Development and analytical validation of a fully-automated platform for quantification of MetaSites to predict systemic metastasis.

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Abstract

Background: Tumor metastasis is responsible for the majority of solid tumor related deaths. Diagnostic assays that accurately predict risk of cancer dissemination provide useful information for optimizing personalized treatment strategies. As previously demonstrated, using manual methods, the number and distribution of MetaSites in the tumor microenvironment significantly predicts metastatic disease in patients with ER-positive early stage invasive breast cancer. A MetaSite is the juxtaposed multicellular structure comprised of a blood vessel, the macrophage immune cell, and an invasive tumor cell. To reduce pathologist counting variability, an objective and reproducible automated laboratory process workflow to identify and quantify MetaSites for a clinical grade assay was developed. **Methods:** Digital pathology imaging coupled with image analysis tools was employed to develop a fully automated, objective method and workflow for quantification of MetaSites in formalin-fixed paraffin embedded tumor samples. Using this method, areas for analysis and quantification of MetaSites were automated by integrating high resolution automated microscopy with multiple image analysis algorithms. A pathologist ensured overall diagnostic quality of the sample in addition to approving individual images for MetaSite scoring. **Results:** In this analytical validation study, the platform was demonstrated to be greater than 97% reproducible with a mean coefficient of variation of 6.6% (n=35) for 3 independent measurements of the same slide. Further, MetaSite scores showed correlation coefficients (Pearson's R) greater than 0.98 between measurements with no significant difference in absolute values by repeated measures analysis. Importantly, MetaSite scoring on independently stained tumor sections showed greater than 90% reproducibility, indicating minimal heterogeneity within the tumor with respect to MetaSite score, section to section. Additionally, day-to-day MetaSite scores showed correlation coefficients (Pearson's R) greater than 0.90 between staining runs with no significant difference in mean MetaSite scores. **Conclusion:** Taken together, these data demonstrate the successful development and analytical validation of a fully automated, highly reproducible MetaSite quantification platform. With development and analytical validation of this test, it is now possible to provide physicians with information regarding the aggressiveness of patient tumors and accurate prediction of cancer metastasis. This method is being further validated in a large (n=481) case control clinical study.

Introduction

MetaSite Breast is a driver-based triple immunohistochemical stain test that identifies sites of intravasation and predicts the likelihood of metastatic recurrence in early stage, node-negative and node-positive, estrogen-positive invasive breast cancer. Breast cancer is one of the most frequently diagnosed cancers in women with an estimated 240,800 new cases diagnosed in the US in 2015. Aggressive cancer leading to metastasis is responsible for the majority of the estimated 40,370 deaths in the US in 2015. Approximately 30% of women diagnosed with early stage invasive breast cancer will progress to metastatic disease[1].

Historically, adjuvant chemotherapy has been used in this patient population as a prophylactic treatment strategy to help prevent metastatic recurrence and improve disease-free survival. Although the overall prognosis is favorable, not all patients with early stage estrogen-positive invasive breast cancer following primary therapy will derive benefit from adjuvant chemotherapy. Patients with less aggressive tumors derive no meaningful benefit from adjuvant chemotherapy while patients with more aggressive tumors have significantly better outcomes[2]. New diagnostic tests that can determine the aggressiveness and metastatic potential of a patient's tumor would be helpful in individualizing oncology treatment by guiding the use of adjuvant chemotherapy to patients that would derive maximal benefit and for those who do not, to avoid the risk of exposure. The MetaSite Score is a quantification of the number of individual stromal tripartite structures consisting of a Mena overexpressing tumor cell, perivascular macrophage and epithelial cell in direct apposition. Previous studies have demonstrated the MetaSite Score predictive for risk of distant metastasis in ER-positive, HER2-negative breast cancer independent of traditional clinical factors[3].

In earlier studies of MetaSite Breast, the MetaSite Score was quantified manually by pathologist subjective counting. It has been well established that manual counting of cellular structures exhibits poor inter- and intra-observer reproducibility (i.e. 71% for Ki67 positivity[4]) due to differences in region selection, counting method, and criteria for positivity. In the absence of arbitration, MetaSite manual counting resulted in only 75% inter-observer reproducibility. In order for MetaSite quantification to be employed in the clinical laboratory setting and to meet the standards required for clinical use it was necessary to develop more reproducible processes for quantification of MetaSites in tumor tissue. Here we demonstrate the development and analytical validation of a robust and objective method for MetaSite quantification in tissue using digital pathology coupled with image analysis and bioinformatics.

Methods

Tissue Staining
Formalin-fixed paraffin-embedded invasive breast cancer samples (n=105) were stained for MetaSites using a modified triple chromogen immunohistochemical stain for CD31-positive blood vessels using a rabbit anti-CD31 monoclonal antibody (AbCam/Epitomics Clone EP3095), CD68-positive macrophages using an anti-CD68 mouse monoclonal antibody (Thermo Scientific Clone PGM1) and Mena-positive tumor cells using an anti-Pan-Mena mouse monoclonal antibody (Metastat, Boston, MA). CD31-positive blood vessels, CD68-positive macrophages, and Mena-expressing tumor cells were visualized using brown, blue, and red chromogens, respectively.

Imaging and Image Analysis
All imaging was conducted with the Perkin Elmer Vectra 2 multispectral microscopy system (Perkin Elmer, Hopkinton, MA). For whole tissue imaging, 20X high resolution images were acquired (up to 100) for areas of invasive tumor using customized inForm (Perkin Elmer) image analysis algorithms (See Figure 1-a). Individual MetaSites were identified using inForm and VisioPharm (VisioPharm, Hoersholm, Denmark) image analysis software. InForm generates spectrally unmixed composite images representing chromogen specific channels for CD31, CD68, and Pan-Mena. These images were analyzed using the VisioPharm MetaSite identification algorithm where individual MetaSites were identified as structures which conform to established criteria.

Pathologist QC
All images used in analysis were reviewed by a pathologist and any images not meeting standard for invasive breast cancer were removed from analysis. Furthermore, the pathologist will review each sample for quality and specific staining patterns. Two pathologists independent QC processes showed 97-100% concordance (data not shown).

Study Design and Statistical Analysis
Sections (n=105) from a cohort of 75 invasive breast cancer tumor specimens was assessed for analytical precision, performance, and accuracy. Due to lack of invasive tumor on some cases, final n=81. Analytical precision was defined as repeated analysis (n=81) of the same slide three (3) times with a primary a priori determined benchmark of ≥95% reproducibility by ROC analysis. Analytical performance was defined as completely independent staining and analysis (n=105) on serial tissue sections on three separate days with a primary a priori determined benchmark of ≥90% reproducibility by ROC analysis. Analytical accuracy was defined as comparison of automated MetaSite quantification with the academic assay reference standard with a primary a priori determined benchmark of ≥80% accuracy by ROC analysis. All statistical analysis was performed in SPSS v. 22 (SPSS Inc., Chicago, IL)

References

1.) http://www.cancer.gov/statfacts/html/breast.html
2.) Yardley DA. Advances in metastatic breast cancer and targeted therapeutic approaches in breast cancer. Breast Cancer Res. 2015 Apr 23;17:60.
3.) Rohan et al., Tumor Microenvironment of Metastasis and Risk of Distant Metastasis of Breast Cancer. 2014. JNCI 106(7)
4.) Polley et al. An International Ki67 reproducibility study. J Natl Cancer Inst. 2013 Dec 18; 105(24):1897-906

Results

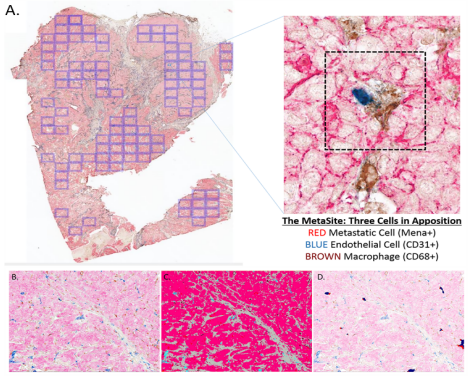

Figure 1: Auto mated field capture and image analysis for objective quantification of MetaSites in breast cancer samples. (A) InForm coupled with the Vectra 2 microscopy system is used to automatically take high resolution 20X fields of view (FOVs) to identify MetaSites. (B) Example of high resolution 20X FOV showing Mena-positive tumor (red), CD31-positive blood vessels (blue) and CD68-positive macrophages (brown). (C) Visiopharm image analysis software is used to identify tumor (red), vessels (blue), and macrophages (brown) and the n (D) MetaSites are identified by determining percentage of juxtaposed cellular interfaces (blue/red interface).

Analytical Precision and Performance Assessment was performed by comparing reproducibility of MetaSite™ Breast scores for multiple three (3) independent scans/analysis of the same slide (Precision) or three (3) independent staining runs and analysis on separate tissue sections (Performance). Figure 2 shows linear regression and means analysis for continuous MetaSite™ Breast score data. Strong correlation (Pearson's R > 0.98) and slopes approaching 1.00 (0.99 -1.01) were observed for all comparisons with no statistically significant difference in mean MetaSite Scores across all 3 repeats (Precision) or staining runs (Performance). Figure 3 shows ROC analysis for categorical (Low v. Intermediate/High (Low Cutpoint) and Low/Intermediate v. High (High Cutpoint)) MetaSite score comparisons. Area Under the Curve (AUC) measurements indicating degree of agreement ranged from 0.97-0.99 for Precision and 0.91-0.96 for Performance with an overall Percent Coefficient of Variation of 6.6%.

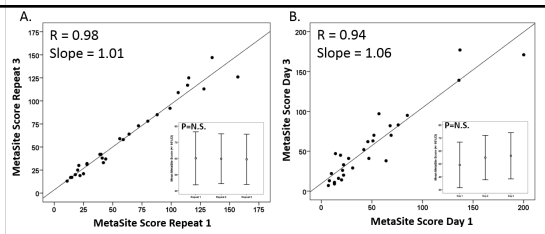

Figure 2: Analytical Precision and Performance Validation. Linear regression and means analysis for (A) Analytical Precision (repeat analysis of the same slide) and (B) Analytical Performance (staining and analysis of serial sections on separate days). Shown are XY Scatterplots (Repeat/Day 1 v. Repeat/Day 3 only) and Means Plot (inset) showing very strong correlation (Pearson's R) and slopes approaching 1 with no significant difference in mean scores by repeated measures analysis. Taken together these data demonstrate a very high level of precision and performance for raw continuous MetaSite scores.

Results Continued

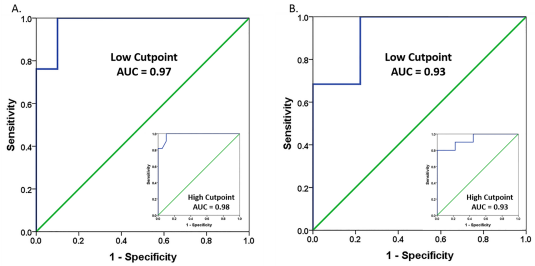

Figure 3: Analytical Precision and Performance Validation continued. ROC analysis for (A) Analytical Precision (repeat analysis of the same slide) and (B) Analytical Performance (staining and analysis of serial sections on separate days) using low and high (inset) cutpoints set by taking tertiles and comparing to subsequent repeat analyses. Shown are comparisons for Repeat/Day 1 versus Repeat/Day 3 with indicated area under the curve (AUC) measurements. Taken together these data demonstrate a high level of reproducibility with respect to group classification both for repeated analysis (Precision) and different sections of the same tumor sample (Performance).

Analytical Accuracy Assessment was performed by comparing automated MetaSite™ Breast scores with the reference standard, semi-manual pathologist TME Mcounting on the same slide. ROC analysis (Figure 4) shows a high level of concordance, 84% for the low cutpoint (low v. intermediate/high) and 90% for the high cutpoint (low/intermediate v. high).

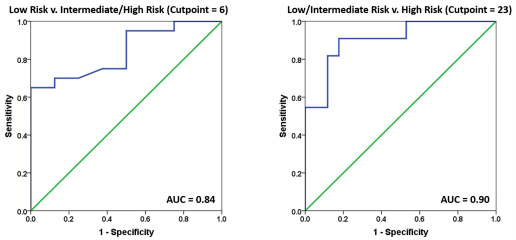

Figure 4: Analytical Accuracy Validation. Continuous automated MetaSite scoring was compared to semi-manual pathologist scoring (reference standard) by ROC analysis using the original Pathologist cutpoints (Rohan et al.[2]) for (A) pathologist low v. intermediate/high risk cutpoint and (B) pathologist low/intermediate v. high risk cutpoints. Data demonstrate a high degree of concordance showing 84% and 90% concordance respectively.

Conclusion

- The MetaSite™ Breast assay, utilizing automated digital pathology methods, demonstrates high reproducibility, accuracy and analytical precision, thus enabling MetaSite™ Breast to be used in the clinical setting.
- With precision and performance of the this assay, specifically %CV < 10%, the MetaSite™ Breast test meets or exceeds current industry standards for analytical performance of *in situ* tissue-based diagnostic tests.

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