonstrated that MUC1 loaded DC vaccine significantly prolonged the survival of the MUC1-positive patients (lung or breast) than the MUC1-negative patients (16.75 versus 3.30 months). In addition, there was clinically reduction in tumor sizes or tumor marker level or disappearance of malignant pleural effusion in most of the MUC1-positive patients (7 out of 9 patients).

The use of fiber-modified adenoviral vector to transfer cDNA of the aberrantly glycosylated MUC1 into iDC has been developed. After addition of GM-CSF and IL-13, iDC were matured normally into mDC. The upregulation of CD40, CD80, CD86 and HLA-DR were preserved and not affected by adenoviral transduction. In addition, the production of IL-12 was increased up to 500 times; and IL12 is contributed to anti-tumor immune response (van Leeuwen et al., 2006a,b). Hence this adenoviral-transduced DC that produced high level of IL-12 may be useful in cancer therapy.

A recent phase I clinical trial using DC pulsed with mannan-MUC1 fusion protein was shown to stabilize the tumor in 2 out of 10 advanced stage ovarian or renal carcinoma patients. This study demonstrated that this DC vaccine can induce T cell response in human (Loveland et al., 2006), whereas mannan-MUC1 fusion protein vaccine produced antibody response in human (as mentioned in previous section). The reason for the antibody response in human immunized with mannan-MUC1 fusion protein was due to the cross reaction of anti-MUC1 peptide with the naturally occurring human anti-Galα(1,3)Gal antibodies in cancer patients (Sandrin et al., 1997; Apostolopoulos et al., 1998). However the DC pulsed with mannan-MUC1 fusion protein could be able to avoid the cross reaction, and therefore inducing T cell response (Loveland et al., 2006).

The limitation of DC vaccine is the high cost, highly specialized facilities and personnel in generating *ex vivo* personalized DC vaccines. In addition, there is a risk of contamination. In future, DC vaccine will benefit more people not just a limited number of cancer patients, if the vaccine does not involve *ex vivo* generation.

Overcome the barrier in immunotherapy

Genetic instability in cancer can cause reduction in expression of tumor-associated antigens and HLA molecules, with disrupted processing and presentation of tumor-associated antigens, allowing malignant cells to elude the surveillance of immune sentinels. In addition, tumor cells do not normally express costimulatory molecules such as B7.1/CD80 and B7.2/CD86, which are found on professional antigen-presenting cells (Mocellin et al., 2004)

Moreover, antigen loss variants (ALV) may develop in cancer cells due to immune pressure (Liu et al., 2005; Sanchez-Perez et al., 2005). Active and passive immunotherapies may contribute to ALV as tumor cells bearing target tumor antigen are eliminated, and other cancer cells containing other antigens are unharmed and allow proliferating. Therefore more than one cancer targets may be able to prevent the development of ALV and eradicate the tumor.

T cell activation may be affected by positive and negative signal from co-stimulatory molecules. B7 binds to CD28 promotes T cell activation, whereas binds to cytotoxic T lymphocyte antigen 4 (CTLA-4) leads to attenuate T cell response. Therefore blockade of CTLA-4 function had shown to enhance antitumor immunity in mice (Davila et al., 2003). In addition, removal of immunosuppressive T cells is another approach to enhance immunity. Cyclophosphamide is a chemotherapeutic agent that was attributed to eliminate immunosuppressive T cell (Bass and Mastrangelo, 1998).

Since MUC1 is a self-antigen, there is a need to overcome the self tolerance in order to enhance antitumor response. Beside eliminating immuno-suppressive T cell, cyclophosphamide was demonstrated to defeat natural and acquired immune tolerance if given prior to an antigen exposure, but promotes the induction of immune tolerance if given along with antigen (Emens et al., 2001).

The elevated level of $CD4^+CD25^+$ T regulatory cells in the peripheral blood of the cancer patients inhibit the proliferation of $CD4^+$ and $CD8^+$ T cell via cell-cell interactions or the release of immunosuppressive cytokines (IL10 and TGFß). Anti-CD25 antibody has shown to enhance the efficiency of the vaccine-induced antitumor immunity by depleting $CD4^+CD25^+$ T cells (Onizuka et al., 1999; Sutmuller et al., 2001). However, CD25 is important in the survival and expansion of effector cells since CD25 is also expressed on the activated $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells (Lizee et al., 2006). Therefore anti-CD25 antibody may not be useful in cancer immunotherapy, and further antigen target unique to T regulatory cells may be needed.

Myelopoiesis defect was shown in tumor-bearing mice and patients which resulting in accumulation of

immature myeloid cells, and leading to immune suppression (Kusmartsev and Gabrilovich, 2002). The immature myeloid cells induced immune suppression by interfering with the T cell proliferation (Bronte et al., 2003). This immune suppression can be reversed by all-trans-retinoic acid that differentiates immature myeloid cells (Kusmartsev et al., 2003).

The starvation of amino acids, L-Arginine (L-Arg) and tryptophan, can impair the T cell function. Indoleamine 2,3-dioxygenase (IDO), which is found in the tumor-draining lymph nodes of cancer patients, catalyses the oxidative degradation of tryptophan. The inhibition of T cells can be reversed by addition of excess tryptophan. L-Arg can be hydrolysed to urea and L-ornithine by arginase, and metabolized to produced citrulline and nitric oxide by nitric oxide synthase (NOS-2). Both argininase and NOS-2 are found in the tumor microenvironment of patients. The low levels of L-Arg lead to induce loss of CD3τ chain and inhibited T-cell proliferation. This inhibition can be overcome by arginine inhibitor and NOS-2 inhibitor or scavenger superoxide dismutase (Rodriguez and Ochoa, 2006).

In addition, some cancer cells may express low density of specific tumor antigens target on their cell surface, and therefore higher concentration of antibodies may be required to induce target cell lysis through ADCC function. However, this may lead to dose-dependent side effects in patients and increase the cost of production. Studies shown that altering the amino acid sequence in the Fc region of the antibodies could enhance the binding affinity of antibodies Fc region to the Fc receptors by at least 10-1000 fold *in vitro* (Shopes, 1992). Nevertheless, there are currently no *in vivo* data on the immmunogenicity and the half-life of these antibodies containing altered amino acid (Schuster et al., 2006). Hence altered amino acid in Fc region may be a potential target in the passive immunotherapy.

Conclusion

MUC1 is highly expressed in breast cancer but weakly expressed in most normal mammary tissue. There are a number of antigens exposed in cancer that can be targeted for cancer vaccine, as they are normally masked by glycosylation in normal mammary tissue. Therefore cancer vaccine targeting at these antigens will only eliminated cancer cells and leave the normal cells untouched. In addition, antigen-based vaccines have been successful in animal models for tumor prevention; however they are less successful in human trial as they are used exclusively as therapeutic agents in advanced disease and often after resistance of the standard therapy. In future, the cancer immunotherapy (i) may combine MUC1 with other cancer antigens in eradicating tumor cells in patients; (ii) may include other chemotherapeutic drug that inhibit cancer tolerance and reduce the proliferation of iDC; (iii) may contain cytokines that activate T cell function.

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