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Tumor-associated antigens identified early in mouse mammary tumor development can be effective vaccine targets

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ABSTRACT

Breast cancer vaccines composed of antigens identified by serological analysis of cDNA expression libraries (SEREX) induce antigen specific immune responses in patients but have had disappointing clinical benefits. While many attempts to modify the adjuvants and vaccine method have been tried, one issue not addressed was whether the SEREX tumor-associated antigens identified from late stages of disease were ideal targets. We questioned in the transgenic TgMMTV-neu mouse model whether the antigen repertoire is distinct between early and late stage breast cancer and whether the antigens identified via SEREX from transgenic mice with early or late stage tumors would elicit differential anti-tumor effects to address this question.

Three early stage antigens, Pdhx, Stk39, and Otud6B, were identified from a SEREX screen of mice prior to development of palpable lesions. Formulated into a vaccine, each early antigen inhibited tumor growth ($p < 0.0001$). The antigens identified from mice with late stage tumors (Swap70, Gsn, and Arhgef2) were unable to inhibit tumor growth when used as vaccines (for example Gsn $p = 0.26$). Each of the three early stage antigens were essential for tumor survival in syngeneic mouse tumor cells and in human breast cancer cell lines across breast cancer subtypes. Silencing protein expression of the early antigens increased apoptosis ($p < 0.0001$ for all antigens in mouse and $p < 0.05$ for all antigens in human triple negative breast cancer) and decreased survival ($p < 0.0001$ for all antigens in mouse and human triple negative and HER2 positive breast cancer). Overexpression of the early stage antigens in women with breast cancer predicted worse prognosis ($p = 0.03$) while overexpression of late stage antigens did not impact prognosis ($p = 0.09$). These data suggest that antigens expressed earlier in breast tumor development and functionally relevant to breast tumor growth may be more effective targets for therapeutic breast cancer vaccines than antigens identified in later disease.

1. Introduction

Tumor-associated antigens have been identified through serological analysis of cDNA expression libraries (SEREX) from autoantibodies present in patients with existing disease as compared to individuals without cancer. Over 2000 breast cancer antigens have been identified by SEREX screening in patients including cancer testis antigens (e.g. MAGE and NY-ESO-1) and overexpressed tumor-associated proteins including HER2, CEA, and NY-BR-62 [1–4]. However, while the cancer vaccines designed from these antigens were immunogenic in early clinical trials, they were not effective at improving clinical outcomes in later phase trials [5–7]. To optimize vaccine efficacy, trials have modified the vaccine design using novel delivery systems and adjuvants [8–10]. Despite improving immunogenicity by changing adjuvants, clinical outcomes were not improved. There has been considerable effort focused on improving vaccine design but little focus on whether the antigens identified in patients with advanced stage disease are the best targets for immune destruction of the tumor [11–13].

The protein repertoire expressed in the tumor changes as a tumor develops through the accumulation of gene activation and inactivation events [14]. Therefore, proteins expressed later in disease may not be associated with common driver pathways as multiple alterations may occur. In evaluating primary component analysis of human breast cancers in metastases and primary tumors, the metastases cluster together and are different from both the primary tumors and genes expressed in normal breast [15]. The genes expressed in metastatic disease that are not found in primary tumors include genes to induce oxidative metabolism,

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activate tissue remodeling, and silence the extracellular matrix and may not be essential to cancer survival [16]. In early primary tumors, genes that are overexpressed are associated with proliferation and growth of the tumor and the genes necessary for metastases, genes of invasion and motility, do not give the primary tumor a selection advantage [17,18].

Since the protein repertoire of early and late tumors differs, the antigens exposed to the immune system in early and late tumor development are also different. In previous studies, we had demonstrated that serum autoantibodies were different between mice with early non-palpable tumors and mice with late stage, palpable tumors [19,20]. In this study, we questioned whether, as proof of principle, 3 antigens identified in the early stage and 3 antigens identified in the late stage breast cancer via SEREX screens could elicit differential anti-tumor effects in the TgMMTV-neu mouse mammary tumor model of breast cancer.

2. Methods:

2.1. Mouse model

TgMMTV-neu mice (FVB/N-Tg(MMTVneu)202Mul/J, strain #002376, Jackson Laboratory, Bar Harbor, ME) were bred under specific pathogen free conditions. The mice have a non-mutated non-activated rat *neu* under the control of the mouse mammary tumor virus (MMTV) promoter [21]. All animal care and use was done in accordance with the University of Washington Institutional Animal Care and Use Committee guidelines. The SEREX studies were performed as described previously [19,20].

2.2. Antigens

The early stage serum samples were collected from mice every 2 weeks starting at 4 to 6 weeks old until development of a palpable tumor (n = 20) [20]. The late stage serum samples were collected from mice that had developed spontaneous, palpable tumors (n = 10) [19]. The early stage tumor antigens (Pdhx, Stk39, and Otud6B) were autoantibodies identified from a SEREX screen of serum from mice 2 weeks prior to development of a palpable tumor. The late stage tumor antigens (Gsn, Swap70, and Arhgef2) were autoantibodies identified from a SEREX screen of serum of mice with palpable tumors [19].

2.3. Immunizations and tumor growth

Positive clones, unreactive to control sera from parental mice, were purified to monoclonality and converted to pBK-CMV phagemid by in vivo excision using XLOLR cells and ExAssist helper phage (Stratagene, LaJolla CA). Plasmid DNA was prepared using a Qiagen Mega kit (Qiagen, Valencia CA). The pBK-CMV3 plasmid with or without the indicated antigens was suspended in 50 μL of PBS with CFA/IFA and 50 μg was given by intradermal injection into the ear. For implant studies, five mice per group for the early stage antigens and three mice per group for the late stage tumor antigens were vaccinated three times approximately 14 days apart. A syngeneic MMC mouse carcinoma line derived from a spontaneous tumor in a TgMMTV-neu mouse was implanted 1 week after the last vaccination. The MMC cell line was harvested using 2 mmol/L EDTA in PBS and washed with PBS before injection. Mice were inoculated with 3×10^5 MMC cells subcutaneously on the mid-dorsum with a 23-gauge needle. For both studies, tumors were measured 3x/week with Vernier calipers and tumor volume was calculated as the product of length × width × height × π/6. Mice were euthanized when the tumor ulcerated, was > 1000 mm^3, or if the animal was ~1 year old. All mice were euthanized when the control mice developed clinically significant tumors.

2.4. IFN-g ELISPOT and Granzyme B ELISA

Mouse splenic cells were evaluated from FVB mice (n = 5) vaccinated with CFA/IFA adjuvant three times 10 days apart with 50 ug of empty vector or 50 ug of each of the plasmids for the early stage antigens (Pdhx, Stk39, and Otud6B) or late stage antigens (Arhgef2, Swap70, and Gsn). Fourteen days after the third vaccination, the mice were sacrificed, splenocytes were isolated, and IFN-g ELISPOT was performed as previously described. [22]. Briefly, 3.5×10^6 splenocytes/mL were stimulated with cell lysates from splenocytes (spleen), the syngeneic mouse mammary tumor line MMC (MMC), or with CONA peptide as positive control in 48 well plates. The T cells were activated with IL2 on day 5 and then re-stimulated with lysates on day 8 when they were re-plated in quadruplicate into 96 well PVDF plates (MAIPS4510; Fisher) that had been incubated overnight with 10 μg/ml anti-mouse IFN-g (clone: AN18, MabTech, Cincinnati OH). The plates were incubated at 37 °C for 48 h and then the supernatant was collected for Granzyme B ELISA. The plates were incubated with 5 μg/ml of biotinylated anti-mouse IFN-g (clone: R4-6A2-biotin, MabTech, Cincinnati OH) for 2 h room temperature and developed with streptavidin–alkaline phosphatase at 1:250 dilution. The colored spots were quantified using an AID ELISPOT High-Resolution reader system and AID ELISPOT Software version 6.0. Cell lysates were made with three cycles of freeze/thaw with sonication and then the protein concentration was determined by the Pierce BCA Protein Assay Kit using BSA as standard. Concanavalin A was used at a final concentration of 5 ug/mL (Sigma) as the positive control.

The Granzyme B ELISA was performed using the mouse Granzyme B ELISA kit (Ebioscience, Vienna Austria). The assay was performed with the kit instructions using pooled supernatant from the replicates for each ELISPOT. For each sample, the supernatant was diluted 1:1 with sample buffer and run in duplicate on the plate. The 10 ug/mL lysate dilution for each sample was evaluated. The plate was evaluated on a VICTOR V3 plate reader (Perkin Elmer, Waltham MA) and evaluated using SOFT-MAX Pro v5.3 software using a 5-parameter best fit standard graph.

2.5. Immune cell depletion studies

TgMMTV-neu mice (n = 3 per group) were vaccinated with the pBK-CMV plasmids containing early stage antigens Stk39, Pdhx, or Otud6B, or empty vector. The mice were implanted with 3×10^5 MMC cells subcutaneously approximately fourteen days after the last vaccine. T cell depletion of $CD3^+$ T cells (clone KT31.3) was performed by using 50 μg of the mouse monoclonal antibody three times a week by intraperitoneal (IP) injection for the first week after tumor implant and then twice a week until sacrifice. $CD22^+$ B cell depletion (clone CT34.1[23]) was performed by giving 100 μg of the monoclonal antibody by IP injection three times in the first week and then twice a week until sacrifice. As a control, 100 μg of a non-specific IgG monoclonal antibody (clone LTF2) was given by IP injection three times in the first week and then twice a week until sacrifice (antibodies from the UCSF monoclonal antibody core, San Francisco CA). Tumors were measured as described above.

2.6. siRNA assays

Four-pooled siRNA for each of the antigens were obtained from mouse and human proteins (Qiagen, Valencia CA; for siRNA identification numbers see supplemental materials and methods). The

apoptosis control was mouse All Stars positive control and human All Stars positive control (Qiagen, Valencia CA). The negative siRNA control was All Stars universal negative control (Qiagen, Valencia CA). The syngeneic mouse mammary tumor cell line MMC and human cell lines MCF10F (non-malignant breast), HCC1500 (ER positive HER2 negative), HCC70 (ER negative HER2 negative), and SKBR3 (ER negative HER2 positive) were seeded in two 96 well plates (ATCC, Manassas VA). Forty pMol of each individual siRNA and the pooled siRNA were transfected in triplicate (40 pMol of each siRNA and the negative and positive controls and 10 pMol of each siRNA when pooled) (Thermo Scientific, Waltham MA). MMC cells were seeded at a density of 2000 cells/well and transfected using Dharmafect liposomes (Dharmacon, Pittsburgh PA). MCF10F, SKBR3, and HCC70 were seeded at 2000 cells/well and HCC1500 was seeded at 4800 cells/well and transfected using lipofectamine liposomes (Thermo-Fisher, Waltham MA). Transfection conditions, cell number, timing, and reagent concentration was determined by feasibility studies that identified optimal assay conditions using control, positive apoptosis control, and universal negative control siRNA. All transfections were performed in triplicate, were corrected for a no cell background luciferase control, and were normalized to wells transfected with liposomes but no siRNA (control).

2.7. Apoptosis assay

Apoptosis was measured with a caspase 3/7 fluorescence assay (Promega, Madison WI). For MMC 50 μL of caspase 3/7 glo reagent was added per well, for MCF10F 100 μL caspase 3/7 reagent was added per well, and for HCC1500, HCC70, and SKBR3 40 μL of caspase 3/7 reagent was added per well. The plates were then incubated for 30 min at 37 °C and read by luciferase intensity at 90 min using the Wallac Envision 2104 Multi-label Detector/plate reader with a 96 well aperture (Perkin Elmer, Waltham MA).

2.8. Cell viability assay

Cell viability was measured by ATP quantification using Cell Titer Glo (Promega, Madison WI). For all cell lines, 20 μL of cell titer glow reagent was added per well and then immediately read using the Wallac Envision 2104 Multi-label Detector/plate reader with a 96 well aperture (Perkin Elmer, Waltham MA).

2.9. TCGA expression data

Publically available data from the 817 breast cancer samples published in Cell [24] from the TCGA database were evaluated using the c-bioportal web-based program [25,26]. Overexpression data was evaluated using both the putative copy number alterations from GISTIC and mRNA expression from RNAseq with z-score of 2.0. The early stage antigens (PDHX, STK39, and OTUD6B) were evaluated separately from the late stage tumor antigens (ARHGEF2, SWAP70, and GSN). The survival data was evaluated with log rank by the c-bioportal program.

2.10. Statistical analysis

Graphs and statistical comparisons were performed in GraphPad Prism v6.05 software. A students T test was used to compare empty vector and vaccinated mice for each peptide. A two-way ANOVA with Bonferroni's post-test was used for comparisons between groups for the mouse tumor growth. A one way ANOVA with Dunnett's comparison was used in rtPCR siRNA knockdown analysis and cell survival/apoptosis analysis. Log-rank analysis was used for survival analysis using TCGA data. Significance was considered at $p < 0.05$ for all statistical tests.

3. Results:

3.1. Vaccination with "early stage" tumor antigens is more effective at inhibiting tumor growth than vaccination with "late stage" tumor antigens.

From the early stage antigen SEREX screen, seven antigens were recovered [20]. Vaccination with each of the seven early stage antigens significantly inhibited tumor growth in the mouse (Fig. 1A). We selected the three early stage antigens that were (1) not identified in both early and late stage antigen screens (2) had previously been identified to be associated with poor prognosis in breast cancer, and (3) autoantibodies against these proteins could be detected in the serum of women up to 150 days prior to breast cancer detection by imaging [20]. Overexpression of STK39 was associated with resistance to Adriamycin and Cytoxan chemotherapy in breast cancer patients and overexpression of PDHX was associated with growth of breast cancer cell lines. [27,28] In control mice vaccinated with empty vector at 27 weeks after tumor implantation, the mean size of the tumor was 534.3 mm^3 (95% CI 373 to 695.5 mm^3). Vaccination with the Pdhx plasmid resulted in lower tumor volume by 38% (95% CI 286 to 337.5 mm^3, $p < 0.0001$), vaccination with Stk39 by 50% (95% CI 164 to 373.6 mm^3, $p < 0.0001$), and vaccination with Otud6b by 77% (95% CI 2.6 to 258.1 mm^3, $p < 0.0001$) (Fig. 1A).

Fifteen tumor antigens were identified by SEREX screen in mice that had late stage tumors. [19] Five of the fourteen antigens were unique to the late stage tumor SEREX screen were evaluated for the ability to inhibit tumor growth in the TgMMTV-neu mouse by vaccination through tumor implant and only one of the five (SRPK1) was able to significantly inhibit tumor growth as compared to empty vector control ($p < 0.0001$). The effect on tumor growth of a sixth late stage tumor antigen KRT 2–8 has previously been reported in the literature and did not significantly inhibit tumor growth as compared to empty vector control. [19] While Lgals8 could inhibit tumor growth, it was not selected because it was common in both screens [19,20]. We selected SWAP70, ARHGEF2, and GSN because overexpression of these targets had been associated with aggressive breast cancer and progression of disease in the literature. Overexpression of SWAP 70 has been associated with malignant transformation of breast cells, overexpression of ARHGEF2 promotes metastases by increasing invasive potential of breast cancer cells through RhoA, and GSN overexpression increases TNF-b expression and is associated with lung metastases in breast cancer patients [29–31]. Mice vaccinated with either Swap70 (95% CI 631.1 to 3642 mm^3, $p = 0.16$) or GSN (95% CI 504.7 to 2563 mm^3, $p = 0.26$) had no difference in tumor growth as compared to empty vector vaccinated mice (Fig. 1B). We also questioned whether the early stage tumor antigens were able to inhibit tumor growth because they had higher expression in the tumor than the late stage tumor antigens. We compared expression to the expression of each of the six target proteins in MMC tumor cell lysate as compared to untreated FVB spleen lysate. All six target proteins were expressed in the MMC cell lysate and not in the FVB spleen lysate suggesting these proteins are expressed on the TgMMTV-neu mouse tumors.(Supplemental Figure 2).

Vaccination with both the early and late stage tumor antigens induced tumor-specific $CD4^+$ and $CD8^+$ T cells. To demonstrate that vaccination with both the early and late stage antigens induced a tumor-specific Th1 T cell response, interferon gamma (IFN-g) ELISPOT was performed comparing vaccination with the early stage tumor antigens and the late stage tumor antigens to negative vector control. T cells were activated by cell lysates from unvaccinated spleen and syngeneic MMC tumor lysate. Fig. 1 D demonstrates the

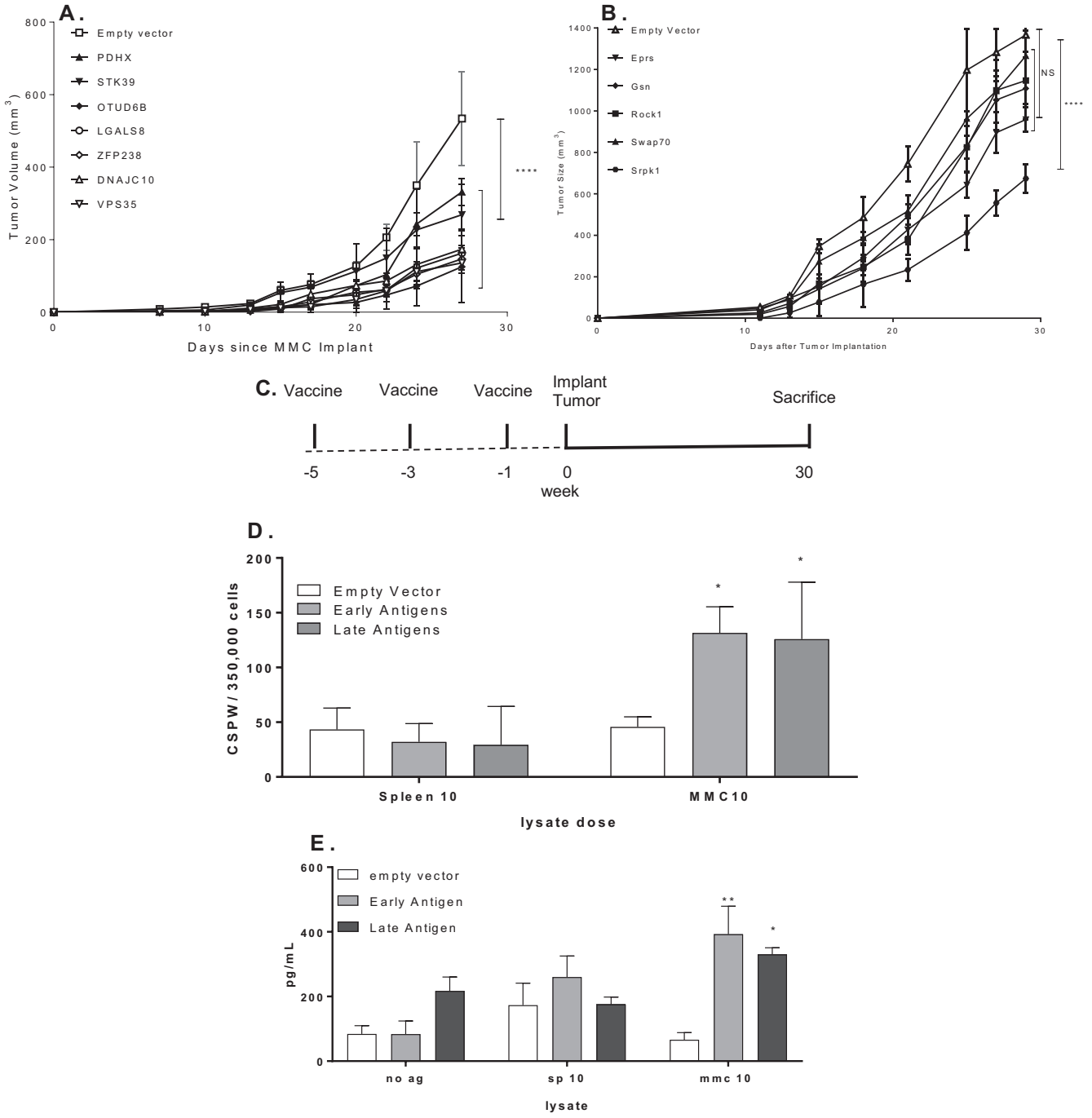

Fig. 1. Vaccination with early and late stage tumor antigens recovered by SEREX from TgMMTV-neu mice induce a T cell immune response but only vaccination with early stage antigens inhibit tumor growth. (A) TgMMTV-neu mice received three vaccinations with empty vector and each of the seven early tumor antigens. MMC tumor cells were implanted on day 0. (B) TgMMTV-neu mice received three vaccinations with empty vector ● and the five late stage tumor antigens that have human homologs. MMC tumor cells were implanted on day 0 ***$p < 0.001$. (C) Vaccination Schema. (D)MMC syngeneic tumor lysate but not lysate from FVB splenocytes induces an IFN-g T cell immune response in mice vaccinated with the early and late stage tumor antigens. IFN-g secreting cells quantified as precursor frequency (y-axis) for FVB mice (n = 5) vaccinated with the plasmids for the early tumor antigens (light gray) or FVB mice (n = 5) vaccinated with the plasmids for the late stage antigens (dark gray) as compared to FVB mice (n = 5) vaccinated with empty control vector plasmid (white). The positive control is concanavalin A (CONA). * $p < 0.05$ (E) Vaccination with the early and late stage tumor antigens induces a $CD8^+$ T cell immune response. Granzyme B ELISA was performed on the pooled supernatants from the ELISPOT assay. ** $p = 0.01$ * $p < 0.05$.

immune response to 10 ug/mL lysate. The positive control CONA was positive for all samples (data not shown). Vaccination with both the SEREX early and late stage tumor plasmids showed an increased immune response compared to mice vaccinated with empty vector alone. Vaccination with the early stage antigen plasmids increased the mean tumor-specific IFN-g T cells from 45.7 ± 9.2 in the empty vector vaccinated mice to 131.5 ± 23.9, $p = 0.03$) while there was no difference in the mean IFN-g tumor-specific T cells induced by the splenocyte lysate ($p = 0.85$). Vaccination with the late stage antigen plasmids increased the mean tumor-specific IFN-g T cells from 45.7 ± 9.2 in the empty vector vaccinated mice to 125.9 ± 26$p = 0.05$) while there was no difference between the IFN-g tumor-specific T cell frequency induced by the splenocyte lysates ($p > 0.79$) (Fig. 1D). There was also a significant increase in Granzyme B produced in mice that were vaccinated with the early and late stage antigens demonstrating

increased tumor-specific $CD8^+$ T cells. 10 μg/mL MMC lysate induced 123.8 pg/mL ± 59.4 granzyme B in empty vector vaccinated mice (n = 5) while inducing 392.9 pg/mL ± 86.1 in mice vaccinated with the early stage antigens (n = 5) (p = 0.005 compared to empty vector) and 330.2 pg/mL ± 20.4 granzyme B (p = 0.04 compared to empty vector) in mice vaccinated with the late stage tumor antigens (n = 5).(Fig. 1E) This demonstrates that vaccination with the SEREX plasmids induces both a tumor-specific IFN-g T cell and $CD8^+$ T cell response and suggests that this type I antigen-specific T cell immune response is responsible for inhibiting tumor growth.

As the early stage antigens were able to delay tumor growth with implanted tumor models, we then evaluated the effect of vaccination on preventing tumors in TgMMTV-neu mice. TgMMTV-neu mice were vaccinated at 6–8 weeks when none of the mice have hyperplasia [32]. Mice vaccinated with the late stage tumor antigens (Swap70, Arhgef2, and Gsn) did not have improved tumor-free survival as compared to empty vector alone (p = 0.23). However, 20% of mice vaccinated with the three early stage antigens (Pdhx, Stk39, and Otud6B) did not develop breast tumors and there was increased tumor free survival even in the animals that did eventually develop tumors (p = 0.02) (Supplemental Figure 4).

Depletion of T cells, not B cells, abrogated the growth inhibition from vaccination with the early stage antigens. For mice vaccinated with Pdhx, Stk39, and Otud6B depletion of $CD3^+$ T cells increased tumor growth (p < 0.0001 compared to empty vector control for all targets). However depleting $CD22^+$ B cells had no impact on vaccine inhibition of tumor growth (p = 0.19 compared to Vaccine + IgG for Pdhx, p > 0.99 for Stk39, and p = 0.68 for Otud6b, Fig. 2A). Adoptive transfer of T cells from mice vaccinated with Pdhx, Stk39, or Otud6b, but not T cells from empty vector vaccinated or unvaccinated mice (naïve), was able to inhibit tumor growth in unvaccinated mice with implanted MMC tumors (Supplemental Fig. 3).

3.2. Silencing expression of the "early stage" tumor antigens in breast cancer cells was more likely to impact breast tumor growth than silencing expression of "late stage" antigens.

The expression of each of the early stage tumor antigens Pdhx, Stk39, and Otud6B was essential for survival of the syngeneic TgMMTV-neu mouse cell line MMC. Silencing Pdhx reduced tumor cell survival by 58% (p < 0.0001) and increased apoptosis by 90% (p < 0.0001), silencing expression of Stk39 reduced MMC survival by 49% (p < 0.0001) and increased apoptosis by 70% (p < 0.0001), and silencing Otud6B decreased MMC survival by 44% (p < 0.0001) and increased apoptosis 80% (p < 0.0001) (Fig. 3A and B). Of the late stage tumor antigens silencing Gsn decreased survival by 78% (p < 0.0001) and increased apoptosis by 120% (p < 0.0001 Fig. 3C and D) but silencing Arhgef2 and Swap70 did not significantly decrease survival (p = 0.08 for Arhgef2) or increase apoptosis (p = 0.99 and p = 0.07, respectively).

The early stage tumor antigens PDHX, STK39, and OTUD6B are overexpressed in both ductal carcinoma in situ (DCIS) and breast cancer [20]. We demonstrated that PDHX, STK39, and OTUD6B were necessary for tumor survival in human breast cancer cell lines across breast cancer subtypes. In the hormone receptor positive cell line HCC1500 (ER positive) silencing STK39 decreased survival (p = 0.03) and silencing OTUD6B increased apoptosis (p = 0.02) (Fig. 4A and B). Silencing all three of the early stage antigens decreased survival (p < 0.0001 for PDHX, STK39, and OTUD6B) and increased apoptosis (PDHX p = 0.03, STK39 p = 0.02, and OTUD6B p = 0.05) in the triple negative breast cancer cell line HCC70. In the HER2 positive cell line SKBR3, silencing all three early stage antigens decreased survival (PDHX and STK39

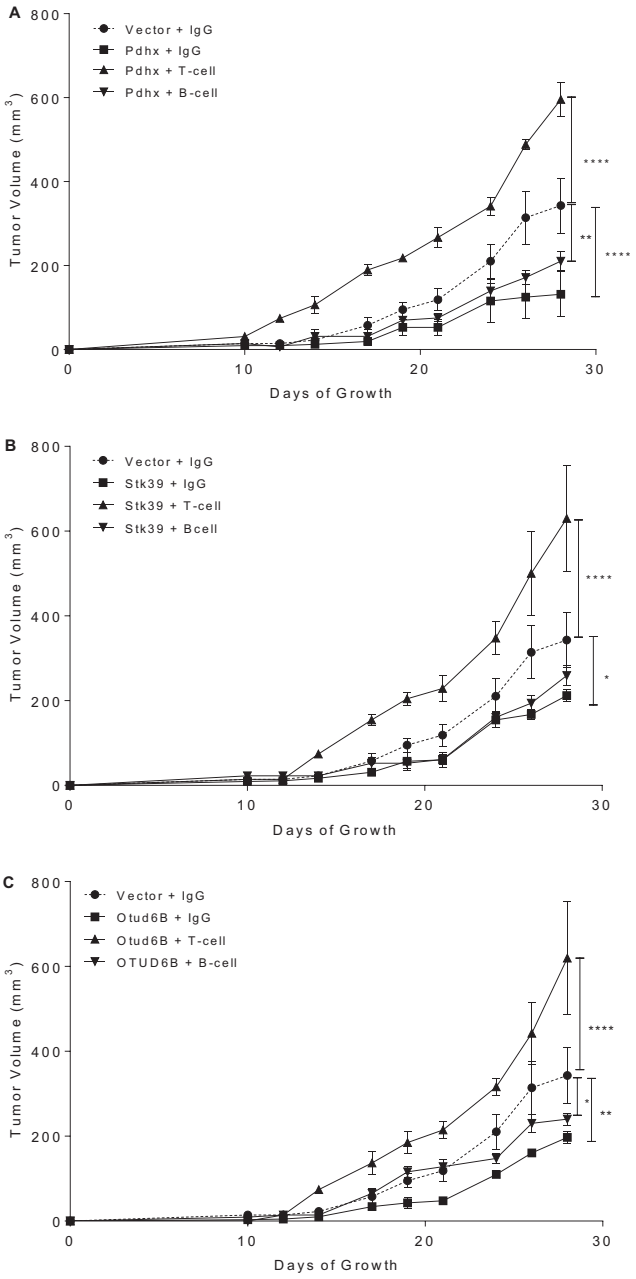

Fig. 2. Vaccination with early stage antigen plasmids induce anti-tumor T cells, not B cells. TgMMTV-neu mice were vaccinated with plasmid DNA encoding tumor antigens (A) Pdhx, (B) Stk39, and (C) Otud6B for 3 vaccinations and then given anti-CD3 (T cell ▲), anti-CD22 (B cell ▼) or control IgG (■) ****p < 0.0001, **p < 0.01, *p < 0.05 as compared to empty vector + IgG ●.

p < 0.0001 and OTUD6B p = 0.05) and both STK39 and OTUD6B increased apoptosis (STK39 p = 0.0002 and OTUD6B p < 0.0001). These responses were specific because they were not seen in cells transfected with liposome (control), cells transfected with a non-specific siRNA control (negative control), or untransfected cells (untransfected, white bars). Each siRNA specifically decreased the expression of the mRNA of the target of interest by RT PCR (Supplemental Figs. 1 and 5).

3.3. Over expression of the "early stage' antigens have a prognostic impact in patients with breast cancer

Publically available data from the TCGA database were evaluated using the c-bioportal web-based program [25,26]. Overex-

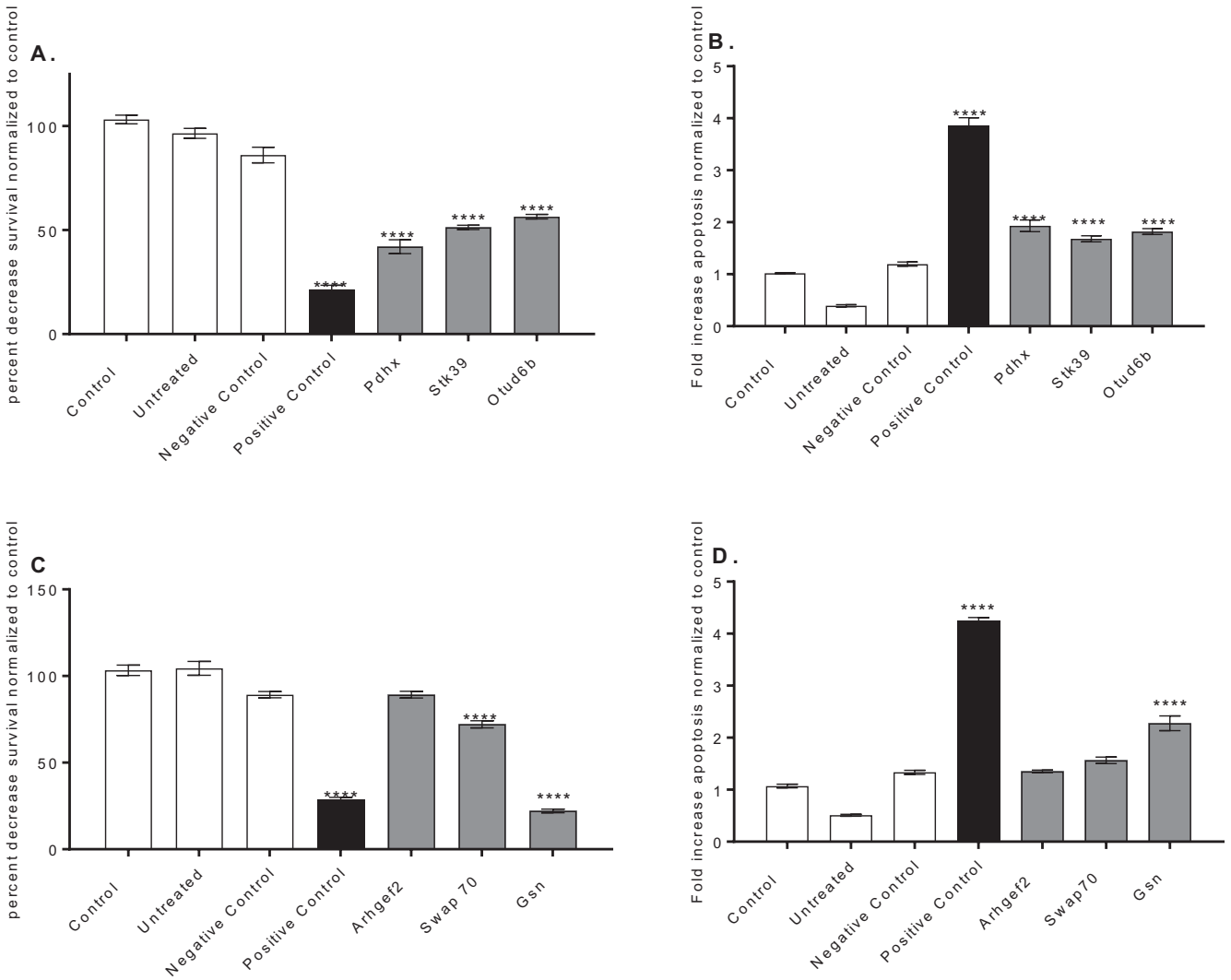

Fig. 3. Expression of all of the early stage antigens, but not late stage antigens, were important for survival of the syngeneic mouse mammary tumor cell line MMC. Using four pooled siRNA for each target in the syngeneic MMC mouse tumor cell line, grey bars are the early stage antigens measuring (A) survival and (B) caspase 3/7 activity as compared to cells transfected with liposomes but no siRNA (control). For the late stage tumor antigens, the grey bars show the late stage tumor antigens measuring (C) survival and (D) caspase 3/7 activity as compared to cells transfected with liposomes but no siRNA (control). White bars include liposome transfected cells (control), non-targeting control siRNA transfected cells (negative control), and untransfected cells (untx). Apoptosis inducing siRNA transfected cells is the black bar (positive control) ** $p < 0.01$ **** $p < 0.0001$ as compared to control.

pression of the early stage antigens predicted worse prognosis in large breast cancer patient cohorts while overexpression of the late stage tumor proteins did not similarly predict worse prognosis [24,33]. From the Cancer Genome Atlas study of 463 patients using microarray data, there was decreased median overall survival of 90.8 months in patients that had overexpression of OTUD6B, STK39, or PDHX (n = 147) as compared to patients without overexpression (n = 316) whose survival was over 50% at the time of evaluation (p = 0.005, data not shown). Overexpression of ARHGEF2, SWAP70, and GSN did not predict worse survival with patients that overexpressed the late stage tumor antigens (n = 125) having a mean overall survival of 129.6 months and patients without overexpression of the late stage tumor antigens (n = 338) having a median overall survival of 113.7 months (p = 0.99) [33]. Similarly, in a study of 807 lobular breast cancer patients, patients with overexpression of the early stage antigens (n = 343) had median survival of 113.7 months while patients without overexpression of the early stage antigens (n = 464) had median survival of 146.4 months (p = 0.03, Fig. 5A). Patients that have overexpression of the late stage tumor antigens (n = 262) had a median survival of 60% at 240 months while patients that do not have overexpression of the late stage tumor antigens (n = 545) had a median survival of 113.7 months (p = 0.09) (Fig. 5B) [24].

4. Discussion:

Not all proteins overexpressed in breast cancer are important for breast tumor development and survival [34,35]. In this study of proteins identified from the TgMMTV-neu mouse, we demonstrated that tumor-associated antigens expressed early in the course of breast tumor development were necessary for tumor survival and more effectively inhibited tumor growth as vaccines than tumor-associated antigens in advanced disease. This suggests that these early stage tumor antigens (PDHX, STK39, and OTUD6B) may be more effective vaccine targets than antigens identified in later stage disease (SWAP70, GSN, and ARHGEF2) because of a greater functional importance.

To improve tumor immune destruction, a T cell immune response should be developed to cells that are important for the growth of the tumor [36,37]. Identifying targets that are necessary

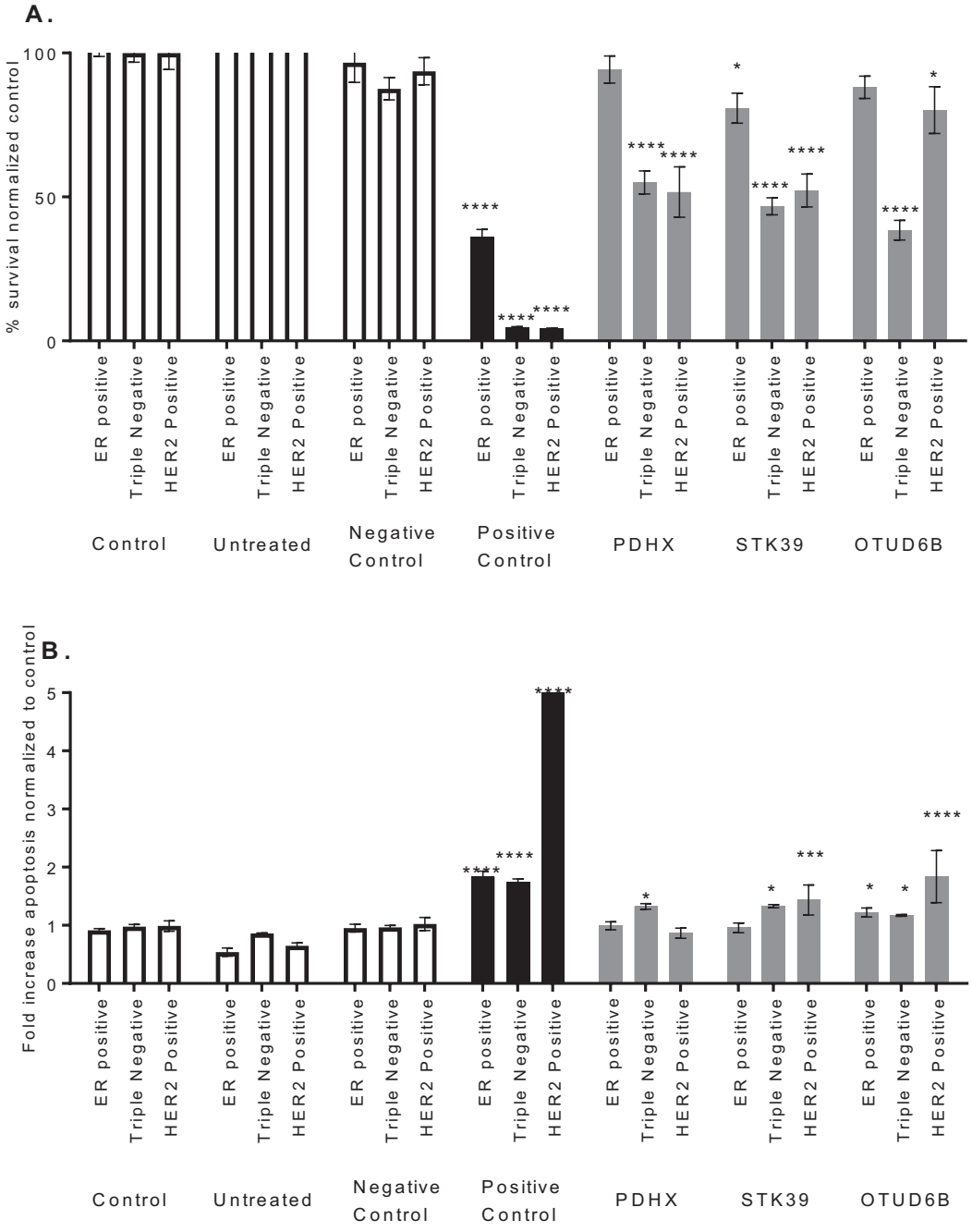

Fig. 4. Expression of early stage antigens are necessary for tumor survival in human breast cancer across breast cancer subtypes. Four pooled siRNA for each early antigen was transfected into HCC1500 (ER positive HER2 negative), HCC70 (triple negative), and SKBR3 (HER2 positive). The effect of knocking down the expression of the early antigens was measured by (A) cell survival and (B) caspase 3/7 activity as compared to cells transfected with liposome but not siRNA (control). White bars include liposome transfected cells (control), non-targeting control siRNA transfected cells (negative control), and untransfected cells (untx). Apoptosis inducing siRNA transfected cells is the black bar (positive control). * $p < 0.05$ ** $p < 0.01$ **** $p < 0.0001$.

for breast tumor survival, particularly across breast cancer subtypes could provide a vaccine response that is widely applicable across breast cancer patients [38]. The expression of the three early stage tumor antigens were essential for breast cancer survival both in the syngeneic mouse tumor cell line and across human breast cancer cell lines of different subtypes. These three proteins have been shown in the literature to be important in enhancing cell growth. PDHX is an ubiquitin E3 ligase that converts pyruvate to acetyl-co-A and regulates glucose metabolism in colon cancer [39,40]. STK39 is a kinase involved in cell survival and is overexpressed in many cancers including non-small cell lung cancer [27,41–43]. OTUD6B is a deubiquitase important in cell survival and proliferation in non-small cell lung cancer [44]. The roles of the late stage tumor antigen proteins are associated with motility and metastases. Keratin 2–8 is involved in breast cancer invasion, Swap70 is associated with malignant transformation of breast cells, and Rock1 and Arhgef2 induce breast cancer metastases and invasion through the RhoA pathway. [29,30,45] Gsn is been associated with increased TNF-b which enhances metastatic activity by inducing stromal invasion and has been associated with increased metastases in the lungs in estrogen negative breast cancer [31,46]. The three early stage tumor antigens are affect tumor

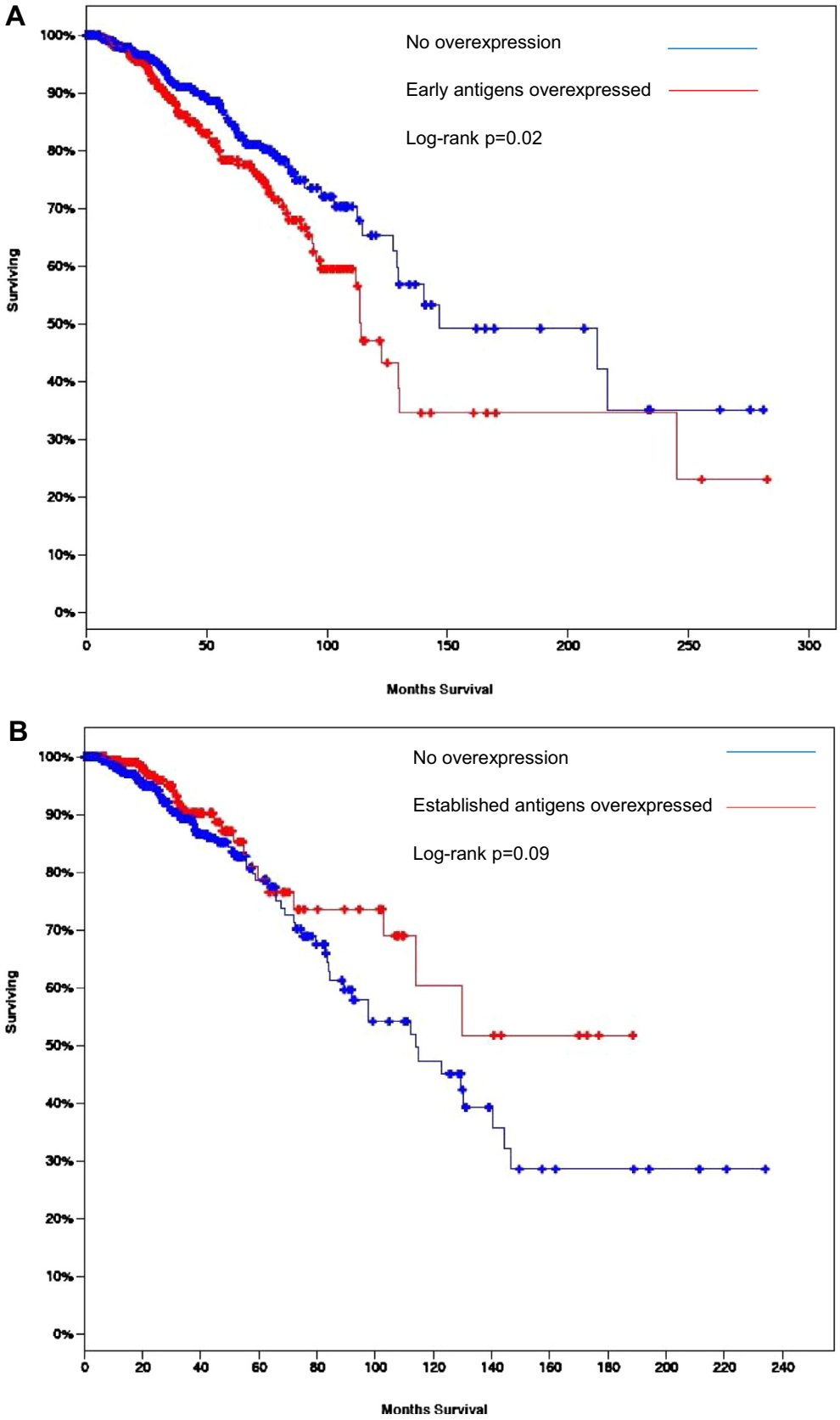

Fig. 5. Overexpression of the early stage antigens is associated with worse survival in patients with breast cancer. mRNA expression and copy number alterations were evaluated from patients with invasive breast cancer (n = 807 patients), cases without the target overexpression in blue and cases with the target overexpression in red (A) PDHX, OTUD6B, and STK39 (without overexpression n = 467 and with overexpression n = 340). Log rank p = 0.02 (B) GSN, SWAP70, and ARHGEF2 (without overexpression n = 550 and with overexpression n = 257). Log rank p = 0.09. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

growth across the subtypes, suggesting that they affect common cancer growth pathways. The expression of the three early stage tumor proteins are associated with metastases in breast cancer suggesting they are involved in invasion. This study suggests vaccination against early stage antigen targets that are associated with tumor growth rather than tumor invasion are targeting earlier stages in tumor development and may be why they may be more effective antigens in this study.

While both the early and late stage tumor antigens induced both a Th1 $CD4^+$ T cell and $CD8^+$ T cell immune response to the tumor, the early stage antigens were more effective in inhibiting tumor growth. However, this study has potential limitations including that the targets recovered were from one mouse mammary tumor model TgMMTV-neu that is a model of luminal B disease. Based on the siRNA knockdown evaluation in human breast cancer cell lines show that these genes are essential for tumor cell survival across subtypes, but evaluation of the protein expression of these targets in tumors across breast cancer subtypes will be important to ensure that they are common breast cancer targets. Another limitation for a therapeutic vaccine is that, while we have shown the early stage antigens are essential for breast cancer survival, we have not demonstrated their efficacy on TgMMTV-neu mice bearing spontaneous tumors and these studies would need to be performed for developing a therapeutic vaccine. A third limitation of the SEREX assay is that we may not have discovered all of the early or late stage antigens and may be missing important targets. This study is to demonstrate proof of principal that the antigens identified earlier in tumor development are more effective vaccine targets but are not an exhaustive panel of early antigens.

This study has demonstrated that three early tumor-associated antigens (Pdhx, Stk39, and Otud6B) identified by a SEREX screen in mouse mammary tumor model TgMMTV-neu prior to palpable tumor development, are superior antigen targets for the inhibition of tumor growth than antigens identified from mice with late stage tumors. This study suggests that transgenic mouse mammary tumor models are powerful tools to identify early antigens that, due to detection limitations, we are currently unable to identify in women and suggests that consideration of the functional relevance of these antigens is important in vaccine design.

Declaration of Competing Interest

Lauren R. Corulli, Hailing Lu, and Ekram Gad have no disclosures to declare. Sasha E. Stanton receives research funding from Precigen. Mary L. Disis receives royalties from patents held with the University of Washington and receives research funding from Pfizer, Celgene, Seattle Genetics, Ephithany, EMD Serono, Precigen, and Jansen

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.05.024.

References

[1] Scanlan MJ, Jager D. Challenges to the development of antigen-specific breast cancer vaccines. Breast Cancer Res 2001;3(2):95–8.
[2] Kostianets O et al. Panel of SEREX-defined antigens for breast cancer autoantibodies profile detection. Biomarkers 2017;22(2):149–56.
[3] Curigliano G et al. Cancer-testis antigen expression in triple-negative breast cancer. Ann Oncol 2011;22(1):98–103.
[4] Obata Y et al. Identification of cancer antigens in breast cancer by the SEREX expression cloning method. Breast Cancer 1999;6(4):305–11.
[5] Melero I et al. Therapeutic vaccines for cancer: an overview of clinical trials. Nat Rev Clin Oncol 2014;11(9):509–24.
[6] Vesely MD, Schreiber RD. Cancer immunoediting: antigens, mechanisms, and implications to cancer immunotherapy. Ann N Y Acad Sci 2013;1284:1–5.
[7] Heemskerk B, Kvistborg P, Schumacher TN. The cancer antigenome. EMBO J 2013;32(2):194–203.
[8] Buonaguro L et al. Translating tumor antigens into cancer vaccines. Clin Vaccine Immunol 2011;18(1):23–34.
[9] Hailemichael Y et al. Persistent antigen at vaccination sites induces tumor-specific CD8(+) T cell sequestration, dysfunction and deletion. Nat Med 2013;19(4):465–72.
[10] Parmiani G et al. Opposite immune functions of GM-CSF administered as vaccine adjuvant in cancer patients. Ann Oncol 2007;18(2):226–32.
[11] Davis ID et al. A pilot study of peripheral blood BDCA-1 (CD1c) positive dendritic cells pulsed with NY-ESO-1 ISCOMATRIX adjuvant. Immunotherapy 2017;9(3):249–59.
[12] Parmigiani RB et al. Characterization of a cancer/testis (CT) antigen gene family capable of eliciting humoral response in cancer patients. Proc Natl Acad Sci USA 2006;103(48):18066–71.
[13] Curigliano G et al. Breast cancer vaccines: a clinical reality or fairy tale? Ann Oncol 2006;17(5):750–62.
[14] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144(5):646–74.
[15] Hartung F et al. A core program of gene expression characterizes cancer metastases. Oncotarget 2017;8(60):102161–75.
[16] Wu JM et al. Heterogeneity of breast cancer metastases: comparison of therapeutic target expression and promoter methylation between primary tumors and their multifocal metastases. Clin Cancer Res 2008;14(7):1938–46.
[17] Chiang AC, Massague J. Molecular basis of metastasis. N Engl J Med 2008;359 (26):2814–23.
[18] Nguyen DX, Massague J. Genetic determinants of cancer metastasis. Nat Rev Genet 2007;8(5):341–52.
[19] Lu H et al. The tumor antigen repertoire identified in tumor-bearing neu transgenic mice predicts human tumor antigens. Cancer Res 2006;66 (19):9754–61.
[20] Mao J et al. Mining the pre-diagnostic antibody repertoire of TgMMTV-neu mice to identify autoantibodies useful for the early detection of human breast cancer. J Transl Med 2014;12:121.
[21] Guy CT, Cardiff RD, Muller WJ. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. Mol Cell Biol 1992;12(3):954–61.
[22] Park KH et al. Insulin-like growth factor-binding protein-2 is a target for the immunomodulation of breast cancer. Cancer Res 2008;68(20):8400–9.
[23] Keren Z et al. B-cell depletion reactivates B lymphopoiesis in the BM and rejuvenates the B lineage in aging. Blood 2011;117(11):3104–12.
[24] Ciriello G et al. Comprehensive Molecular Portraits of Invasive Lobular Breast Cancer. Cell 2015;163(2):506–19.
[25] Cerami E et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2012;2 (5):401–4.
[26] Gao J et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal 2013;6(269):p. pl1.
[27] Cleator S et al. Gene expression patterns for doxorubicin (Adriamycin) and cyclophosphamide (cytoxan) (AC) response and resistance. Breast Cancer Res Treat 2006;95(3):229–33.
[28] Eastlack SC et al. Suppression of PDHX by microRNA-27b deregulates cell metabolism and promotes growth in breast cancer. Mol Cancer 2018;17 (1):100.
[29] Li P et al. The ectopic expression of IFN regulatory factor 4-binding protein is correlated with the malignant behavior of human breast cancer cells. Int Immunopharmacol 2009;9(7–8):1002–9.
[30] Liao YC et al. Overexpressed hPTTG1 promotes breast cancer cell invasion and metastasis by regulating GEF-H1/RhoA signalling. Oncogene 2012;31 (25):3086–97.
[31] Chen ZY et al. Involvement of gelsolin in TGF-beta 1 induced epithelial to mesenchymal transition in breast cancer cells. J Biomed Sci 2015;22:90.
[32] Disis ML et al. A multiantigen vaccine targeting neu, IGFBP-2, and IGF-IR prevents tumor progression in mice with preinvasive breast disease. Cancer Prev Res (Phila) 2013;6(12):1273–82.
[33] Cancer Genome Atlas, N., Comprehensive molecular portraits of human breast tumours. Nature, 2012. 490(7418): p. 61–70.
[34] Rajendran BK, Deng CX. Characterization of potential driver mutations involved in human breast cancer by computational approaches. Oncotarget 2017;8(30):50252–72.
[35] Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. Immunity 2013;39(1):1–10.
[36] Lollini PL, Forni G. Cancer immunoprevention: tracking down persistent tumor antigens. Trends Immunol 2003;24(2):62–6.
[37] Finn OJ. Premalignant lesions as targets for cancer vaccines. J Exp Med 2003;198(11):1623–6.

[38] Cecil DL et al. T-helper I immunity, specific for the breast cancer antigen insulin-like growth factor-I receptor (IGF-IR), is associated with increased adiposity. Breast Cancer Res Treat 2013;139(3):657–65.
[39] Chen B et al. MicroRNA-26a regulates glucose metabolism by direct targeting PDHX in colorectal cancer cells. BMC Cancer 2014;14:443.
[40] Park YH, Patel MS. Characterization of interactions of dihydrolipoamide dehydrogenase with its binding protein in the human pyruvate dehydrogenase complex. Biochem Biophys Res Commun 2010;395(3):416–9.
[41] Johnston AM et al. SPAK, a STE20/SPS1-related kinase that activates the p38 pathway. Oncogene 2000;19(37):4290–7.
[42] Huang YT et al. Genome-wide analysis of survival in early-stage non-small-cell lung cancer. J Clin Oncol 2009;27(16):2660–7.
[43] Balatoni CE et al. Epigenetic silencing of Stk39 in B-cell lymphoma inhibits apoptosis from genotoxic stress. Am J Pathol 2009;175(4):1653–61.
[44] Sobol A et al. Deubiquitinase OTUD6B isoforms are important regulators of growth and proliferation. Mol Cancer Res 2017;15(2):117–27.
[45] Maskey N et al. MicroRNA-340 inhibits invasion and metastasis by downregulating ROCK1 in breast cancer cells. Oncol Lett 2017;14(2):2261–7.
[46] Padua D et al. TGFbeta primes breast tumors for lung metastasis seeding through angiopoietin-like 4. Cell 2008;133(1):66–77.