

Development and Validation of a Novel Radiosensitivity Signature in Human Breast Cancer

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Abstract

Purpose: An unmet clinical need in breast cancer management is the accurate identification of patients who will benefit from adjuvant radiotherapy. We hypothesized that integration of post-radiation clonogenic survival data with gene expression data across breast cancer cell (BCC) lines would generate a radiation sensitivity signature (RSS) and identify patients with tumors refractive to conventional therapy.

Experimental Design: Using clonogenic survival assays, we identified the surviving fraction (SF-2Gy) after radiation across a range of BCC lines. Intrinsic radiosensitivity was correlated to gene expression using Spearman correlation. Functional analysis was performed *in vitro*, and enriched biologic concepts were identified. The RSS was generated using a Random Forest model and was refined, cross-validated, and independently validated in additional breast cancer datasets.

Results: Clonogenic survival identifies a range of radiosensitivity in human BCC lines (SF-2Gy 77%-17%) with no

significant correlation to the intrinsic breast cancer subtypes. One hundred forty-seven genes were correlated with radiosensitivity. Functional analysis of RSS genes identifies previously unreported radioresistance-associated genes. RSS was trained, cross-validated, and further refined to 51 genes that were enriched for concepts involving cell-cycle arrest and DNA damage response. RSS was validated in an independent dataset and was the most significant factor in predicting local recurrence on multivariate analysis, outperforming all clinically used clinicopathologic features.

Conclusions: We derive a human breast cancer-specific RSS with biologic relevance and validate this signature for prediction of locoregional recurrence. By identifying patients with tumors refractory to standard radiation this signature has the potential to allow for personalization of radiotherapy. *Clin Cancer Res*; 21(16); 3667–77. ©2015 AACR.

Introduction

Breast cancer is the most common form of cancer in women and the second most common cause of cancer-related death (1). Traditionally, breast cancer has been treated with surgery, radiation, and chemotherapy and while these modalities are effective for many early-stage breast cancers, local control remains a significant issue for some. While previous meta-analyses have consistently shown a clinically significant risk reduction with the addition of adjuvant radiotherapy, it is clear

that a significant percentage of patients are either over- or undertreated (2).

Recent gene expression profiling studies in breast cancer have identified clinically significant heterogeneity among breast tumors in terms of gene and protein expression that is not fully accounted for by the standard histopathologic classification of breast cancer (3–5). Furthermore, studies detailing the poor response of basal-like (often ER-negative, PR-negative, and HER2/*neu*-negative) and HER2/*neu*-positive tumors (in the pre-trastuzumab era) to adjuvant radiotherapy further underscore the biologic differences and as yet undefined oncogenic drivers of these particular types of tumors (6, 7).

Previous attempts to identify radiation-specific signatures to predict likelihood of benefit to adjuvant radiotherapy have either failed independent validation or have not been disease-specific resulting in inconsistent performance in independent cohorts (8–12). A recent study demonstrated the feasibility of this approach in the postmastectomy setting using patient-derived gene expression data from the DBCG82bc trials (13). We hypothesized that the integration of post-radiation clonogenic survival data with gene expression data across a large spectrum of breast cancer cell (BCC) lines would generate a breast cancer-specific radiation sensitivity signature (RSS) that would predict radiation response in breast cancer patients and allow identification of patients with tumors refractive to conventional therapy. We then validate this signature on an independent clinical breast cancer

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Translational Relevance

Current trimodality therapy is effective for many women with breast cancer. Despite its efficacy, some women will develop locoregional recurrence after standard treatment and some women do not benefit from radiation. Currently, there is no test that can accurately identify which women may need treatment intensification and which women are at sufficiently low risk that adjuvant radiotherapy is not needed. Here we use the intrinsic radiosensitivity of human breast cancer cell lines to develop a molecular signature that accurately discriminates women likely to develop local recurrence after radiotherapy from those likely not to recur. Our results suggest that this intrinsic radiosensitivity is not subtype-dependent and performs better than any available parameter currently used to predict likelihood of recurrence. Such a signature has the potential to identify patients who need treatment intensification and those who have a low risk of disease recurrence with standard therapy.

dataset and demonstrate that our signature accurately identifies patients likely to develop recurrence despite standard of care therapy. This is the first study to identify and validate a radiation-specific signature of response in patients with breast cancer and may aid in the identification of patients for whom standard therapy is insufficient and require treatment intensification.

Materials and Methods

Patient cohorts

Two publicly available clinical cohorts were utilized. A multi-institutional training cohort consisted of 343 patients from the Netherlands and France with early-stage breast cancer treated with breast-conserving surgery with postoperative radiation (Servant and colleagues; ref. 11). A validation cohort consisted of 295 patients from the Netherlands with early-stage breast cancer treated surgically with radiation as indicated (van de Vijver and colleagues; ref. 14). Of note, there were 67 patients in common between the two datasets and the validation studies were all performed after exclusion of the patients included in the training set, leaving 228 patients in the validation dataset. All appropriate Institutional review board (IRB) protocols were followed in the acquisition and analysis of the data from these clinical datasets. Please refer to the original cited publications for full details of the IRB approval.

In vitro studies

Clonogenic survival assays. Exponentially growing cells were transfected with control siRNA oligos or gene-specific siRNAs for 24 hours and then treated with radiation at doses as indicated. Cells were grown for up to 14 days and then fixed and stained with methanol-acetic acid and crystal violet, respectively, and scored for colonies of 50 cells or more. Radiation survival data from siRNA-treated cells were corrected for siRNA cytotoxicity, as previously described (15). Cell survival curves were fitted using the linear-quadratic equation as previously described (16). The radiation enhancement ratio (EnhR) was calculated as the ratio of the mean inactivation dose under control conditions divided by the mean inactivation dose under siRNA-treated conditions

Cell culture. Breast cancer cells were propagated from frozen samples in cell culture media and passaged when reaching confluence. Cell lines were chosen to include an appropriate representation of all molecular subtypes. All cell lines were purchased between July 2012 and January 2014 from ATCC (except the ACC cell lines) and the remainder (all ACC cell lines) from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. All cell lines were authenticated, characterized, and genotyped immediately before evaluation at the University of Michigan DNA Sequencing core facility by fragment analysis and ProfilerID utilizing the AmpFLSTR Identifier Plus PCR Kit (Life Technologies; Cat #4322288) run on an Applied Biosystems AB 3730XL 96-capillary DNA analyzer. Sample fragments were compared against cell line standards provided by ATCC and DSMZ. ZR75-30, MDA-MB-231, MDA-MB-453, BT474, BT20, AU565, HCC 1954, HCC 1806, HCC38, HCC70, and HCC 1937 breast cancer cell lines were grown in RPMI1640 (Invitrogen) supplemented with 10% FBS (Invitrogen) in a 5% CO₂ cell culture incubator. ACC-231 cells were grown in 90% RPMI medium (Invitrogen) supplemented with 10% FBS (Invitrogen) in a 5% CO₂ cell culture incubator. ACC-302 cells were grown in 80% DMEM (Invitrogen) supplemented with 20% FBS (Invitrogen) in a 5% CO₂ cell culture incubator. ACC-422 cells were grown in 85% minimum essential medium (MEM; Invitrogen) supplemented with 15% FBS (Invitrogen) in a 5% CO₂ cell culture incubator. BT549 and T47D cells were grown in RPMI1640 (Invitrogen) supplemented with 10% FBS (Invitrogen) and 0.023 IU/mL insulin in a 5% CO₂ cell culture incubator. ACC-459, ACC-440, and CAMA-1 were grown in DMEM (Invitrogen) supplemented with 10% FBS in a 5% CO₂ cell culture incubator. MCF-7 cells were grown in modified MEM (Invitrogen) with 0.023 IU/mL insulin in a 5% CO₂ cell culture incubator. All cultures were maintained with 50 U/mL of penicillin/streptomycin (Invitrogen). siRNA, gene expression, and irradiation studies were performed as previously described (17–19) and a detailed description of Methods is found in the Supplementary Methods section.

Statistical analyses

Publicly available normalized microarrays for the breast cancer cell lines were obtained as described in the Supplementary Methods section and were not generated by the authors but were made freely available for research use. This normalized gene expression was used and the genes that were highly correlated genes with the SF-2Gy with a minimum internal range of expression were selected for further use in the model. Briefly, normalized expression data for the cell lines was downloaded from the Wellcome Trust Sanger Institute where all of the data has been deposited and made freely available to researchers. It is freely available at the following links: <http://www.cancerrxgene.org/>; <ftp://ftp.sanger.ac.uk/pub4/cancerrxgene/releases> (20). Normalized expression data for the training cohort was downloaded from the Gene Expression Omnibus (GSE30682; ref. 21). Normalized expression data for the validation cohort was obtained from http://microarray-pubs.stanford.edu/wound_NKI/explore.html (22). All expression data was log transformed and median centered and scaled to the same minimum/maximum to make the disparate platforms comparable. These genes were used to train a Random Forest model in the training cohort and refined using a backward stepwise feature reduction algorithm. The model predictions were then validated in an independent publicly available validation cohort. Receiver

operating characteristic (ROC) and Kaplan–Meier curves were generated, and univariate and multivariate analysis was performed using Cox regression. R software packages (<http://www.r-project.org>) were used for all data and statistical analysis. Ingenuity Pathway Analysis (Ingenuity) was used to assess for enrichment of biologic processes and pathway contextualization. The radiosensitivity index was generated as described previously (8).

A detailed description of the methods (*in vitro* and statistical) can be found in the Supplementary Experimental Methods section.

Results

Development of a radiation response signature

To develop a molecular biomarker signature for radiation response, we employed a novel strategy to investigate the intrinsic radiosensitivity of 16 breast cancer cell lines chosen to represent the heterogeneity found within human breast cancer. This selection included 5 luminal, 4 basal A, 4 basal B, and 3 HER2/*neu*-amplified cell lines as defined by previous gene expression profiling studies (23, 24). An overview of the experimental design is depicted in Supplementary Fig. S1. We characterized the long-term intrinsic radiation sensitivity of the cell lines by performing clonogenic survival assays after varying doses of ionizing radiation. The surviving fraction after 2 Gy (SF-2Gy; the typical daily dose used for breast cancer treatment) of radiation was calculated and represents the intrinsic radiosensitivity of these BCC lines (Fig. 1A). Clonogenic assays identified a broad range of intrinsic radiosensitivity across the cell lines with SF-2Gy values ranging from 17% to 77%.

Intrinsic breast cancer radiosensitivity is independent of subtype

We then determined whether cell line radiosensitivity was correlated to the previously defined intrinsic subtypes of human breast cancer, as significant correlation would suggest an existing method for predicting radiosensitivity and limit the benefit of further radiation signature development. Indeed there exists conflicting data regarding the degree with which intrinsic subtype is associated with clinical radiosensitivity with previous groups demonstrating an association when intrinsic subtype is defined by hormone receptor status (17, 18). While limited in the number of cell lines available for statistical analysis, our analysis revealed no significant association ($P = 0.48$) between the radiosensitivity of the cell lines and their respective intrinsic subtype (Fig. 1B). Thus, it was anticipated that a radiation response signature developed based on gene expression from these cell lines would not be a simple recapitulation of the previously defined intrinsic breast cancer subtypes but would identify a novel response-dependent signature.

RSS development

To develop the RSS, we used the gene expression data from the breast cancer cell lines and calculated the correlation coefficient of the basal gene expression values with the radiation sensitivity metric (SF-2Gy) on a gene-by-gene basis. Gene expression values that were correlated with radiation sensitivity (Spearman correlation; $P < 0.05$, fold-change > 2 fold) were retained within the signature (representative gene shown in Fig. 1C). This analysis identified 147 genes (80 genes negatively correlated, 67 genes positively correlated) whose expression was significantly corre-

lated with SF-2Gy (Fig. 1D). A full list of these genes is included in the Supplementary Table S1.

We next performed unsupervised hierarchical clustering to evaluate the strength of association between gene expression and radiosensitivity. Unsupervised hierarchical clustering identifies two distinct groups of cell lines and appropriately clusters the radioresistant cell lines (SF-2Gy $> 45\%$) distinct from the radiosensitive cell lines (SF-2Gy $< 45\%$; Supplementary Fig. S2).

Signature development identifies novel radiation sensitivity-related genes

We then validated a number of top predictive genes in our signature as being differentially expressed in radioresistant versus radiosensitive cell lines at both the RNA and protein level (Fig. 2A and B), and undertook experiments to determine whether any of the identified associated genes played a role in the radioresistance phenotype. As our analysis identified a number of genes previously reported to be associated with radiation sensitivity (*ATM*, *BUB1*, *RAD51*), we hypothesized that our signature would identify additional radiation sensitivity-related genes. To determine whether this expression-to-radiosensitivity correlation played a meaningful role in the radiation-resistant phenotype, we interrogated the effect of gene expression knockdown in the radioresistant cell lines to determine the radiosensitizing effect of single gene manipulation. Our studies identify *TACC1*, *RND3*, and *DTL* as being involved in the radiosensitivity of human breast cancer cell lines (Fig. 2C). Expression of *TACC1* and *RND3* was increased in the cell lines that were found to be the most radioresistant in clonogenic survival assays. Knockdown of *TACC1* and *RND3* in the radioresistant cell line MDA-MB-231 using siRNA approaches identified significant radiosensitization with single gene knockdown in these cell lines (radiation enhancement ratios of 1.31–1.43 to 1.22–1.32, respectively; Fig. 2D).

Refinement of the RSS

Having utilized a correlative approach to identify genes whose expression was significantly correlated with radiation sensitivity *in vitro*, we sought to further refine our signature by identifying genes contributing most significantly to the signature's performance and incorporating the outcomes data from a clinical cohort. To that end, we identified a human breast tumor dataset for which gene expression levels were known and for which there was long-term follow-up, including local recurrence information provided (11). This training dataset included 343 patients with 10-year follow-up and long-term locoregional recurrence events captured. The majority of these patients had early-stage node-negative disease managed with breast conserving surgery, treated with radiation, without adjuvant chemotherapy (see Supplementary Table S3 for details). Given the need for an increased number of locoregional recurrence events to train our signature, this cohort intentionally was selected with this bias to improve the training of our signature for future validation in independent datasets.

We used this training cohort to train and refine our RSS to 51 genes. In an effort to define the underlying mechanisms of radiation sensitivity in breast cancer, we analyzed biologic concepts that were enriched in these 51 genes. We found that biologic

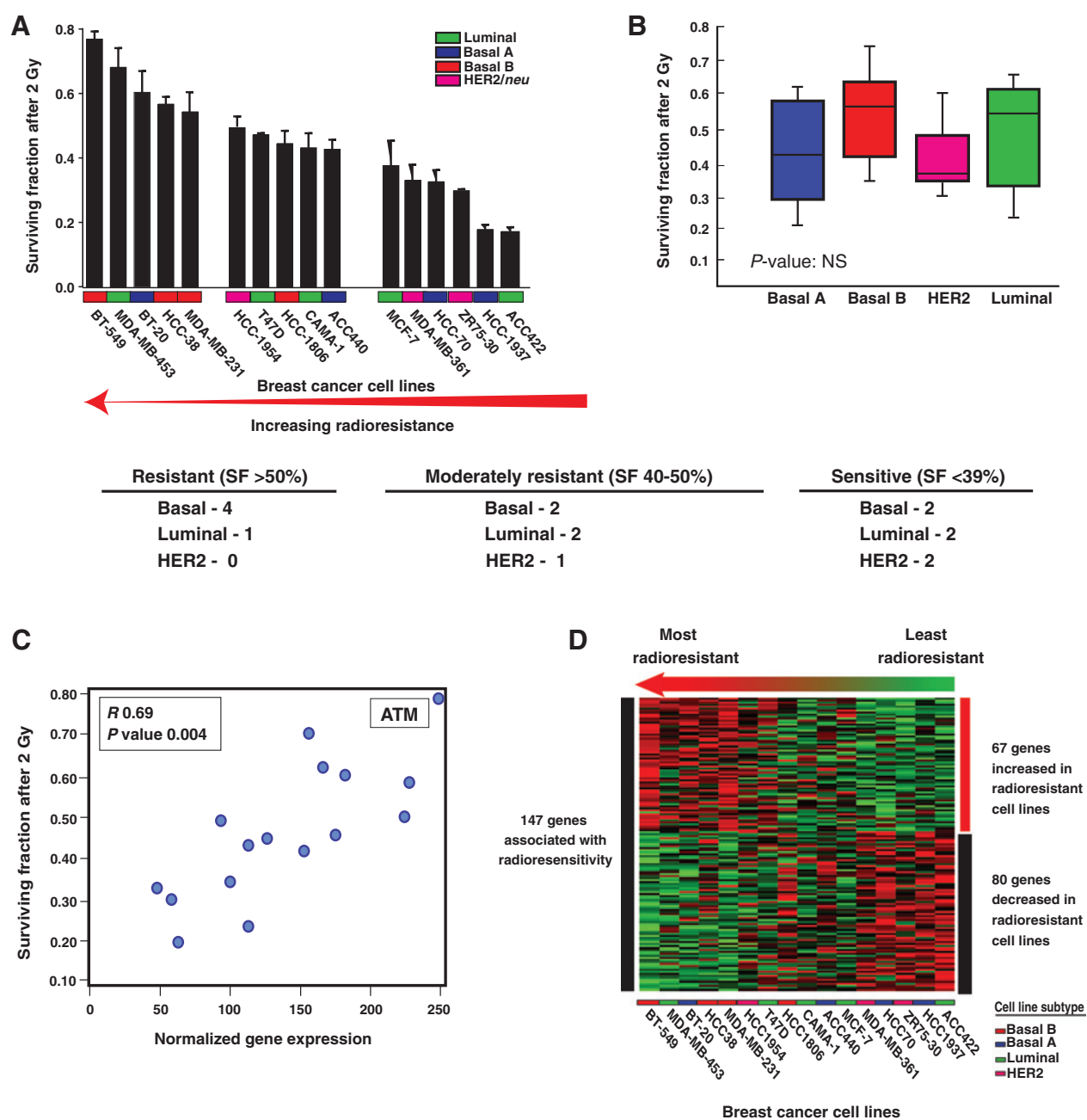


Figure 1. Clonogenic survival assays were performed to assess the baseline radiation sensitivity in a panel of 16 breast cancer cell lines. Clonogenic survival assessing the surviving fraction of cells after exposure to a standard fraction of radiation (SF-2Gy; A) identifies a range of radiation sensitivity in human breast cancer cell lines (SF-2 Gy 77%-17%). Box and whisker plot depicting minimum and maximum SF-2Gy values. ANOVA testing shows no significant difference in radiosensitivity ($P = 0.48$) between the intrinsic breast cancer subtypes (B). Grouping of breast cancer cell lines into resistant, moderately resistant, and radiation-sensitive cell lines shows a mixture of each intrinsic subtype in these categories. The RSS was generated by selecting genes whose expression was significantly correlated with intrinsic radiation sensitivity (SF-2Gy) with a Spearman correlation $P < 0.05$ and 2-fold difference in expression with representative correlation scatter plot depicted for the gene *ATM* (C). On the basis of these selection criteria, 147 genes were identified as being correlated with radiation sensitivity, with the expression level of 67 genes correlated with radiation resistance and 80 genes negatively correlated with radiation resistance. A heatmap depicting relative gene expression of these 147 genes ordered by the intrinsic radiosensitivity of the 16 breast cancer cell lines is depicted (D).

concepts related to the cell cycle, DNA damage, and DNA repair were significantly enriched (Fig. 3).

As expected the performance of the RSS in the training cohort was perfect (Fig. 4A) with complete separation of the Kaplan–

Meier survival curves (Fig. 4C). A random forest out-of-bag (OOB) predictions technique was used for cross-validation which demonstrated an ability to distinguish those patients with recurrence after radiation from those without recurrence with a

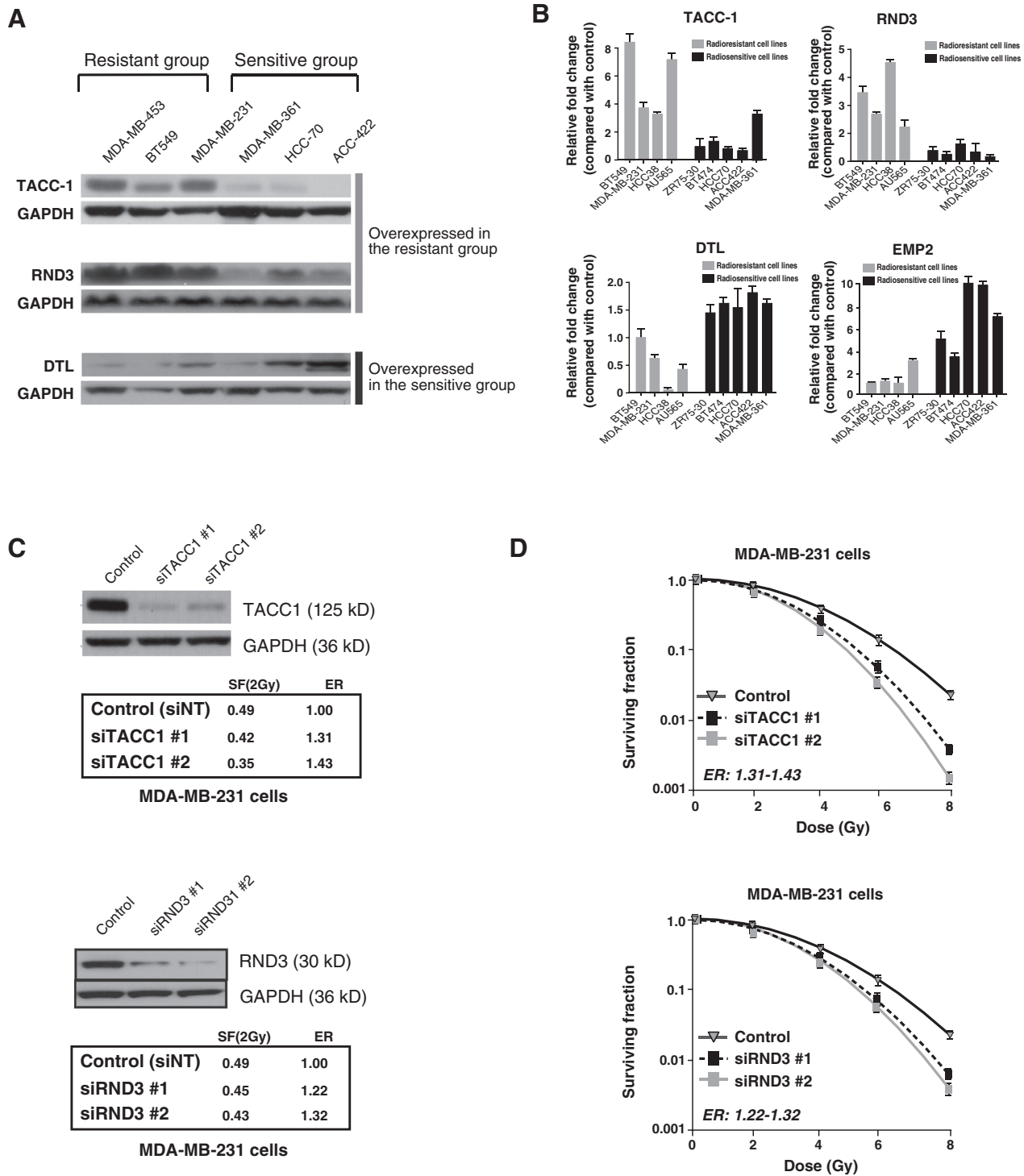


Figure 2. Gene overexpression was validated in breast cancer cell lines by assessing protein and RNA expression. The expression of selected genes identified in the array profiling were validated as being more highly expressed in the radiation resistant cell lines compared with the radiation sensitive cell lines by Western blotting (A). Expression data for 6 representative genes (*TACCI*, *DDR2*, *RND3*, *DTL*, *ATM*, *RAD51*) from a panel of radiation-resistant and radiation-sensitive breast cancer cell lines are shown (B). Asterisks indicate $P < 0.01$. Data are represented as mean \pm SEM. Clonogenic survival assays validates several genes identified as being overexpressed in the radioresistant signature are involved in radioresistance. siRNAs designed against *TACCI* and *RND3*, effectively knock down gene expression (C). Clonogenic survival assays in MDA-MB-231 breast cancer cells after siRNA knockdown of *TACCI* and *RND3* show significant sensitization of these cells to ionizing radiation with an enhancement ratio of 1.22-1.43 (D).

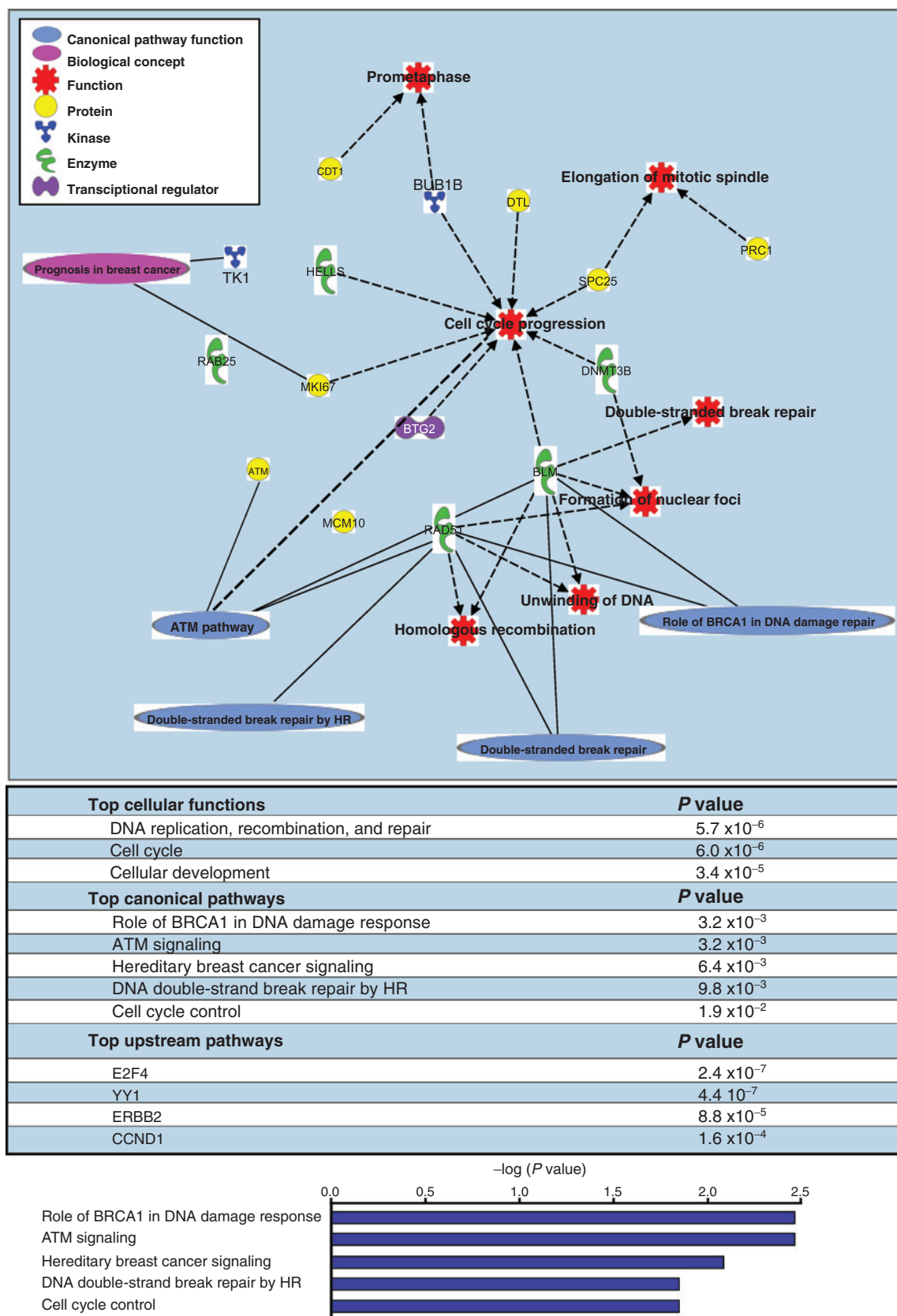


Figure 3. Ingenuity Pathway Analysis and gene ontology enrichment analysis demonstrates that the radiation sensitivity signature is significantly enriched for concepts related to radiation response including DNA repair, cell cycle, and DNA damage response.

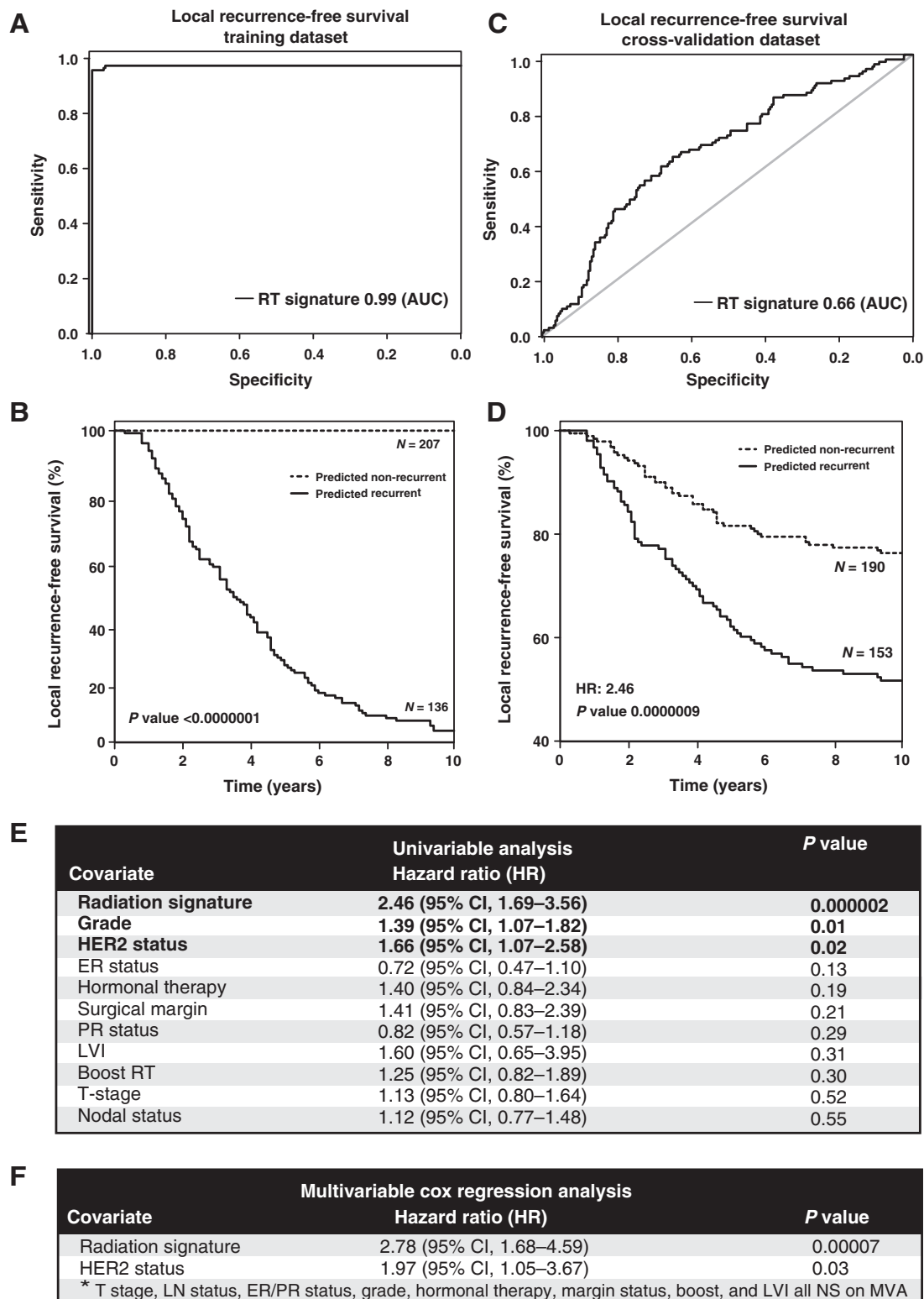


Figure 4. Ten-year receiver operating characteristic (ROC) curves and Kaplan-Meier survival analysis in training and cross-validation dataset with univariable and multivariable analysis. AUC values from the training set show perfect performance (A) and Kaplan-Meier analysis demonstrates complete separation of the curves between those predicted to recur and not predicted to recur (B). Performance in a cross-validation cohort is also shown with an AUC of 0.66 (C) and significant separation of the Kaplan-Meier survival estimate curves (D). Univariable (E) and multivariable (F) analysis identifies the radiation signature score as the variable most strongly associated with local recurrence.

log-rank $P < 10^{-6}$ and HR = 2.5 (Fig. 4B and D). On univariable and multivariable Cox regression analysis, the OOB RSS score predictions were the single most predictive clinical or pathologic variable, even with the inclusion of breast cancer intrinsic subtype (Fig. 4E and F and Supplementary Table S3).

Independent validation of the RSS

We then identified an independent human breast tumor dataset with gene expression and long term clinical outcomes that included locoregional recurrence in which to validate our RSS (14). The validation cohort included 228 nonoverlapping patients with a minimum of 5-year follow-up with locoregional recurrence and OS endpoints. Similar to the training set, all the patients had early-stage disease ($\leq pT2$) and the majority received adjuvant radiotherapy and did not receive systemic chemotherapy as standard of care (see Supplementary Table S4).

Evaluation of the performance of the RSS in the validation dataset demonstrated a 10-year ROC AUC comparable with the out-of-bag cross-validation at 0.72 and was better than any other clinical or pathologic parameter (Fig. 5A). The RSS predicted those who would develop recurrence remarkably well ($P < 0.001$, HR = 5.3; Fig. 5B) with a sensitivity of 84% and a negative predictive value (NPV) of 89% for locoregional recurrence at 10 years. On univariable and multivariable analysis, the RSS score outperforms all other prognostic clinical or pathologic variables, including the intrinsic breast cancer subtype ($P < 0.01$, HR = 6.1; Fig. 5C and D). C-indices were also calculated in the training and validation cohorts and are included in Supplementary Table S3 as is the HRs for the intrinsic breast cancer subtype. In addition, calibration curves using logistic regression analysis for local recurrence with curves were generated in both the out-of-bag cross validation cohort as well as the external validation dataset and are included in the Supplementary Data (Supplementary Fig. S5). Interestingly, we found that the RSS score was also highly prognostic for OS on univariable and multivariable analysis, with a cox regression $P < 0.05$ with a HR = 1.8 even after accounting for clinicopathologic variables (Fig. 5C and D). Thus, we not only identify a RSS that is highly sensitive and specific for local recurrence, but also is prognostic for overall survival in an independent breast cancer dataset.

RSS outperforms previously described radiation-related signatures

Previous groups have also attempted to develop predictive radiation signatures. These signatures, though not derived in a breast cancer-specific manner, have been applied to breast cancer datasets to assess the ability to risk-stratify breast cancer patients after radiation treatment (8). We evaluated the performance of our signature against one such previously reported signature in the training and validation cohort and find that our breast cancer-specific signature outperforms other signatures in both of these datasets (ref. 8; Supplementary Fig. S3 and S4).

Discussion

In this study, we demonstrate significant heterogeneity in the intrinsic breast cancer radiosensitivity of breast cancers and demonstrate that this radiosensitivity is independent of intrinsic breast cancer subtype. We develop a molecular signature of radiation

response in breast cancer that is enriched for biologic concepts implicated in response to radiotherapy including DNA damage repair and cell-cycle regulation. Furthermore, we identify novel genes previously unreported to be associated with radiation sensitivity and show that perturbation of these genes is sufficient to confer alterations in the response to radiation. After refinement of the signature, we demonstrate the prognostic import of this signature in an independent dataset. We show that this radiation response signature is able to, with great sensitivity and specificity, discriminate patients unlikely to develop local recurrence after radiotherapy from those patients at high likelihood of recurrence despite standard radiotherapy. For the first time, we also demonstrate that the RSS, a local recurrence molecular signature, is also prognostic for OS, consistent with the previously appreciated finding in breast cancer-specific meta-analyses that local recurrence benefit translates into an overall survival advantage in human patients (2, 25). This molecular signature, then, may have the ability to identify patients likely to do well with current local treatment and those patients who may need treatment intensification because they remain at high risk for local recurrence despite currently available therapies.

This is the first report to identify a breast cancer-specific molecular signature of response to radiation that provides potentially clinically relevant data regarding the prognosis of patients who receive adjuvant radiotherapy for management of breast cancer. Interest in the intrinsic radiosensitivity of cancer cell lines and the potential utility of this information in predicting patient outcome is not new, but the utilization of this information in a tissue-specific manner has not been previously performed (19). In addition, despite previous attempts to generate a radiation-only response signature, no single signature has performed well in validation using cohorts of patients with breast cancer. This is not surprising, given the diversity and heterogeneity of genomic, transcriptomic, and proteomic alterations common to cancers of different origins. Recent analysis of the mutation landscapes across various cancer types further characterizes the heterogeneity of various cancer types (26, 27). Thus, previous signature attempts, which rely on radiation sensitivity data from a diverse range of human cancers, may have identified conserved pathways implicated in generic cellular response to ionizing radiation, but these have performed poorly in individual cancer types, including breast cancer. Previous studies demonstrated that the clinical and biologic features of human breast tumors and human breast cancer cell lines derived from these tumors are remarkably well conserved (24, 28). Capitalizing on these similarities, we utilized the intrinsic radiosensitivity of human breast cancer cell lines to develop a radiation response signature that is breast cancer-specific.

The limitation of this study includes a reliance on datasets that included patients not randomly accrued and treated on clinical trial. The training and validation datasets come from patients treated per standard of care at the time in a nonrandomized setting. In addition, we were limited by the number of available clinical datasets with the complementary gene expression data. This meant that the microarray platforms from discovery, training, and validation datasets were not identical, making the reproducibility of the results more remarkable given the technical differences and cross-platforming complexities. Finally, our validation cohort had a lower event rate than the training set, though this event rate is more consistent with modern series with current chemotherapeutic regimens radiation techniques.

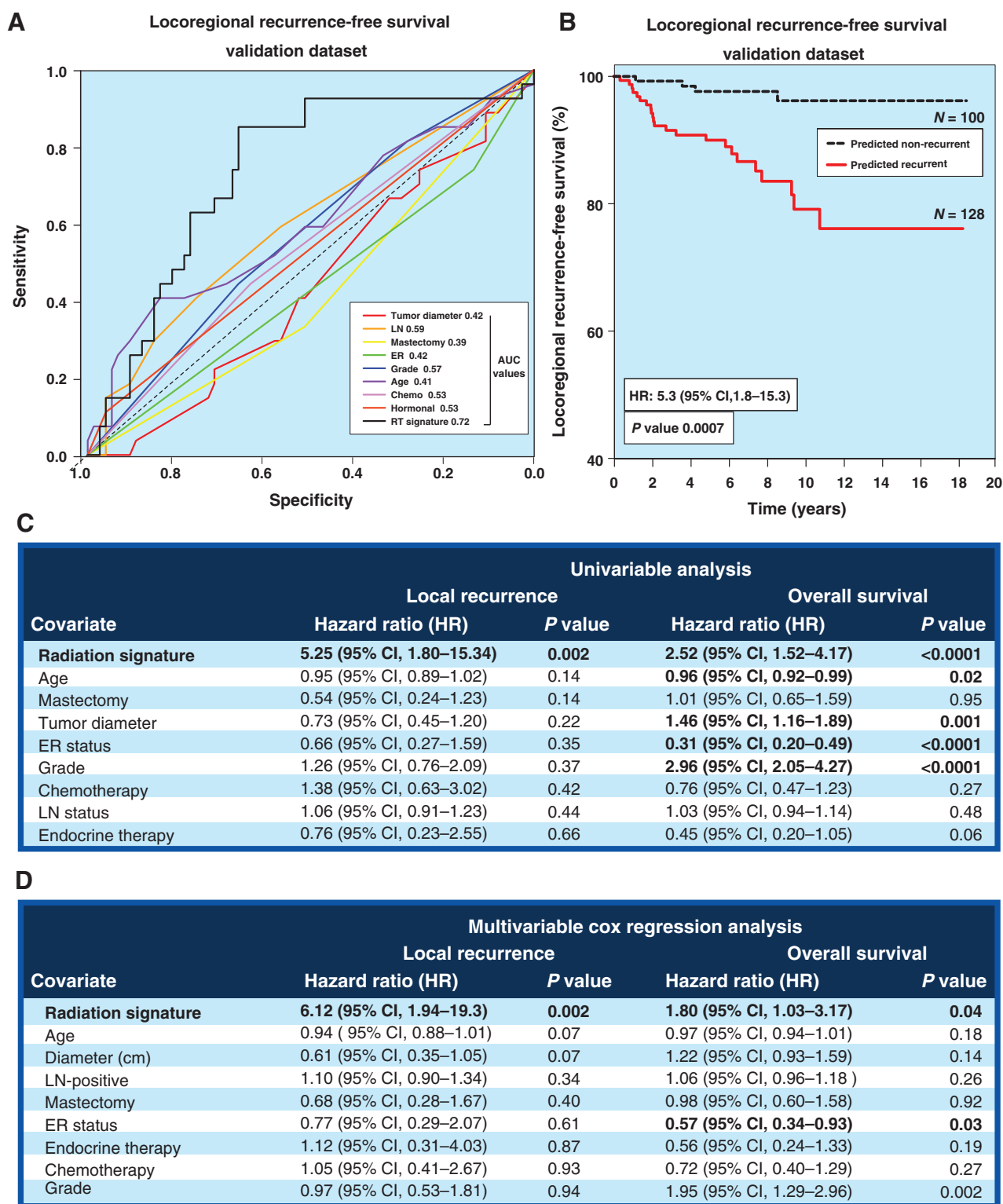


Figure 5. Ten-year receiver operating characteristic (ROC) curves and Kaplan-Meier survival estimate analysis in validation dataset with univariable and multivariable analysis. AUC values from the validation dataset show the radiation signature score outperforms every other clinical and pathologic parameter (A). Kaplan-Meier survival estimate analysis and HRs are depicted in B. Univariable (C) and multivariable (D) analysis identifies the radiation signature score as the variable most strongly associated with local recurrence and overall survival. C-indices were also calculated and included in Supplementary Table S3.

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This study identifies a number of novel radiation-sensitivity related genes. While little is known about these genes, especially as it relates to response to ionizing radiation, the degree of radiation sensitivity seen with individual gene knockdown is comparable with the effects of cisplatin, a known and well-characterized radiation sensitizer, in cancer cell lines (29, 30). Future experiments are underway to understand the molecular mechanisms underlying their involvement in the radiation response pathway.

In the era of personalized medicine, the development of molecular signatures for the prediction of clinical benefit from intervention is of critical importance. Specifically, there remains an unmet need for predictive biomarkers to identify which patients are likely to benefit from radiotherapy. Predictive biomarkers (and signatures) are defined as a marker which can be used to identify subpopulations of patients who are most likely to respond to a given therapy. Unlike prognostic biomarkers that provide information on the likely course of the disease in an untreated individual, predictive biomarkers provide information regarding a patient's likelihood of benefiting from a given treatment. Within this context, a purely predictive radiation signature would be one that accurately predicts which patients benefit from radiation therapy but provides no meaningful information in patients who received no radiation treatment. Our signature was validated in a cohort of patients who all received standard radiotherapy and thus cannot be considered a predictive signature but rather a prognostic signature. While this signature provides clinically relevant information regarding which patients are likely to do poorly with standard radiotherapy, it has not yet been validated in the predictive setting to identify which patients derive the greatest benefit from radiation treatment. Those patients predicted to do poorly with radiotherapy should be the subject of prospectively designed clinical trials to determine whether they may benefit from treatment intensification through dose-escalation. Alternatively, this signature may predict patients for whom alternative modalities of treatment should be considered. Perhaps more interestingly, it is currently unclear whether the patients predicted to do well (i.e., not develop a local recurrence after radiation treatment based on the radiation signature) derive a significant clinical benefit from ionizing radiation in the adjuvant setting, or whether the biology of their disease was so indolent that they were destined to not develop a recurrence regardless of the utilization of adjuvant radiotherapy. Given the current inability

to accurately identify these patients based on clinical, pathologic, or molecular (or combinations of the three) parameters, future efforts should be aimed at developing such prognostic and predictive biomarkers. Indeed, current efforts are underway in our group to validate the signature described herein in just such a patient population. These patients will have been enrolled in a phase III randomized trials testing the efficacy of the addition of radiotherapy to surgical resection and will thus pass the rigors of clinically relevant biomarker testing as previously proposed (31)

Disclosure of Potential Conflicts of Interest

C. Speers, S. Zhao, L.J. Pierce, and F.Y. Feng are co-inventors of a provisional patent on compositions and methods for the analysis of radiosensitivity that has been filed by the University of Michigan. H. Bartelink is a consultant/advisory board member for AGENDIA. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Bartelink, F.Y. Feng
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Speers, S.G. Zhao, M. Liu, L.J. Pierce, F.Y. Feng
Writing, review, and/or revision of the manuscript: C. Speers, S.G. Zhao, H. Bartelink, L.J. Pierce, F.Y. Feng
Study supervision: S.G. Zhao, L.J. Pierce, F.Y. Feng

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