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EXPRESSION AND IMMUNOLOGICAL CHARACTERIZATION OF HERV-K TRANSMEMBRANE ENVELOPE PROTEIN IN HUMAN BREAST CANCER

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A

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The human endogenous retrovirus K (HERV-K) *env* gene encodes envelope protein comprising surface (SU) and transmembrane (TM) domains. Having shown the exclusive expression of SU in human breast cancer and the stimulation of SU-specific immune responses in patients with breast cancer, our research here confirmed and extended the data by investigating the expression of HERV-K TM envelope domain and the induction of specific immune responses against TM in breast cancer patients. We found HERV-K TM mRNA and protein expression only in human breast cancer cells but not in normal controls. The specific immune responses against TM domain were induced in mice determined by enzyme-linked immunosorbent assay (ELISA) and IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assay. Furthermore, ELISA detected higher titers of anti-HERV-K TM Env IgG antibodies in sera of breast cancer patients. In addition, the magnitude of the anti-HERV TM B cell response was correlated with the disease stage. Peripheral blood mononuclear cells (PBMCs) before and after *in vitro* stimulation (IVS) with HERV-K TM from patients with breast cancer as well as healthy controls were tested for T cell responses against HERV-K TM domain by ELISPOT assay. Breast cancer patients (n=21) had stronger HERV-K TM-specific cellular responses than healthy controls (n=12) ($P < 0.05$). These findings suggest, for the first time, that HERV-K TM expression was enhanced in human breast cancer cells and was

able to induce specific B cell and T cell immune responses in breast cancer patients. This study provides support for HERV-K TM as a promising source of antigen for anti-tumor immunotherapy, prevention, diagnosis, and prognosis.

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ABBREVIATIONS

6-His	histidine hexamer
BLV	bovine leukemia virus
cDNA	complementary DNA
CFA	complete Freund's adjuvant
CMV	cytomegalovirus
CTL	cytolytic lymphocyte
DAPI	4',6-Diamidino-2-phenyindole
DC	dendritic cell
DCIS	ductal carcinoma in situ
ddH_2O	distilled deionized water
DEPC	diethylpyrocarbonate
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunosorbent spot
env	envelope
FBS	fetal bovine serum
FLV	feline leukemia virus
gag	group-specific antigen

GM-CSF	granulocyte macrophage colony-stimulating factor
HBV	hepatitis B virus
HCV	hepatitis C virus
HERV-K	human endogenous retrovirus K
HPV	human papillomavirus
HTLV-1	human T lymphotropic virus type 1
IDC	invasive ductal carcinoma
IFA	incomplete Freund's adjuvant
IFN	interferon
IFS	immunofluorescence staining
IL	interleukin
IPTG	isopropyl-β-D-thiogalactoside
ISD	immunosuppressive domain
IVS	*in vitro* stimulation
KLH	Keyhole limpet hemocyanin
KSHV	Kaposi's sarcoma-associated herpesvirus
LTR	long terminal repeat
MLV	murine leukemia virus
MMTV	mouse mammary tumor virus

MoMLV	Moloney murine leukemia virus
ORF	open reading frame
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PLZF	promyelocytic leukaemia zinc finger
pol	polymerase
pro	protease
RSV	Rous sarcoma virus
RT-PCR	reverse transcription-polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SU	surface
TE	transposable element
TM	transmembrane
TNF- α	tumor necrosis factor-α

Chapter 1 Introduction

Since a virus, later named as Rous sarcoma virus (RSV), was defined as the transforming agent to propagate tumors in chickens by Peyton Rous in 1911, more evidence has accumulated demonstrating the association between viruses and cancers. Approximately 15-20% of human cancers can be linked to viral infection worldwide (1). At least six viruses have been known to contribute to human malignancy. Epstein-Barr virus (EBV), for example, the first discovered human cancer virus in 1965, is considered as the etiologic agent of Burkitt's lymphoma, nasopharyngeal carcinoma, and Hodgkin's lymphoma. Other notable associations are between Hepatitis B virus (HBV) and hepatitis C virus (HCV) with hepatocellular carcinoma, high risk strains of human papillomavirus (HPV) with cervical cancer, human T-cell lymphotropic virus type 1 (HTLV-1) with adult T-cell leukemia/lymphoma, and Kaposi's sarcoma-associated herpesvirus (KSHV) with Kaposi's sarcoma (2).

Retroviruses are exceptionally important cancer-causing agents in animals and were initially named as 'RNA tumor viruses'. The original identification of oncogenic animal retroviruses in chicken and mice stimulated intense interest to search for retroviruses in humans. HTLV-1 was the first human exogenous retrovirus identified to be the etiological agent of adult T-cell leukemia and lymphoma in 1980. As there is an analogy of human exogenous retrovirus in the human genome to these viruses, human endogenous retroviruses (HERVs) have recently been proposed to play a role in cancers, including human breast cancer. In this section, we will focus on the general features of HERVs and the association with human breast cancer.

1.1 Human endogenous retrovirus (HERV)

1.1.1 Structure of HERV

HERVs are considered as the remnants of ancient exogenous retrovirus infection in the host genome. HERVs are enveloped RNA viruses, characterized by two characteristics of their replication: the ability to convert genetic information from viral RNA into complementary DNA (cDNA) and the integration of the newly synthesized DNA copy of viral RNA into the host genome. The process of the conversion of viral RNA into cDNA is called reverse transcription, using the viral-encoding enzyme reverse transcriptase. The latter feature provides a means for retroviruses to persist in the host genome forming the endogenous retroviruses. The majority of the integrated provirus becomes inactive given the accumulated random mutations during amplification in the infected progeny host cells.

HERVs are typically 80 to 100 nm in diameter. The RNA genome, approximately 8-10 kb in size, is a dimer consisting of two identical linear (+) sense copies and is associated with the nucleocapsid forming a ribonucleoprotein complex (Figure 1). The viral genome are flanked by two copies of the long terminal repeat (LTR), which contain important cis elements such as the promoter, enhancer, primer binding site, and polyA sequences. The LTRs are important in initiating reverse transcription. Open reading frames (ORFs) are located between the two LTRs and consist of four main genes coding for the basic translated regions: *gag*, *pro*, *pol*, and *env*.

The *gag* gene codes for gag proteins composed of nucleocapsid, capsid, and matrix. The *pro* gene codes for protease which is able to degrade viral precursor polyproteins to make mature gag and pol proteins. The *pol* gene codes for reverse transcriptase and

integrase which are essential for the reverse transcription process and the integration of the proviral DNA into the host genome. The *env* gene codes for env proteins which are composed of surface (SU) protein and transmembrane (TM) protein. SU protein is responsible for the viral binding to the host cell receptor and TM protein is capable of holding SU domain in the envelope and plays a role in the membrane fusion. The ribonucleoprotein complex and the enzymes encoded by retroviruses are surrounded by capsid protein and then matrix protein. The viral outer layer is made up of envelope mediating viral entry (Figure 2).

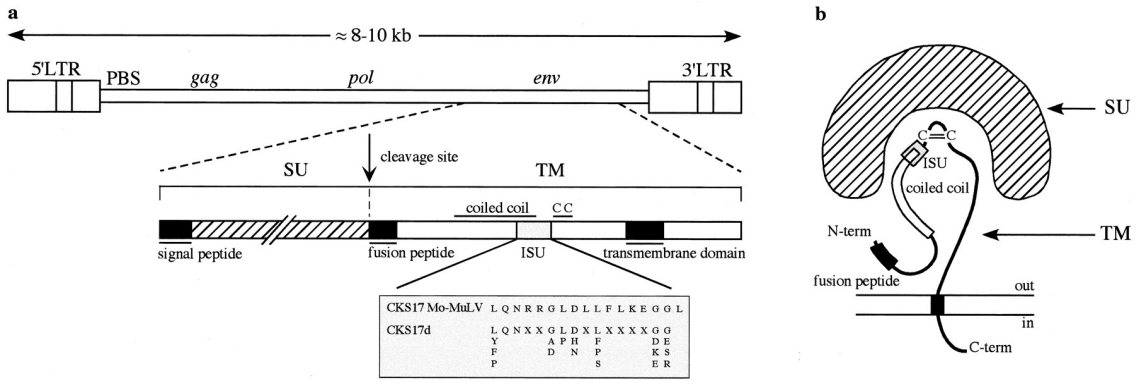

Figure 1 Schematic representation of the proviral form of a retrovirus and its *env* gene products. Reprinted from reference (3) with permission from American Society for Microbiology.

(a) Genomic proviral structure and delineation of the SU and TM subunits encoded by the *env* gene. (b) schematic structure of a retroviral envelope protein products. CC, disulfide bond; ISD, immunosuppressive domain.

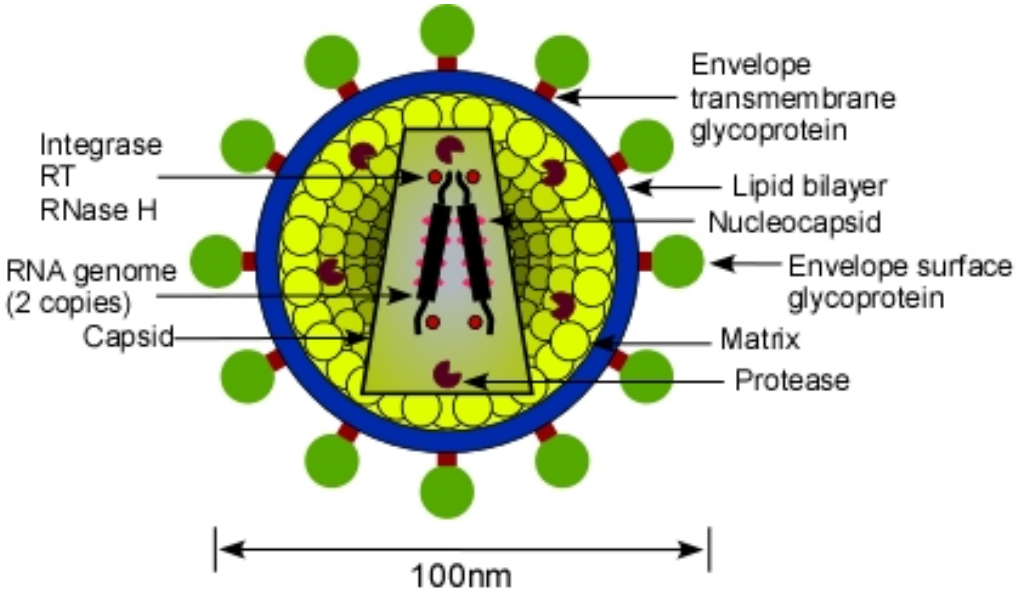

Figure 2 Schematic structure of a retroviral particle. Reprinted from reference (4) with permission from BioMed Central.

1.1.2 Classification of HERV

Almost half of the human genome is derived from transposable elements (TEs) including DNA-transposons (2.8%), non-LTR retroelements (33.9%), and LTR retroelements (8.3%) (5). HERVs belong to the LTR retroelements which are divided into groups by using different classification systems.

The most common way to classify HERVs is based on sequence of the primer binding sites (PBSs) in LTRs. PBS is the region which is hybridized with a tRNA used to initiate reverse transcription. The HERVs can be named by adding the single letter amino acid code for tRNA specificity. For example, HERV-K would use a lysine tRNA as its primer. But this method may contain some limitations when the same tRNAs are used by distantly related HERV groups or when no tRNA information is available due to mutations.

HERVs are also classified into three classes based on the phylogenetic analysis, Class I, II, and III, based on sequence similarity to the exogenous retroviruses (Figure 3). Exogenous retroviruses, belonging to the virus family Retroviridae, contain seven genera: α retrovirus, β retrovirus, γ retrovirus, δ retrovirus, ε retrovirus, spumaretrovirus, and lentivirus.

Class I HERVs are close to exogenous γ retroviruses such as murine leukemia virus (MLV) and include 6 groups such as HERV-W, HERV-FRD, and HERV-H. HERV-W and HERV-FRD proviruses have maintained expression and fusogenic capacity of the envelope protein in trophoblast cells in the placenta, named syncytin-1 and syncytin-2, respectively. The fusion mediated by these envelope proteins are thought to create a layer of fused cells in the placenta, called syncytiotrophoblasts, which is important for

placentogenesis and pregnancy. Class II HERVs include 10 groups which are close to β retroviruses such as mouse mammary tumor virus (MMTV) and are known as HERV-K when the lysine tRNA binding site sequence is used. Class III HERVs are distantly related to spumaretroviruses and include a few groups like HERV-L and HERV-S (5).

Most HERVs appear to be ancient and are found in both Old and New World monkeys, suggesting that the first integration occurred more than 35 million years ago before the divergence of New World and Old World monkeys. HERV-K is the youngest HERV and is the one most likely to be active. The age of an endogenous retrovirus can be roughly estimated by comparing its two LTRs. At time of integration, both of the LTRs should be identical. But over time, the accumulating mutations during the host DNA replication process would generate one difference between two LTRs every approximately 200,000 to 450,000 years. Studies show that some HERV-K members have less than five differences between the two LTRs, suggesting that the integration occurred only less than 1 million years ago.

The erratic way for the HERV classification and naming may cause some problems. It may lead to, for example, different name designations for the same sequence. HERV-K10, HTDV, and HERV-K, once named differently by various research groups, are finally confirmed as the same HERV subfamilies. Recently, some new revised classification systems are being developed based on a combination of similarity, structure, function, and previous naming which are thought to be able to overcome the classification problems (6, 7).

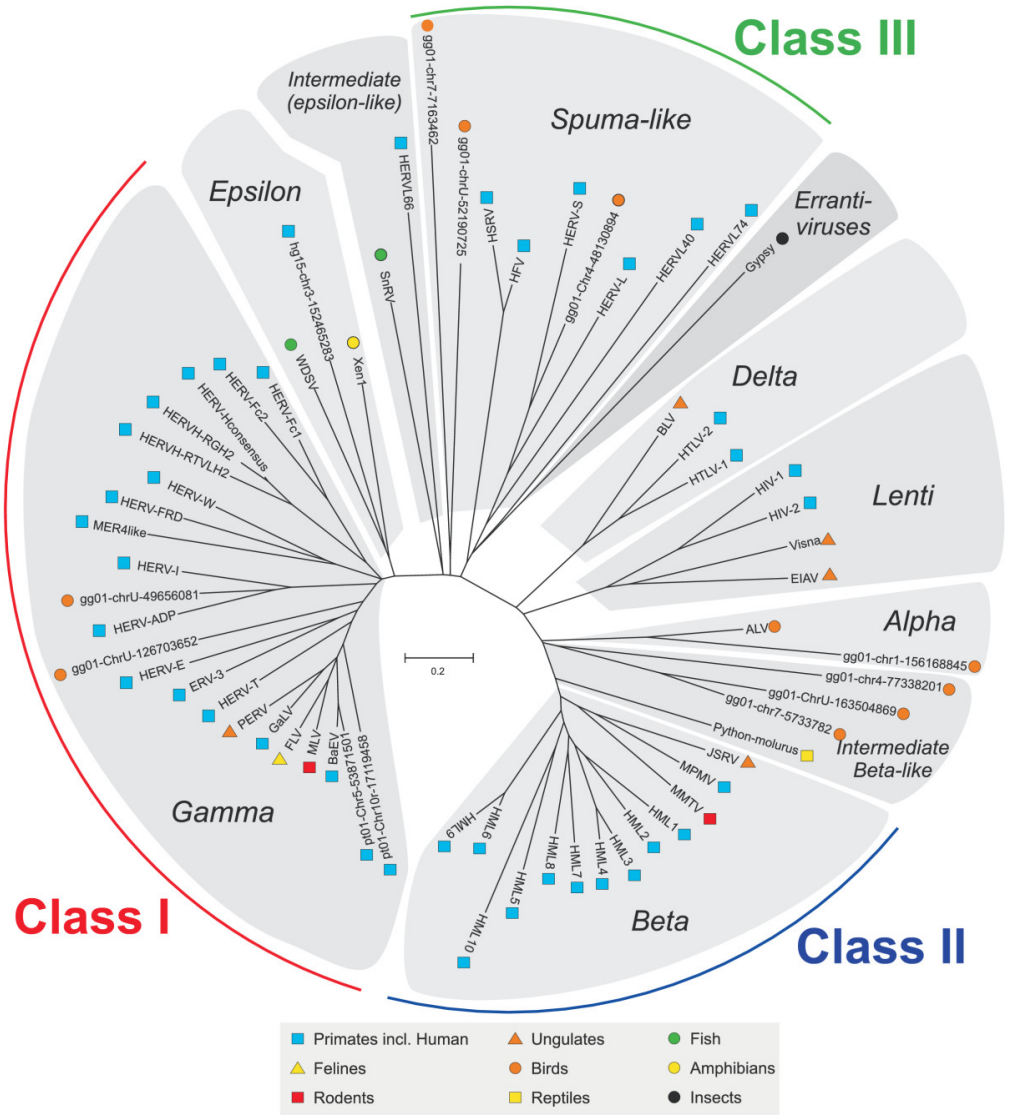

Figure 3 General tree of retroviruses based on the Pol proteins of exogenous and endogenous retroviral sequences. Reprinted with reference (8) with permission from BioMed Central.

1.1.3 Physiological effects of HERV

Considering the retention of HERVs for over millions of years in the human population during evolution, HERVs may provide beneficial functions for their hosts.

First, HERVs, as well as other TEs, may play a role in the shaping and plasticity of the human genome. For instance, it has been found that HERV LTRs constitute regulatory elements which provide promoter, enhancer, or polyadenylation signals for nearby host DNA genes. HERV LTRs often contain the p53 regulatory binding sites and may be involved in anti-tumor activity through the p53 pathway (9). Telomerase, considered to stabilize the chromosomes, is shown to be derived from RTs.

Second, HERV-W envelope proteins can protect host cells from infections by closely related exogenous retroviruses via receptor interference (10).

Third, some HERVs are associated with placenta formation. HERV-W and HERV-FRD *env* genes, encoding the protein syncytin, namely syncytin-1 and syncytin-2, respectively, are highly expressed in the placenta. They are thought to be function as fusogenic activities by mediating the fusion of the cytotrophoblast into the syncytiotrophoblast. Besides the function in placenta morphogenesis, the expression of syncytin-2 may also contribute to prevention of allogeneic rejection of the fetus at the fetal syncytiotrophoblast boundary by immunosuppressive activity; this is not the case for syncytin-1 (11).

1.1.4 HERV and human diseases

Considering the pathologic properties of human exogenous retroviruses, it is easily understood that the primary effects of HERVs for the host is not beneficial but

detrimental. The scientific literature has shown that HERVs are tentatively involved in several types of human diseases including autoimmune diseases and cancers (12).

Based on the observation of retroviral antibodies in the plasma of patients and the presence of amplified products homologous to HERVs, HERVs have been considered to be associated with several types of human autoimmune diseases such as lupus erythematosus, insulin-dependent diabetes mellitus, multiple sclerosis, and rheumatoid arthritis. Several mechanisms have been documented to be involved in the dysregulation of the host immune system by HERVs (13). The insertion of the proviral HERVs into the certain host genes associated with immune regulation may contribute to the diseases (14). Moreover, HERVs may express some 'foreign' proteins which would trigger immune responses to produce antibodies causing cross-reaction against our own proteins (15). HERVs can also encode some superantigen proteins which are able to trigger strong immune responses by expansion of autoreactive T lymphocytes (16). It is also shown that the envelope proteins encoded by class I HERVs are capable of activating the innate immune cells to cause the production of several cytokines, which, in turn can act on T lymphocytes (17). In addition, the HERV SU envelope proteins can activate dendritic cells and further promote Th1-like immune responses (18).

HERVs are transcriptionally and translationally active in many different types of human cancers such as germ cell tumors, melanoma, and breast cancer (Table 2). Even though cumulative evidence has shown that HERVs may be involved in the process of carcinogenesis, the contributions of HERVs to human cancers appear more complex. Several potential mechanisms of HERV in human cancer development have been discussed (19).

First, HERVs are assumed be able to play a role in human cancers by insertional mutagenesis indicating retroviral movement in the genome. But it must be emphasized that there is no replication-competent HERVs identified in present-day human individuals.

Second, HERVs may contribute to human cancers by the action of two accessory oncoproteins encoded by HERV-K. They are termed Rec and NP9. Rec is functionally related to the regulatory proteins HTLV Rex and HIV Rev, responsible for the nuclear export of retroviral mRNA into the cytoplasm. It has been found that both Np9 and Rec are involved in cellular transformation under certain conditions, and Rec is able to cause carcinoma in transgenic mice (20). They can directly bind the promyelocytic leukaemia zinc finger (PLZF) which is known as a tumor suppressor of c-myc. The inhibition of the transcriptional inhibiting activity of PLZF by Rec or NP9 results in the elevated expression of c-myc and of c-myc responsive genes such as p53, PCNA and IkBa. Moreover, NP9, in contrast to Rec, is involved in the Numb/Notch pathway by binding to and interfering with the ligand of Numb protein X (LNX), a RING-Type E3 ubiquitin ligase. Notch signaling has been suggested to be involved primarily in breast cancers, leukemias, and germ cell cancers (21).

Third, endogenous retroviral envelope proteins may mediate immunosuppressive activity resulting in tumor escape considering the evidence from research in animals (22). Further studies are needed to clarify whether the HERV envelop proteins are immunosuppressive and expressed in human cancer. Moreover, it has not been demonstrated up to now whether the increased expression of some elements of HERVs

precedes the human cancers or whether they are the results of altered gene regulation in tumor cells.

Table 1 HERV-encoded proteins previously detected in human tumors and tumor cell lines.

Tumor type	HERV protein*
Germ cell tumor tissue	HERV-K(HML-2) Gag
Germ cell tumor cell lines (GH, Tera-1, NCCIT, 2102Ep)	HERV-K(HML-2) Gag HERV-K (HML-2) Env HERV-K(HML-2) Rec HERV-K(HML-2) Np9
Melanoma tissue, melanoma cell lines (SK-Mel-28, Mel-Juso)	HERV-K (HML-2) Env (TM) HERV-K(HML-2) Gag HERV-K(HML-2) Rec
Breast cancer tissue, breast cancer cell lines (MCF-7, MDA-MB-231)	HERV-W Env
Ovarian cancer tissue	HERV-K Env
Endometrial carcinoma tissue	HERV-W Env
Astrocytoma tissue	HERV-W Env
Neuroblastoma cell line (IMR32)	HERV-W Gag HERV-W Env

* HERV-encoded proteins were detected by immunohistochemistry and/or Western blot.

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1.2 Human breast cancer

1.2.1 General features of breast cancer

Breast cancer arises from the epithelial cells of the breast and is the most common cancer among women. It is estimated that around one in ten women will develop breast cancer in their lifetime worldwide. In 2011, about 288,130 new breast cancer cases are expected to occur among US women (American Cancer Society. Cancer Facts & Figures 2011 [online]). Whereas the mortality from breast cancer has largely decreased since 1990 as a result of earlier diagnosis and improved treatment, breast cancer is the second cause of cancer death in women, exceeded only by lung cancer. Around 39,520 women are expected to die from breast cancer in 2011, accounting for 15% of all cancer death in US women.

A wide variety of risk factors influencing an individual's likelihood of developing breast cancer has been established. Two most important risk factors are being female and increasing age. Other potential risk factors include being overweight or obese, hormone therapy, low physical activity, dense breast tissue, previous chest radiation, a long menstrual history, oral contraceptive usage, never having children or late age at first pregnancy, and a previous personal history of breast cancer. Moreover, women with positive family histories of breast cancer have a 2- to 3-fold increased risk of developing malignancy. An inherited mutation in the BRCA1 or BRCA2 tumor-suppressor genes is the strongest known indicator of hereditary breast cancer risk. Genetic mutations in either of these two genes are responsible for a 40-80% chance of developing inherited breast cancer (23).

1.2.2 Viral etiology of breast cancer

Human breast cancer is a highly heterogeneous malignancy with different biological, histopathological, genetic, epidemiological, and clinical features. This heterogeneous characteristic could well suggest that multiple causal agents play significant roles in the initiation and development of human breast cancer (24). An immense current research, although not conclusive, shows that three viruses are potentially involved in causing breast cancer: human papillomavirus (HPV), MMTV-like virus, and EBV (25). MMTV, belonging to β-retroviruses, has been proved to be the cause of mammary cancer in mice. So, similar viruses have long been investigated as a potential cause of human breast cancer. Recently, MMTV-like genetic material and viral particles have been identified in human breast cancer (26). Moreover, more evidence shows that HERV-K, approximately 57% homologous to MMTV gene sequence, is expressed in human breast cancer. In addition, a few more viruses have recently been shown to be associated with human breast cancer. These include bovine leukemia virus (BLV) and cytomegaloviruses (CMV) (27).

1.3 Involvement of HERV-K in human breast cancer

As mentioned above, almost all the HERVs are defective due to the accumulating mutations, premature stop codons, truncations, etc. But, HERV-K is the only one possessing the replication activity capable of encoding all viral proteins such as gag, pro, pol, and env proteins. HERV-K is comprised of two types based on the presence or absence of a 292-bp at the *pol-env* boundary. Type I HERV-K displays the absence of a 292-bp at the *pol-env* boundary, whereas type II is presence of this region. Type II, the HERV-K prototype, expresses the accessory protein Rec, which functions in transporting viral RNA from nucleus into cytoplasm. Type I, instead, encodes the

synthesis of another accessory protein NP9, which is considered to be associated with tumorigenesis (28).

HERV-K SU *env* mRNA and proteins have been found to be overexpressed in many breast cancer cell lines and breast tumor tissues but not in normal breast tissues (29, 30). Beyond the expression of retroviral gene fragments, researchers also focus on the discovery of HERV-K viruses and hope to find a replication competent virus. An earlier study shows that HERV-K viral-like particles were identified from the human breast cancer cell line T47D (31). A recent study suggests that HERV-K viral-like particles could be found in the sera from lymphoma patients under an electron microscope (EM) (32). Moreover, reanimated HERV-K viral-like particles constructed by correcting the mutations are capable of replicating (33). These experiments demonstrated the potential capability of HERV-K to replicate in the past, but they do not directly address whether HERV-K can still produce infectious viruses in modern humans. In addition, HERV-K components are documented to be immunogenic. Antibodies against HERV-K SU envelop protein have been detected by western blot and ELISA in a proportion of breast cancer patients (30). Cytolytic lymphocytes (CTLs) have been found to be able to kill the HERV-K SU envelop-expressing breast cancer cells (30).

1.4 Hypothesis and specific aims of this study

As mentioned above, the findings that HERV-K SU envelope protein is specifically overexpressed in human breast cancer and immune responses against HERV-K SU envelope protein are induced in breast cancer patients but not in normal donors have indicated the possibility that HERV-K may represent an ideal target for diagnosis, prevention, and treatment of breast cancer. In this regard, we further focused on the other

domain of HERV-K *env*, namely the HERV-K TM, to analyze the reactivity in breast cancer. Some evidence has shown that HERV TM protein was especially strongly expressed in human melanoma biopies (34) and in some human cancer cell lines including colon and prostate carcinoma cell lines (35). Furthermore, anti-TM antibody in sera was detected in 22% of melanoma patients but not in healthy individuals (34).

Based on these previous studies **we hypothesized that HERV-K TM envelope protein was a potentially valuable source of tumor-associated antigen which was exclusively expressed and elicits specific immune responses in human breast cancer, and could thereby be used for breast cancer immunotherapy or diagnosis.** The hypothesis was tested by completing the following two specific aims.

Specific aim 1 To determine the expression of HERV-K TM envelope mRNA and protein in human breast cancer.

Specific aim 2 To determine TM-specific humoral and cellular immunological responses in breast cancer patients.

Chapter 2 Materials and Methods

2.1 Cell lines and clinical samples

A number of human breast cancer cell lines including SKBR3, T47D, MDA-MB-231, and MDA-MB-453 were obtained from American Type Culture Collection (Rockville, MD). The breast cancer cell line MCF-7 was a kind gift from Dr. Melinda Hollingshead (Developmental Therapeutics Program, DCTD, NCI). The normal human breast epithelial cell line MCF10A was a kind gift from Dr. Robert Pauley (Karmanos Cancer Institute). MCF10A was cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) medium supplemented with 5% horse serum, 10 µg/mL insulin, 20 ng/mL EGF, and 1× pen/strep. SKBR3 was cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1× pen/strep. The other cell lines were cultured in complete RPMI 1640 medium (i.e., PRMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, and 1× pen/strep). T47D cells were treated with 10 µg/mL insulin.

With the informed consent, blood samples, breast tissue biopsies, and healthy female controls were obtained from human subjects at the M. D. Anderson Cancer Center according to the approved Institutional Review Board (IRB) protocols. Sera from breast cancer patients at different stages as well as healthy controls were a kind gift from collaborators at NIH.

2.2 RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the cultured cell lines in Tri-reagent (Sigma, St. Louis, MO) according to the manufacturer's instruction. The resulting RNA was treated with RNase-free DNase I (Ambion, Austin, TX) at 37°C for 30 min to prevent the

contamination of genomic DNA, followed by heating at 75°C for 5 min to inactivate residual DNase.

RT-PCR to amplify TM gene was performed as described previously (29). Briefly, a first strand cDNA was synthesized from the RNA template using Ready-To-Go™ You-Prime First-Strand Beads (GE healthcare). 10 µg RNA was added into 32 µL Diethylpyrocarbonate (DEPC)-treated H_2O and heated at 65°C for 10 min. 1 µL random primers (Promega) was added to the quenched RNA solution and then incubated with the beads at 37°C for 1 hr. 3.3 µL cDNA, corresponding to 1 µg of input RNA, was amplified in a final volume of 50 µL reaction system containing 5 µL of 10× PCR buffer (Fisher), 0.25 µL of Taq DNA polymerase (1.25 units; Fisher), and the TM outer primer pair at 1 mM each (sense primer:5' AGT CGG TAA ACT TTG TTA ATG 3' ; antisense primer: 5' TCT CCT ATG TCT ACT TCT TT 3'). β-actin was synthesized in parallel with human β-actin primers to show the same amount of cell loaded. After the initial denaturation step at 94°C for 3 min, 30 cycles of amplification were performed as follows: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The amplified genes were finally extended at 72°C for 10 min. The RT-PCR product was subjected to electrophoresis on a 1% agarose gel by ethidium bromide staining to check the insert size.

2.3 Construction and characterization of HERV-K TM expression vector

The DNA fragment encoding TM was amplified by RT-PCR from a human melanoma patient sample using the same conditions as described above and then amplified by nested PCR with a TM inner primer pair incorporating the restriction enzyme sites BamHI and HindIII (i.e., sense primer 5' CGG GAT CCA TTG GCA AAA

GAA TTC TG 3'; anti-sense primer 5' CCC GGG AAG CTT CTA CAC AGA CAC AGT AAC 3'). The nested PCR product was purified using a Gel Extraction Kit (Qiagen Inc., Chatsworth, CA) and further cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA). The recombinant plasmid was amplified by PCR and subjected to gel electrophoresis. The bacterial clones containing the insert fragment with expected size were combined and the plasmids were purified by mini-prep using a QIAprep Spin Miniprep Kit (Qiagen Inc., Chatsworth, CA). The TM fragment was digested from the recombinant plasmid pCR2.1-TM by double restriction digestion with BamHI and HindIII, purified from agarose gel, and then subcloned in frame into the same restriction sites downstream of the histidine hexamer (6-His) in prokaryotic expression vector pQE30 followed by transformation in *E. Coli* BL21 (Qiagen Inc., Chatsworth, CA). The transformed bacteria were cultured in LB broth containing 100 µg/mL ampicillin and 25 µg/mL kanamycin. The recombinant plasmids were screened by PCR and again subjected to double restriction digestion. The candidate recombinant clones containing the insert with the predicted size were induced on a small-scale using 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) at 18°C overnight. The ability of the candidate clones to produce TM fusion protein was assessed by Coomassie blue staining and Western blot. One recombinant pQE30-TM clone expressing the expected fragment was chosen to be further confirmed by sequencing using the recommended sequencing primers (Qiagen Inc., Chatsworth, CA). The clone with the complete TM sequence was retained for TM protein purification as described below.

2.4 Expression and purification of recombinant fusion TM protein in *E. coli*

To express the TM fusion protein, the recombinant pQE30-TM bacterial clone, confirmed by western blot and sequencing, was grown in LB broth containing both ampicillin (100 μg/mL) and kanamycin (25 μg/mL) at 37°C with vigorous shaking (i.e., 250 rpm) until an OD_{600} of 0.8 was reached. The culture was induced overnight by adding 1 mM IPTG at 18°C. The induced culture was spun down at 6,000 × g for 10 min at 4°C and frozen at -20°C overnight.

The 6×His-tagged fusion protein was purified using Ni-NTA affinity chromatography under the recommended conditions (Qiagen Inc., Chatsworth, CA). Briefly, the frozen bacterial pellet was thawed on ice and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) at 3 mL/g net weight. Lysozyme was added to 1 mg/mL. After 30 min incubation on ice, the cell suspension was sonicated with six 10 s bursts at 80-100 W with a 10 sec cooling period between each burst and then spun down at 10,000 × g for 10 min at 4°C to pellet the cellular debris. The Ni-NTA slurry, pre-equilibrated with the same lysis buffer, was added to the cleared lysate and incubated overnight by gently shaking (100 rpm) at 4°C. The lysate-Ni-NTA mixture was added into a column pre-equilibrated with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0). After washing the column twice with the same wash buffer, the bound protein was eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The protein product was dialyzed in cold phosphate buffered saline (PBS) overnight at 4°C. After sterilization, endotoxin removal from the protein was performed using Detoxi-gel endotoxin removing gel (Thermo Scientific, Rockford, IL) as described by the manufacturer. Purified fusion

protein was analyzed by Coomassie blue staining and western blot. Protein concentration was measured by Bradford assay using the Quick Start Bradford 1× Dye Reagent.

2.5 Coomassie blue staining and Western blot

The purified TM protein (typically 10 µg) for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was preheated at 100°C for 5 min in a 6× Laemmli buffer. The sample was run on 12% SDS-PAGE gel at 120 V for about 2 hr in a Laemmli sample buffer (1 g/L SDS, 3.03 g/L Tris base, and 14.4 g/L glycine). The gel was fixed with de-stain buffer (40% v/v methanol and 10% v/v acetic acid) for 30 min and then stained with Coomassie blue stain, which contained 0.1% (w/v) Coomassie blue R250, 40% methanol, and 10% acetic acid at room temperature for 1 hr with gentle agitation.

For Western blot, the protein in the gel was transferred to a nitrocellulose membrane (Bio-rad laboratories, Hercules, CA) at 100 mA for 2 hr in a transfer buffer (3.03 g/L Tris base, 14.4 g/L glycine, and 20% v/v methanol). After washing in Tris buffered saline (TBS, 17.53 g/L NaCl and 4.85 g/L Tris base, pH 7.5) for 5 min, the membrane was blocked in blocking buffer containing 3% (w/v) non-fat dried milk in TBST (i.e., TBS containing 0.2% Tween 20) for 1 hr at room temperature with gentle shaking (100 rpm). After washing three times with TBST for 10 min, the membrane was then incubated overnight with the primary anti-mouse RGS·His antibody (Qiagen Inc., Chatsworth, CA) at 1:1000 dilution in the same blocking buffer at 4°C. After washing shown as above, the membrane was incubated with the secondary 1:5000 diluted anti-mouse IgG antibodies conjugated with horseradish peroxidase in the blocking buffer at room temperature for 1 hr. The membrane was washed again, developed using

Visualizer™ Spray & Glow™ detection reagent (Millipore, Billerica, MA) and exposed to X-ray film.

2.6 Immunization, mouse polyclonal antibodies and spleen cells

Female HLA-A2 transgenic C57BL/6 mice (6-8 week old) were subcutaneously immunized with 100 µL volume containing 100 µg purified recombinant HERV-K TM fusion protein each mixed with the same volume of complete Freund's adjuvant (CFA). Three more boosters were given at the same dose in incomplete Freund's adjuvant (IFA) at weeks 4, 5, and 6. Another group of mice, considered as mock mice, were inoculated with a control consisting of 100 µL PBS mixed with the same volume of adjuvant.

Ten days after the last booster, the mice were sacrificed using CO_2 and bled from the heart. To collect the serum, the blood was left to coagulate at room temperature for more than 30 min and spun down at maximum speed for 5min at 4C. The harvested serum was stored at -80°C prior to analysis by enzyme-linked immunosorbent assay (ELISA) and immunofluorescence staining (IFS) assay.

The spleen cells from the immunized mice were also collected. Briefly, the spleen was removed aseptically and homogenized with homogenizer. The viable spleen cells were isolated by Histopaque 1083 density gradient centrifugation (Sigma, St. Louis, MO) following the manufacturer's instructions. Red blood cells were completely removed from the spleen cells using red blood cell lysis buffer (8.3 g/L NH_4Cl, 1 g/L $KHCO_3$, and 0.09 g/L EDTA). After washing with PBS, the spleen cells were frozen in liquid nitrogen and analyzed by enzyme-linked immunosorbent spot (ELISPOT) assay.

2.7 Primary cell isolation from human breast cancer clinical samples

Breast tumor or nonmalignant tissue was thoroughly washed 3 times with PBS and then minced into small pieces in a Petri dish using a sterile scalpel (Feather #10) in 5mL complete NOE culture medium (i.e., DMEM/M199 supplemented with 10% FBS and 1× Pen/Strep). Contents of the Petri dish were transferred into a 50mL conical tube. 30 mL of the complete culture medium was added to the sample and then mixed and spun down at 2,000 rpm for 5 min. The supernatant was decanted and the pellet was washed once using PBS. The pellet was digested with AccuMax (Sigma, St. Louis, MO) at 37°C for 1 hr. After spinning down at 2,000 rpm for 5 min, the pellet was resuspended in 40 mL complete culture medium. The sample was homogenized by inserting a Stomacher 80 bag containing the sample into a Stomacher 80 (Seward, West Sussex, UK) and running the Stomacher 3 times for 2 min at high speed. The sample was transferred to a new 50 mL conical tube and spun down again. After washing, the primary human breast tumor cells were placed onto a 6-well plate in 12 mL of complete culture medium and incubated in a 5% CO_2 incubator at 37°C.

2.8 Peripheral blood mononuclear cell (PBMC) isolation from human blood samples

PBMCs were isolated from the whole blood samples by density gradient centrifugation using Histopaque-1077 (Sigma, St. Louis, MO). Briefly, 10 mL of blood from a heparinized tube was transferred to a 50 mL Falcon tube and spun down at 2,000 rpm for 5 min at room temperature. The upper layer (i.e., plasma) was collected without disturbing the lower layers and stored at -80°C for future analysis. 30 mL of PBS was added to the remaining blood and mixed well by gentle pipetting. The diluted blood sample was then carefully and very slowly added to a 50 mL Falcon tube previously

loaded with 10 mL Ficoll-1077 without breaking through the Ficoll layer. The tubes were spun down at 1,500 rpm for 30min at room temperature using slow acceleration and deceleration rates. PBMCs were recovered by collecting the all three layers (i.e., PBS, PMBCs, and Ficoll layers) without the bottom layer of erythrocytes and granulocytes with a 10 mL pipette and placed to a new 50 mL tube. The cells were washed by adding 30 mL PBS and spun down at 1,000 rpm for 10 min at room temperature. The supernatant was discarded. 5mL of RBC Lysis Solution (8.3g NH_4Cl, 1.0g $KHCO_3$, and 1.8ml of 5% EDTA in 1L distilled H_2O)was added to the cells and the mixture was incubated for 5 min at room temperature. After washing three times with PBS, the PBMCs were re-suspended in complete RPMI 1640 culture medium at 5×10^6/mL, seeded in a 6-well plate, and cultivated in a 37°C incubator overnight for dendritic cell (DC) preparation as described below.

2.9 DC and *in vitro* stimulation (IVS) cell preparation

For generating DCs, non-adherent cells were removed from the overnight culture of PMBCs with a pipette. The wells containing the adherent cells were gently washed with 2 mL/well of RPMI 1640 kept at 37°C to remove the remaining non-adherent cells. The washing step was repeated once. 2 mL/well of fresh complete RPMI 1640 was added to the plate and cells were incubated with 1,000 units/mL each of granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4. Six days later, the immature DCs were harvested from the plate with a pipet and pulsed with HERV-K SU or TM protein using BioPORTER protein delivery reagent (Genlantis) according to the manufacturer's instructions. DCs were incubated with a protein/bioporter mixture for 4 hrs at 37°C. After washing, the pulsed DCs were cultured overnight in complete RPMI

1640 medium containing 7.5 ng/mL tumor necrosis factor-α (TNF-α) to induce DC maturation.

To prepare *in vitro* stimulation (IVS) cells, autologous PBMCs were added to protein-pulsed DCs at a DC to PBMC ratio of 1:30. The cells were cultured in the presence of 10 units/mL of rhIL-2 and the culture medium with rhIL-2 was exchanged every 2-3 days. After 7 days, IVS cells were collected and ready for analysis.

2.10 Immunofluorescence staining (IFS) assay

Indirect IFS assay for the analysis of HERV-K TM Env protein expression was performed by seeding cultured cells (e.g., MCF10A, MDA-MB-231, SKBR3, and primary cell culture from human breast cancer tissue as well as uninvolved breast tissue) on sterile glass cover slips in 6-well plates (50,000 cells per well) and incubation overnight in a 37°C incubator. After washing once with PBS, the cells were fixed and permeablized in 100 µL of Cytofix/Cytoperm solution (BD) per slip for 20 min at room temperature. The cells were then washed twice with Perm/Wash buffer (BD) and incubated with 100 µL blocking buffer (3% BSA in BD Perm/Wash buffer) per slip for 30 min at room temperature to block the nonspecific binding. After washing, the cells were stained by sequential incubation with mouse antisera against TM diluted 1:100 in blocking buffer for 1 hr at room temperature and anti-mouse IgG conjugated with Alexa Fluor-488 diluted in 1:200 for 30 min at room temperature. Control staining was performed with the corresponding sera from mice injected with PBS. After washing again, the cellular nuclei were stained with 4',6-Diamidino-2-phenyindole (DAPI; Invitrogen) according to the manufacturer's instructions. The cells were mounted with

90% glycerol in water and examined under a laser-scanning confocal microscope (LeicaSP5) with appropriate filters.

The protocol above was modified to characterize the epithelial phenotype of primary cell culture from breast cancer patient samples. Anti-vimentin (1:100) and anti-pan-cytokeratin (1:100) polyclonal antibodies (Sigma) were used as the first antibodies.

2.11 Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to detect anti-HERV TM IgG antibodies in sera from human samples or mice as described previously (36). Briefly, the wells of a 96-well plate were coated with 100 µL/well of 10 µg/mL purified HERV-K SU or TM fusion protein in PBS and incubated overnight at 4°C. The coating solution was discarded and the plate was washed six times with PBS. The coated wells were blocked by adding 100 µL/well blocking buffer (3% BSA in PBS) for 2 hrs at room temperature. 100 µL/well diluted sera were added and incubated overnight at 4°C. After washing, 100 µL/well species-specific anti-IgG antibody conjugated with HRP (1: 4,000 dilution, Sigma) was added and incubated for 1 hr at room temperature. After washing again, 100 µL/well freshly-made ABTS substrate solution (200 µL ABTS and 10 µL 30% H_2O_2 in 10 mL citrate buffer) was added and the absorbance was measured with a 1420 multilabel counter (PerkinElmer, Shelton, CT) at 405 nm at different time points. The ELISA was performed in triplicate for each dilution.

2.12 Enzyme-linked immunosorbent spot (ELISPOT) assay

Direct ELISPOT assay was performed on a 96-well ELISPOT plate (Millipore) to quantify antigen dependent IFN-γ secreting mouse spleen cells. Briefly, the ELISPOT plates were pre-wetted with PBS for 45 min at room temperature and coated with 100

µL of 5 µg/mL anti-mouse IFN-γ antibody (Mabtech) per well by incubating the plate overnight at 4°C. The plates were washed once with PBS and blocked with 100 µL of 10% fetus bovine serum (FBS) in PBS per well for 40 min at room temperature. After washing with PBS, test spleen cells from immunized mice were added at 5×10^5 per well and re-stimulated with 20 µg/mL HERV-K TM protein in a total volume of 100 µL complete RPMI medium. The assay was performed in triplicate in all experiments. The seeded cells were incubated in a 37°C incubator for 18-24 hrs. After incubation, the plates were washed five times with PBS and incubated with 100 µL/well biotinylated anti-mouse IFN-γ antibody (Mabtech) at 1 µg/mL in PBS containing 0.5% FBS at room temperature for 2 hrs. After washing again with PBS, 100 µL of alkaline-phosphatase streptavidin (1/1000 in PBS containing 0.5% FBS; Sigma) was added to each well and incubated at room temperature for 1.5 hrs. The plates were washed again and developed for less than 10 min at room temperature by adding 100 µL/well of NBT/BCIP substrate (Sigma, St. Louis, MO) in distilled deionized water (ddH_2O) prepared following the manufacturer's instructions. The reaction was stopped by washing the plate three times with tap water. Spots were counted with the aid of an ELISPOT reader (C.T.L., Shaker Heights, OH). Only the number of TM-dependent IFN-γ secreting cells is reported in each case. The spleen cells from the mice immunized with PBS were run in triplicate as negative control to ensure that spot formation requires the immunization with the antigen. Keyhole limpet hemocyanin (KLH) was also used as a negative control in this assay. The results are expressed as the number of IFNγ secreting cells per 5×10^5 cells.

Anti-human IFN-γ ELISPOT assay was used for monitoring specific cellular immune responses against HERV-K SU and TM proteins in breast cancer patient

samples (30). The pre-wet ELISPOT plates were coated with 5 µg/mL of anti-human IFN-γ antibody (Mabtech) and incubated overnight at 4°C. The plates were then blocked with complete RPMI medium for 2 hrs at room temperature. PBMC, IVS TM or IVS SU cells were plated at 50,000 cells per well with the autologous DCs pulsed by corresponding proteins at 2,500 cells per well. The plates were incubated for 18-24 hrs at 37°C and developed and read as described above.

2.13 Statistical analysis

Analyses were done using GraphPad Prism version5. Statistical significance between groups was determined by a Student's t test. Difference among the populations with cancer at various phases was determined by a ANOVA analysis. A difference was regarded as significant if the p value was less than 0.05.

Chapter 3 Results

3.1 Production and characterization of TM protein

To experimentally characterize the TM envelope domain of HERV-K, we first developed the TM-expressing vector and generated the purified TM fusion protein. The construction of protein expression vector containing the HERV-K TM protein DNA sequence (pQE30-TM) was carried out according to the flowchart shown in Figure 4. Total RNA was derived from melanoma cell culture from a melanoma patient. A 591 bp DNA sequence coding for TM was amplified by RT-PCR and nested PCR, and then ligated with TA cloning vector pCR2.1 to form the recombinant plasmid pCR2.1-TM. The recombinant was identified by PCR and the fragment was analyzed by 1% agarose gel electrophoresis. Nineteen of twenty screened clones showed PCR product with the expected size.

To increase the possibility of obtaining translationable sequence without the premature stop codon, all of the nineteen successfully cloned recombinants were combined to prepare a small amount of plasmid mixture (Figure 5). After double digestion, gel extraction, and ligation reaction, the sequence coding for HERV-K TM was cloned from the mixed pCR2.1-TM into the prokaryotic expression vector pQE30 in *E. coli* host strain BL21. Among the twenty-four recombinants screened, ten were identified that contained the inserted sequence with the expected size by PCR and double digestion (Figure 6A and 6B). Furthermore, Coomassie blue staining and Western blot were carried out to confirm the ability of the recombinant vector to express the fusion protein of interest. Results showed that seven clones were positive for protein production (Figure 7). One clone (clone#23) was chosen for sequencing and sequence comparison

with several HERV-K TM sequences showed the cloned nucleic acid sequence most closely resembled (99% homology) the TM of HERV-K 37 (accession number: DQ112095; Figure 8). All the three positions in nucleic acid sequence from the clone 23, which are different from KERV-K37, occurred in the third base of the triplet codon, so there are no amino acid differences between them. The sequence is also highly homologous to the TM gene of HERV-K113 (96%; accession number: AY037928) and HERV-K115 (97%; accession number: AY037929), which are known full-length, intact HERV-K elements and which are polymorphic in human genome.

After induction with IPTG, the recombinant clone #23 containing HERV-K TM was expressed in *E.coli* BL21 to produce the 6-His TM fusion protein. The TM protein was purified using Ni-NTA coupled to agarose chromatography beads and purity was demonstrated by SDS-PAGE. A unique protein band with the predicted molecular mass (about 25 kDa) was observed using Coomassie blue staining and Western blot (Figure 9).

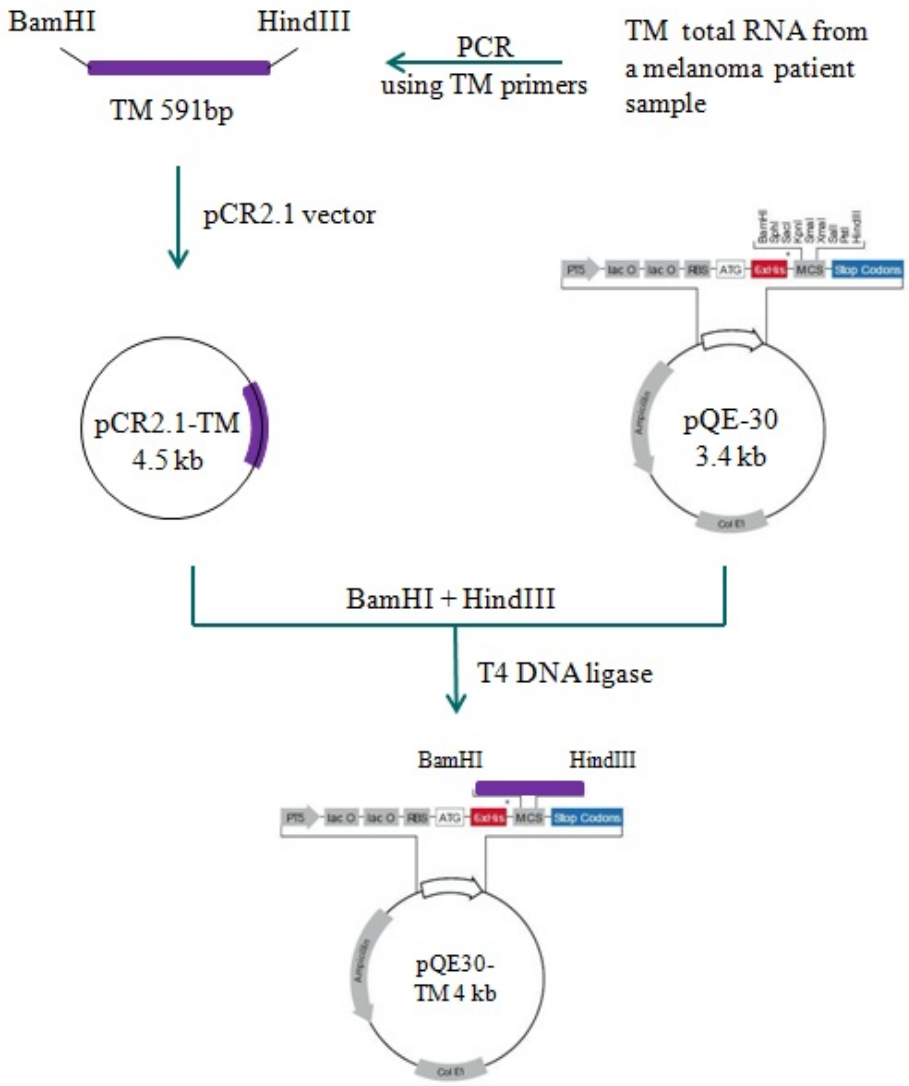

Figure 4 Flowchart of cloning and construction of the recombinant plasmid pQE30-TM expressing TM fusion protein of HERV-K.

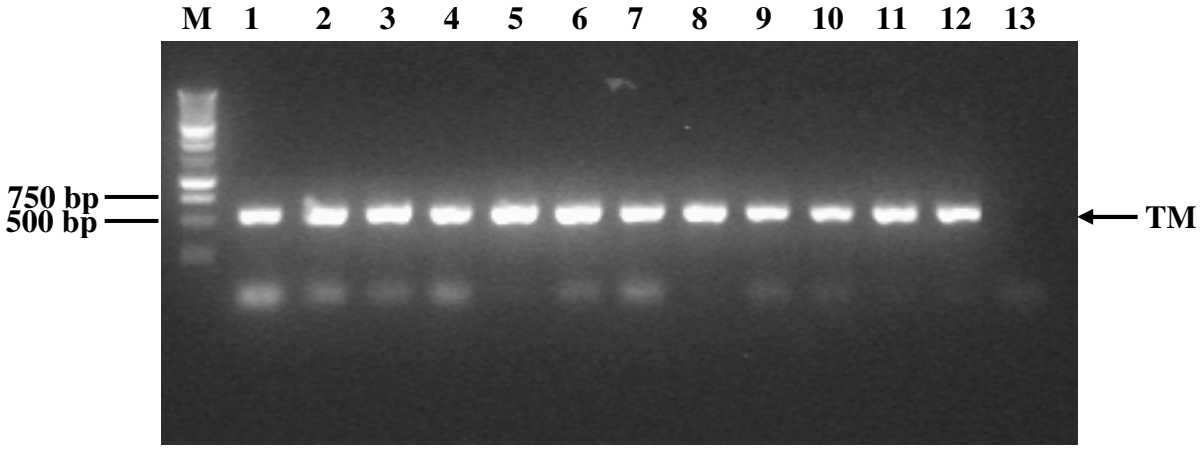

Figure 5 Screening of recombinant plasmid pCR2.1-TM by PCR.

Using standard procedures for ligation, HERV-K TM fragment amplified from a melanoma patient was cloned into pCR2.1 vector. The candidate recombinant clones were screened by PCR using TM outer primers. This figure shows that a DNA fragment with the expected size was amplified from the majority of the recombinant clones (clone #1-13) except for clone #13.

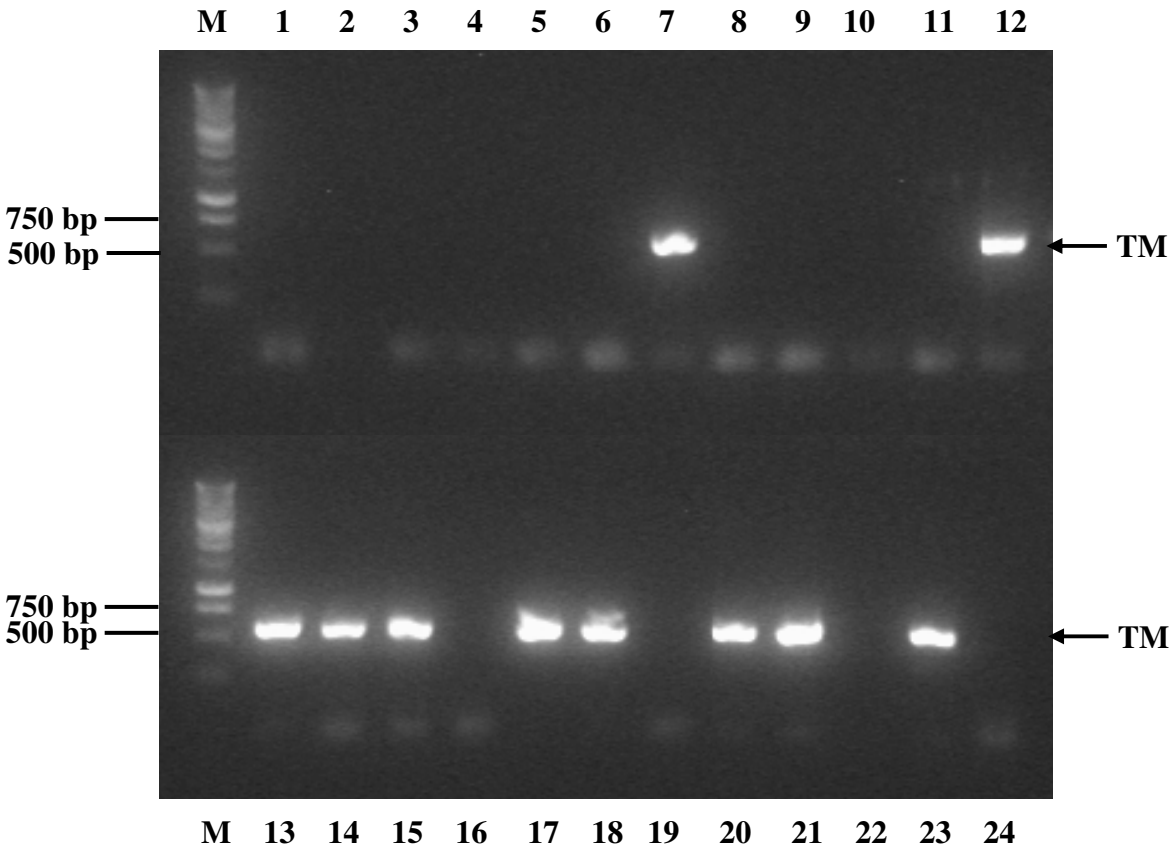

Figure 6 Screening and identification of pQE30-TM by PCR and double digestion reaction.

(A) The TM gene cloned into the pCR2.1 vector was ligated with the prokaryotic expression vector pQE30 using BamHI and HindIII sites to construct the recombinant plasmid pQE30-TM. After transformation, twenty-four clones of the candidate recombinant vector were screened by regular PCR with the TM inner primer pair under the conditions shown in the text. The figure shows ten of these clones (clone #7, #12, #13, #14, #15, #17, #18, #20, #21, and #23) that contained an insert fragment with the expected size.

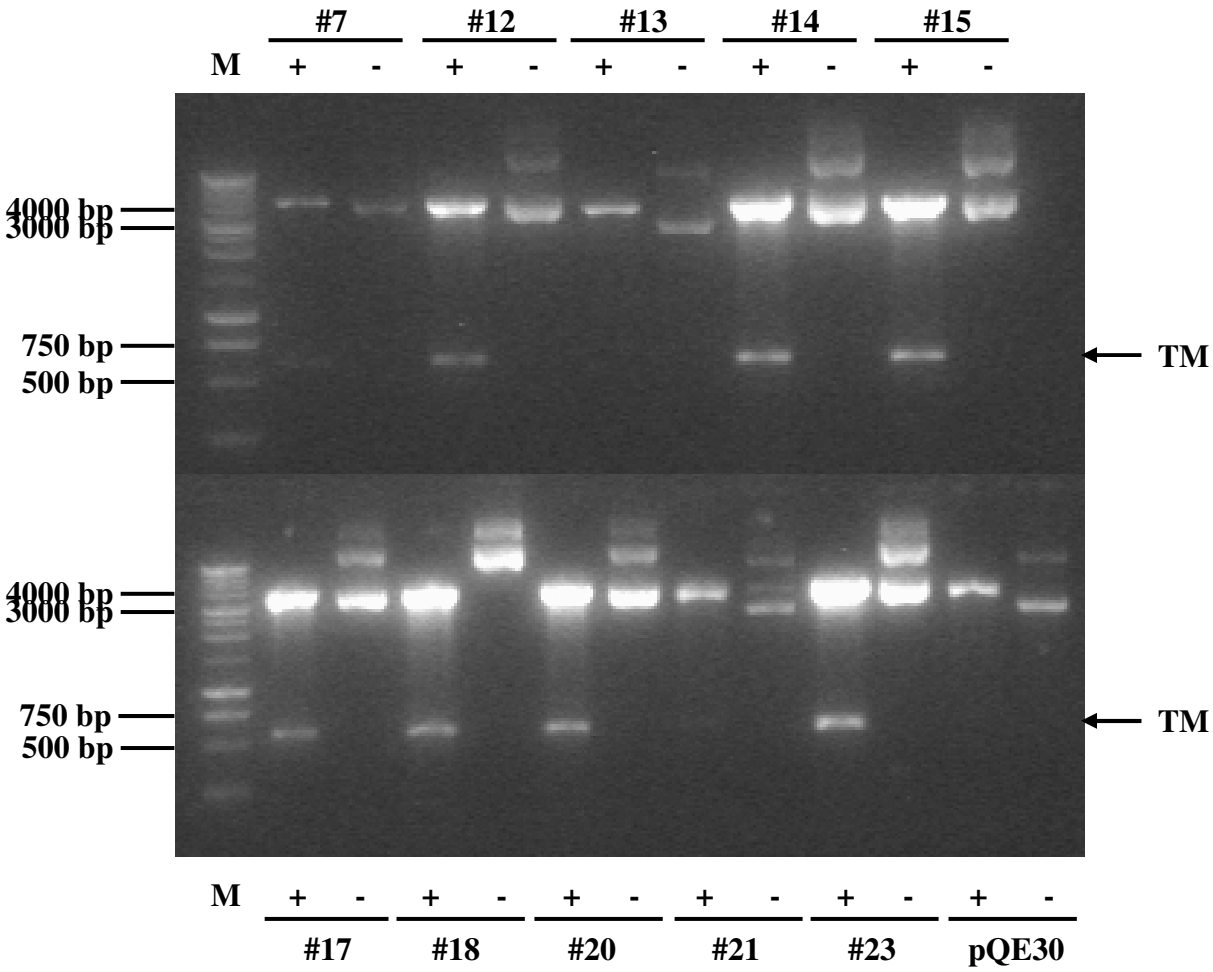

Figure 6 Screening and identification of pQE30-TM by PCR and double digestion reaction.

(B) The ten PCR-positive recombinant vectors were further characterized by double digestion reaction with BamHI and HindIII. This figure shows that all clones contained the insert fragment with the expected size. The empty vector pQE30 was also digested as negative control. (+: clones after digestion; -: clones before digestion)

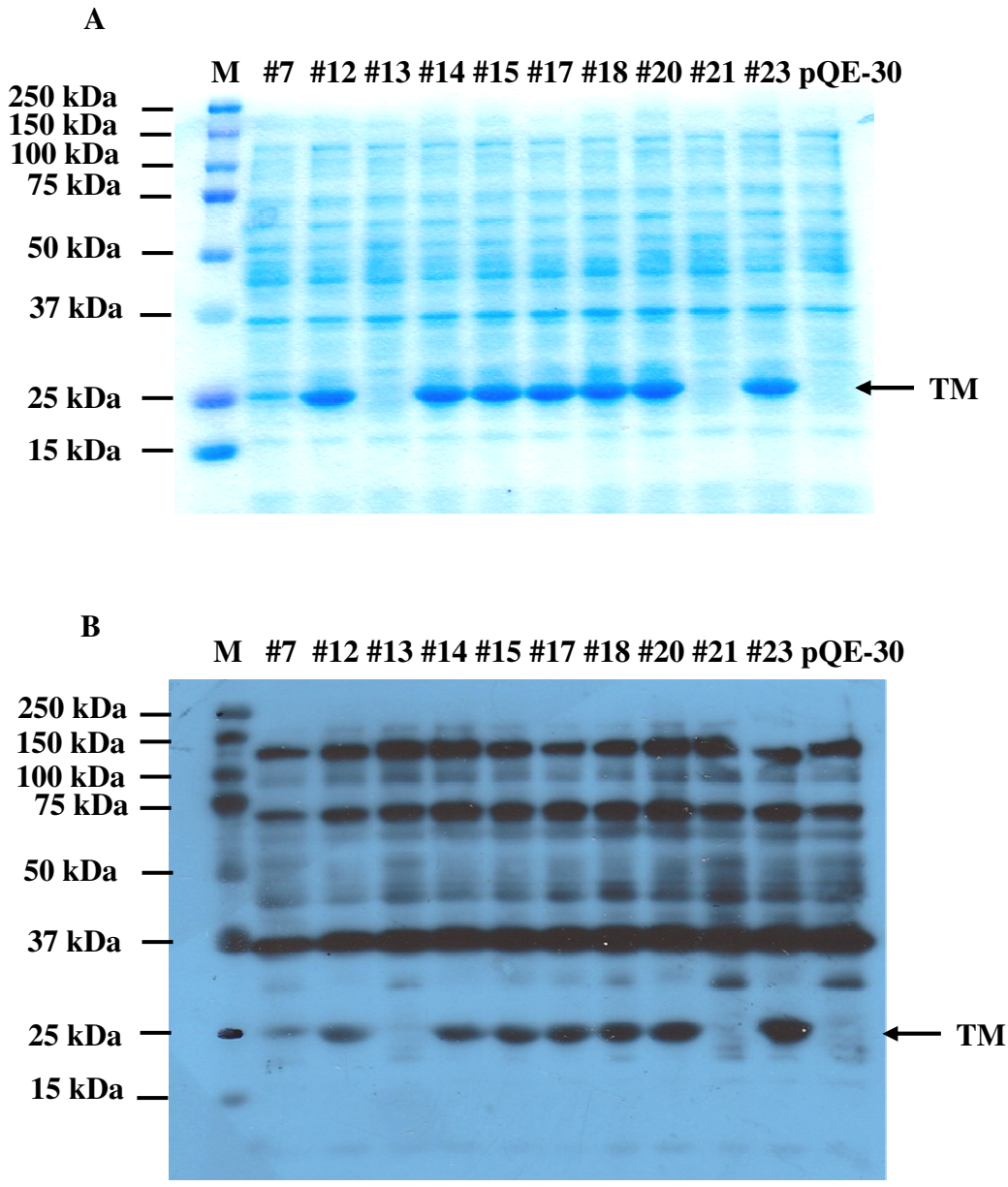

Figure 7 Expression of the recombinant TM fusion protein detected by Coomassie blue staining and Western blot.

The ten candidate recombinant clones identified by both PCR and digestion reaction were induced in the *E. coli* strain BL21 on a small scale by IPTG to express His-tag-TM fusion protein. The Coomassie blue staining (A) and Western blot (B) analysis demonstrated that seven of the ten clones were able to generate 25 kDa His-tag-TM fusion protein.

Figure 8 Nucleotide and amino acid sequence of HERV-K TM domain induced from clone #23 comparing the sequence of the TM fusion protein with that of HERV-K37 (GeneBank accession number: DQ112095). Three base pair differences are enclosed in boxes.

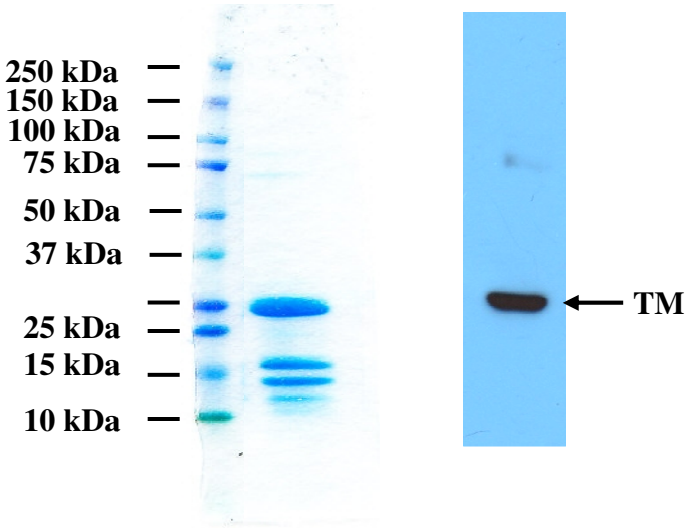

Figure 9 Purified TM fusion protein detected by Coomassie blue staining and Western blot.

The *E. coli* strain BL21 containing the pQE30-TM clone #23 was selected to express the TM fusion protein. The bacteria were induced by 1 mM IPTG overnight at 18°C and the His-tag-TM fusion protein was purified with affinity chromatography on a Ni^{2+}-NTA resin column. Coomassie blue staining (left panel) and Western blot (right panel) results demonstrated the purity of the TM fusion protein with the predicted size.

3.2 Humoral and cellular immune responses against TM protein in mice

We then asked if HERV-K TM envelope protein could be immunogenic in mice. To investigate whether the TM fusion protein could induce a humoral TM-targeted immune response in mice, we performed ELISA to analyze the antibody production by mice immunized with the recombinant TM fusion protein. The HERV-K TM fusion protein was produced in *E. coli* and purified. The HLA-A2 transgenic mice were immunized subcutaneously followed by three boosts at 1-week intervals. Mice mock-injected with PBS were used as a control. Sera were collected 10 days after the last boost and a serial dilution was prepared for testing the anti-TM antibodies by ELISA using TM fusion protein as the antigen. As illustrated in Figure 10, the HERV-K TM fusion protein was able to induce much higher TM-specific IgG titers ($p<0.0001$) in all of the immunized mice (n=3) than in the mock-immunized mice (n=3).

We next determined the HERV-K TM-specific T cell immune responses induced in mice assessed by anti-mouse IFN-γ ELISPOT assay. Spleen cells were isolated from the immunized mice and directly re-stimulated *in vitro* with TM fusion protein for 18-24 hrs in ELISPOT plates. Spleen cells from the mock mice were used as a control. As shown in Figure 11, significantly ($p<0.5$) higher numbers of IFN-γ secreting cells were observed in the mice immunized with TM protein than in the mock mice. The number of IFN-γ secreting cells was calculated by subtracting the average number of spots in wells of spleen cells re-stimulated *in vitro* by KLH protein from that of spots in wells of spleen cells stimulated by KTM.

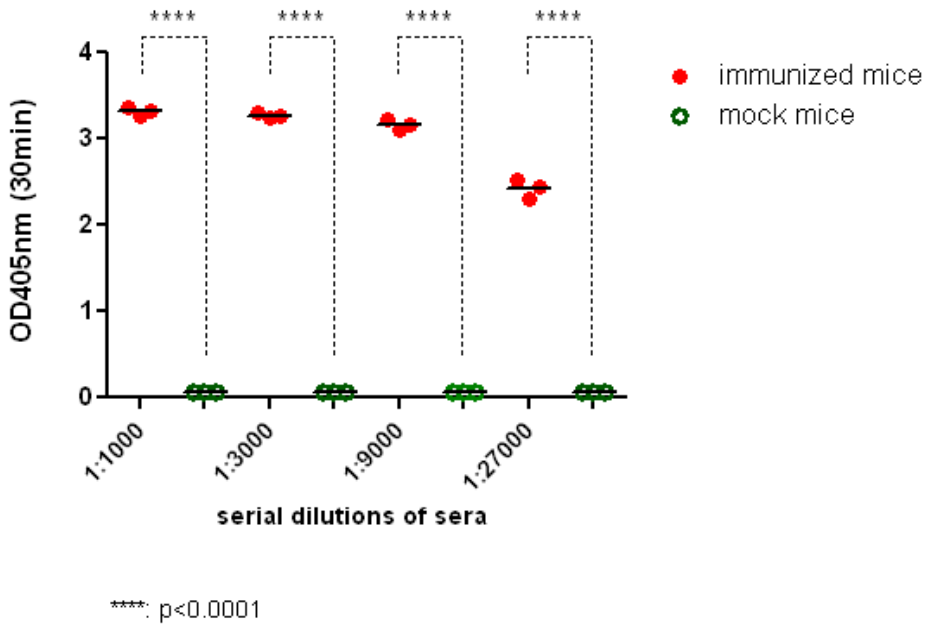

Figure 10 ELISA analysis of humoral immune response in mice immunized with HERV-K TM fusion protein.

Mice were immunized four times with 100 µg purified HERV-K TM protein per mouse in the presence of adjuvant. Ten days after the last boost, the mice were sacrificed and sera were collected. To test the anti-TM antibody in the serum, sera were diluted and analyzed by ELISA using the TM protein of HERV-K as antigen. This figure shows that anti-HERV-K TM antibody titer is significantly higher in mice immunized with TM protein than in mock-immunized mice.

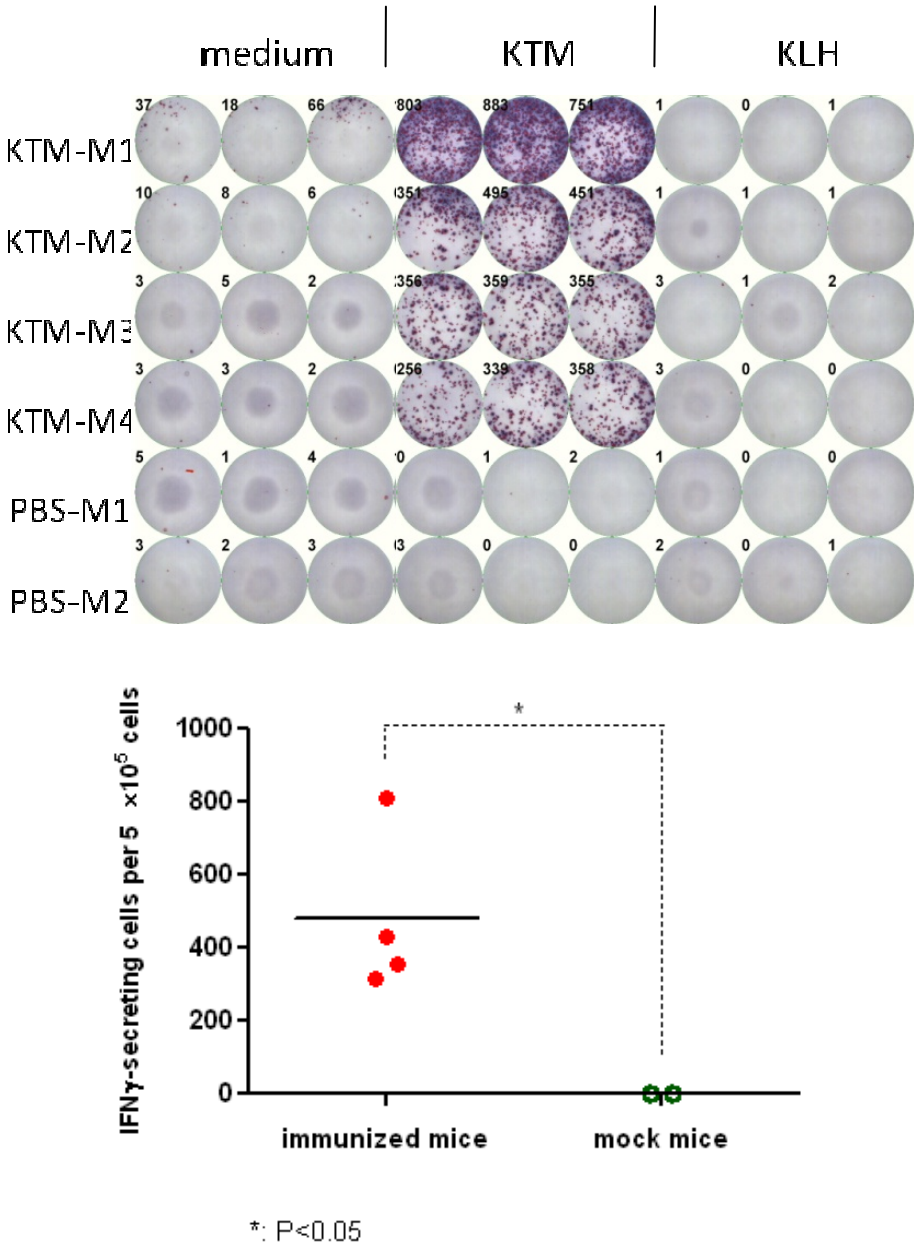

Figure 11 Anti-mouse IFN-γ ELISPOT analysis of the cellular immune response in mice immunized with HERV-K TM fusion protein.

Spleen cells were isolated from mice immunized with HERV-K TM protein or were mock-immunized (PBS) and then re-stimulated with TM protein (20 µg/mL) in ELISPOT plates (5×10^5 cells per well) for 18-24 hrs prior to spot development and counting. Data are presented as IFN-γ secreting spleen cells per half million spleen cells in TM-stimulated samples minus that measured in KLH samples. Significantly more IFN-γ secreting spleen cells were detected in the immunized mice than in the mock mice.

3.3 Expression of HERV-K TM envelope mRNA and protein in human breast cancer cells

To investigate the expression of HERV-K TM transcripts in human breast cancer cell lines, RT-PCR analysis was performed using HERV-K TM outer primer pair, which allowed the amplification of a 591bp DNA product. As shown in Figure 12, the expression of HERV-K TM mRNA was detected in all five tested human breast cancer cell lines (i.e., SKBR3, MDA-MB-453, MDA-MB-231, T47D, and MCF7) cultured in our laboratory, but not the normal breast epithelial cell line MCF10A, which showed a weak expression of TM mRNA.

We next determined whether HERV-K TM envelope protein was expressed in human breast cancer cell lines. To do so, we used a polyclonal antibody specific for the TM envelope protein of HERV-K and analyzed the cellular distribution of viral components by IFS staining assay. The HERV-K TM-specific antiserum was generated by immunization of mice with the purified TM protein produced by the recombinant clone pQE30-TM #23. Both MDA-MB-231 and SKBR3 cells were shown to express the HERV-K TM protein at approximately the same level (Figure 13 d and f), whereas cultured MCF10A cells were negative (Figure 13 b). These data are in agreement with the differences in expression of HERV-K TM-specific mRNA in all three cell lines by the RT-PCR method. None of the corresponding mock sera was reactive with any of the cells tested (Figure 13 a, c, and e). As can be clearly observed in Figure 13 d and f, the TM protein was broadly distributed in the cytoplasm, as expected for a protein synthesized in the endoplasmic reticulum.

To determine whether HERV-K TM protein could be detectable in human breast cancer, we performed IFS on clinical samples from breast cancer patients with detection using mouse anti-TM sera. Our results show that expression of HERV-K TM protein was specific for tumor cells (Figure 14). In contrast, the adjacent, non-involved breast cancer cells showed no TM protein expression. The epithelial phenotype of primary cell culture was characterized with anti-vimentin and anti-cytokeratin antibodies by IFS (Figure 15).

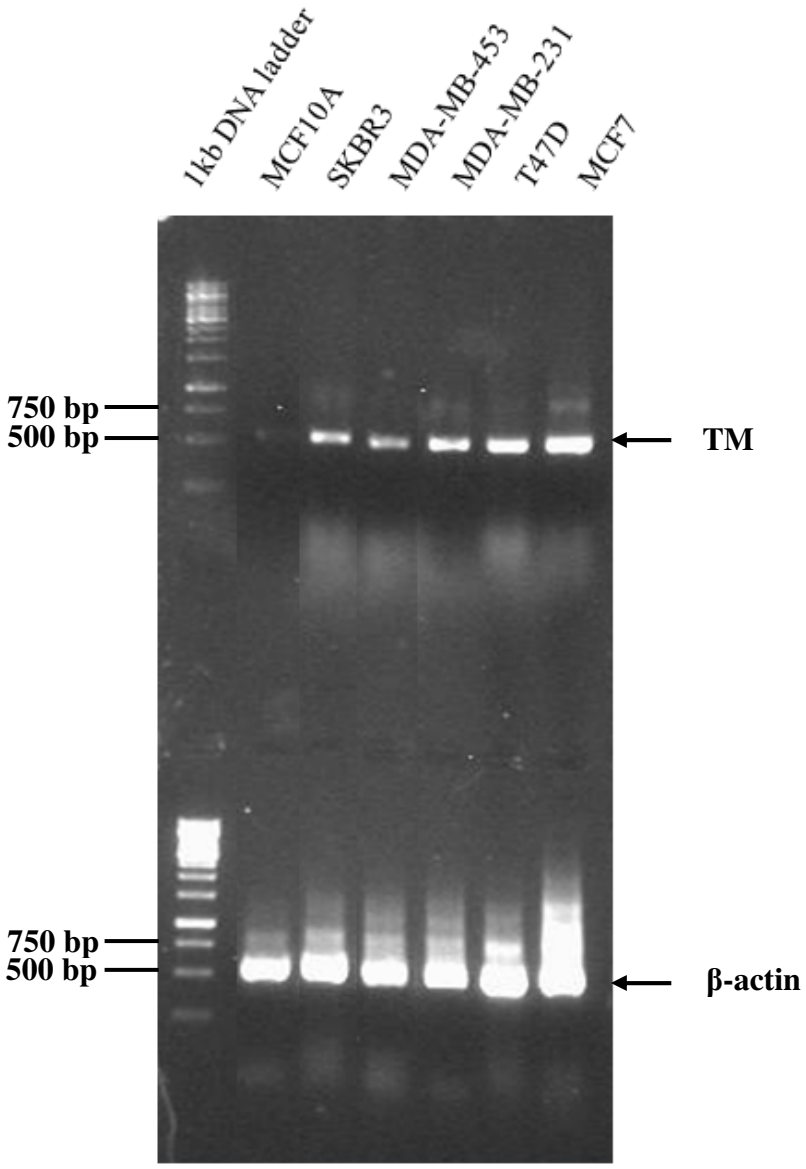

Figure 12 Expression of HERV-K TM mRNA in human breast cancer cell lines as well as a normal breast epithelial cell line by RT-PCR assay.

Cells from six human breast cancer cell lines (SKBR3, MDA-MB-453, MDA-MB-231, T47D, and MCF7) and one normal human breast epithelial cell line (MCF10A) were collected for total RNA isolation using Tri-reagent. Reverse transcriptase PCR was performed with the TM outer primer pair under the conditions described in the text. All of the six human breast cancer cell lines presented much stronger expression of HERV-K TM mRNA compared to that of the normal breast cell line. β-actin was amplified to confirm equivalent loading amounts of each sample.

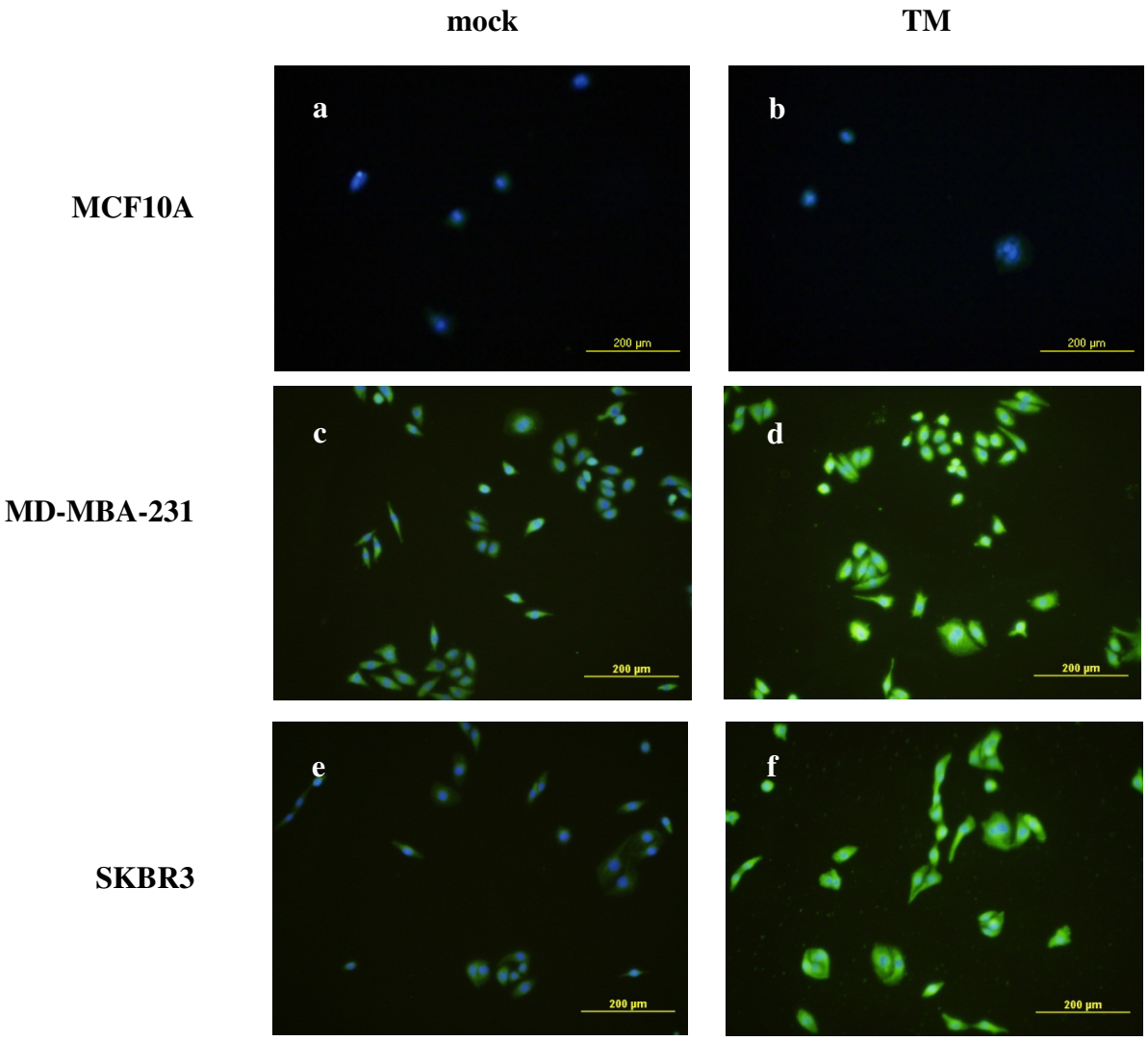

Figure 13 Expression of HERV-K TM envelope protein in human breast cancer cell lines as well as a normal breast epithelial cell line by IFS assay.

An immunofluorescence staining assay was performed approximately 24 hr post inoculation on the human breast cancer and normal breast cell lines grown on the glass cover slips. MCF10A, MDA-MB-231, and SKBR3 cells were fixed, permeabilized, and stained for HERV-K TM envelope protein expression with mouse anti-TM serum (b, d, and f) and with mock serum (a, c, and e), both at a dilution of 1:100 (whole-cell staining). The figure shows that HERV-K TM protein was expressed in human breast cancer cell lines, but absent in normal breast cancer cell line. Mock: serum from mice immunized with PBS; TM: serum from mice immunized with HERV-K TM protein.

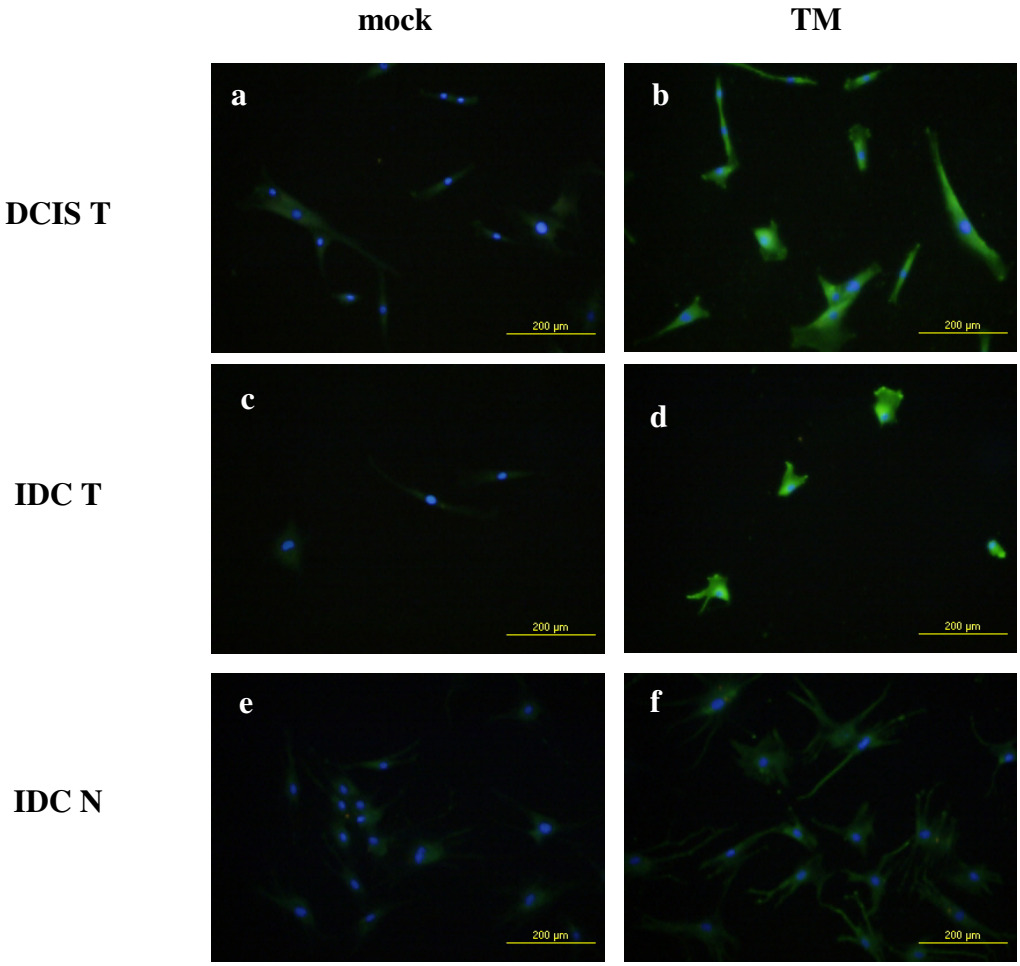

Figure 14 Expression of HERV-K TM envelope protein in human breast cancer patient samples by IFS assay.

An immunofluorescence staining assay was performed with HERV-K anti-TM antibodies on primary cells cultured from breast cancer patients. The figure shows the presence of HERV-K TM protein in breast cancer samples (b and d) but its absence from non-involved adjacent breast tissue samples (f). T: breast tumor tissue; N: uninvolved breast tissue; DCIS: ductal carcinoma in situ; IDC: invasive ductal carcinoma; mock: serum from mice immunized with PBS; TM: serum from mice immunized with HERV-K TM protein

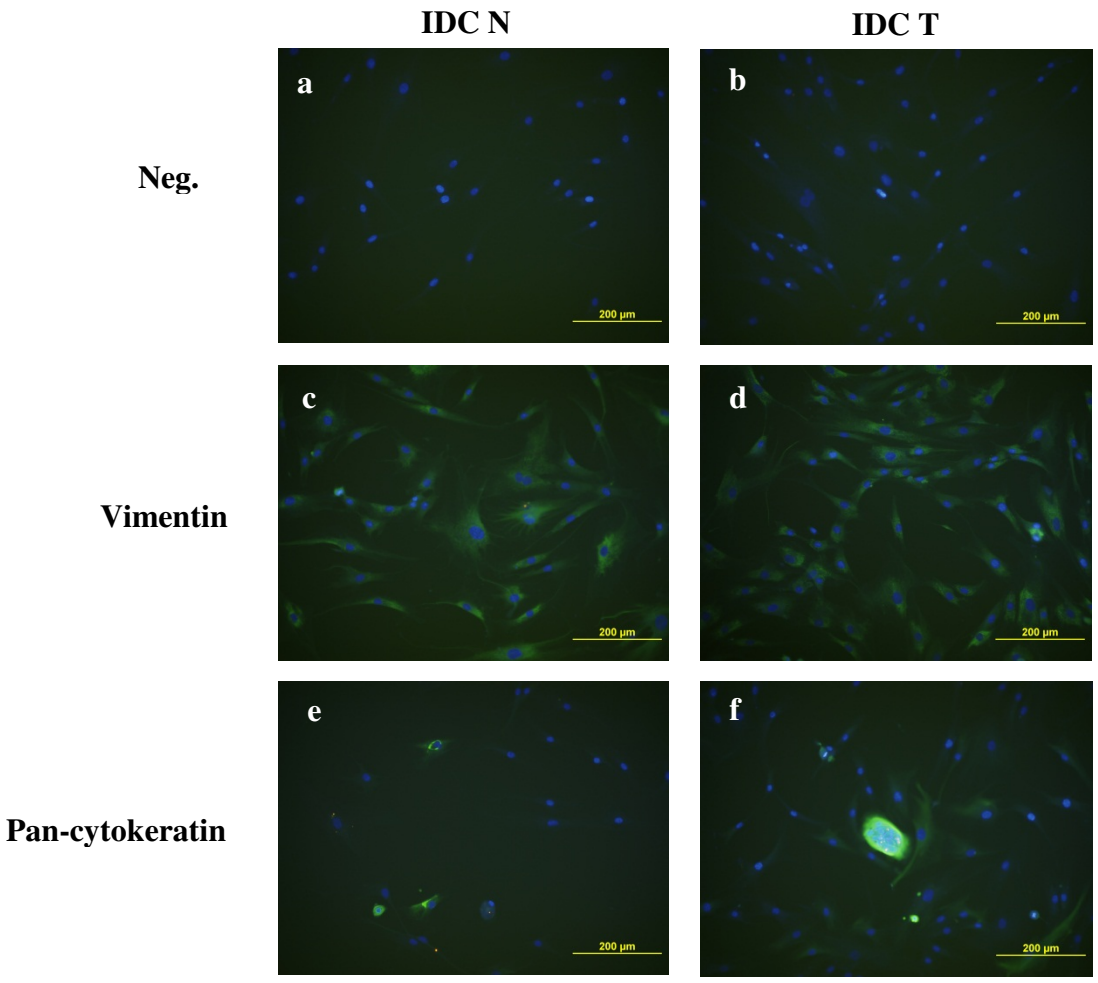

Figure 15 Characterization of epithelial cells and stromal cells of human breast cancer patient sample by IFS assay.

A representative IFS assay was performed with anti-vimentin and anti-pan-cytokeratin antibodies on primary cells cultured from one breast cancer patient. The figure shows the presence of epithelial cells from breast cancer sample (d and f) and non-involved adjacent breast tissue samples (c and e). T: breast tumor tissue; N: uninvolved breast tissue; IDC: invasive ductal carcinoma; neg.: no primary antibody used

3.4 Humoral and cellular immune responses against HERV-K TM envelope domain in breast cancer patients

We next determined the HERV-K TM-specific immune response induced in patients with breast cancer. First, we tested the antibodies against TM domain in sera from patient with breast cancer. To evaluate the correlation between the specific humoral immune response and severity of the disease, ELISA was performed with sera from breast cancer patients at different stages. Overall, the titer of TM-specific IgG antibodies was elevated in sera from patients compared to healthy donors. 46% of breast cancer patients (23 in 50) were positive for antibodies against HERV-K TM envelope protein (OD>0.5). It was to be noted that all of the tested patients with stage III breast cancer were positive for TM-specific antibodies (Figure 16). Whereas only around 11.1% of healthy donors (2 in 18) were positive for TM and the mean absorbance was below 0.5. Moreover, Figure 16 revealed an increased tendency for anti-TM IgG antibody titers in patients with breast cancer at higher stage, even though no significant difference was observed except between patients in groups with cancer in situ and stage III breast cancer. Similar results were documented for the IgG responses against HERV-K SU domain produced in the same cohort of breast cancer patients (Figure 17).

Second, we analyzed the T cell responses against HERV-K TM domain induced in breast cancer patients. To do so, PBMCs were isolated from the blood of breast cancer patients (see Table 2 for clinic information of patients) and corresponding healthy female donors. After *in vitro* stimulation of PBMCs by co-culturing with autologous DCs pulsed with HERV-K TM fusion protein for 1 week in the presence of rhIL-2, anti-human IFN-γ ELISPOT assay was performed as described in Materials and Methods. A representative ELISPOT experiment done with two breast cancer patients and two

healthy donors was shown in Figure 18. For the two breast cancer patient samples, a significantly increased number of spots was observed in the wells with IVS/TM cells re-stimulated with autologous DCs pulsed with HERV-K TM protein compared to those co-cultured with the DCs pulsed with control KLH protein. In contrast, in the two healthy donors, obviously higher number of IFN-γ secreting cells was not observed in the IVS/TM cells co-cultured with TM-pulsed DCs than in the IVS/TM cells with KLH-pulsed DCs. Furthermore, a much higher IFN-γ response was induced after IVS using autologous TM-pulsed DCs in the two patient samples than in the two normal donors. The similar increased IFNγ production was evident in the IVS/SU cells re-stimulated by protein-pulsed DCs in the two cancer patients compared to the two healthy individuals. The IFNγ ELISPOT data with 21 breast cancer patients and 12 healthy normal donors was summarized in Figure 19. Compared to the healthy donors, the results from the breast cancer patient samples showed that a significantly enhanced number of IFN-γ secreting cells was observed in IVS TM cells (Figure 19A) as well as IVS SU cells (figure 19B) after re-stimulation with autologous DCs pulsed with corresponding protein in the ELISPOT plates.

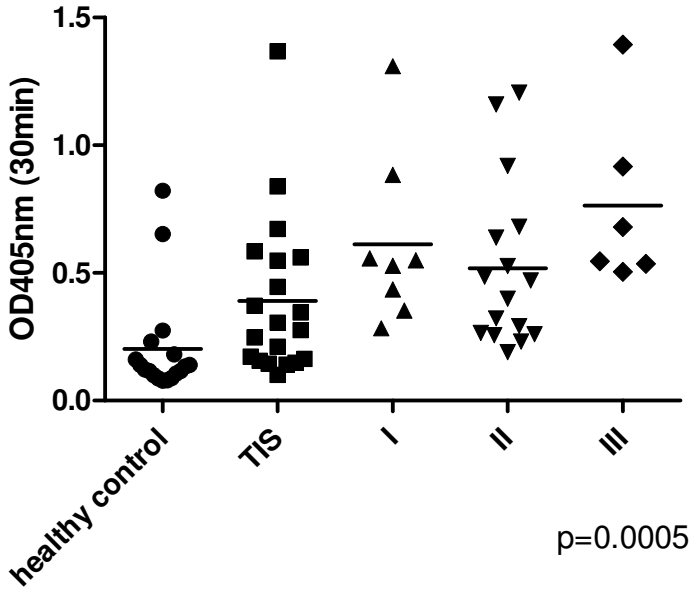

Figure 16 ELISA for anti-HERV-K TM antibody levels in serum from human breast cancer patients. ELISA assays to detect anti-HERV-K TM antibody levels were carried out in serum at a dilution of 1:100 from patients with breast cancer at different stages or from healthy donors. Purified HERV-K TM fusion protein was used as the antigen. Horizontal bars are shown as the mean and each point represents an average of three measurements for each sample. This figure shows that the tendency for the TM antibody level to be increased was likely related to the severity of breast cancer. The significant difference among the groups was analysed by ANOVA test.

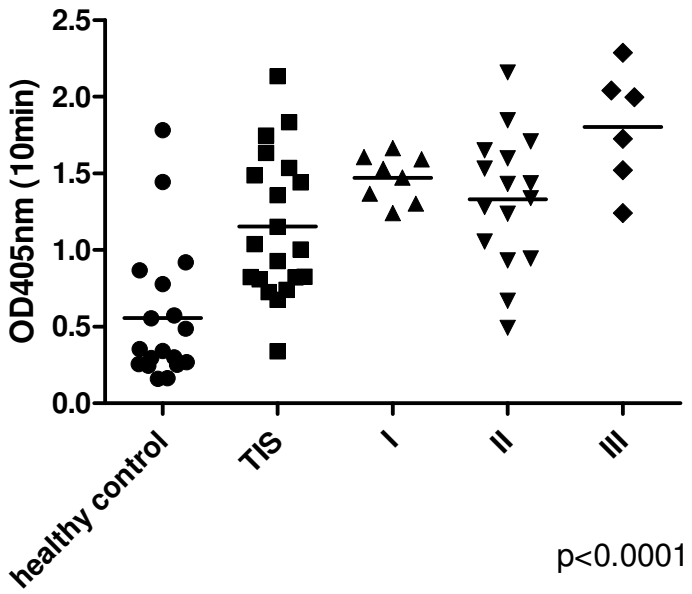

Figure 17 ELISA for anti-HERV-K SU antibody levels in serum from human breast cancer patients. ELISA assays to detect anti-HERV-K SU antibody level were carried out in serum at a dilution of 1:100 from patients with breast cancer at different stages or from healthy donors. Purified HERV-K SU fusion protein was used as the antigen. Horizontal bars are shown as the mean and each point represents an average of three measurements for each sample. This figure shows that the tendency for the SU antibody level to be increased was likely related to the severity of breast cancer. The significant difference among the groups was analysed by ANOVA test.

Table 2 Summary of clinical information for the 21 patients with breast cancer.

Patient sample	Age/Race/Sex	Diagnosis	ER	PR	Her-2/neu	LN status	Nuclear grade	Tumor Size (cm)
BC01	44/w/f	ILC	+	+	-	None	1	7.2
BC02	46/h/f	ILC	+	+	-	Left	2	1.5
BC03	67/w/f	IDC	+	-	-	None	1	5.0
BC04	83/w/f	IDC	+	+	-	None	1	1.5
BC05	60/w/f	IDC	+	+	+	None	2	2.5
BC06	50/b/f	IDC	+	-	-	None	2	1.0
BC07	64/w/f	IDC	-	-	-	None	3	1.7
BC08	44/w/f	IDC	-	-	+	None	3	3.4
BC09	55/w/f	IDC	+	+	-	Right	3	6.0
BC10	63/w/f	IDC	+	+	-	None	3	4.3
BC11	67/w/f	IDC	+	+	-	None	3	2.0
BC12	59/w/f	DCIS	+	+	n/a	None	2	n/a
BC13	50/w/f	DCIS	+	+	-	None	2	1.4
BC14	46/w/f	DCIS	+	+	-	None	2	2.7
BC15	68/w/f	DCIS	+	+	n/a	None	2	0.10
BC16	52/w/f	DCIS	+	+	n/a	None	2	2.3
BC17	60/w/f	DCIS	+	+	-	None	3	2.9
BC18	41/w/f	DCIS	+	+	n/a	None	3	2.0
BC19	58/w/f	DCIS	+	+	-	None	3	n/a
BC20	39/h/f	DCIS	+	+	-	None	3	1.9
BC21	55/w/f	DCIS	+	-	+	None	3	8.0
BC22	64/w/f	DCIS+IDC	+	+	+	None	3	n/a

Abbreviations: ILC, invasive lobular carcinoma; IDC, invasive ductal carcinoma; DCIS, ductal carcinoma in situ; LN, lymph node; PR, progesterone receptor; ER, estrogen receptor; n/a, not available

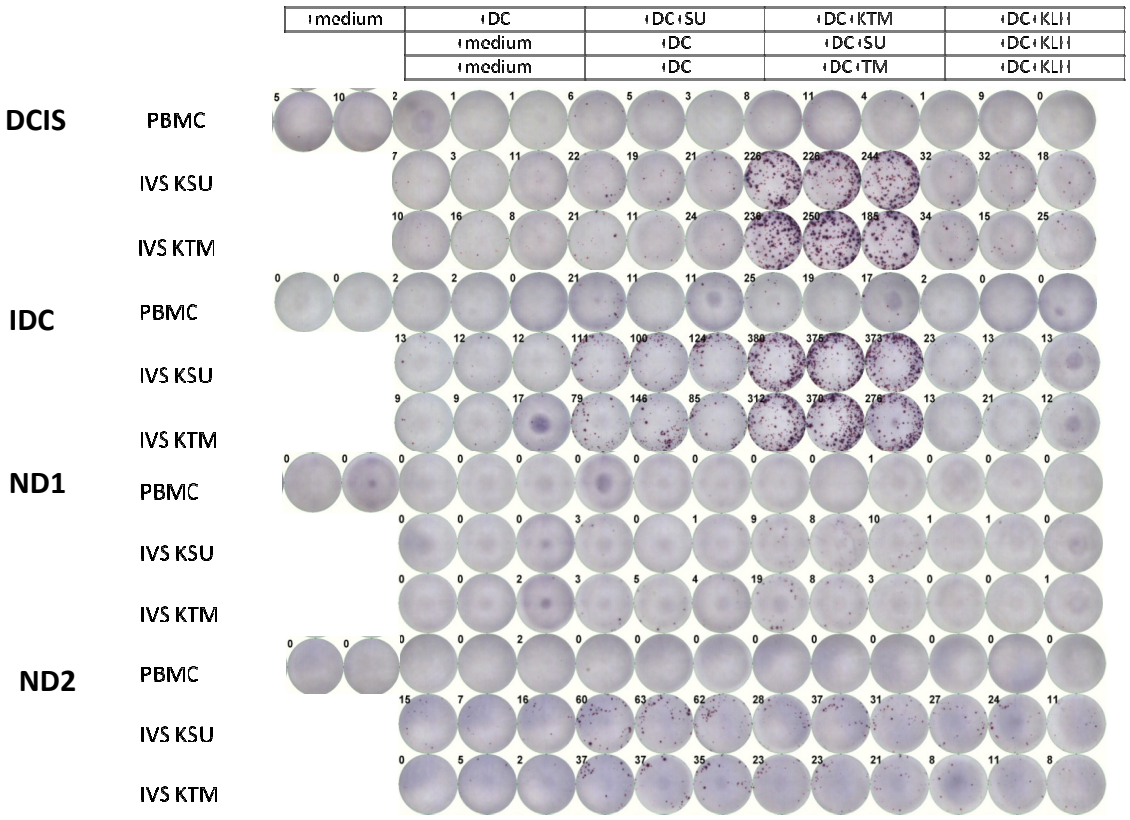

Figure 18 A representative ELISPOT assay for testing HERV-K SU/TM specific T-cell responses in human breast cancer patients and healthy donors.

PBMCs, IVS SU, and IVS TM cells (5×10^4 cells per well) from two patients with breast cancer and two healthy donors were co-cultured with autologous protein-pulsed DCs for 18-24 hrs as detailed in Materials and Methods. All samples were tested in triplicate. This figure shows that many more spots were detected in both IVS SU and IVS TM cells from two patients than from the two healthy donors. ND: normal donor; DCIS: ductal carcinoma in situ; IDC: invasive ductal carcinoma

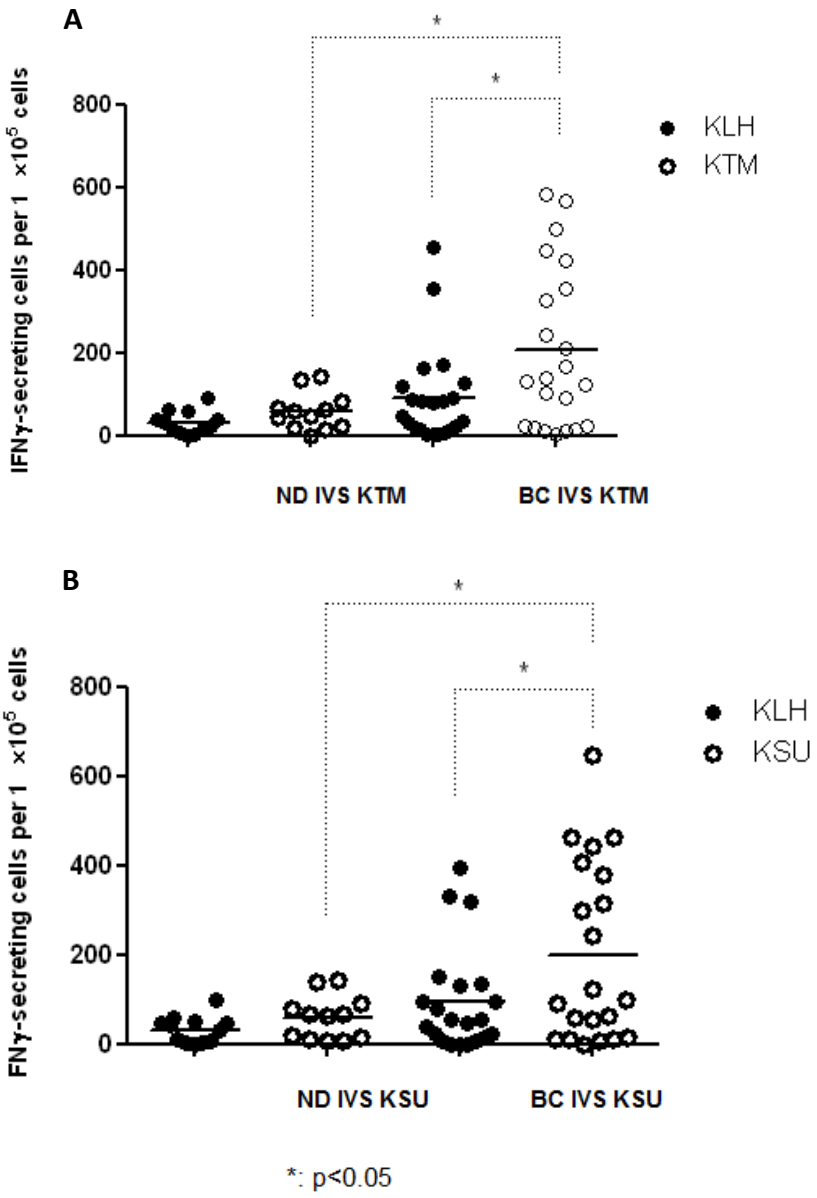

Figure 19 Quantitative assessment of HERV-K SU/TM specific T-cell responses in human breast cancer patients and healthy donors by anti-human IFN-γ ELISPOT assay.

These two graphs summarize the ESLIPOT data testing the HERV-K SU/TM specific T-cell responses in 21 patients with breast cancer as well as 12 healthy donors. Each circle represents the average amount of IFN-γ-secreting cells of three measurements for each sample and the horizontal bars show the mean value. The data suggest that specific T-cell responses against both TM (A) and SU (B) protein were induced in breast cancer patients. The black circles refer to the IFN-γ secreting IVS cells co-cultured with KLH-pulsed DCs and the white circles refer to those co-cultured DCs pulsed with HERV-K TM (A) or SU (B) protein. ND, normal donors; BC, breast cancer

Chapter 4 Discussion

In humans, the majority of the endogenous retroviruses are defective and not expressed although they make up about 8% of the human genome. Only HERV-K, however, has maintained intact ORFs for *gag*, *pro*, *pol* and *env*. The expression of mRNA and proteins of HERV-K and even the production of retroviral particles have been detected in some types of human cancers including breast cancer (30, 34, 37). Although enhanced levels of HERV-K SU *env* expression have been found in human breast cancer (30, 38), little is known about the expression of endogenous retroviral TM Env protein in human cancers (Table 1). Here, we confirmed and extended data on the expression and the immunological characterization of HERV-K TM envelope domain and revealed the potential value of TM as a promising tumor target for breast cancer diagnosis and immunotherapy.

In this work, we have isolated a gene encoding the HERV-K TM envelope domain from a human melanoma patient and expressed the purified TM protein. The TM gene sequence possesses extremely high identity (99%) with the sequence of the TM gene of HERV-K37 (Figure 8) and the purified TM protein possesses exactly the same amino acid sequence as the HERV-K 37 since all three variant nucleic acids are located in the third position of the triplet codons. HERV-K 37 was estimated to integrate into the germline genome approximately 0.5 million years ago and thus is considered an evolutionary young HERV-K family (39).

Although it was previously shown by our group and others that HERV-K SU *env* gene was expressed at both the mRNA and protein levels, to our knowledge, so far there has been no report showing evidence of TM domain expression in human breast cancer.

We have shown here that the HERV-K TM mRNA was expressed in all tested human breast cancer cell lines but not in the normal human breast cell line MCF10A by RT-PCR (Figure 12). Moreover, TM protein was exclusively expressed in human breast cancer cell lines as well as in human breast cancer specimens by IFS analysis (Figure 13, 14). These results supported the assumption that the HERV K TM env domain is specific to human breast cancer.

The detection of retroviral protein expression in breast cancer patients further raises the question about the association between the expression of retroviruses and the transformation and progression of human breast cancer. It is estimated that the re-activation of retroviral proteins in human cancers would be the consequence of increased transcriptional and translational activation during tumor development and progression. As a transposable element, HERV-K may also be actively involved in malignant transformation by insertional mutagenesis. In addition, there is recent evidence that two accessory proteins encoded by HERV-K, Rec and NP9, are potential oncogenes contributing to cellular transformation and induction of tumor formation in mice (40).

Another possible reason for retroviral protein overexpression in cancers concerns their correlation with the immunosuppressive activity of the retroviruses. Many retroviruses have been found to be capable of inducing immunosuppression to protect from immunologic rejection consequent of transformed cells (41). The expression of the envelope protein of retroviruses, including infectious retroviruses such as Moloney murine leukemia virus (MoMLV) and HTLV as well as endogenous retroviruses like HERV-H, enables the transformed cells to grow in allogeneic immunocompetent mice (22, 41, 42). It has been verified that the immunosuppressive activity is mediated by the

conserved transmembrane envelope protein of retroviruses, for example, p15E of MoMLV and feline leukemia virus (FLV) and gp21 of HTLV-1 and HTLV-2, which can inhibit lymphocyte proliferation and cytolytic activity of human natural killer cells, and modulate cytokine production (22, 43). A seventeen amino acid peptide CKS-17 (LQNRRGLDLLFLKEGGL), a synthetic peptide corresponding to a highly conserved domain (i.e., ISD) of the TM protein, has been found to be the exact domain presenting the immunosuppression activity (44). Some reports have verified that the retroviral ISD can induce very low IgG titers in mice, whereas the mutant non-IS form induces much higher humoral response (41, 45).

However, in this present study, we discovered that the HERV-K TM envelope protein is capable of triggering strong humoral immune responses. The ELISA results with mice showed that the TM protein induced significantly high titers of TM-specific antibodies in sera from all TM-immunized mice (Figure 10). The HERV-K TM envelope protein was further characterized in patients with breast cancer. Our results show that around 50% of the patients (n=50) with breast cancer produced antibodies directed against the TM protein of HERV-K (Figure 16). Such antibodies were detectable at a lower frequency in the healthy donors (11.1%, n=18) with lower mean absorbance value. However, it should be noted that a few healthy individuals had unexpected anti-HERV-K TM antibody, suggesting that events other than breast cancer may lead to the aberrant HERV-K TM expression and anti-TM immunity. Furthermore, research by another group suggests that antibodies directed against HERV-K TM envelope protein are also present in melanoma patients (34, 46). Such antibodies were detected at a very low frequency in normal blood donors. This discrepancy may be explained by phylogenetic

analysis based on the TM proteins. Although the TM domain is conserved and the ISD can be found in the majority of the known retroviruses, there are a few exceptions which do not possess the motif (including MMTV and HERV-K) (3).

It was documented that the detection of antibodies against HERV-K proteins was of diagnostic and prognostic value in patients with germ cell cancer and melanoma (34, 47). Consistently, our ELISA study performed here reveals a positive correlation between anti-HERV-K TM serum reactivity and tumor progression. Considering the limited population tested, however, further prospective studies are needed to confirm this result with a larger sample size.

Having identified the humoral immune response against HERV-K TM protein, it was important to determine the TM-specific T cell response. ELISPOT assay is a common used method to monitor T cell immune response in humans and animals. Here, we performed IFN-γ ELISPOT assay to determine the cellular immune response in immunized mice and breast cancer patients. Anti-mouse IFN-γ ELISPOT results show that a putative specific T cell response against HERV-K TM protein was identified in TM-immunized mice (Figure 11). Moreover, anti-human IFN-γ ELISPOT assay was performed using PBMCs from breast cancer patients after a single *in vitro* stimulation; results showed that HERV-K TM protein could induce significantly higher T cell response in breast cancer patients than in healthy donors (Figure 18, 19).

Although the exact role of HERV-K TM envelope protein in tumorigenesis remains unclear, the exclusive overexpression in breast cancer cells and the specific immune responses induced in patients with breast cancer make it a potentially valuable source of

tumor antigens for cancer vaccination and immunotherapy. Anti-HERV-K TM env antibodies may also be used as a screening tool for breast cancer.

Chapter 5 Conclusion and Future Studies

5.1 Conclusion

We constructed a prokaryotic expression plasmid containing the gene encoding HERV-K TM envelope domain (pQE30-TM) and generated purified 6-His tagged TM fusion protein. We found TM mRNA and protein were exclusively expressed in human breast cancer cells. The HERV-K SU immunity profile confirmed our previous findings (30); however, our research challenged the prevailing data regarding the immunity of retroviral TM protein in breast cancer. Our results suggest that HERV-K TM protein can induce both humoral and cellular immune responses in human breast cancer patients and immunized mice. Moreover, the data in our study clearly document a strong association between HERV-K TM antibodies and the clinical manifestations of breast cancer patients. Our findings expand the current knowledge of HERV-K in breast cancer and suggest HERV-K TM may represent a novel source of breast cancer antigen for cancer immunotherapy, prevention, diagnosis, and prognosis.

5.2 Future studies

Future research is required to validate our findings in a larger cohort of breast cancer patients and corresponding healthy donors. In addition, more assays, such as CTL assay and T cell proliferation assay, will be needed to confirm the immunogenic characteristics of HERV-K TM protein in breast cancer. Furthermore, determination of potent immunogenic peptides within the TM domain would provide the basis for generation of anti-tumor vaccines.

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