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In our grant application, we detected HERV-K viral particles by transmission electron microscopy (TEM) in sera from an invasive ductal carcinoma (IDC) patient and in BC cell culture media. In fact, HERV-K viral like particles have been found in a variety of tumor cells, as reported by others. These viral particles would be expected to have RT activity. The RT activity of various breast cell culture media was compared, and we found that all cancer cells had higher RT, compared with MCF-10AT cells. In addition, two IBC cell lines (KPL-4 and SUM149) had increased RT activity.

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Project 1: To identify infectious retroviruses in patient specimens:

1.1 Expression of HERV-K env mRNA or protein in human inflammatory breast cancer (IBC) cells and tissues: Prior to determination of reverse transcriptase (RT) activity in blood or tissue samples, expression of human endogenous retrovirus type K (HERV-K) env RNA and protein was evaluated in additional breast cancer (BC) tissues and cell lines by RT-PCR, western blot, and immunofluorescence (IFS). We found that HERV-K env mRNA (Fig. 1A) and protein (Fig. 1B and Fig. 1C) are expressed in IBC cells (SUM-149, KPL-4, and MDA-IBC3) or primary BC biopsies (Acc177, Acc185, Acc168, and Acc204), but not in MCF-10A cells and in normal breast tissues (ND263 and ND273).

![Fig. 1](image)

**Figure 1** (A) The expression of HERV-K env type 1 or type 2 mRNA (ty1 and ty2) was detected in invasive ductal carcinoma (Acc177, Acc185, Acc168, and Acc204), but not in normal breast tissues (ND263 and ND273) by RT-PCR. β-actin was used as control. (B) The expression of HERV-K env protein was detected in BC cells (MCF-7, SUM-149, and MDAMB231) but not expressed in MCF-10A benign human breast cells by Western blot using anti-HERV-K mAb (6H5). β-actin was used as control. (C) The surface expression of HERV-K (green fluorescence) was demonstrated on malignant cells (KPL-4, MDA-IBC3, and SUM149) by IFS using 6H5. mlgG was used as control.

1.2 We will determine reverse transcriptase (RT) activity in blood or tissue samples: (months 0 to 48)

In our grant application, we detected HERV-K viral particles by transmission electron microscopy (TEM) in sera from an invasive ductal carcinoma (IDC) patient and in BC cell culture media. In fact, HERV-K viral-like particles have been found in a variety of tumor cells, as reported by others. These viral particles would be expected to have RT activity. The RT activity of various breast cell culture media was compared, and we found that all cancer cells had higher RT, compared with MCF-10A or MCF-10AT cells. In addition, two IBC cell lines (KPL-4 and SUM149) had increased RT activity (Fig. 2). The highest RT activity was demonstrated in density gradient fractions (DGF) 5 to 6 in both cell lines. Importantly, RT activity was demonstrated in RNA, not DNA using SYTO RNA selected stain.

![Fig. 2](image)

**Fig. 2** (A) RT activity was compared in cell culture media from the IBC cell lines KPL-4 and SUM149. Serial dilutions of murine leukemia virus RT (Stratagene) were used as calibrators (data not shown). (B) RT activity was demonstrated in RNA, not DNA using SYTO RNA selected stain.

We also found increased RT activity in blood plasma samples from breast cancer patients. RT activity in DGF obtained from various donors including patients with invasive breast cancer (BC), or cancer patients with ductal carcinoma in situ (DCIS), or normal female donors was determined (Fig. 3). Fraction A (fractions 1 to 5) and B (fractions 6 to 10) were compared between normal donors vs. cancer donors (BC or DCIS). RT units were significantly higher in fraction A (Fig. 3A) or B (Fig. 3B) obtained from BC or DCIS patient plasma, compared with fractions obtained from normal female donors. In addition, RT activity was compared in tumor tissues vs.
uninvolved normal breast tissues obtained from the same donors. Significantly higher RT activity was demonstrated in most tumor tissues compared with matched uninvolved normal breast tissues (Fig. 3C). We reported RT activity in our grant application, and verified this increased activity in additional samples here, including IBC cells; we are preparing a manuscript to report all of these results.

In addition, HERV-K viral env mRNA or proteins with RT activity localized in gradient centrifugation fractions of cell culture media or patient plasma was detected by RT-PCR using HERV-K primers (data not shown), or by Western blot using anti-HERV-K env monoclonal antibody (6H5; Fig. 4). HERV-K viral env proteins were detected in only BC patient plasma (Fig. 4A), but not in plasma from normal female donors (Fig. 4B). The density of each fraction was labeled. Our data indicate that fractions with higher RT activity indeed express HERV-K.

1.3 Identification of infectious viruses: (months 6 to 42)
We determined the synthesis of infectious viruses in density gradient fractions after ultracentrifugation of plasma obtained from BC patients, and compared to fractions having the same density as that obtained from other cancer patients with high or low RT activity. Plasma samples obtained from women without cancer were used as controls. Viruses were labeled in buffer containing AlexaFluor 546 – dUTP and other reagents necessary for the endogenous reverse transcription (endo-RT) to proceed. Then viruses from plasma (0.2% of the fraction volume) (0.6 µl) or from BC tissues (2% of the fraction volume) (6 µl) were used to infect target cells (C3.555 feline cells) in media containing 4 µg of Polybrene/ml for 3 h at 37°C. Cells were washed and incubated in regular medium for 3h, 8h, or 96 h after infection. Viruses that integrated into target cells were observed between 8 hr and 96 hr (Fig.5C and Fig.5D).

This technique in Fig.5 allows us to visualize the viral genome once it is delivered in target cells. We are the first to demonstrate that HERV-K viruses isolated from BC patients with IDC are able to infect target cells. Our data provide strong evidence for infection and activation of HERV-K virus in BC, which may impact BC development and tumorigenesis.

Project 2: To evaluate anti-viral antibodies and viral RNA as detection and progression biomarkers:

2-1 Evaluation of anti-HERV antibodies as biomarkers for BC: (months 3 to 46)

In our grant application, RT-PCR was used to confirm viral load in sera by qRT-PCR, demonstrating that HERV-K viral mRNAs were indeed present in cancer patient sera. Our data indicate that HERV-K antibodies (Fig.6A) and even viral particles are present in BC patient sera, especially in patients with DCIS.

2.2 Determine the viral load as a potential biomarker for early detection: (months 6 to 42)

HERV-K was quantitated by qRT-PCR using HER-K env SU primers from RNA isolated from donor sera from patients with different stages of breast cancer. The viral load was compared with viral load in normal female control sera (Fig. 6B).
Serum antibodies were also detected as markers of viral load. We synthesized and tested several HERV-K multiple antigen peptides (MAPs), which are protease and peptidase resistant, as early detection markers of BC. We compared normal female donors and patients with tumor in situ (TIS). The MAPs-based assays were able to discriminate between TIS and controls (Fig. 7) as well as our HERV-K protein-based assays, suggesting that synthetic peptides can be used instead of HERV-K proteins for detecting serum antibodies in ELISA assays.

**Fig. 7** Anti-HERV-K antibodies were detected using various HERV-K multiple antigen peptides (MAPs) (10 µg/ml) in BC patient (tumor in situ, TIS) and normal female control sera (1:200 dilution) by ELISA. Significantly higher titers were observed in TIS patients including MAPs 95-96 (P=0.033), 75-76 (P=0.0136), 92-93 (P=0.0266), and 73 (P=0.006), but not in MAPs 108 and 82-83. The PC+HC group is also a control group (female women without breast cancer).
2.3 Determine whether viral load can be used as a biomarker for predicting BC metastasis: (months 12 to 48)

Recently, sera taken from two groups of BC patients at diagnosis were matched for ER, PR and HER2 status. One group was metastatic BC patients (MBC) who developed metastatic breast cancer 3 years after diagnosis, and the other group (NED) showed no evidence of disease after 3 years. Significantly (P = 0.0415) increased expression of HERV-K in MBC (N=56) over NED (N=56; Fig. 8A) was demonstrated in our lab using the Wilcoxon rank-sum test (Fig. 8). These results validate previous findings from a pilot study, where MBC (N=21) had higher expression than NED (N=21) (P = 0.034), and provide strong evidence that HERV-K is a predictive biomarker of metastatic BC. Sites of metastasis of these MBC are shown in Fig. 8B.

2.4 Determine whether HERV-K viruses promote tumor growth:

MCF-10AT cells were transfected with pLVX-Kenv or empty vector (pLVX), to overexpress HERV-K. The expression of HERV-K env RNA and protein in both cell lines was determined by RT-PCR, qRT-PCR, and Western blot (Fig. 9A). Cell proliferation and transformation was determined in both cell lines (Fig. 9B and 9C). Significantly increased proliferation (p=0.0307) and colony number (p<0.0001) was observed in MCF-10AT[pLVX-Kenv compared in the cells transfected pLVX. These results indicate that HERV-K env gene promotes non-cancer cell proliferation and transformation. Our next planned set of experiments will be to determine whether HERV-K overexpression promotes tumorigenicity in vivo in mouse models.

Fig. 8 (A) Significantly higher HERV-K env RNAs was demonstrated in patient sera with MBC compared in patient sera without metastasis (NED). (B) Metastasis sites from MBC patients were compared. Higher metastasis sites were found in brain, lung, bone, and others than liver and pleura.

Fig. 9 HERV-K env cDNA isolated from infectious viruses was cloned into a pLVX Lentivirus (Lenti) vector and transfected into MCF-10AT cells to generate pLVX-Kenv. MCF-10AT cells transfected with pLVX-Lenti vector only (pLVX) were used as control. (A) The expression of HERV-K env RNA was compared between pLVX and pLVX-Kenv, and there was significantly increased expression of HERV-K in MCF-10AT cells transfected with Kenv, compared with cells transfected with vector only (P=0.0002). The expression of HERV-K env protein was increased in MCF-10AT cells transfected with pLVX-Kenv, in comparison to MCF-10AT cells transfected with pLVX vector only cells (1.83 fold increased by Image J analysis), by Western blot using anti-HERV-K mAb (6H5). (B) Cell proliferation was compared in cells cultured for 3 days post-transfection. A significantly increased cell proliferation (p=0.0307) was observed in MCF-10AT cells transfected with pLVX-Kenv than in cells transfected with pLVX only. (C) A soft agar assay revealed a significantly higher number of colonies (p=0.0001) in MCF-10AT cells transfected with pLVX-Kenv than in the same cell line transfected with vector only.
2.5 Determine whether HERV-K viruses promote metastasis: (months 18 to 46)

A.

![Fig. 10 (A)](image)

Tumor sizes were compared between two groups of MDA-MB-231 cells stably transfected with shRNA targeting HERV-K env mRNA (shRNAenv) or with control shRNA (shRNAc) (Top, regular light. Bottom under fluorescent light). (B) Significantly reduced tumor weights were evident in the HERV-K knockdown group (p < 0.0005). (C) Lung tissues were compared between the two groups. Many more metastatic tumor cells were observed in MDA-MB-231 cells transfected with control shRNA (shRNAc), compared to MDA-MB-231 cells transfected with HERV-K env mRNA (shRNAenv).

After we observed increased proliferation and transformation in MCF-10AT cells (Fig. 9), we further explored HERV-K involvement in tumorigenicity and metastasis. Toward this end, MDA-MB-231 cells were stably transfected with shRNA targeting the HERV-K env gene or control shRNA with a scrambled insert (shRNAc). The tumor growth in NOD/SCID gamma female mice was compared in mice xenografted with MDA-MB-231 cells stably transfected with shRNA targeting HERV-K env mRNA (shRNAenv), compared with mice injected with control shRNA (shRNAc) (Fig. 10). Significantly reduced tumor sizes (Fig. 10A) and weights (Fig.10B) were demonstrated in MDA-MB-231 cells stably transfected with shRNAenv compared with shRNAc. These results indicate that HERV-K plays an important role in tumorigenicity. Furthermore, lung metastasis was compared in these mice bearing xenografts of cells transfected with shRNAenv or shRNAc. Lung tissues were harvested from both groups and the number of metastases formed was determined (Fig. 10C). Stronger green fluorescence was observed in pieces of lung tissue from mice injected with MDA-MB-231 cells transfected with shRNAc than with shRNAenv (bottom two panels). More metastatic cells were also observed in MDA-MB-231 cells transfected with shRNAc than with shRNAenv (top two panels).

In addition, MDA-MB-231 or MCF-7 breast cancer cell lines will be transfected with pLVX-Kenv to determine whether HERV-K overexpression promotes tumor growth. Cell proliferation and soft agar assays will be used to determine the changes in growth rates and cell transformation. Also, these cells will be injected through the tail vein and in situ, and metastasis to lung, liver, and other organs will be examined.

2.6 Determine associations of HERV-K with the p53 pathway: (months 8 to 47)

To establish that HERV-K is involved in oncogenic signaling pathways, we propose to investigate p53 and other mechanisms by which HERV-K triggers changes that result in the development of BC. In this Aim, we will continue to explore our finding of p53 involvement in HERV-K signaling, and focus on the p53 pathway in BC cell lines treated with anti-HERV-K monoclonal antibodies or shRNA to down-regulate HERV-K expression. We found that knockdown of HERV-K env with shRNA resulted in down-regulation of H-Ras, MDM2 and c-Myc mRNAs. c-Myc induces the tumor suppressor ARF, which inhibits MDM2 and leads to stabilization of the p53 protein 19. In this grant, qRT-PCR was performed to determine changes in expression of p53, MDM2 and c-myc in cancer cell lines stably transfected with shRNA targeting the HERV-K env gene, to evaluate whether HERV-K is affecting signaling via the p14ARF/MDM2/p53 axis (Fig. 11A). Changes in expression of these genes at the protein level were also determined using flow cytometry with various antibodies (Fig. 11B). A possible pathway involving HERV-K signaling in cancer is shown in Fig. 11C.
Fig. 11 (A) qRT-PCR was used to determine the changes in expression of several genes including HERV-K env, H-Ras, MDM2, and Tp53 RNAs in cancer cells transfected with shRNA targeting HERV-K env RNAs, relative to control shRNA. Down-regulation of HERV-K env RNA by shRNA targeting led to down-regulation of H-Ras, MDM2, and c-Myc, and upregulation of Tp53 in cancer cells transfected with shRNA. (B) Anti-HERV-K monoclonal antibody (6H5; 10μg/ml) was used to treat various breast cell lines in a single assay and changes in expression of various proteins including HERV-K, Caspases 3, 8, and 9, CIDEA, TNFSF8, TNFRSF10D, TP53, TP53AIP1, GML, and MYOD1 were determined. Increased expression of multiple proteins was observed in BC cell lines compared with non-BC cell lines (MCF-10A and MCF10A(T)), with the exception of TP53AIP1 in MCF-10A. 6H5 thus has an effect on expression of cancer-relevant proteins in BC cell lines. (C) Possible pathways through which anti-HERV-K antibody blocks BC proliferation and progression.

Project 3: HERV-K viral targets for immunotherapy:

3.1. Screening PBMCs from BC patient blood for T-cell responses: (Months 3–42).
An HERV-K specific chimeric antigen receptor (CAR) was generated from an anti-HERV-K single chain antibody generated from anti-HERV-K 6H5 antibody. PBMCs from BC (Fig.12A) or OC patient blood (Fig.12B) or other donors (Fig.12C) that were bearing the CAR were used for adoptive therapy (Fig. 12). MDA-MB-231 cells transfected with shRNA targeting HERV-K env RNA (shRNA) or control shRNA (cont) were used as target cells for CTL assays to determine whether or not the cytotoxicity depend on HERV-K expression. HERV-K specific CAR T cells were cytotoxic toward MDA-MB-231 cells treated with control shRNA, but not cells treated with shRNA targeting HERV-K env RNA, which down-regulated HERV-K env RNA expression in MDA-MB-231 cells. Our results indicate that HERV-K specific CAR killing depends on target cell expression of HERV-K.

Fig. 12 HERV-K specific CAR cytotoxicity toward breast cancer cells (MDA-MB-231) was determined using a CTL assay. HERV-K specific CAR T cells were generated from PBMC cells isolated from BC (Acc108; A), OC (Acc153; B), and a normal female control (ND#427478; C), and were used for CTL assays. MDA-MB-231 breast cancer cells stably transfected with HERV-K shRNA (MDAMB231 shRNA) or control shRNA (MDAMB231 control) were used as target cells for the CTL assays. Only MDA-MB-231 control cells, but not MDAMB231 shRNA cells, were killed by HERV-K specific CAR T cells obtained from BC, OC, and a normal female donor.

Furthermore, HERV-K specific T cells were generated from BC (Fig.13A) or a normal female donor (Fig.13B) and MDA-MB-435eb1 BC cells served as target cells (Fig.13). HERV-K specific T cells generated from a BC patient (Fig.13A) showed greater cytotoxicity toward MDA-MB-435eb1 cells than did T cells generated from a
normal female donor (Fig. 13B). CD3 and CD8 specific T cells from HERV-K specific T cells were determined by flow cytometry (Fig. 13C). These data indicate that greater numbers of HERV-K specific T cells were only generated in BC patients, but not in normal female donors who do not express HERV-K.

### 3.2. Development of tumor infiltrating lymphocytes for T-cell adoptive therapy: (months 4 to 44)

Tumor specimens from BC surgery patients were collected and tumor infiltrating lymphocytes (TIL) or normal uninvolved tissue infiltrating lymphocytes (NIL) were cultured. Mononuclear cells were cultured and expanded to lymphocytes according to the Statement of Work provided in “Supporting Documentation”. These cells were stored and banked for future use.

### 3.3. We will generate TCRs that target the HLA-A*0201-restricted epitopes of HERV-K env protein, and we will compare its antitumor effect with HERV-K CARs side-by-side: (months 12 to 46)

- mRNA will be isolated from sorted, highly avid CD8 T cells using a Oligotex Direct mRNA Mini kit.
- the anchor-sequence-containing cDNA will be synthesized from the mRNA obtained with a SMARTer PCR cDNA Synthesis kit (Clontech).
- TCR gene amplification will then be performed on the single-stranded cDNA using primers complementary to the TCR constant region and the SMART oligonucleotide.
- The PCR products will be cloned into pCRII vector and the inserts will be sequenced.
- The anti-tumor effects of HERV-K specific CAR vs. HERV-K specific TCR will be compared side-by-side in autologous cancer cells in vitro and in vivo.

### Project 4: Viral activation of the innate immune response in BC: (months 3 to 44)

4.1. Determine if HERV-K modulation effects (Toll-like receptor) TLR and RIG-I-like receptors (RLR) activation and signaling, and if so which TLRs and RLRs.

In this project, we investigate whether or not HERV-K has the ability to activate the innate immune response TLR and RLR families similar to exogenous viruses and other endogenous danger signals. First, the expression of TLR including TLR3 (green), 7 (red), and 9 (red) in various breast cell lines was determined by Western blot and IFS using their antibodies. One result is shown in Fig. 14. We will carry out these same assays after cells are infected with HERV-K viruses isolated from BC patients who have elevated RT activity.
4.2. We will assess the effect of HERV-K on antiretroviral genes and retroviral restriction factors in BC and normal breast: (months 4 to 45)
- Analyze the extent to which these restriction factors alter retroviral replication as a function of HERV-K status.
- Express Bst-2/tetherin in 293T cells, infect the cells with HERV-K and measure recovery of HERV-K from supernatants of tetherin-expressing cells.
- Block HERV-K with siRNA to see if the effect on restriction is specific for HERV-K and can reverse tetherin or APOBEC3 restriction.

4.3. We will evaluate changes occurring at very early stages of BC and during BC progression that have an impact on TLRs or RLRs: (months 5 to 46)
- Investigate the relationship between BC stem cells (CSCs) and HERV-K expression, evaluate the influence of HERV-K knockdown on various parameters associated with metastasis, and determine whether TLRs or RLRs are required for HERV-K to exert its effects in CSCs or during metastasis.
- The involvement of Polo-like kinases (Plks) as essential components of antiviral pathways will be determined in vitro and in vivo.

Co-localization (yellow; left panel) of TLR 9 (red; right panel) and HERV-K (green; center panel) was compared in MCF-10A and MDA-MB-231 cells (Fig. 15). Co-localization was prominent in MDA-MB-231 BC cells but not in the transformed but non-tumorigenic breast cell line MCF-10A, which showed low expression of both HERV-K and TLR9. Our data demonstrate for the first time co-expression of TLR9 and HERV-K in cancer cells. TLRs may thus be required for HERV-K to exert its effects on breast tumorigenicity. We will focus on evaluating interactions between TLR9 and HERV-K in upcoming studies.
5.1. We will develop rapid screening of high-affinity, highly cytotoxic human antibodies against BC from the memory B-cells of pre-screened BC patients using nanowell screening: (months 3 to 40)

In this project, a microengraving technique for validation of cytotoxicity of antibodies has been developed in our lab (see Fig. 16A). Anti-HERV-K mAb 6H5 hybridoma cells were used for testing our hypothesis before using memory B cells from BC patients. Single hybridoma cells were added single MDA-MB-231 cells and a CYTOX marker was used for measuring cytotoxicity. Single 6H5 hybridoma cells that included A1, A7, B3, B5, and C3 were selected as depicted in Fig. 16A and PCR was carried out as in Fig. 16B. Sequence analysis was further employed to confirm their sequences (data not shown).

A. B.

![Diagram showing nanowell screening process](image)

**Fig. 16 (A)** A nanowell screen has been developed in our lab which can rapidly screen for high-affinity single cell antibodies. Single cells obtained from 6H5 hybridoma cells were tested using this method. The single cells A1, A7, B3, B5, and C3 were selected using nanowell screening for their ability to kill target MDA-MB-231 BC cells. **(B)** Heavy chain and light chain fragments from the above single cells were produced by PCR using their primers, with the exception in C3, for which the light chain is missing.

- Using standard single-cell RT-PCR techniques we will amplify and clone the paired variable regions corresponding to these antibodies.

5.2. We will carry out molecular characterization of human anti-HERV-K env antibodies and quantify effector functionality *in vitro* using BC cell lines: (months 6 to 46)

- Clone into an Ig expression vector carrying the constant region of human γ1, Ck, and Cα, sequence and transiently express in 293T cells.
- We will carry out standard characterization (ELISA/Biacore) for affinity measurement.
- We will quantify antibody dependent cytolysis (ADCC) and complement dependent cytotoxicity (CDC) using annexin V and caspase assays and antibody mediated induction of apoptosis using caspase assays.

5.3. We will characterize therapeutic antibodies in a mouse model for BC: (months 12 to 46)

- Anti-tumor effects of human anti-HERV-K monoclonal antibodies (mAbs) will be tested *in vitro* and *in vivo*.
- Multiple assays will be carried out to determine the relationship between the expression of HERV-K env protein after treatment with human or murine anti-HERV-K antibodies (or control antibodies) and cell growth, apoptosis and tumorigenic potential of BC cell lines (soft agar assay).