Published OnlineFirst July 7, 2015; DOI: 10.1158/1078-0432.CCR-15-0630

Personalized Medicine and Imaging

Clinical Cancer Research

Prediction of Response to Neoadjuvant Chemotherapy Using Core Needle Biopsy Samples with the Prosigna Assay

Aleix Prat[1,2], Patricia Galván[1], Begoña Jimenez[3], Wesley Buckingham[4], H. Arthur Jeiranian[4], Carl Schaper[4], Maria Vidal[1,2], Martina Álvarez[3], Sherley Díaz[5], Catherine Ellis[6], Paolo Nuciforo[7], Sean Ferree[4], Nuria Ribelles[3], Barbara Adamo[2], Santiago Ramón y Cajal[5], Vicente Peg[5], and Emilio Alba[3]

Abstract

Purpose: Most hormone receptor (HR)$^+$/HER2$^-$ breast cancer patients respond unfavorably to neoadjuvant chemotherapy (NAC); however, genomic tests may identify those patients who are likely to benefit. Using the Prosigna assay, we first evaluated the technical performance of core needle biopsy (CNB) tissues. We then determined whether Prosigna risk of relapse (ROR) score and intrinsic subtype predicted response to NAC in HR$^+$/HER2$^-$ patients using CNB samples.

Experimental Design: Using the NanoString's nCounter Dx analysis system and a development tissue sample set, we established tissue requirements and assay output variance. We then evaluated the concordance in subtype and correlation in ROR between CNBs and corresponding surgical resection specimens (SRS) in a second independent sample set. Finally, we analyzed 180 independent CNB samples from HR$^+$/HER2$^-$ patients who were treated with NAC and correlated ROR and intrinsic subtype with pathologic response.

Results: Intra- and interbiopsy variabilities were 2.2 and 6.8 ROR units, respectively. Subtype concordance within multiple CNBs was high for the 4- and 3-subtype classifications (k = 0.885 and 0.889, respectively). Correlation in Prosigna ROR score observed between paired CNBs and SRS was high ($r \geq 0.90$), and subtype concordance was also high for the 4- and 3-subtype classifications (kappa = 0.81 and 0.91, respectively). Prosigna results obtained from the HR$^+$/HER2$^-$ patient samples showed that both ROR ($P = 0.047$) and intrinsic subtype (OR LumA vs. non-LumA = 0.341, $P = 0.037$) were significant predictors of response to NAC.

Conclusions: Prosigna ROR and intrinsic subtype are readily obtained from CNB samples in normal practice and reliably predict response to NAC in HR$^+$/HER2$^-$ patients. *Clin Cancer Res; 22(3); 560–6. ©2015 AACR.*

Introduction

The likelihood of a successful and minimally invasive surgical resection can be greatly increased in a subset of breast cancer patients by administering neoadjuvant chemotherapy (NAC; ref. 1). In addition, recent studies have also shown that pathologic surrogate endpoints for NAC efficacy, such as pathologic complete response (pCR) or residual cancer burden (RCB), are significantly associated with improvement in disease-free and overall survival (2–4). Administration of multiple cycles of neoadjuvant anthracyclines and taxanes has been shown to achieve reasonable pathologic responses in some subgroups of patients; however, patients with hormone receptor–positive, human growth factor receptor–negative (HR$^+$/HER2$^-$) tumors are of particular concern due to their limited response rates (4–6). Next-generation diagnostic tests may be able to identify those patients with negligible response rates who may be spared ineffective and costly treatments. As in the adjuvant setting where the clinicopathologic features are commonly supplemented with genomic information in order to tailor treatment decisions to the patient, genomic information may also be useful in the neoadjuvant setting for similar decisions.

During the last decade, several prognostic and predictive multigene tests have been developed in an effort to tailor specific treatment strategies for patients with breast cancer (6, 7). NanoString's PAM50-based Prosigna assay on the nCounter Dx Analysis System has recently been introduced as a novel and decentralized formalin-fixed, paraffin-embedded (FFPE)-based classifier to identify the intrinsic molecular subtypes of breast cancer (i.e., Luminal A, Luminal B, HER2-enriched, and Basal-like) and to estimate the 10-year risk of recurrence (ROR) in postmenopausal patients treated with adjuvant endocrine therapy alone (7–10). In two large randomized phase III trials (ATAC and ABSCG8) involving 2,485 patients treated only with adjuvant endocrine therapy for 5 years, the Prosigna test provided

[1]Translational Genomics Group, Vall d'Hebron Institute of Oncology (VHIO), Barcelona, Spain. [2]Medical Oncology Department, Hospital Clínic, Universitat de Barcelona, Barcelona, Spain. [3]University Hospital Regional and Virgen de la Victoria of Malaga, Malaga, Spain. [4]NanoString Technologies, Seattle, Washington. [5]Department of Pathology, Hospital Universitari de la Vall d'Hebron, Barcelona, Spain. [6]GlaxoSmithKline, Collegeville, Pennsylvania. [7]Molecular Oncology Group, VHIO, Barcelona, Spain.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Aleix Prat, Hospital Clínic de Barcelona, Villarroel 170, 08036 Barcelona, Spain. Phone: 646847709; Fax: 34932100860; E-mail: aprat@vhio.net

doi: 10.1158/1078-0432.CCR-15-0630

Published OnlineFirst July 7, 2015; DOI: 10.1158/1078-0432.CCR-15-0630

Translational Relevance

Most hormone receptor (HR)$^+$/HER2-negative breast cancer patients respond unfavorably and do not benefit from chemotherapy; however, next-generation genomic tests may identify patients who are likely to benefit from chemotherapy while sparing the rest from a costly and ineffective treatment. The Prosigna Breast Cancer Gene Signature Assay identifies the intrinsic subtypes and utilizes gene expression data, weighted together with clinical variables, to generate a risk of relapse (ROR) score that assesses a patient's individual risk of distant recurrence. In this study, we first evaluated the technical performance of formalin-fixed paraffin-embedded biopsy tissues with the Prosigna assay, and then evaluated the ability of Prosigna ROR score and intrinsic subtype as predictors of response to neoadjuvant multiagent chemotherapy in HR$^+$/HER2$^-$ patients. Findings from this study demonstrate the feasibility of performing Prosigna in biopsy tissues and its ability to identify HR$^+$/HER2$^-$ patients who benefit the most from chemotherapy.

significant information above and beyond all common clinical–pathologic variables for the prediction of distant recurrence (8, 9). In these two studies, the ability of the Prosigna test to predict late recurrence (i.e., outcome after 5 years of endocrine therapy) has also been demonstrated (10, 11). In the United States, Prosigna ROR score was cleared by FDA in 2013 as a continuous predictor of distant recurrence in stage I node-negative and stage II node-negative and node-positive (1–3 nodes) postmenopausal HR$^+$ breast cancer patients. Although there have been studies demonstrating the effectiveness of PAM50 and intrinsic subtyping in predicting outcome to (neo)adjuvant treatment (9, 12, 13), the chemopredictive value of Prosigna ROR score and intrinsic subtyping based on the Prosigna test has remained uncharacterized.

In order to evaluate the performance of next-generation genomic tests for the purposes of guiding treatment prior to surgery, the following criteria can be used to lay the foundation for their clinical adoption: First, it is necessary to demonstrate the analytical validity of the testing platform with the associated tissue type. Second, when analytical performance has been established, the ability of the assay to predict response to contemporary therapies is examined. In this study, we use these criteria to assess the performance of the Prosigna assay in predicting response to chemotherapy in the neoadjuvant setting using core needle biopsy (CNB) samples.

Materials and Methods

CNB development study population

A total of 122 FFPE tumor blocks containing CNB samples from 95 patients with newly diagnosed invasive breast carcinomas from 2008 to 2014 from a single institute were retrospectively evaluated and designated as the CNB development study sample set (Table 1). This sample set was further divided and used to establish and subsequently test all technical feasibility requirements for assaying CNBs on the NanoString nCounter Dx Analysis System. Of the 122 FFPE tissue blocks, 30 were used to establish tissue requirements, 33 for determining assay variability within multiple RNA extractions performed on the same CNBs, 29 for determining assay variability across CNBs, and the final 30 for comparing assay output between CNBs and corresponding surgical resection specimens (SRS; Supplementary Fig. S1). Retrospective evaluation revealed that ROR and subtype distributions between sample sets were similar. A pathologist reviewed a hematoxylin and eosin (H&E) stain of a slide-mounted tumor section to identify the tumor surface area and the percentage of viable tumor cells for each sample. Tissue selection required a minimum of $\geq$2 mm^2 and $\geq$10% tumor cells. To ensure representation of all the subtypes, tumors falling in the following four categories were selected: estrogen receptor (ER)–positive/HER2-negative/Ki67-low, ER-positive/HER2-negative/Ki67-high, ER-negative/HER2-positive, and ER-negative/HER2-negative. The ER and the HER2 statuses as well as the proliferation activity measured by Ki-67 were extracted from pathology reports. The receptor status was determined during routine diagnosis by immunohistochemistry; in case of HER2 2$^+$ (DAKO score), HER2 positivity was followed by silver enhanced *in-situ* hybridization. The CNBs were taken under ultrasound guidance with 14G needles. This study was approved by the Vall d'Hebron Institutional Review Board (IRB) in Barcelona, Spain.

Metastatic study population

A total of 40 FFPE tumor blocks containing either CNB or excisional biopsy samples from metastatic lesions were

Table 1. Clinical–pathologic characteristics of primary breast cancers

	CNB development study		Chemo-prediction validation study	
Characteristic	***N***	**%**	***N***	**%**
All cases[a]	95	100	180	100
Histologic type				
Ductal carcinoma	85	89.5	130	72.2
Lobular carcinoma	8	8.4	20	11.1
Other carcinoma	2	2.1	30	16.7
Tumor size (mm)				
≤20	61	64.2	18	10
>20	34	35.8	162	90
Nodal status				
N0	56	58.9	67	37.2
N1	25	26.3	60	33.4
N2	7	7.4	40	22.2
N3	4	4.2	13	7.2
Nx	3	3.2	0	
Histologic grade				
G1	10	10.5	27	15
G2	57	60	96	53.3
G3	28	29.5	46	25.6
GX			11	6.1
HER2 status				
Positive	16	16.8	0	0
Negative	79	83.2	180	100
ER status				
Positive	83	87.4	179	99.4
Negative	12	12.6	1	0.6
PR status				
Positive	71	74.7	153	85
Negative	24	25.3	24	13.3
Unknown			3	1.7
Ki67%				
≤14	35	36.8	4	2.2
>14	60	63.2	174	96.7
Unknown			2	1.1

[a]Only cases with clinical data (chemo-prediction validation set).

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collected from a single institute and retrospectively evaluated. Ten independent breast cancer samples were collected from each of four metastatic sites: skin, lymph node, lung, and liver. RNA extraction followed the minimum tissue requirements determined from the CNB development study population. To correlate the Prosigna subtype results with pathologic data, ER (DAKO; cat#IS-657), progesterone receptor (PR; DAKO; cat#IS-068), and HER2 statuses (DAKO; cat#K5331 ± silver enhanced *in-situ* hybridization) were determined on each metastatic tumor sample (except one due to lack of tissue) following the 2010 and 2013 ASCO/CAP guidelines (14, 15).

SRS study population

From the CNB development sample set, 30 independent samples were selected that also had a corresponding SRS. The time between CNB and SRS was 14 to 21 days for all patients. Since these were banked samples, a new section was cut for H&E staining for each SRS and compared with the initial H&E-stained section performed on the same SRS block, and also compared with the paired CNBs. In order to correct for extensive specimen block usage, we ensured that the banked tumor blocks contained sufficient tissue representative of the original tumor.

Chemo-prediction validation population

A total of 216 locally assessed $HR^+/HER2^-$ banked breast tumor samples from breast cancer patients in an independent multicenter Spanish cohort were selected for the chemo-prediction validation study and retrospective analysis. Selection criteria required that tissue samples were collected within the last 10 years, the patient had completed a neoadjuvant chemotherapy treatment regimen, and the tissue sample was reviewed by a pathologist prior to testing with Prosigna. All patients had been prescribed a standard NAC regimen consisting of 8 to 10 cycles of anthracyclines and taxanes. The RNA extraction procedure for this sample set used a modified version of the minimum tissue requirements established from the CNB development study population due to the presence of multiple cores embedded in the same tissue block. A total of 180 patients within this population with passing assay results also had an RCB classification. Mean patient age in this population was 50.1 ± 11.1 years. We included pre-, peri-, and postmenopausal status in order to test as many samples from the tissue bank as possible. Although we acknowledged that previous studies evaluating the ability of Prosigna to provide prognostic information were performed in postmenopausal patients, this study aimed to evaluate the ability of Prosigna's ROR score and intrinsic subtype to predict response in a representative cohort of patient's eligible for neoadjuvant therapy. An RCB status of RCB-0 or RCB-I was classified as responding to chemotherapy, whereas an RCB status of RCB-II+ was classified as chemoresistant. These classifications are commonly used to differentiate between responders and nonresponders. Previous studies have shown that classifications of RCB-0 or RCB-I are associated with similarly high rates of distant relapse-free survival (DRFS), whereas classifications of RCB-II or RCB-III are associated with significantly lower rates of DRFS (2). All 216 patients were consented for participation in this study, and the study was approved by the IRB at IBIMA (Institute of Biomedical Research of Málaga).

RNA extraction

RNA was extracted from slide-mounted breast tissue sections using a RNA extraction kit manufactured by Roche according to NanoString's procedures (16). Following removal of genomic DNA, RNA was eluted (30 μL volume) and tested to ensure it met the specifications for concentration (≥12.5 ng/μL) and purity (OD 260/280 nm, 1.7–2.3). Tissue requirements for SRS followed the PAM50 Package Insert specifications (16).

Assay output data

For the CNB development studies, the Prosigna assay was performed as previously described (16–18). The following Prosigna data were evaluated: 4-class subtype classification (Luminal A, Luminal B, HER2-enriched, and Basal-like), 3-class subtype classification (Luminal A/B, HER2-enriched, and Basal-like), ROR score (0–100), proliferation score (the geometric mean expression of 18 proliferation-related genes of the Prosigna assay), and the correlation to each of the 4-subtype centroids.

For the chemo-prediction validation study, Prosigna results including ROR score and intrinsic subtype were obtained for each patient sample. In this population, nodal status had been determined via standard institutional procedures, and initial tumor size had been obtained by imaging methods. Mammographic tumor size (≤2 cm or >2 cm) was used for the calculation of the Prosigna ROR score. The Prosigna assay procedure, as utilized in the clinical validation of Prosigna (TransATAC, ABSCG.8), included the gross tumor size factor (≤2 cm, >2 cm) in the ROR algorithm (7, 8, 16).

Statistical analysis

For the CNB development studies, the tissue volume requirements of the Prosigna test in CNBs were determined to reflect the lower 95% confidence limit for obtaining a nucleic acid concentration of >20 ng/μL. To estimate the variability of each test output, linear mixed models were used to estimate the SD for multiple RNA extractions and Prosigna tests within a single CNB, as well as RNA extractions and Prosigna tests on multiple CNBs from the same tumor. For these analyses, variability in the various components of the ROR score (subtype correlation and proliferation score) was reported in order to illustrate that variability was not attributable to any single component. We then estimated correlation and concordance using Pearson coefficients and multirater kappa values, respectively, to compare Prosigna results between paired CNBs and SRS. For the chemo-prediction validation study, we evaluated whether Prosigna ROR score or intrinsic subtype was correlated with RCB status using univariate logistic regression models.

Results

Identification of tissue requirements for CNBs

To identify the minimum tissue requirements for CNBs, we evaluated the tumor surface area and cellularity of 30 independent CNBs from the CNB development population. The baseline median surface area and cellularity of these samples were 10.2 mm^2 (range, 4–16) and 45% (range, 20–90), respectively. FFPE sections (10-μm-thick) per sample were obtained (except for 2 cases with 5 and 8 sections each), and RNA was extracted. Median RNA yield concentration and purity (i.e., 260/280 ratio) were 155.3 ng/μL (range, 28.0–278.9) and 2.0 (range, 1.9–2.04), respectively. Correlation between surface area and RNA concentration, or total surface area and RNA concentration, was

Published OnlineFirst July 7, 2015; DOI: 10.1158/1078-0432.CCR-15-0630

Table 2. SD and subtype concordance between multiple CNBs

	SD						Concordance	
	ROR	Basal-like	HER2E	LumA	LumB	Proliferation score	4-subtype classification	3-subtype classification
Multiple RNA extractions from the same CNB	2.2	0.02	0.02	0.02	0.02	0.05	94% (k = 0.889)	100%
Multiple CNBs of the same tumor	6.8	0.08	0.09	0.10	0.08	0.22	93.2% (k = 0.885)	100%

low [Pearson correlation coefficient (r) of 0.13 and 0.24). All cases met the Prosigna minimum RNA specification (16).

Based on the results of these first 30 specimens, the minimum tissue requirements to reflect the lower 95% confidence limits of obtaining a minimum RNA concentration of >20 ng/μL were as follows: >12 mm^2 = two 10 μm slides; 6 to 12 mm^2 = four 10 μm slides; <6 mm^2 = eight 10 μm slides. The RNA yield and assay pass rate of the established tissue volume requirements were then tested on an independent set of 30 CNBs. Median RNA concentration and purity were 45.46 ng/μL (range, 15.5–97.2) and 1.98 (range, 1.83–2.15), respectively. All cases met the Prosigna RNA minimum specifications (16).

Assay output variability in CNB

In order to evaluate the variability of the Prosigna assay outputs (i.e., subtype call and ROR score) within CNBs, multiple extractions from a single CNB (i.e., same tumor cylinder) were tested with the assay. A total of 84 extractions from 32 independent CNBs (10 patients) from the CNB development population were tested (average of 2.6 extractions per CNB). Subtype calls were 98% concordant (82/84), and the average SD for the ROR score was 2.2 units (Table 2). Subtype call discordance in the 2 cases was limited to switching between Luminal A and Luminal B resulting from minor variability between different RNA extractions. Variability in the Pearson correlation coefficients for the various components of the ROR score did not exceed 0.05 (Table 2).

To determine the Prosigna assay output variability across different CNBs of the same tumor, a total of 79 CNBs from 30 independent tumors (average of 2.7 CNBs per tumor) were evaluated. Subtype calls were 94% concordant (74/79), and the SD for the ROR score was 6.8 units (Table 2). All 5 subtype discordant cases were between Luminal A and B subtypes. Variability in the Pearson correlation coefficients for the various components of the ROR score did not exceed 0.10 (Table 2). Given that the SD for the ROR score within multiple RNA extractions of the same CNB is similar to that reported for ROR score within an SRS (17), we hypothesize that the increased variance observed across multiple CNBs of the same tumor is likely due to molecular heterogeneity within the tumor tissue (18, 19).

Assay output concordance between paired CNBs and SRS

In order to further evaluate the integrity of results captured from CNBs using the tissue requirements established by this study, we tested the Prosigna assay output concordance between paired CNBs and SRS in an independent, retrospective dataset of 30 paired samples from the CNB development population. None of these samples were tested in the experiments described previously. Comparison between paired CNB and SRS samples resulted in a correlation coefficient of ≥0.90 (range, 0.90–0.98) for ROR score, proliferation score, and the 4-subtype centroid correlations. Intrinsic subtype concordance between paired CNBs and SRS was 87.1% (kappa = 0.81) and 96.8% (kappa = 0.91) for the 4-subtype and 3-subtype classifications, respectively (Table 3). Three of 4-subtype discordances were between Luminal A versus B calls. The other discordant case was between a Luminal B and Basal-like disease.

Individual gene correlations between paired CNB and SRS

We then evaluated the correlation coefficients of the expression of each gene contained in the Prosigna assay in CNB versus SRS (Supplementary Table S1). Gene expression normalization was performed using a proprietary algorithm and a synthetic reference sample (unpublished data). The median correlation coefficient was 0.91 (range, 0.62–0.99). The correlation coefficients of *ERBB2*, *ESR1*, and *PGR* were 0.99, 0.98, and 0.97, respectively. Only 4 genes (i.e., *KRT5*, *KRT14*, *KRT17*, and *MMP11*) showed a correlation coefficient below 0.80. Interestingly, high expression of *KRT5*/14/17 and low expression of *MMP11* are characteristic of normal breast tissue (20), which may indicate the presence of normal breast contamination among a portion of the paired specimens.

Prosigna in metastatic breast cancer specimens

The RNA yield and assay pass rate of the established tissue volume requirements were then tested in 40 independent metastatic breast cancer specimens from 4 organs (skin, lymph node, lung, and liver). Median RNA yield concentration and purity were 120.5 ng/μL (range, 16.9–637.9) and 1.96 (range, 1.70–2.01), respectively. All cases but one met the Prosigna RNA minimum specifications (16). Of note, the median RNA concentration in metastatic tissues was higher compared with the median RNA concentration in CNB (i.e., 45.46 ng/μL) since most metastatic tissues were obtained from surgical resections.

In this metastatic breast cancer 40-sample dataset, all the intrinsic molecular subtypes were identified [Basal-like (n = 5), HER2-enriched (n = 4), Luminal A (n = 8), and Luminal B (n = 22)]. As expected, 6 of 7 ER-negative samples (85.7%) were identified as Prosigna nonluminal (i.e., Basal-like or HER2-enriched), 29 of 32 ER-positive samples (90.6%) were identified as Prosigna luminal (i.e., Luminal A or B), and 3 of 3 HER2-positive samples (100%) were identified as HER2-enriched (Supplementary Table S2). Taken together, these data suggest that reliable Prosigna assay results can be obtained from tissue biopsies.

Table 3. Correlation coefficients and subtype concordance between paired CNBs and SRS

Pearson correlation coefficient						Concordance	
ROR	Basal-like	HER2E	LumA	LumB	Proliferation score	4-subtype classification	3-subtype classification
0.90	0.98	0.94	0.96	0.97	0.92	87.1% (k = 0.813)	96.8% (k = 0.918)

Published OnlineFirst July 7, 2015; DOI: 10.1158/1078-0432.CCR-15-0630

Table 4. Clinical response and intrinsic subtype data for patient population

Characteristic	Chemo-prediction validation study *N* (%)
Total cases	216 (100)
Assay passed	207 (95.8)
Clinical data available	180
CR	
RCB-0/I (responder)	34 (18.9)
RCB-II+ (nonresponder)	146 (81.1)
Luminal A	54 (30)
Nonresponder	49 (90.7)
Responder	5 (9.3)
Luminal B	105 (58.3)
Nonresponder	84 (80.0)
Responder	21 (20.0)
HER2-enriched	7 (3.9)
Nonresponder	6 (85.7)
Responder	1 (14.3)
Basal	14 (7.8)
Nonresponder	7 (50)
Responder	7 (50)

Assay pass rate

The Prosigna assay pass rate of CNBs evaluated in all of the development studies using the predefined tissue requirements criteria was 234/238 (98.3%). The tissue requirements defined in the development studies guided the methods that were used to test the CNB samples in the chemo-prediction validation study. In the chemo-prediction validation study, modifications to the tissue requirements were made to account for tissue blocks containing multiple cores. The Prosigna assay pass rate of CNBs evaluated in the chemo-prediction validation study was 207/216 (95.8%).

Univariate analysis of ROR and intrinsic subtype and RCB status

The overall neoadjuvant chemotherapy response rate, defined by an RCB status of RCB-0/I, in the chemo-prediction validation study population was 18.9% ($n = 34$; Table 4). We evaluated the chemopredictive value of the results from the Prosigna assay in this population. We found that the continuous ROR score was a significant predictor of response to NAC ($P = 0.047$; Table 5). When the patient population was limited to N_0–N_1 patients (as used for Prosigna's clinical validation), ROR remained a strong predictor of response to NAC using pCR as the endpoint (P value $= 0.027$). The ROR score is a composite measure of the tumor's gene expression profile relative to that of the prototypical intrinsic subtypes as well as the tumor's relative expression of genes involved in proliferation. An increase in the expression of proliferation genes is associated with an increase in ROR score. Further analysis revealed that for every 20 point increase in ROR score, a patient was 59.1% more likely to respond to chemotherapy in the neoadjuvant setting (Fig. 1).

Table 5. Chemo-predictive value of ROR score and intrinsic subtype

Predictor	Chemo-prediction OR	Validation study *P*
ROR score		0.047
10 point increase	1.261	
20 point increase	1.591	
P score	1.416	0.005
Subtype		
Luminal A versus other	0.341	0.037

As a component of ROR score and a measure of the proliferative capacity of the tumor, we evaluated the ability of proliferation score (P score) to predict response to NAC. Analysis of P score as a continuous variable revealed that P score is also significantly associated with response to chemotherapy ($P = 0.005$; Table 5). Mean KI-67 (IHC) and mean proliferation score (gene expression) values were significantly different between responders and nonresponders ($P < 0.01$, respectively). Subtype classification in this population and response to chemotherapy is shown in Table 4. Categorical analysis of intrinsic subtype using a logistic regression model revealed that Luminal A tumors are significantly less likely to respond NAC relative to the other intrinsic subtypes (OR Luminal A vs. non-Luminal A $= 0.341$, $P = 0.037$; Table 5).

Discussion

Given that genomic testing for breast cancer in the neoadjuvant setting requires compatibility of the test with CNB tissue, we first evaluated the feasibility of the Prosigna assay in FFPE CNBs and compared CNB results to corresponding FFPE surgical breast cancer sections. Our results show that the tumor tissue requirements for performing Prosigna in CNBs are practical in daily clinical practice (19). All the CNBs evaluated in this portion of the study were from retrospective diagnostic specimens that had already been used for standard histopathologic workup including determination of the status of ER, PR, HER2, and Ki67. Importantly, we focused on the extreme case of only a single core (i.e., 1 tumor cylinder) per tumor block, whereas in current clinical practice, $\geq$2 diagnostic CNBs are usually obtained from the same tumor. We also found that the Prosigna assay in CNBs was highly reproducible when multiple extractions from the same CNBs were compared. The measured total SD was 2.2 ROR units on a 0 to 100 scale. This SD is similar to the previously reported SD of 2.9 ROR units observed with SRS (17), suggesting that the tissue collection method does not introduce additional variability into the assay output. As expected, when different CNBs of the same tumor were compared, the measured SD increased to 6.8 ROR units. The observed increase in variability is likely due to different areas of the tumor being biopsied (i.e., intratumor heterogeneity; refs. 18, 19). It is also possible that the quality of a subset of the biopsy pairs was biased with regard to tissue quality (e.g., more or less normal tissue contamination). Nonetheless, the robustness of the ROR score and the subtype concordance rate between different CNBs of the same tumor were very high. Pathologic review of the CNB and SRS of the single Luminal B to Basal subtype switch revealed that the CNB (Luminal B) was Luminal B-like [ER-positive (100%)/PR-positive (100%)/HER2-negative/Ki67 (30%)], whereas the SRS (Basal-like) had lower luminal features and higher proliferation [ER-positive (100%)/PR-negative (0%)/HER2-negative/Ki67 (60%)] than the corresponding CNB sample. Identification of Basal-like disease within ER-positive breast cancer has been previously reported (21). Considering the differences in precision between multiple biopsies from a single tumor and multiple extractions from a single biopsy, these discordant results likely reflect intratumoral heterogeneity (18, 19).

The distributions of intrinsic subtype as determined by Prosigna for the metastatic biopsy samples given their HR and HER2 statuses were of the expected frequencies. Furthermore, the Prosigna subtype concordance and the Prosigna signature score

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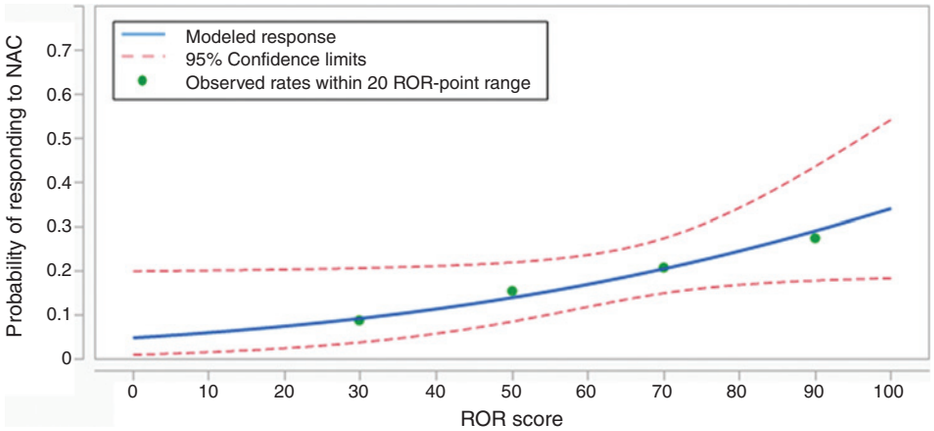

Figure 1.
Probability of responding to NAC plotted as a function of ROR score.

correlations between paired CNBs and SRS were high ($\geq$0.81 kappa scores and $\geq$0.90 correlation coefficients). Taken together, these data confirm that Prosigna test outputs obtained from diagnostic CNBs are both reliable and reflective of the primary SRS.

We then used diagnostic CNBs to evaluate the chemopredictive performance of Prosigna in an independent sample set. We found that both ROR score and intrinsic subtype are significant predictors of response to chemotherapy in the neoadjuvant setting. The patients tested in this portion of the study were all centrally confirmed $HR^+/HER2^-$ from a multicenter Spanish cohort, and all patients had received multiple cycles of a standard anthracycline and taxane regimen prior to surgery.

A tumor's intrinsic subtype, as defined by the Prosigna algorithm, describes the relative similarity of the tumor's gene expression profile to that of the prototypical profiles of Luminal A, Luminal B, HER2-enriched, or Basal-like tumors. Each of these subtypes is associated with the upregulation or downregulation of clusters of genes associated with largely distinct proliferative and survival pathways. The subsets of Prosigna signatures that are directly and primarily involved in proliferation are typically downregulated in Luminal A tumors relative to the other subtypes. Indeed, the gene expression profile of Luminal A tumors is largely similar to normal tissue with the exception of upregulation of the ER-related cluster of genes. This estrogen dependence and indolent nature indicate that Luminal A tumors would be less sensitive to chemotherapeutic agents that target replicative processes in tumor cells, an observation that is confirmed by the results of this study.

The ROR score is the product of an algorithm that measures the relative similarity between the gene expression profile of a patient's tumor and the expected profile for the four intrinsic subtypes. Genes involved in proliferation are additionally weighted and tend to collectively increase the ROR score when upregulated. A key characteristic of tumors that are identified as Luminal B, HER2-enriched, and Basal-like is the upregulation of proliferation genes, whereas tumors identified as Luminal A are more indolent. In practice, Luminal A tumors have lower ROR scores than other subtypes. Previous studies have shown that the sensitivity of a tumor to chemotherapeutic agents is associated with its proliferative state (22–24). Thus, it is not surprising that ROR score, as an inclusive measure of a tumor's proliferative potential, can predict those tumors that would be more sensitive to chemotherapeutic agents.

Given that we did not select for age, menopausal status, or cancer stage within this study, the results presented here highlight the value of consideration of tumor biology, in the form of intrinsic subtype and proliferation information delivered by the Prosigna assay, in therapy selection. The heterogeneity of the HR-positive subgroup of patients may not be adequately characterized by assays that do not consider the underlying aggressive nonluminal gene expression profiles.

One drawback of our study is the lack of long-term outcomes on these patients, which calls into question whether prediction of NAC sensitivity based on the ROR score or intrinsic subtype will also lead to prediction of a survival benefit. There are data supporting the fact that response to neoadjuvant therapy can be a surrogate to long-term outcome, such as RFS or overall survival (2). In fact, the FDA recently accepted the use of pathologic responses observed in the neoadjuvant setting as a surrogate endpoint for approval for neoadjuvant use of pertuzumab (25–27) based on the observed high correlation between pathologic response and long-term outcome in early breast cancer as confirmed in the NSABP B-18 trial and by others (28–30). In conclusion, the results of this study indicate that accurate Prosigna results can be reliably obtained from diagnostic CNBs. Prosigna ROR score and intrinsic subtype are strong predictors of response to chemotherapy in the neoadjuvant setting.

Disclosure of Potential Conflicts of Interest

A. Prat and C. Schaper are consultants/advisory board members for Nanostring Technologies. W. Buckingham and S. Ferree have ownership interests (including patents) in NanoString Technologies. E. Alba reports receiving speakers bureau honoraria from NanoString Technologies, Novartis and Roche. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: A. Prat, W. Buckingham, C. Schaper, C. Ellis, S. Ferree, V. Peg, E. Alba

Development of methodology: A. Prat, P. Galván, B. Jimenez, W. Buckingham, M. Álvarez, S. Ferree, V. Peg, E. Alba

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Prat, P. Galván, B. Jimenez, M. Vidal, P. Nuciforo, N. Ribelles, B. Adamo, S.R.y. Cajal, V. Peg

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Prat, P. Galván, W. Buckingham, H.A. Jeiranian, C. Schaper, P. Nuciforo, S. Ferree, B. Adamo, V. Peg, E. Alba

Writing, review, and/or revision of the manuscript: A. Prat, P. Galván, B. Jimenez, W. Buckingham, H.A. Jeiranian, M. Vidal, C. Ellis, S. Ferree, B. Adamo, S.R.y. Cajal, E. Alba

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P. Galván, H.A. Jeiranian, S. Díaz
Study supervision: A. Prat, B. Jimenez, S. Ferree, E. Alba

Grant Support

This study was funded by a research contract with NanoString Technologies, by a Career Catalyst Grant from the Susan Komen Foundation (to A. Prat), by GlaxoSmithKline and by funds from the Banco Bilbao Vizcaya Argentaria (BBVA) Foundation (to A. Prat).

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Received March 16, 2015; revised June 2, 2015; accepted June 24, 2015; published OnlineFirst July 7, 2015.

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Published OnlineFirst July 7, 2015; DOI: 10.1158/1078-0432.CCR-15-0630

Clinical Cancer Research

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Aleix Prat, Patricia Galván, Begoña Jimenez, et al.

Clin Cancer Res 2016;22:560-566. Published OnlineFirst July 7, 2015.