Breast Cancer Prognostic Biomarker Using Attractor Metagenes and the

**FGD3-SUSD3 Metagene**

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Abstract

**Background:** The winning model of the Sage Bionetworks/DREAM Breast Cancer Prognosis Challenge made use of several molecular features, called attractor metagenes, as well as another metagene defined by the average expression level of the two genes *FGD3* and *SUSD3*. This is a follow-up study towards developing a breast cancer prognostic test derived from and improving upon that model.

**Methods:** We designed a feature selector facility calculating prognostic scores of combinations of features including those that we had used earlier, as well as those used in existing breast cancer biomarker assays, identifying the optimal selection of features for the test.

**Results:** The resulting test, called BCAM (Breast Cancer Attractor Metagenes) is universally applicable to all clinical subtypes and stages of breast cancer and does not make any use of breast cancer molecular subtype or hormonal status information, none of which provided additional prognostic value. BCAM is composed of several molecular features: the breast cancer specific *FGD3-SUSD3* metagene, four attractor metagenes present in multiple cancer types (CIN, MES, LYM, and END), three additional individual genes (*CD68*, *DNAJB9* and *CXCL12*), tumor size and the number of positive lymph nodes.

**Conclusions:** Our analysis leads to the unexpected and remarkable suggestion that ER, PR and HER2 status, or molecular subtype classification, do not provide additional prognostic value when the values of the *FGD3-SUSD3* and attractor metagenes are taken into consideration.

**Impact:** Our results suggest that BCAM's prognostic predictions show potential to outperform those resulting from existing breast cancer biomarker assays.
Introduction

Several prognostic models for breast cancer using molecular features have been used in biomarker products (1-3), which have also proven to be of value to medical decision making, such as predicting whether an early-stage patient will benefit from adjuvant chemotherapy. A recent crowd-sourced research study, the Sage Bionetworks-DREAM Breast Cancer Prognosis Challenge (BCC) (4) used the METABRIC data set (5) containing molecular and clinical features from 1,981 breast cancer patients. The winning model (6, 7) as well as all five top-scoring models made use of several molecular features, called attractor metagenes (8), as well as the FGD3-SUSD3 metagene defined by the average of the expression levels of the two genes, FGD3 and SUSD3, which are located directly adjacent to each other at Chr9q22.31.

To make a prognostic tool useable in a clinical setting derived from our newly identified metagenes, we optimized a new model based on the disease-specific survival information included in the METABRIC data set, providing an estimate of the breast cancer specific 10-year survival rate for each patient. Here we present the derivation of the model to which we refer as the BCAM (Breast Cancer Attractor Metagenes) biomarker. We derived the model using the uniformly renormalized (4) 1,981-sample METABRIC data set, in which the two genes whose high expression is most associated with good prognosis are FGD3 and SUSD3; in fact the most prognostic molecular feature that we had found (6) was the FGD3-SUSD3 metagene. At the other extreme, the genes whose high expression is most associated with poor prognosis were members of the mitotic chromosomal instability ("CIN") attractor metagene, which we previously identified (8) as a "pan-cancer" molecular signature using unsupervised analysis of other data sets from different cancer types.

Because many models submitted in the BCC were similar to those used in existing biomarker products, we hypothesized that the features that we used in our models can improve the accuracy of existing prognostic assays. We therefore compared our features with those used in the 21-gene Oncotype DX (1), 70-gene MammaPrint (2), and 50-gene PAM50 (3) assays, using several breast cancer data sets. Given the lack of the actual implemented biomarker scores for the patients in these data sets, we used the available microarray values to produce an
estimated performance. Based on these comparisons, our results suggest that the combination of features used in the BCAM model may compare favorably with those used in these products, and therefore we conclude that it is promising and deserving of formal evaluation within the context of several ongoing adjuvant clinical trials.

Materials and Methods

Data Sets, pre-processing, end points of survival analysis

Because most breast cancer data sets do not include the number of positive lymph nodes, we relaxed the requirements for acceptable validation data sets to allow for those that merely provide a binary (negative/positive) lymph node status. Still, we found only four data sets in addition to METABRIC (5), available through Sage Synapse under accession number syn1710250, with the requirements that they include probes for genes *FGD3* and *SUSD3*, tumor size, lymph node status and disease-specific survival or recurrence data, from which we could extract at least one statistically significant ($P < 0.05$) comparison between the BCAM formula and those used in other genomic assays. We refer to these four data sets as Loi (9), Buffa (10), Wang (11), and Miller (12), and they are available from the Gene Expression Omnibus under accession numbers GSE6532, GSE22219, GSE19615, and GSE3494, respectively. Only the Buffa data set provides the number of positive lymph nodes; in the other data sets we used the BCAM formula setting the number of positive lymph nodes for lymph node positive patients to 1. The tumor size and the lymph node number were logarithmically transformed.

The data sets generated from Affymetrix U133A/B, and Plus2.0 arrays were renormalized using Robust Multi-array Average (RMA), as implemented in the *affy* package in Bioconductor (www.bioconductor.org) in the R software. If there was more than one platform provided for each patient, the measurements were combined and renormalized using RMA. The METABRIC data set was renormalized by Sage Synapse (4). Because the BCAM formula is the linear combination of heterogeneous covariates, we corrected the distribution of genomic assays in each data set by multiplying the size and the lymph node number with the ratio of the standard deviations of the genomic assays in each data set to the standard deviation of the genomic assays in the METABRIC data set.
For survival analysis, because each data set uses different end point for censoring, we used the end point defined closest to disease-specific survival available in the METABRIC data set and in the Miller data set. We used time to recurrence in the Loi and Wang data sets and distant-relapse free survival in the Buffa data set.

**Comparison of predictive models**

The concordance index (13) is used to assess the accuracy of the rankings of patients’ risk. It is defined as the relative frequency of accurate pairwise predictions of survival ranking over all pairs of patients for which such a determination can be achieved. To compare the performances of the predictive models, we estimated the distribution of the concordance index as the overall C-index (13) for each model on each subset of samples. Since the overall C estimator is proven to be asymptotically normal (13), the null distribution of the C-index can be approximated by a normal distribution with mean 0.5 and the sampling variance of C-index when the sample size is sufficiently large. Standardized by the mean under the null hypothesis and estimated variance from data, the C-index follows a Student’s t distribution approximately. The difference between two estimated C-indices, after standardization, also follows a t distribution approximately under the null hypothesis that the two C-indices are equal. Therefore, the comparison between two overall C-indices can be carried out by a Student’s t-test and the P value is evaluated accordingly. The overall C-index estimation and t-test were performed by the *survcomp* package (14) in the R software.

**Feature selector facility**

The prognostic score displayed for each combination of selected features was designed to be resistant to overfitting. It is evaluated as the asymptotic average of the concordance indices resulting from random 2-fold cross-validation experiments in the METABRIC data set. Each experiment uses the selected features as covariates to train a Cox proportional hazards model on half of the data set based on random splitting, and evaluates the corresponding concordance index of the fitted model on the other half. Each experiment is also repeated by reversing the training/validation roles of the same subsets.
Estimation of survival rate

The final BCAM score between 0 and 100 is generated as the corresponding percentile value from the Cox model formula against the 1,981-sample METABRIC data set. The breast cancer specific 10-year survival rate associated with the BCAM score is found by calculating the Kaplan-Meier hazard ratio at ten years for the METABRIC subpopulation inside a sliding window containing 20% of the samples (10% in each side) with the closest BCAM scores. If there are not enough patients on one side of the window, we reduce the window size so that it remains symmetric.

Other Breast Cancer Prognostic Formulas

We compared BCAM with four biomarkers used in other genomic assays: The 21-gene Oncotype DX signature (1), the 70-gene MammaPrint signature representing a good prognosis gene expression profile (2), the 50-gene ROR-S signature whose different expression profiles constitute centroids for four intrinsic PAM50 subtypes (3); and the ROR-C signature combining the PAM50 subtypes with original tumor size (3). The definition of each of the four groups in the 21-gene signature and the formula for combining them were obtained in (1) without applying the cut-off thresholds, as the expression levels of the groups for the microarray values and RT-PCR values were not compatible. The score of the 70-gene assay was derived as described in the original papers (2, 15). The centroids of intrinsic subtypes were obtained from the Bioconductor package genefu. The formula of combining the individual scores for the four subtypes and tumor size were obtained from the original paper (3).

Results

Validation of the FGD3-SUSD3 metagene

We first confirmed that the breast cancer-specific FGD3-SUSD3 metagene, which was the most prognostic molecular feature in METABRIC, remains highly prognostic in all other data sets. Figure 1A shows the Kaplan-Meier survival curves of the FGD3-SUSD3 metagene demonstrating statistical significance in all five data sets. The gene most associated with the FGD3-SUSD3 metagene in METABRIC (also among the most associated ones in all the other data sets) is the estrogen receptor ESR1, which is less prognostic than FGD3-SUSD3 in all
five data sets (Figure 1B).

**Feature selection**

We then defined the features, which, when combined, would optimize prognostic performance in the METABRIC data sets. The *FGD3-SUSD3* metagene was an obvious such candidate feature. We also included the metagenes that we had used in the top-ranked models of the BCC, namely CIN (mitotic chromosomal instability), MES (mesenchymal transition) and LYM (lymphocyte infiltration) and two conditioned versions: MES*, restricted to early-stage tumors defined as lymph node negative with tumor size less than 30mm, and LYM*, restricted to samples with more than three positive lymph nodes (During our participation in the BCC we had found (6) that MES was prognostic only in early-stage cancers and that LYM, though protective overall, was associated with poor prognosis in the presence of multiple positive lymph nodes). Following our additional results analyzing other data sets from 12 different cancer types (16) in collaboration with The Cancer Genome Atlas (TCGA) Pan-Cancer project (17), we also considered the next top-ranked attractor metagene, END, a newly discovered multi-cancer molecular signature of endothelial markers. We also included all the molecular features whose combination is used in existing breast cancer prognostic assays: Oncotype DX (proliferation, invasion, estrogen, HER2 groups, *CD68, GSTM1, BAG1* genes); PAM50 defined molecular subtypes (Basal, Luminal A, Luminal B, HER2 features); the single 70-gene Mammaprint feature; and the three genes *ESR1, PGR, ERBB2* used (18) in the TargetPrint assay. Finally we included the number of positive lymph nodes and tumor size, as we had identified them in the BCC as two of the most prognostic clinical features.

We designed a feature selection web-based facility (www.ee.columbia.edu/~anastas/featureselector), which evaluates a prognostic score after selecting a specified number among the above features. The score was designed so that it will cease increasing when overfitting has occurred. We include logarithmic versions for the number of lymph nodes and the tumor size, because we found that the score becomes consistently higher if we include these versions rather than the direct values. The purpose of the overall facility is to provide an estimate of the performance of each of the existing assays by selecting the corresponding features, as well as to provide insight on the relative contribution of individual features when combined with other ones, leading to the
selection of an optimal biomarker. Instructive results, noted in the facility, are the identified best selection of a given number $N$ of features.

For $N = 1$, the most prognostic feature among those listed in the facility is the "Luminal A" feature of PAM50, which measures the degree of correspondence with a good prognosis subtype. However, the Luminal A feature is eliminated from the best choice of features when $N = 2$, in which case the optimal choice is the $FGD3-SUSD3$ metagene combined with the number of positive lymph nodes. At $N = 3$ the CIN metagene is also selected, followed in increasing order by tumor size, MES*, LYM, LYM*, $CD68$ and $END$, each of which increases the score, at which point ($N = 9$) it reaches the value of 0.741. Following this selection of nine features, no additional feature increases the score. To further increase performance, we then implemented a heuristic optimization algorithm by including randomly chosen single genes in combination with some or all of the selected features, retaining genes with both high cross-validation scores and known roles in cancer literature. We thus identified two additional genes, $DNAJB9$ and $CXCL12$, for a total number of eleven features increasing the score to 0.747. $DNAJB9$ has the property that, if included among the potential features, is selected as early as $N = 4$ (www.ee.columbia.edu/~anastas/featureselector2). The other gene, $CXCL12$, is selected at $N = 7$. Both of these genes are known to play important roles in cancer (19, 20).

**BCAM biomarker**

We thus defined our model based on the Cox model formula defined by the full METABRIC data set using the eleven features $FGD3-SUSD3$, CIN, MES*, LYM, END, LYM*, $CD68$, $DNAJB9$, $CXCL12$, number of positive lymph nodes, and tumor size (Supplementary Methods and Materials).

The final BCAM score between 0 and 100 is generated from the Cox model formula as the percentile value against the 1,981-sample METABRIC data set. Figure 2 shows the estimated breast cancer specific 10-year survival rate as a function of the BCAM score.
Validation in other data sets

We compared the prognostic performance of the BCAM formula with formulas of other genomic assays: Oncotype DX, MammaPrint, ROR-S (using PAM50 subtype information alone), and ROR-C (using PAM50 subtype information and tumor size). We used other breast cancer data sets appropriate for evaluating prognostic values to which we refer as Loi (9), Buffa (10), Wang (11) and Miller (12). For each data set, we also consider the two subsets, 1) lymph node-negative (LNN) patients, and 2) estrogen receptor-positive (ERP) patients (regardless of PR and HER2 status). Additional intersection of these sets would not lead to results of statistical significance.

BCAM outperformed the other genomic assays in all cases in which comparisons had statistical significance (Table 1). In most of these comparisons (except when comparing BCAM with ROR-C in the LNN subsets) BCAM makes use of clinical information not used in the other assays. These results demonstrate the advantage of integrating clinical stage with molecular feature information into one product with enhanced prognostic power.

Discussion

The results of our analysis of the METABRIC data set lead to the unexpected and remarkable suggestion that breast cancer subtype classification, as well as estrogen/progesterone receptor and HER2 status do not provide any additional prognostic information as long as the expression levels of the $FGD3$-$SUSD3$ and the attractor metagenes are known and taken into consideration. This suggestion is strengthened by the fact that the uniformly renormalized 1,981-sample METABRIC data set is uniquely rich and useful for reaching results of statistical significance in survival analysis.

In support of the above suggestion, using the publicly available web-based feature selector facility, for all feature combinations that we tried:

(a) Selecting the Oncotype DX Estrogen group, or any of genes $ESR1$ and $PGR$, in addition to any selected
feature combination that includes metagenes $FGD3$-$SUSD3$ and CIN, does not increase, and in most cases decreases the score.

(b) Replacing the selection of the Oncotype DX Estrogen group or any of genes $ESRI$ and $PGR$ (including any multiple selection of these features) with $FGD3$-$SUSD3$, in any selected feature combination, increases the score.

The expression values of Her2, PR and ER and subtype designation are correlated with the metagenes of BCAM and therefore are indirectly taken into account. They obviously provide vital information and improved understanding of breast cancer biology, which has led to effective treatments. However, our results suggest that an optimum breast cancer biomarker product does not need to include them.

Many early versions of microarray platforms, notably the popular Affymetrix U133A, do not contain probes for $FGD3$ and $SUSD3$, which may provide some explanation as to why these genes were not found earlier as highly prognostic in breast cancer. The two genes are genomically adjacent to each other and are correlated with $ESRI$ and $PGR$. The simultaneous silencing of $FGD3$ and $SUSD3$ is strongly associated (6) with poor prognosis. Furthermore, a recent study (21) identified $SUSD3$ as the single most predictive gene (more than $ESRI$) of response to aromatase inhibitor therapy. Based on the above facts, we believe that existing breast cancer prognostic assays should be reevaluated, and that the biological mechanism responsible for $FGD3$-$SUSD3$ silencing, as well as the corresponding phenotypic associations, should be priority research topics.

The alternative offered by the BCAM biomarker is one universal prognostic assay applicable to all breast cancer subtypes and stages, integrating tumor biology across stages. Indeed, as evidenced by the feature selector facility, the LYM and MES metagene would not be prognostic in the absence of stage information, and the conditioned LYM* and MES* features add significantly to the overall prognostic power. BCAM is also independent of tumor grade, since the CIN metagene is a proxy for, and more prognostic than, grade, or the expression of the $Ki67$ gene.
We also observed that inclusion of gene \textit{CD68}, used in the Oncotype DX assay, improves the prognostic performance of our model, of which we were not aware during our BCC participation. The expression of gene \textit{CD68}, a marker of tumor associated macrophages, is associated with worse prognosis, although it is positively correlated with the protective LYM lymphocyte infiltration signature, and their combination improves prognostic ability.

The CIN, MES and LYM and END features were previously identified and precisely defined by multi-cancer analysis (8, 16) as representing attributes of cancer in general. Furthermore, they were all found without any use of the METABRIC data set, and their prognostic power was independently confirmed in METABRIC. This raises the exciting possibility that they will also prove to be useful serving as "building blocks" of biomarkers in other types of cancer as well.

There are a few limitations to this study, one being the lack of large independent datasets that have outcomes as well as the necessary data for applying the BCAM model, such as the expression levels of genes \textit{FGD3} and \textit{SUSD3}. The four available independent data sets that we used have relatively small sample sizes and are difficult to pool. Furthermore, the estimated performance of existing biomarker assays on platforms different from those used in the actual product implementation is only suggestive and cannot be used as a definitive proxy of the actual performance. However, as a general justifying principle, relative gene expression has been shown to be reliable across platforms and measurement techniques. For example, a high level of interplatform concordance in terms of genes identified as differentially expressed was demonstrated (22) by comparing three RT-PCR platforms and seven microarrays including Illumina, Affymetrix, and Agilent.

There are several published examples of across-platform comparisons of breast cancer biomarker products: The predictions from five gene-expression-based models, including those used in Oncotype DX, MammaPrint and intrinsic subtypes have been compared with each other (23) using Agilent microarray data in all cases.
Furthermore, the ability of six genomic signatures, including Oncotype DX, MammaPrint and PAM50-ROR to predict relapse in breast cancer patients with ER+ tumors treated with adjuvant tamoxifen, was evaluated (24) using only four Affymetrix microarray data sets. As the authors acknowledge in that work, one important caveat to their analyses that must be recognized and always kept in mind when interpreting across platform genomic studies, such as the one presented here, is that "although we strove to implement each predictor as published, signatures developed on platforms other than the Affymetrix U133A [used in their work] were suboptimally implemented." Such imperfect comparisons, however, are still valuable. Many other publications including some recently published ones (25, 26) also use microarray values to compare breast cancer prognostic signatures.

Accordingly, the aim of our study is to demonstrate that our results are promising and to suggest that they should be rigorously evaluated in the context of larger-scale clinical studies either underway or being planned.

Acknowledgements

This study makes use of the METABRIC data set (5), generated by the Molecular Taxonomy of Breast Cancer International Consortium. Funding for that project was provided by Cancer Research UK and the British Columbia Cancer Agency Branch. These data were renormalized by Sage Bionetworks and accessed through Synapse (synapse.sagebase.org).
References

Table 1: List of scores, measured by the corresponding concordance index, after applying the formula of each prognostic assay on cancer data sets and their lymph node-negative (LNN) and ER-positive (ERP) subsets. Highlighted in blue are the values achieving highest score in each case. Highlighted in yellow and boldface are the scores for which the corresponding $P$ value of comparison with the BCAM score is less than 0.05. The last set of rows contains the scores from the METABRIC data set and the listed BCAM scores result from applying the formula on the entire data set. These cannot be compared with other scores because METABRIC was used for BCAM training. ROR-S uses the gene expression based PAM50 assay; ROR-C uses the gene expression plus tumor size based PAM50 assay; 21-gene uses the Oncotype DX 21-gene assay; and 70-gene uses the Mammaprint 70-gene assay.

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Figure captions

**Figure 1**: Kaplan-Meier survival curves on the basis of A, the FGD3-SUSD3 metagene B, the ESR1 gene, in five data sets. *P* values were derived using the log-rank test after dividing each data set into two equal-sized subgroups.

**Figure 2**: Breast cancer specific 10-year survival rate as a function of the BCAM score normalized as the percentile value against the 1,981-sample METABRIC data set.
Breast Cancer Prognostic Biomarker Using Attractor Metagenes and the FGD3-SUSD3 Metagene


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