Evaluating Tissue Expression of RANTES With Subtype Specific Breast Cancer Biomarkers Found Elevated in Plasma

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Abstract

Background: A previous analysis of 23 plasma proteins found that levels increased in cancer patients compared to benign-breast-disease controls when the cancer was analyzed based on tumor subtypes. Circulating levels of RANTES were significantly increased in cancer patients and were commonly associated with two or more of the following proteins; amphiregulin, EGF, HB-EGF, TGFa, PDGF, and VEGF in a tumor subtype specific pattern. However, it is unknown if expression of these proteins correlate in the tumor tissue of breast cancer subtypes thus contributing to the elevated expression seen in plasma. In this study, we evaluate the expression of 5 of plasma biomarkers in a 70-subject breast cancer tissue microarray (TMA) to determine if these proteins are increased differentially by tumor subtypes.

Design: BioChain TMA containing 70 duplicated cores covering the common types of breast cancer, 5 normal, and 5 benign cases are assayed using GBI Labs triple stain kits to evaluate subtype specific expression of AREG and HB-EGF or PDGF and VEGF with RANTES. Imaging and quantitative assessment of the distribution and intensity of each stain of VEGF and TFGα evaluated using a multi-spectral imaging (MSI) approach, automated morphologic analysis software and compared to a visual screen.

Results: Results indicate that an algorithm can be developed to accurately recognize tumor versus stromal tissue within each core. Furthermore, as compared to visual evaluation, the MSI approach has the added capability to separate the color of the three immunostains from the triple stain for precise quantification of each protein without crosstalk. The automated scoring algorithm shows the limitations of visual assessment. Especially in the case when three proteins are localized in the cytoplasm.

Conclusion: The knowledge gained from correlating these subtype-specific patterns of tissue antigens with circulating levels in plasma may provide important information about the contribution of specific markers of breast cancers. Application of MSI to the triple staining in the TMA screen provided a superior quantitative method for evaluation of multiple chromogens linked proteins within a tumor core compared to visual observations/grading of the complex multicolor immunostaining in this study.

Introduction

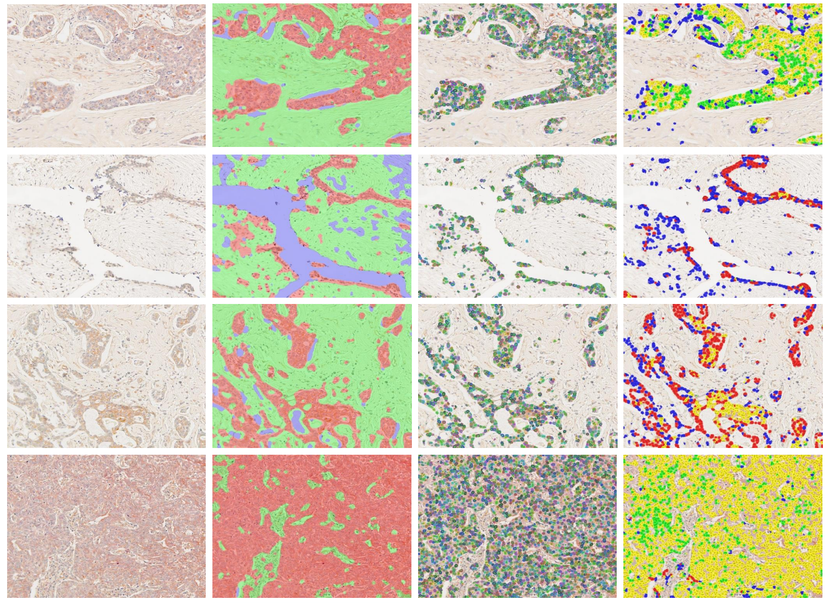
Sandwich ELISA microarray platform was use to evaluate candidate biomarkers in plasma samples from women with newly diagnosed breast cancer (*Cancer Epidemiol Biomarkers July 2011 20; 1543*). In our previous study, the disease data set was compared with plasma samples of women with benign breast neoplasia. We showed that RANTES significantly increased in women with newly diagnosed breast cancer independent of breast cancer subtype. However other biomarkers whose expression changed such as AREG, EGF, HB-EGF, PDGF, RANTES, TGFα, and VEGF did so in breast disease subtype dependent manner as defined by ER and HER2 expression levels. Studies have shown that these biomarkers play a role in other diseases, so we can not conclude that the breast tumors are directly contributing to change noted of these cytokines in the plasma. In this study, our goal is to evaluate the tumor tissue expression pattern of the plasma biomarkers to see if the tumors expression of the biomarker correlated with the biomarker expression seen in the plasma of our earlier study. Additionally we evaluated if the expression of multiple biomarkers were expressed in the same tumor cell or not. Using BioChain's Breast Cancer tissue microarray (TMA) which has a good representation of three of the breast cancer subtypes (double negative tumors; ER+; and Her2+) and benign/normal breast tissue, we found that, indeed, the markers were expressed in the tumors; however they did and did not seem to follow the pattern found in the earlier study.

Methods

The 70 case breast cancer TMA was obtained from BioChain Cat# Z7020004. Slides were de-waxed in xylene and re-hydrated using graded alcohols then rinsed in tap water. Endogenous peroxidase was blocked with 3% H2O2 for 10 minutes and washed in several changes of water. If Heat Induced Epitope Retrieval was required for primary antibody, this step was done with 10mM Citrate buffer pH6.0 for 15 min at 98C with a cool-down to 45C. The double and triple staining procedure is highly dependent on the primary antibody combination with respect to animal species. Mouse-Rabbit or Mouse-Rabbit-Goat primary antibody combinations were incubated on the tissue together. Mouse-Mouse-Rabbit or Rabbit-Rabbit-Mouse primary antibody combinations were stained sequentially according to manufactures protocols with GBI Triple Stain kits listed in table. Most primary antibody combinations were incubated for 30 minutes at room temperature unless otherwise indicated by primary antibody source. Manual scoring using Olympus BX40 Light microscope was assessed on total percent positive cells and intensity of stain. Each core of the TMA was imaged multispectrally using a Vectra™ Automated Imaging System. Multispectral images were unmixed using spectral signatures developed from singly stained control samples. The unmixed images were analyzed using inForm™ Tissue Finder, which was trained to automatically find tumor regions. Within the tumor regions, cell segmentation was performed, and each marker scored for positive/negative according to a manually selected intensity threshold. Double positivity scoring was also performed.

Results

Figure 1
Multispectral imaging of double-stained VEGF and TGFα Breast Cancer Tissue Array



Original Image From Scope
VEGF-DAB & TGFα-AP-Red

Map =Tumor Red / Normal
Green/ Blue Empty Space

Individual Cell Assessment

Yellow is double positive
Green is single positive TGFα
Red is single positive VEGF
Blue is double negative

BioChain -Z7020004 2x70 cores
Breast Cancer Tissue Array

Vectra® Automated Imaging System

Table1 Antibody Source

Antibody Used	Source/Cat#
Gt anti-EGF	SantaCruz/SC-1343
Gt anti-HB-EGF	SantaCruz/SC-1414
Ms anti-Amphiregulin	SantaCruz/SC-74501
Ms anti-VEGF	SantaCruz/SC-3462
Rb anti RANTES	Abcam /Ab9678
Rb anti-TGFalpha	Abcam /Ab9585
Rb anti-PDGF	SantaCruz/SC-128
Mult-Stained Kits	**Source/Cat#**
Mouse, Rabbit	GBI Labs/DS201
Rabbit,Mouse, Mouse	GBI Labs/TS301
Rabbit, Rabbit, Mouse	GBI Labs/TS302
Mouse, Rabbit, Goat	GBI Labs/TS303

Figure 2

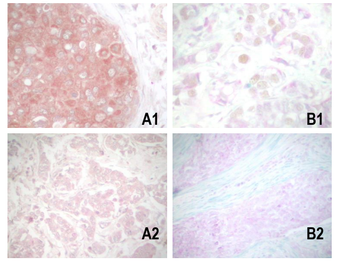
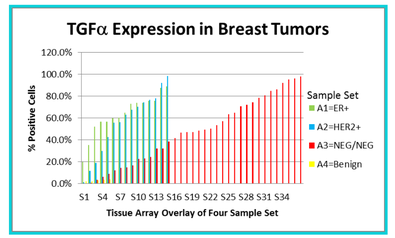


Fig.2 Image A1 and A2 are from tissue array stained with Ms anti-AREG and Goat anti-HB-EGF. In A1 you see strong expression of both Amphiregulin-DAB and HB-EGF-AP Red. In A2 AREG is expressed but much less of HB-EGF is expressed. Images B1 and B2 are from tissue arrays stained with Ms anti-ER DAB , Rb anti-RANTES AP-Red, and Gt anti-EGF Emerald . B1 shows expression of all three proteins, B2 is an ER negative tumor showing expression of RANTES and EGF. The EGF was rarely detected in the tumor cells but was often increased in the surrounding stromal cells of the tumor.



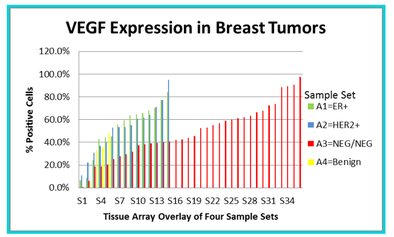


Figure 3 The graphs show increased expression and different subtype of TGFα and VEGF when measured by Vectra Automated Imaging system. In order to see the pattern of staining, tumors cores were sorted to show expression from least to highest and then graphed. Double negative tumors are represented with twice as many samples (red). TGFα was not expressed at all in the benign tissue but was significantly increased in all tumor types.

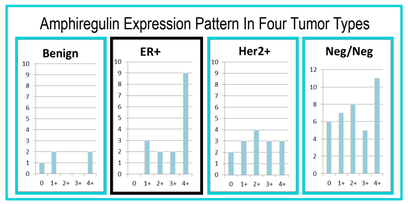


Figure 4 The graphs show that AREG increased significantly only in the ER tumor. In our previous study, AREG increased only in plasma of ER+ tumors. Scored on intensity and number of positive cells.

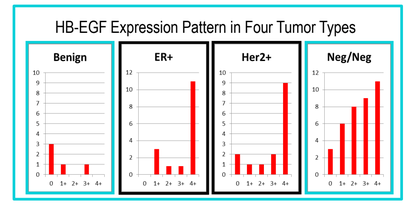


Figure 5 The graphs show that HB-EGF increased significantly in the ER and Her2+ tumor subtypes similar to early stage breast cancer plasma samples. Scored on intensity and number of positive cells.

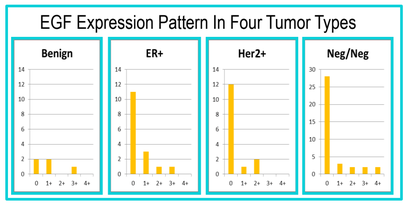


Figure 6 These graphs show that EGF was rarely expressed in any type of breast tumor epithelial cells. In our previous study, EGF was significantly increased in the plasma samples from HER2+ breast tumors. The question is why did we see significant increase in plasma samples? The answer may lie in the increase expression of EGF in the tissue surrounding the tumor as seen in Fig.2 B1 and B2 to the left of this panel. One could hypothesize that the tissue surrounding the tumor cells is receiving signals from the tumor.

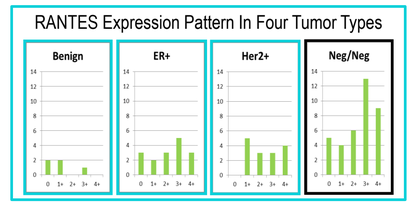


Figure 7 These graphs show that RANTES was expressed in all type of breast tumor epithelial cells. However the ER+ and Her2+ breast tumors showed ~30% of the tumors with low expression. It is hard to know if the tissue results correlates to the plasma study which found RANTES significantly since the benign cases number was low on the tissue array. However if the expression pattern seen in this study holds true in larger sent of benign and tumor cases the results would show that RANTES may be contributed from diseased breast tissue.

Conclusion:

This study evaluated breast tumor expression of the EGFR ligands screened in the plasma samples of early stage breast cancers. The subtype dependent tumor tissue expression of AREG, HB-EGF and TGFa mirrored the plasma expression patterns for the EGFR ligands of our previous study however HB-EGF seem to be increased in double negative tumors as well. We can not rule out that HB-EGF plays a larger role in double negative tumors in later stage tumor development as compared to early stage breast cancer. RANTES was found to be significantly increase in the four major types of breast cancer but not the benign set. With the benign number of 5 it is to small to conclude that RANTES increase seen here is significant however a significant body of literature to support the RANTES increase in breast cancer. The double negative breast tumors showed the most significant increase in RANTES expression within the tumor group when comparing the number of tumors that are not expressing RANTES to those that are expressing RANTES. It was rare to see EGF in the tumor epithelial cytoplasm but we did see significant increase in the stroma surrounding the tumor as seen in fig 2B1 and 2B2. Future goal is to evaluate the expression of the EGF in the stroma using the Vectra Automated Imaging system to see expression of EGF is alter in the stroma in a subtype specific manner.