Molecular profile of a GM-CSF overexpressing breast cancer whole-cell vaccine with systemic anti-tumor activity

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#2369

ABSTRACT

BACKGROUND AND PURPOSE OF STUDY:
The allogeneic whole-cell cancer vaccine BriaVax™ (formerly SV-BR-1-GM) is an ER/PR negative, HER2/neu positive breast cancer cell line (SV-BR-1) we engineered to stably overexpress GM-CSF. BriaVax, rendered proliferation incompetent by irradiation, has thus far been applied to 4 advanced stage cancer patients (3 subjects with breast cancer, 1 subject with ovarian cancer). One breast cancer subject responded to BriaVax with complete remission of a measurable lung lesion and near complete remission of multiple breast lesions after only 3 inoculations. Nevertheless, she relapsed 3 months after completing the protocol, with brain metastases as well as multiple breast lesions. We obtained FDA permission to resume vaccinations, and, upon doing so, all metastatic sites responded with a prompt tumor regression after only 3 inoculations. To prospectively identify patients with a high likelihood of benefiting from BriaVax therapy we began a program to identify molecular factors of diagnostic potential. Here, we describe a gene expression signature that might both be informative about BriaVax' mechanism of action and helpful for developing diagnostic or monitoring biomarkers.

METHODS:
To prospectively identify patients with tumors responsive to BriaVax we began a molecular analysis of both the BriaVax cell line and cells obtained from the special clinical responder's blood. BriaVax gene expression profiles were obtained through Illumina BeadArray and NanoString nCounter technologies and compared to gene expression data sets publically available through the Gene Expression Omnibus (GEO; National Center for Biotechnology Information) portal.

RESULTS:
BriaVax™ expresses a gene signature consistent with a mechanism of action involving not only the activation of cytotoxic T cells but also the induction of a humoral response. In addition, BriaVax™ expresses known cancer antigens. Notably, blood-derived cells of the special clinical responder expressed genes complementing BriaVax' gene expression signature, thus possibly explaining the unusually prompt and robust clinical response to BriaVax™.

CONCLUSIONS:
Our findings suggest that BriaVax exerts its therapeutic effects via multiple modes. We identified both candidate immunogens overexpressed in BriaVax™ compared to normal breast cells and unraveled a potential mechanism of action explaining the encouraging clinical response observed.

PERSPECTIVE

- BriaVax™ is a whole-cell "GVAX" vaccine prepared from a breast cancer cell line with an unusual variety of cytogenetic abnormalities (Wiseman and Kharazi, 2006 and 2010).
- In a small initial clinical trial, one "Special Responder" (SP) experienced prompt, widespread, and replicable regression at multiple sites of metastatic breast cancer (Wiseman and Kharazi, 2006).
- Molecular analysis of HLA, known tumor-associated antigens, and immune response genes is a necessary effort in addressing the BriaVax™ mechanism of action. We analyzed gene expression profiles of BriaVax™ using Illumina BeadChip microarray and NanoString nCounter technologies, and compared the data with published results from 16 established breast cancer cell lines.
- Histocompatibility allele match(es) between BriaVax™ and patients may be a requirement for therapy efficacy assuming a mechanism of action in which patient T cells are activated via cancer antigens co-expressed in BriaVax™ and patient tumors and displayed on BriaVax™ MHCs.

Hypothetical Mechanism of Action

BriaVax™ expresses complete sets of genes predicting presence of both MHC class I (β2-microglobulin, HLA-A, HLA-B) and class II (HLA-DRA, -DRB3, -DMA, -DMB) complexes and presents with high transcript levels of CD83, CD74, and IL6, i.e., factors with established roles in T cell activation.

Strikingly, both BriaVax™ and the special responder carried HLA-A*11:01 and HLA-DRB3*02:02 alleles. Furthermore, compared to normal human breast cells, BriaVax™ overexpresses several genes known to encode TAAs, such as PRAME, a cancer/testis antigen gene.

Because BriaVax™ 1) expresses genes known for their immune stimulatory roles, and 2), encodes identical MHC I and II alleles as the special responder, we hypothesized that:

- BriaVax™ cells can act directly as allogeneic APCs.
- patient DCs can cross-dress with BriaVax™ pMHCs.
- patient DCs can cross-present BriaVax™ antigens on their MHC system.

ABBREVIATIONS

APC:	Antigen-Presenting Cell	MCB:	Master Cell Bank
CP:	Cell Product	MHC:	Major Histocompatibility Complex
CTA:	Cancer/Testis Antigen	NCBI:	National Center for Biotechnology Information
CTL:	Cytotoxic T Lymphocyte	pMHC:	Peptide-loaded MHC
DC:	Dendritic Cell	SP:	Special Responder
GEO:	Gene Expression Omnibus	TAA:	Tumor-Associated Antigen
HLA:	Human Leukocyte Antigen	T_H cell:	T helper cell

METHODS

Microarray gene expression profiling

- BriaVax™ cells, obtained directly from cryovials following recovery from liquid nitrogen, or harvested from cultures, were subjected to total RNA extraction (RNeasy Mini kits; Qiagen, Valencia, CA). Gene expression profiles were established using HumanHT-12 v4 Expression BeadChip arrays (Illumina). Array hybridization and data acquisition was conducted at the University of Minnesota Genomics Center.

- Non-normalized data was analyzed with various modules of *GenePattern* using the public server portal (http://www.broadinstitute.org/cancer/software/genepattern/) (Reich et al., 2006). For some analyses, BriaVax™ samples were compared to other gene expression data sets also generated via HumanHT-12 v4 BeadChip arrays and available through the GEO (NCBI) portal. Data sets to be compared to one another were merged with the *MergeColumns* module then quantile-normalized using the *IlluminaNormalizer* module (beta version), or, for Affymetrix data sets (*in silico* validation), using the *ExpressionFileCreator* module. Normalized data was further processed in Microsoft Excel. Hierarchical clustering was conducted via the *HierarchicalClustering* module. Heat maps of clusters were visualized using the *HierarchicalClusteringViewer* module and *Visualizer* (Gene Pattern).

NanoString nCounter-based gene profiling

BriaVax™ cells obtained directly from cryovials following recovery from liquid nitrogen were lysed in Buffer RLT (RNeasy Mini kit, Qiagen) then total RNA isolated from a portion of the lysates via RNeasy Mini Kit technology (Qiagen). Samples were hybridized onto the nCounter® PanCancer Immune Profiling Panel (NanoString Technologies, Seattle, WA). Data was analyzed using nSolver Analysis Software (NanoString Technologies) and Microsoft Excel.

HLA Typing

BriaVax™ and peripheral blood lymphocyte (PBL) samples from 4 subjects of a clinical study (FDA protocol BB-IND 10312) were subjected to high-resolution HLA typing for HLA-A, HLA-B, and HLA-DRB3 (City of Hope Laboratories, Duarte, CA).

RESULTS

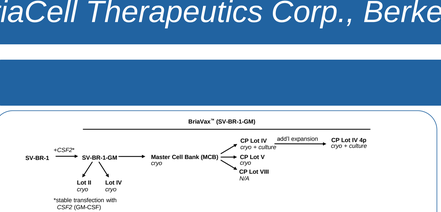


Figure 1. BriaVax™ samples used for this study. SV-BR-1-GM (BriaVax™) was derived from SV-BR-1 breast cancer cells following stable transfection with *CSF2*, encoding granulocyte-macrophage colony-stimulating factor (GM-CSF) (Wiseman and Kharazi, 2006 and 2010). The diagram only shows BriaVax™ samples used for this study. "cryo" refers to samples lysed for RNA extraction directly from cryovials, "culture" indicates culturing prior to lysis.

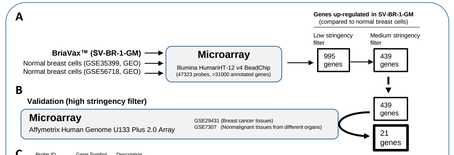


Figure 2. Identification of candidate immunogens. A. BriaVax™ RNA samples were hybridized onto Illumina HumanHT-12 v4 BeadChip arrays then expression values compared to those of normal human breast cells provided in the Gene Expression Omnibus (GEO, NCBI) database as DataSets GSE35399 and GSE56718. Two serial filters were applied to the quantile-normalized expression values to enrich for genes likely differentiating BriaVax™ from normal breast cells. After the first, low stringency, filter, 995 genes (including 3 unannotated Illumina probes) were retained, of which after the second, medium stringency, filter, 439 genes were selected. **B.** The 439 genes retained after the medium-stringency filter were *in silico* validated on GEO DataSets GSE29431 (breast cancer tissues) and GSE7307 (nonmalignant tissues representing various organs). This high stringency filtration step served to identify genes with expression levels higher in breast cancer than in a variety of nonmalignant tissues. 21 genes were retained in the high-stringency filter.

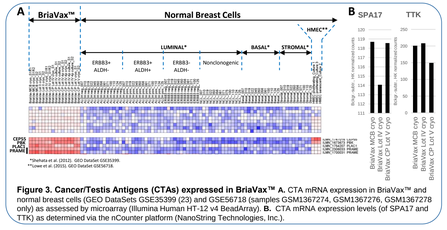
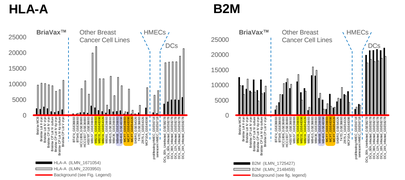
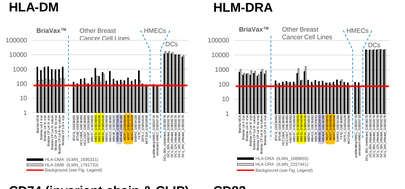
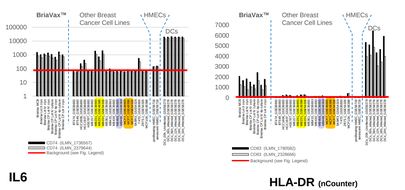


Figure 3. Cancer/Testis Antigens (CTAs) expressed in BriaVax™ A. CTA mRNA expression in BriaVax™ and normal breast cells (GEO DataSets GSE35399 (23) and GSE56718 (samples GSM1367274, GSM1367276, GSM1367278 only) as assessed by microarray (Illumina Human HT-12 v4 BeadArray). **B.** CTA mRNA expression levels (of SPA17 and TTK) as determined via the nCounter platform (NanoString Technologies, Inc.).







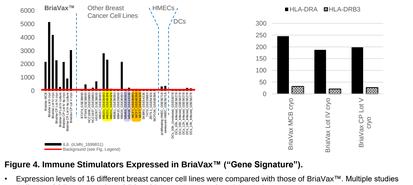


Figure 4. Immune Stimulators Expressed in BriaVax™ ("Gene Signature").

- Expression levels of 16 different breast cancer cell lines were compared with those of BriaVax™. Multiple studies (different "GSE numbers" from GEO, NCBI) were included for MB231 (MDA-MB-231), MB468 (MDA-MB-468) and MCF7. HMECs: human mammary epithelial cells (GSE56718), DC: dendritic cells (GSE58278).
- Background was defined as the median expression value across all human RNA targeting, non-control, probes (very rough estimation that approx. 50% of the genes in a tissue are expressed (Jongeneel et al., 2005)).
- Values on the Y-axes represent normalized gene expression levels. Shown are arithmetic means across replicates per study.
- "HLA-DR (nCounter)" refers to expression levels (counts) as determined via the nCounter (NanoString) technology. Other panels show expression levels as determined via Illumina microarrays.

HLA Types of BriaVax™ and Phase I Clinical Trial Subjects

Subject ID	Survival (months)	Tumor regression	HLA-A		HLA-B		HLA-DRB3	
A001	40.7	No	02:01	**24:02**	13:02	41:01	03:01	-
A002	33.7	Yes	02:01	**11:01**	18:03	44:02	**02:02**	-
A003	35.6	No	02:01	03:01	07:02	13:02	Negative	-
B001	7.0	No	**11:01**	-	35:01	40:01	Negative	-
BriaVax	N/A	N/A	**11:01**	**24:02**	35:08	55:01	01:01	**02:02**

Table 1. BriaVax™ and Subject A002 with tumor regressions even at metastatic sites (reported in Wiseman and Kharazi, 2006) share both MHC class I (HLA-A) and class II (HLA-DRB3) alleles.

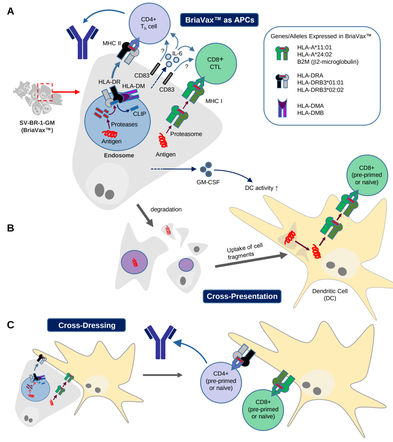


Figure 5. Model of Proposed Mechanism of Action.
Expression of the gene signature shown for BriaVax™ and outlined in Figure 4 is consistent the following mechanisms of anti-tumor immune stimulation:

A. BriaVax™ as a direct activator of pre-primed T cells.
Factors and (some of) their known roles as immune stimulants identified by mRNA gene expression profiling. Expression of MHC class I and II genes in BriaVax™ is consistent with a model in which BriaVax™ directly stimulates CD8+ CTLs and CD4 T helper (T_H) cells and thereby, potentially, induces both cytotoxic and humoral (antibody-based) responses.

B. Cross-presentation of BriaVax™ peptides on DCs
BriaVax™ is degraded and fragments of apoptotic cells taken up by dendritic cells (DCs) from the patient. BriaVax™ antigens are proteolytically degraded inside such DCs then presented on cell surface MHCs to patient T cells.

C. Cross-dressing of DCs with BriaVax™ Peptide-MHCs
Allogeneic BriaVax™ cell surface MHCs loaded with BriaVax™ antigens are directly transferred onto the cell surface of patient DCs by trogocytosis. T helper (T_H) cells (CD4+) recognizing MHC II complexes may induce a humoral anti-tumor response and CTLs (CD8+) recognizing MHC I complexes a cytotoxic response.

D. Patient tumor attack
Directly (A) or indirectly (C and D) by BriaVax™ activated T cells recognize and kill tumor cells if they express and present cancer antigen(s) that are also expressed in BriaVax™. Additionally, tumor destruction may occur via antibodies.

DISCUSSION

SUMMARY

The cancer vaccine BriaVax™ expresses Class I and Class II MHC alleles:
- HLA-A: 11:01 and 24:02
- HLA-B: 35:08 and 55:01
- HLA-DRB3: 01:01 and 02:02

2 of these alleles were also found in blood cells of the Special Responder
- HLA-A: 11:01
- HLA-DRB3: 02:02

BriaVax™ overexpresses ERBB2 (Her2/neu) and at least 20 other candidate tumor-associated antigens, and expresses several cancer/testis antigens including PRAME.

Several immune response mediators were also identified in the BriaVax™ cell line:
- CD83: a dendritic cell marker
- IL6: a secreted factor with both pro- and anti-inflammatory properties
- CD74: promotes MHC class II presentation of exogenous antigens via its invariant chain (Ii) and CLIP products. Expression in BriaVax™ ~10X higher than in other breast cancer cell lines and ~10X lower than in DCs. No/low expression possibly better than high expression for whole-cell vaccines (Thompson et al, 2008).

CONCLUSIONS

While confirmatory studies will be required, the data implies the following:

1. The presence of Class I and Class II HLA alleles is consistent with the development of both cell-mediated and humoral immune responses.
2. The double match of HLA alleles between the Special Responder and BriaVax™ is consistent with stringent MHC restriction.
3. The BriaVax™ cancer vaccine may influence the host immune response by:
   - **acting as allogeneic antigen-presenting cells (APCs)** *and/or*
   - **up-regulating T cell cytotoxicity** by cross-presentation and/or "cross-dressing" *and/or*
   - **secreting IL6 and GM-CSF**

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ACKNOWLEDGEMENTS

We are grateful to Gerhard Bauer, Brian Fury, Emily Lynn Fledderman, Tye Daniel Petrie, Dane P. Coleal-Bergum, and Tia M. Hackett from the UC Davis GMP facility (Sacramento, CA) for their BriaVax™ manufacturing efforts. We thank Darrell Johnson and his team from the University of Minnesota Genomics Center for their outstanding microarray service.