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Mesothelin, a novel immunotherapy target for triple negative breast cancer

Julia Tchou^{1,2,*}, Liang-Chuan Wang³, Ben Selven¹, Hongtao Zhang⁴, Jose Conejo-Garcia⁵, Hossein Borghaei⁶, Robert H Vondeheide^{2,7}, Steven M. Albelda³, Carl H June^{2,4,7}, and Paul J Zhang⁴

¹Division of Endocrine and Oncologic Surgery, Department of Surgery, and the Rena Rowan Breast Center, Perelman School of Medicine, University of Pennsylvania, Philadelphia

²Abramson Cancer Center, Perelman School of Medicine, University of Pennsylvania, Philadelphia

³Thoracic Oncology Research Laboratory, Pulmonary Division, Perelman School of Medicine, University of Pennsylvania, Philadelphia

⁴Department of Pathology and Lab Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia

⁵Tumor Microenvironment and Metastasis Program, The Wistar Institute, Philadelphia

⁶Fox Chase Cancer Center, Philadelphia

⁷Abramson Family Cancer Research Institute, Perelman School of Medicine, University of Pennsylvania, Philadelphia

Abstract

Mesothelin is a cell-surface glycoprotein present on mesothelial cells and elicits T-cell responses in a variety of cancers including pancreatic and ovarian cancer. Breast cancer is not known to express mesothelin. We postulated that mesothelin may be a unique tumor associated antigen in triple negative breast cancer (TNBC), a less common breast cancer subtype which may have been underrepresented in prior studies that characterized mesothelin expression. Therefore, we screened 99 primary breast cancer samples by immunohistochemistry analysis using formalin fixed paraffin embedded archival tumor tissues subtypes and confirmed that mesothelin was overexpressed in the majority of TNBC (67%) but only rarely in < 5% ER(+) or Her2-neu (+) breast cancer respectively. To determine whether mesothelin may be exploited as a novel immunotherapy target in breast cancer, an *in vitro* cell killing assay was performed to compare the ability of genetically modified T cells expressing a chimeric antibody receptor (CAR) specific for mesothelin (mesoCAR T-cells) or non-transduced T-cells to kill mesothelin-expressing primary breast cancer cells. A significantly higher anti-tumor cytotoxicity by mesoCAR T-cells was observed (31.7% vs. 8.7%, $p < 0.001$). Our results suggest that mesothelin has promise as a novel immunotherapy target for TNBC for which effective targeted therapy is lacking to date.

*Corresponding author: Julia Tchou, Perelman Center for Advanced Medicine, 3rd Floor West, 3400 Civic Center Boulevard, Philadelphia, PA 19104-1693, Tel: 215-615-1693, Fax: 215-615-0555, Julia.tchou@uphs.upenn.edu.

Introduction

Mesothelin is a glycosyl-phosphatidyl inositol-linked cell surface glycoprotein present on mesothelial cells and over-expressed in a variety of cancer including pancreatic and ovarian cancer [1–3]. Given its limited expression in normal tissues and that it is immunogenic, mesothelin is an ideal tumor antigen for the development of targeted therapy [4]. Breast cancer is not known to express mesothelin [5]. However, we postulate that mesothelin may be a unique tumor associated antigen in triple negative breast cancer (TNBC), a less common breast cancer subtype which may have been under-represented in prior studies that characterized mesothelin expression [5].

The impetus to explore the role of mesothelin as a tumor antigen in TNBC is driven by 1) the lack of targeted therapy for women in TNBC; and 2) promising preclinical and ongoing clinical studies which have demonstrated that mesothelin may be an effective immunotherapy target for several epithelial tumors (reviewed in [4,6,7]). More recently, our group has demonstrated that genetically modified T-cells expressing a chimeric antigen receptor (CAR) that recognized mesothelin could eradicate pre-established tumors in mouse bearing tumor xenograft that expressed mesothelin [6]. A similar treatment strategy may be explored if mesothelin is indeed expressed in breast cancer. Therefore, in this study, we evaluated mesothelin expression in 99 breast cancer tissue specimens comprising three major breast cancer subtypes stratified by expression of estrogen (ER), progesterone (PR) and Her2-neu receptors. We confirmed that mesothelin was frequently expressed in TNBC but not in ER(+) or Her2-neu (+) breast cancer. Furthermore, *in vitro* cell killing assay using a genetically modified T cells expressing a chimeric antibody receptor (CAR) specific for mesothelin (mesoCAR T-cells) demonstrated specific cytotoxicity on a primary breast cancer cell line that expresses mesothelin. Our results suggested that mesothelin is a novel breast tumor antigen which may be exploited as an immunotherapy target specific for TNBC in the future.

Materials and Methods

Patients and tissue specimens

After approval from our institutional review board was obtained, we selected all known cases of TNBC from a cohort of women who had undergone breast cancer surgery at our institution between 2009 and 2010 to be included in this study (n=43). We also included two other major subtypes of breast cancer, i.e. ER(+) (n=29) and Her2(+) (n=27) defined as ER or PR(+) but Her2-neu (-); and ER and PR(-) but Her2-neu(+) respectively, in our study cohort.

Immunohistochemistry staining Protocol

Estrogen, progesterone, and Her-2/neu receptor expression were performed by standard immunohistochemistry (IHC) staining techniques on formalin-fixed paraffin-embedded (FFPE) breast cancer tissue samples for all participants as part of standard pathology evaluation at our institution. The FDA approved PharmDx ER and PR test kits (DAKO, Carpinteria, CA) and HercepTest (DAKO, Carpinteria, CA) were used to evaluate ER, PR

and Her2 expression strictly following the manufacture guidelines. The tests were reported as negative if Allred score was 2 or less for ER and PR and 0 or 1+ for Her2. Tumors with specific ER/PR/Her2 phenotype were selected by clinical chart review and only cases with sufficient tumoral tissue were studied. Fluorescent in situ hybridization (FISH) was performed using PathVision HER2 DNA probe kit (Abbott, Late County, IL) on all TNBC to evaluate HER2 amplification. No Her2 amplification (HER2/CEP17 ratio <1.8) was detected in all TNBC in the series. Expression of mesothelin was evaluated on formalin fixed paraffin embedded tissues sections by immunohistochemical (IHC) staining using a mouse monoclonal antibody specific for mesothelin (clone 5B2, 1:100) (Thermo Scientific MS-1320) on fully automated Leica Bond™ instrument using the Bond Polymer Refine Detection System. Slides were pre-treated with Bond ER2 solution for 20 minutes at 100 degrees C. Mesothelioma tissue was used as positive control. Positive mesothelin staining was scored by proportion of tumor cells with positive stain (%) and intensity of the stain (1+, 2+, 3+), and by H score (0 – 300), defined as the product of % positive tumor cells and intensity (1, 2, or 3).

Mesothelin expression in breast cancer tumor cell lines

Mesothelin expression was evaluated in several commercially available (ATCC) breast cancer cell lines (BT20, MCF-7, MDA-MB-231) and one primary breast cancer cell line, TB129, derived from a patient with metastatic breast cancer. TB129 tumor cell line was established from breast cancer tissue derived from a patient with an ulcerating locally advanced ER (–) and Her2 (+) breast cancer with documented distant metastasis. Fresh tumor tissue was mechanically and enzymatically dissociated and resuspended in culture medium (DMEM supplemented with 10% FBS and penicillin and streptomycin). By passage 2, attached cells (>95%) were confirmed by flow cytometry to express the epithelial cell specific marker, EpCAM. TB129 tumor cells formed tumors when injected into the flanks of immunocompromised mice (data not shown).

To stain for mesothelin, tumor cells (10^6) were stained using standard protocol with the following antibodies: unconjugated mouse anti-human mesothelin antibody (1:40 dilution; CAK1 (K1) clone, Sig-3623-1000, Convance Research Products, Inc. Dedham, Massachusetts), mouse IgG_{2b} isotype antibody as negative control (1:40 dilution; clone A-1, SouthernBiotech, Birmingham, Alabama), R-phycoerythrin-conjugated sheep anti-mouse IgG (whole molecule) F(ab')₂ fragment antibody (1:50 dilution; P8547, Sigma Aldrich, St Louis, Missouri). Cells were analyzed by flow cytometry (CyAn™ ADP flow cytometer; Beckman Coulter Inc., Brea, California). EM-meso, a human mesothelioma cell line, as positive control, was transduced with lentivirus to stably express mesothelin as described [6,7].

***In vitro* cell killing assay of mesothelin expression breast cancer cell by mesoCAR human T-cells**

Transduction of human T cells isolated from PBMC with mesoCAR lentiviral vectors to generate mesoCAR T-cells was performed as described [7]. EM-meso and TB129 were stably transduced to express green fluorescence protein (GFP), and plated in a 96-well white flat-bottom plate at 5,000 cells per well in triplicate and incubated overnight at 37°C and 5%

CO₂. MesoCAR T cells were then added to the tumor cells at a 20:1 effector/target ratio. After 6 hours of incubation at 37°C and 5% CO₂, the wells were washed with PBS twice and resuspended with 100 µl of PBS. Remaining tumor cells were determined by measuring amount of GFP using a fluorescence plate reader (Spectra Max Gemini XS Fluorometer, Molecular Devices, LLC). The proportion of cell killing (%) was calculated as the GFP fluorescence difference between treated vs. untreated tumor cells.

Statistical analyses

For the *in vitro* killing assays, we used unpaired Student's t-tests. When comparing more than 2 groups, we used 1-sided ANOVA with appropriate post hoc testing. Differences were considered significant when $P < 0.05$.

Results

Mesothelin is frequently overexpressed in TNBC

Using mesothelioma as our positive control (Fig 1a), we noted that the majority of the TNBC cases (67%) exhibited mesothelin expression in at least 5% of the tumor cells with 19% of TNBC samples expressing mesothelin in over 50% of the tumor cells. In contrast, only one of each ER(+) and Her2(+) breast cancer case expressed mesothelin in 3% and 80% tumor cells, respectively (Table 1). The proportion of tumor cells expressing mesothelin in TNBC varied between tumor samples ranging from 3–5% (Figure 1b) to 80% of tumor cells (Figure 1c) with a median of 10% tumor cells staining positive for mesothelin in our cohort. In addition, the intensity of tumor cells staining positive for mesothelin also varied. An H-score of > 50 was noted in 12 TNBC (28%). This varied pattern of expression is reminiscent of the heterogeneous Her2-neu expression in breast cancer. Of note, mesothelium expression was not detected in normal or non-neoplastic mammary epithelium in all cases (Figure 1d).

In vitro killing of a mesothelin expressing primary breast cancer cell line directed by mesoCAR T-cell

As our laboratory has previously demonstrated that adoptive transfer of T cells expressing a chimeric antigen receptor specific for mesothelin (mesoCAR T-cell) could eradicate pre-established mesothelin expressing tumor xenografts in mice [6,7], we hypothesized that meso CAR T-cells would have similar efficacy on breast cancer cells *in vitro*. Flow cytometry analyses were used to evaluate cell-surface mesothelin expression in a primary breast cancer cell line (TB129) which was previously established in our laboratory and several commercially available breast cancer cell lines, MCF-7, BT20, MDA-MB-231, using EM-meso cells [7] as positive control. Mesothelin was not expressed in MCF-7, an ER (+) cell line or BT20 or MDA-MB-231 (both are triple negative breast cancer cell lines [8]) but was expressed in TB129 (Fig. 2A). We then compared the specificity and cytotoxicity of mesoCAR T-cells vs. non-transduced T-cells in their ability to kill TB129 using an *in vitro* cell kill assay. As shown in Fig. 2B, incubating mesothelin-expressing tumor cells, EM-meso or TB129, with mesoCAR T-cells, resulted in a significantly higher cell killing than achieved with non-transduced T-cells (42.1 % vs. 6.2% and 31.7% vs. 8.7%, $p < 0.001$ respectively).

Discussion

Triple negative breast cancer lacks expression of the conventional prognostic markers (estrogen, progesterone, and Her2 receptors) and therefore will not respond to current therapy targeting these receptors. Although TNBC comprises of only 15–20% of all breast cancer subtypes, its aggressive biology and the lack of targeted therapy mandates the search for novel treatments specific for TNBC [9–12]. Mesothelin, a cell surface tumor antigen, has shown promise as an immunotherapy target in preclinical and on-going clinical trials in ovarian cancer and mesothelioma [6,7]. A previous report has suggested that mesothelin is not expressed in breast cancer but that study may have been limited by a small sample size in which TNBC was underrepresented. We therefore evaluated mesothelin expression in women diagnosed with TNBC at our institution between 2009 and 2010. We report here, for the first time, that mesothelin is frequently and usually markedly expressed in TNBC. Mesothelin expression in other breast cancer subtypes was infrequent but future work to include a larger sample set will be needed to confirm our observation. Our results have identified mesothelin as a candidate molecule for targeted therapies in TNBC.

Recent advances in the use of adoptive transfer of genetically modified autologous T cells expressing chimeric antigen receptors (CAR) specific for CD19 to treat patients with refractory chronic lymphoid leukemia suggest that similar strategies may be useful in the treatment of epithelial tumors [13]. Our *in vitro* cell kill assay results support the notion that mesothelin has promise as a novel immunotherapy target for breast cancer, specifically for TNBC which currently lacks effective targeted therapy. In previous studies we have developed mesothelin specific CAR T cells that have potent effects in pre-clinical models [6,14]. Therefore, results from this study and prior preclinical studies would accelerate the translation of the use of mesoCAR T-cells as a potential novel targeted therapy to treat TNBC in the near future.

Our finding that TNBC frequently expresses mesothelin suggested that a myriad of treatment options may be available for women diagnosed with TNBC [4]. One of the many tasks now needed is to define the threshold for determining mesothelin positivity so that future work could explore the efficacy of mesothelin targeted therapy in TNBC. Related to this is the question of the frequency and magnitude of elevated serum mesothelin levels in patients with TNBC [15].

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Figure 1. TNBC frequently expresses mesothelin. 20× magnifications views of light microscopy of stained sections were depicted in a, b, and c. a) mesothelioma positive control; b) TNBC with ~ 5% tumor cells staining positive for mesothelin; c) TNBC with ~80% tumor cells staining positive for mesothelin ; d) normal terminal lobular ductal units are mesothelin negative: TNBC (5× magnification) with ~ 70% tumor cells staining positive for mesothelin (black arrow) and normal ductal lobular glands staining negative for mesothelin (red arrow).

Figure 2.

A) Histograms depicting mesothelin expression in various breast cancer cell lines using EM-meso cells as positive control. **B)** *in vitro* cell killing assay to evaluate proportion of tumor cells lysed by mesoCAR T-cells vs. non-transduced T cells on EM-meso cells and TB129.

Table 1

Mesothelin is frequently expressed in triple negative breast cancer

% tumor cells expression mesothelin	TNBC (n=43)		ER (+) (n=29)		Her2 (+) (n=27)	
	n	%	n	%	n	%
0	14	33	28	97	26	96
1-4	0	0	1	3	0	0
5-10	10	23	0	0	0	0
11-25	8	19	0	0	0	0
26-50	3	7	0	0	0	0
51-80	8	19	0	0	1	4