Ras Signaling Is a Key Determinant for Metastatic Dissemination and Poor Survival of Luminal Breast Cancer Patients

Katherine L. Wright1,2, Jessica R. Adams1,2, Jeff C. Liu3, Amanda J. Loch1, Ruth G. Wong1, Christine E.B. Jo1, Lauren A. Beck1, Divya R. Santhanam1, Laura Weiss1, Xue Mei1, Timothy F. Lane4, Sergei B. Koralov5, Susan J. Done6, James R. Woodgett7, Eldad Zacksenhaus3, Pingzhao Hu8, and Sean E. Egan1,2

Abstract

Breast cancer is associated with alterations in a number of growth factor and hormone-regulated signaling pathways. Mouse models of metastatic breast cancer typically feature mutated oncogenes that activate PI3K, Stat3, and Ras signaling, but the individual and combined roles of these pathways in breast cancer progression are poorly understood. In this study, we examined the relationship between oncogenic pathway activation and breast cancer subtype by analyzing mouse mammary tumor formation in which each pathway was activated singly or pairwise. All three oncogenes showed cooperation during primary tumor formation, but efficient dissemination was only dependent on Ras. In addition, transcriptional profiling demonstrated that Ras induced adenocarcinomas with molecular characteristics related to human basal-like and HER2+ tumors. In contrast, Ras combined with PIK3CAH1047R, an oncogenic mutant linked to ERα+/luminal breast cancer in humans, induced metastatic luminal B-like tumors. Consistent with these data, elevated Ras signaling was associated with basal-like and HER2+ subtype tumors in humans and showed a statistically significant negative association with estrogen receptor (ER) signaling across all breast cancer. Despite this, there are luminal tumors with elevated Ras signaling. Importantly, when considered as a continuous variable, Ras pathway activation was strongly linked to reduced survival of patients with ERβ disease independent of PI3K or Stat3 activation. Therefore, our studies suggest that Ras activation is a key determinant for dissemination and poor prognosis of ERα+/luminal breast cancer in humans, and hormone therapy supplemented with Ras-targeting agents may be beneficial for treating this aggressive subtype. Cancer Res; 75(22); 4960–72. ©2015 AACR.

Introduction

Ras genes are mutated in a relatively small percentage of human breast cancers. However, Ras signaling can be activated through copy number changes or mutation of many genes, including those coding for tyrosine kinase receptors like HER2, as well as genes coding for tyrosine phosphatases, for regulators of Ras-GTP loading or for kinases functioning downstream of Ras. Indeed, 28% of human breast tumors have deletions or loss-of-function mutations in NF1, which codes for a Ras GTPase activating protein (Gap, ref. 1). Other tumors show reduced expression of RASAL2, a distinct RasGap gene. While RASAL2 is not commonly deleted or mutated, reduced expression is associated with luminal B breast cancer (2).

Ras was first linked to metastatic transformation in fibroblasts (3). Subsequently, a number of mouse models of human breast cancer were generated using activated Ras, and some of these develop metastatic tumors (4, 5). For example, activated Ras cooperates with loss of Par3 to induce metastatic mammary tumors in mice (6).

Breast cancer represents a collection of diseases, most of which express the estrogen receptor (ERx). Transcriptional profiling has been used to identify common breast cancer subtypes, including luminal A and luminal B (both of which express ERx), HER2+ (which express very high levels of HER2/Neu, typically as a result of gene amplification) as well as basal-like and claudin-low tumors (7, 8). Additional and refined subtypes have been identified through incorporation of data on mutations and copy number alterations (9) and comparison to specific mammary epithelial cell types (10). These different forms of breast cancer progress through different mechanisms, and poor prognosis in each subtype can be linked to alterations in distinct signaling pathways or transcriptional programs. For example, the “Bach1-pathway metastasis gene

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K.L. Wright and J.R. Adams share first authorship of this article.

Corresponding Author: Sean E. Egan, The Program in Developmental and Stem Cell Biology, The Peter Gilgan Center for Research and Learning, The Hospital for Sick Children, Toronto, Ontario, Canada. Phone: 416-813-5267; Fax: 416-813-8823; E-mail: segan@sickkids.ca
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signature" (BPMS) is associated with metastasis and poor disease-free survival of patients with basal-like breast cancer, but not with luminal and HER2 tumors (11).

PIK3CA, which codes for the p110α catalytic subunit of Class I phosphoinositide 3-kinase (PI3K), is the most commonly mutated gene in ER+ luminal tumors and in metastatic breast cancer (8, 12). We recently described the first mouse model for PIK3CA-mutant breast cancer (13). Most mammary tumors that form in this model are metastatic adenocarcinomas or adenomyoepitheliomas. Pik3caH1047R mice do not develop mammary tumors that mimic the much more common ER+ luminal breast tumor types (13, 14).

While crossing these mice to p53 conditional mutants dramatically enhanced tumor formation, very few luminal type tumors were seen in double mutant animals. Also, the tumors that form in Pik3ca, as well as in Pik3ca/p53 double mutant mice, do not metastasize at a high rate. These data suggest that PIK3CA-mutant alleles may cooperate with distinct signaling pathways to induce metastatic ER+ breast cancer. Muller and colleagues have developed two mouse models for breast cancer, both of which metastasize to lungs (15, 16). Interestingly, each model involves enhanced Ras signaling (17). Metastatic dissemination of tumors in MMTV-Neu mice is dependent on Stat3 signaling and can be enhanced through expression of a dominant allele of Stat3 (Stat3+; refs. 18-20) or through deletion of Pten, which activates PI3K signaling (21).

In an effort to define the relationship between oncogenic pathway activation and breast cancer subtype, as well as metastatic dissemination, we generated mouse models for breast cancer with elevated PI3K, Stat3, and/or Ras signaling. We also used pathway-specific gene signatures to test for linkage of elevated PI3K, Stat3, and Ras to relapse and survival in humans.

Materials and Methods

Mouse colony maintenance

Mice were housed at the Toronto Centre for Phenogenomics and received care according to guidelines defined by the CCAC (Canadian Council on Animal Care). Strains were genotyped using primers listed below. Males were not studied. Genotyping primers used in this work are as follows: (i) for Cre recombinase using primers listed below. Males were not studied. Genotyping primers used in this work are as follows: (i) for Cre recombinase

Molecular subtype classification

Microarray analysis on mouse tumor models was carried out using Affymetrix Mouse Gene 2.0 ST (Centre for Applied Genomics, Hospital for Sick Children, Toronto, Ontario, Canada). The GEO accession number is GSE73073. Microarray data were normalized using RMA method via Partek software and log2-transformed gene expression values were obtained. Published datasets containing multiple mouse models (GSE42640) and human breast cancer subtypes determined by PAM50 (GSE18229) were downloaded from the GEO database. Data from our mouse models were integrated with the above GSEs using "Distance Weighted Discrimination" (DWD). Unsupervised hierarchical clustering (complete linkage) with the intrinsic genes signature (24) was used to group tumor samples for subtype classification.

Statistical analysis

Statistical analysis for Kaplan-Meier survival curves was done in R (25). These curves were generated using the "survival" library and the "survfit" function. Survival statistics were calculated as nonparametric log rank P values for censored survival data using the "survdiff" function (26). Censored data (represented by "вин" on Kaplan-Meier survival curves) represents a mouse that was removed from the study before 18 months. For overall survival, censored mice were removed prematurely from the study for a variety of reasons such as unresolved infections. For mammary tumor-free survival analysis, censored mice were removed prematurely due to any non-mammary tumor endpoint or death. Means and SEs and t tests were also calculated using R. In each case, significant statistical differences were defined as P-values.

Histology and immunostaining

Five micron thick paraffin sections were stained with hematoxylin and eosin (H&E) for histology. For immunohistochemistry and immunofluorescence, sections were deparaffinized in xylene and rehydrated through an alcohol series. Antigen retrieval was performed in a digital decloaking chamber (Biocare Medical; Set-Point1 = 125°C for 5 minutes, Set-Point2 = 90°C for 10 seconds). Using heat-induced epitope-retrieval solution (Reveal Decloaker pH 6.0, Biocare Medical RF1000G1). Slides were rinsed in running tap water for 5 minutes and then mounted onto a Tescan FreedomEvo liquid-handling robot. Staining was performed according to standard procedures (13, 27). Images were captured with an AxioCam HR digital camera (Zeiss) and AxioVision (release 4.6.3) software. Immunocytochemistry images using anti-ERα antibodies (Santa Cruz Biotechnology; Cat# sc-542 at 1:100 dilution) were captured using Velocity software (Perkin-Elmer, release 6.2.1).

Testing for relationship of signaling pathway activation with relapse/survival in breast cancer patients

Patient samples from three independent gene expression datasets (GSE1456, GSE2034 and GSE3494) stored in the GEO database were analyzed as survival information of the samples include either relapse, death, or both. We first analyzed the disease-free survival (DFS) based on datasets GSE1456 and GSE2034, where survival event is relapse. The combined dataset includes 445 samples and 147 of them relapsed. We then performed overall survival (OS) based on datasets GSE1456 and GSE3494, where survival event is death. The combined dataset
Figure 1.
Activated Ras promotes mammary adenocarcinoma formation in cooperation with dominant alleles of Pik3ca$^{H1047R}$ or Stat3$^c$. A–C, Kaplan–Meier mammary tumor-free survival curves for control (MMTV-Cre and Cre-negative R26-Pik3ca$^{H1047R}$, R26-Stat3$^c$, and K-Ras$^{G12D}$) and experimental mice. Six cohorts of MMTV-Cre-positive experimental mice were used: R26-Pik3ca$^{H1047R}$, R26-Stat3$^c$, both Pik3ca$^{H1047R}$ and Stat3$^c$ at the R26 locus, K-Ras$^{G12D}$, R26-Pik3ca$^{H1047R}$ with K-Ras$^{G12D}$, and R26-Stat3$^c$ with K-Ras$^{G12D}$. D, number of mammary tumors that developed per mouse. (Continued on the following page.)
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includes 410 samples and 84 have died from breast cancer. In both cases, the patients who did not have the event are considered censored. We accessed the pathway activation information for each of the patient samples in PI3K, Stat3, and Ras signaling from Supplementary Table S1 in Gatza and colleagues (28). The pathway activation is represented as relative probability (0–1). The higher the probability, the more active the pathway in a given sample. Survival analyses were performed by constructing Kaplan–Meier survival curves for a given pathway, and the log-rank test was used to examine the differences between groups (26). These have been done for high active and low active patients in a given pathway. The high active patients are those that have pathway activation probability larger than or equal to 0.5 while the low active patients are those that have pathway activation probability smaller than 0.5. A Cox proportional hazard regression model (29) was also used to evaluate the association between the survival information and pathway activation for each of the candidate pathways, where pathway activation has not been binarized and was treated as a continuous variable. We performed the analyses using Survival R package (25).

Testing for relationship of combinations of signaling pathway activation with relapse/survival in breast cancer patients

For a combination of two or three pathways among PI3K, Stat3, and Ras, high active patients are those that have pathway active probability larger than or equal to 0.5 in the combination while low active patients are those that have pathway active probability smaller than 0.5 in either PI3K, Stat3, or Ras pathway. Survival analyses were performed by constructing Kaplan–Meier survival curves for the combination of pathways given the defined high and low active patients.

Results

Ras cooperates with mutant/activated Pik3ca or Stat3 to induce ERα tumor formation

As transformation by mutant Pik3ca can be dependent on tyrosine phosphorylation of Stat3 (30), and activated Stat3 is associated with metastatic dissemination in some contexts (26–28), we tested for cooperation between Pik3ca and Stat3 in a given context (31). This allele, commonly known as Pik3caH1047R (13) and Pik3caStat3 (32–34). The R26 Cre-conditional transgenic system has been used to express Pik3caH1047R (13) and Stat3C (35, 36). We therefore intercrossed these lines to link induction of both alleles to expression of Cre recombinase. Specifically, we compared mammary tumor formation in female mice from the following cohorts: (i) R26-Pik3caH1047R/MMTV-Cre-Stat3C; (ii) R26-Pik3caH1047R/MMTV-Cre-Stat3C; (iii) R26-Stat3C/MMTV-Cre; and (iv) negative controls (including transgenics without Cre as well as MMTV-Cre only mice). No statistically significant difference in tumor formation was seen between mice in double and triple transgenics (cohorts i and ii, respectively). Finally, overall survival, cause of death, and number of mammary tumors per mouse were also unaffected by expression of Stat3C in Pik3ca model mice (Fig. 1D and Supplementary Fig. S1A and Supplementary Table S1).

Analysis was based on observational data collected at necropsy. E–l, mammary tumors from R26-Pik3caH1047R/MMTV-Cre mice (E) were mostly adenocarcinomas (PDA), papillary adenocarcinomas (Pap), complex adenocarcinomas (CAC), and adenomyoepitheliomas (Fig. 1E). Mammary tumors from R26-Pik3caH1047R/MMTV-CreNLST mice were mostly keratinized tumors (such as adenosquamous carcinomas (ASC) or adenomyoepitheliomas (AME)). A smaller number of tumors were squamous cysts (SC), poorly differentiated adenocarcinomas (PDA), papillary adenocarcinomas (Pap), complex adenocarcinomas (CAC), squamous tumors/radial scars (ST/RS) or squamous tubular carcinomas (STC). R26-Stat3C/MMTV-Cre mice did not develop mammary tumors. R26-Pik3caH1047R/MMTV-CreNLST female mice (F) also developed mostly adenosquamous carcinomas (ASC) or adenomyoepitheliomas (AME). A smaller number of tumors were squamous cysts (SC), poorly differentiated adenocarcinomas (PDA), papillary adenocarcinomas (Pap), complex adenocarcinomas (CAC), squamous tumors/radial scars (ST/RS) or squamous tubular carcinomas (STC). R26-Stat3C/MMTV-Cre female mice (G) were mostly adenosquamous carcinomas (ASC) or adenomyoepitheliomas (AME). A smaller number of tumors were squamous cysts, squamous cell carcinomas (SCC), keratoacanthomas (KA), or squamous tubular carcinomas. When R26-Pik3caH1047R and K-RasG12D were expressed together (H), mice developed mostly poorly differentiated adenocarcinomas, complex adenocarcinomas, squamous tubular carcinomas; a smaller number of acinar tumors, adenosquamous carcinomas, squamous cysts, squamous cell carcinomas, keratoacanthomas, and adenomyoepitheliomas also formed. When K-RasG12D and R26-Stat3C were expressed together (I), mice developed many poorly differentiated adenocarcinomas, complex adenocarcinomas, and squamous tubular carcinomas, suggesting expression of mutant K-Ras determines mammary tumor pathology. A smaller number of acinar tumors, adenosquamous carcinomas, squamous cysts, squamous cell carcinomas, keratoacanthomas, solid nodular carcinomas, and adenomyoepitheliomas also formed.

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(Fig. 1C and Supplementary Fig. S1A; Supplementary Table S1). Once again, cooperation was not seen at the level of mammary tumors per mouse (Fig. 1D). As with other Ras models (K-Ras<sup>G12D</sup> alone, or K-Ras<sup>G12D</sup> with Pik3ca<sup>H1047R</sup>, K-Ras<sup>G12D</sup>;R26-Stat3<sup>C</sup>;MMTV-Cre<sup>N</sup>LST mice developed adenocarcinomas at a high frequency (Figs. 1C and I and 2C). As all models with mutant Ras developed PDAs and CACs, we tested for estrogen receptor (ER<sub>a</sub>) expression in these lesions. Indeed, the majority of tumors that formed in K-Ras<sup>G12D</sup> alone, K-Ras<sup>G12D</sup> plus Pik3ca<sup>H1047R</sup> and K-Ras<sup>G12D</sup> plus Stat3<sup>C</sup> cohorts were ER<sub>a</sub><sup>+</sup> (Fig. 2A–D; Supplementary Fig. 2). Interestingly, these tumors stained positive for Stat3<sup>pY705</sup> and express phospho-MapK (Supplementary Figs. S3A and S3B), which is consistent with findings in mammary tumors from MMTV-Ras mice (37). Thus, either oncogenic Ras or oncogenic events that cooperate with Ras, activate Stat3 phosphorylation in this context.

**Luminal oncogene, Pik3ca<sup>H1047R</sup>, shifts the effect of Ras towards luminal B subtype tumors**

Gene expression profiling has been used to analyze mouse models of breast cancer. Indeed, when 27 models were analyzed, they partitioned into 17 distinct molecular subtypes with characteristic relationships to major human subtypes (38). For example, tumors from our R26-Pik3ca<sup>H1047R</sup>;K-Ras<sup>G12D</sup>;MMTV-Cre<sup>N</sup>LST model were mostly adenomyoepithelomas with a Class14Ex signature or adenosquamous carcinomas with a Squamous-like Ex signature (38). In contrast, tumors with activated Ras had NeuEx or Class8Ex signatures, with similarity to luminal A breast cancer in humans and with features related to normal alveolar function. Mammary tumors from K-Ras<sup>G12D</sup>;MMTV-Cre<sup>N</sup>LST mice formed adenocarcinomas with some squamous differentiation. These clustered near Pik3ca<sup>H1047R</sup> tumors with a Squamous-like Ex signature.
In comparison with human molecular subtypes, they clustered near normal human breast cancers but within the HER2 subtype (Fig. 4). Most strikingly, when activated K-RasG12D and Pik3caH1047R were combined, the mammary tumors that formed had a distinct gene expression signature, with a high degree of similarity to luminal B breast cancer. Thus, Ras and PI3K pathways cooperate to induce luminal B type tumors.

(Fig. 3). In comparison with human molecular subtypes, they clustered near normal human breast cancers but within the HER2 subtype (Fig. 4). Most strikingly, when activated K-RasG12D and Pik3caH1047R were combined, the mammary tumors that formed had a distinct gene expression signature, with a high degree of similarity to luminal B breast cancer. Thus, Ras and PI3K pathways cooperate to induce luminal B type tumors.
Ras cooperates with mutant Pik3ca or Stat3 to induce metastatic mammary adenocarcinoma

Mammary tumors that form in Pik3caH1047R mice had a very low rate of lung metastasis (3/51 mice had one or two mets each, see Fig. 5A). Given the published link between Stat3 and metastasis (18, 19), we also screened tumor bearing mice from Pik3caH1047R;Stat3c transgenics for disseminated disease. Metastases occurred in a small percentage of tumor bearing mice (see Fig. 5A).

Figure 4.
Comparison of Ras and Pik3ca/Ras mouse models with human breast cancer subtypes. Cluster analysis of Ras and Pik3ca/Ras tumors using an intrinsic gene signature (24) in comparison with human breast cancer samples from GSE18229 (solid boxes; Basal-like, Luminal-A, Luminal-B, HER2, and normal-like (NL)). Samples were stratified by unsupervised hierarchicall clustering with complete linkage.
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Figure 5. Mammary tumors that form in K-Ras<sub>G12D</sub> model mice are metastatic. A, mammary tumors from R26-Pik3ca<sup>H1047R</sup>;MMTV-Cre mice were mostly nonmetastatic, while 24% of R26-Pik3ca<sup>H1047R</sup>;Stat3<sup>C</sup> mice had lung metastases. B, an example of lung metastases from K-Ras<sup>G12D</sup>;MMTV-Cre mice (left) and R26-Stat3<sup>C</sup>;K-Ras<sup>G12D</sup>;MMTV-Cre mice (right). Both cohorts had mice with less than ten lung metastases (as in the top figures) and others with more than ten (as in the bottom panel).

R26-Pik3ca<sup>H1047R</sup>;Stat3<sup>C</sup>;MMTV-Cre<sup>NST</sup> triple transgenics (mets detected in 24% or 5/21 mice; Fig. 5A). Once again, one or two small metastatic lesions were found in these mice. Finally, we screened Ras cohorts for metastasis. Indeed, half of Ras-alone mammary tumor-bearing mice had lung metastases (7/14; Fig. 5A and B). 44% (11/25) of K-Ras<sup>G12D</sup>;Pik3ca<sup>H1047R</sup> tumor bearing mice and 58% (11/19) of K-Ras<sup>G12D</sup>;Stat3<sup>C</sup> tumor bearing mice had metastasis (Fig. 5A and B). Twenty percent and 32% of tumor-bearing mice from each Ras-expressing/double-oncogene cohort (K-Ras<sup>G12D</sup>;Pik3ca<sup>H1047R</sup> and K-Ras<sup>G12D</sup>;Stat3<sup>C</sup>, respectively) had greater than 10 lung metastases (Fig. 5A and B). Thus, activated Ras can cooperate with a Pik3ca mutant or activated Stat3 to induce transformation, as well as metastatic dissemination of tumor cells. However, as noted above, Ras-alone tumors were also metastatic. Next, histology was compared between lung metastases and primary tumors from the same animals. In 4 of 5 K-Ras<sup>G12D</sup>;MMTV-Cre mice analyzed, the histology of lung metastases matched the histology of mammary tumors from the same mouse (both were poorly differentiated adenocarcinomas or PDA). In one case, lung metastases were PDA, whereas mammary tumors in this mouse were squamous cyst and keratoacanthoma. In 5 of 5 R26-Pik3ca<sup>H1047R</sup>;K-Ras<sup>G12D</sup>;MMTV-Cre mice, lung metastases histology matched that of mammary tumors from the same mouse (2 were PDA and 3 were a mix of PDA and spindle cell tumors or SCT). Finally, in 3 of 3 R26-Stat3<sup>C</sup>;K-Ras<sup>G12D</sup>;MMTV-Cre mice, lung metastases histology matched mammary tumors from the same mouse (2 were PDA and 1 was SCT).

High-level Ras signaling is associated with relapse and death of luminal breast cancer patients

Gatza and colleagues have reported on gene expression signatures associated with activation of 18 different signaling pathways in human breast cancer (28). To establish these signatures, they infected cells with adenoviral vectors expressing pathway activating signaling proteins and used transcriptional profiling to find genes that were induced or repressed in response to each pathway (see Supplementary Data in Gatza and colleagues; ref. 28). To test for an effect of PI3K, Stat3, and Ras signaling on breast cancer relapse and death, we tested for activation of these pathways in publicly available gene expression datasets with linked follow-up data. Indeed, Ras pathway activation was associated with a significant increase in relapse [HR, 2.0; 95% confidence interval (CI), 1.4–2.8; P = 2.3 × 10⁻⁵; Fig. 6A]. Also, high level Ras signaling showed a significant association with increased chance of death [HR, 2.5; 95% CI, 1.6–3.9; P = 1.9 × 10⁻³; Fig. 6B]. As for high PI3K or Stat3 signaling, we did not see a significant correlation with relapse (Fig. 6A) or death (Fig. 6B) for either pathway. Linkage of Ras activation to PI3K or Stat3 pathway activation did not enhance the effect of Ras (Supplementary Fig. S4). Similar, but much more dramatic results were obtained if we treated pathway activation information as a continuous variable in a multivariate Cox proportional hazard regression model (Table 1A and B; for relapse: HR = 8.2, 95% CI, 2.9–24; P = 8.4 × 10⁻⁵; for survival: HR, 22.4; 95% CI, 5.4–93; P = 1.8 × 10⁻⁶).

Next, we tested for effects of PI3K, Stat3, or Ras signaling on relapse or death within each molecular subtype. Previous transcriptional profiling showed an association between elevated Ras signaling and basal-like as well as HER2<sup>+</sup> subtypes (28). Consistent with these findings, we saw a negative correlation between ER pathway activity and Ras signaling (activity correlation = 0.44, Wilcox test P = 9.3 × 10⁻¹¹). With respect to patient outcome, elevated PI3K signaling was found to be associated with relapse in basal-like breast cancer (HR, 2.50; 95% CI, 1.0–6.3; P = 0.044), but not in HER2<sup>+</sup>, luminal (A or B), or normal breast cancer subtypes. It was not associated with death in any cancer subtype (Supplementary Fig. S5). Stat3 pathway activation was also not associated with relapse or death in any subtype (Supplementary Fig. S5). In contrast, Ras pathway activation was linked to relapse, specifically in patients with luminal A (HR, 2.7; 95% CI, 1.1–6.4; P = 0.024) and luminal B (HR, 1.9; 95% CI, 1.3–3.0; P = 0.0026) subtype tumors (Fig. 6C). It was also associated with death in luminal B breast cancer (HR, 2.5; 95% CI, 1.4–4.5; P = 0.0016; Fig. 6D). Subtype analysis is based on binarization of pathway activation data. These relatively small effects are consistent with the previously identified link between high Ras pathway activation and poor survival when activation of pRaf and pMapk were assessed in ER<sup>+</sup> breast cancer (39).

Once again, similar but much more dramatic results were obtained if we treated pathway activation data as a continuous variable in a Cox proportional hazard regression model. In this case, PI3K pathway activation showed a significant association.
Figure 6. Kaplan–Meier analysis shows that Ras pathway activation is associated with relapse and survival of patients with luminal breast cancer. A, relapse of breast cancer patients with low (red) versus high (green) pathway activity for PI3K, Stat3, and Ras pathways as determined by pathway-specific gene signatures. B, survival of breast cancer patients with low (red) versus high (green) pathway activity for PI3K, Stat3, and Ras pathways. C, relationship between relapse and pathway activity for patients with distinct breast cancer subtypes as determined by pathway-specific gene signatures. Kaplan–Meier curves show earlier and more frequent relapse in luminal A and luminal B breast cancer patients with high (green) as compared with low (red) Ras pathway activation. D, relationship between survival and pathway activity for patients with distinct breast cancer subtypes. Kaplan–Meier curves show earlier and more frequent relapse/death in luminal A and luminal B breast cancer patients with high (green) as compared with low (red) Ras pathway activation.
with relapse of basal-like breast cancer patients (HR, 6.7; 95% CI, 0.35–48; \( P = 0.057 \)) and death of HER2 \(^+\) breast cancer patients (HR, 73; 95% CI, 1.5–3,500; \( P = 0.03 \)). Notably, Ras pathway activation was linked to relapse, specifically in patients with luminal A (HR, 315; 95% CI, 8.1–120,000; \( P = 0.0021 \)) or luminal B tumors (HR, 6.2; 95% CI, 1.3–29; \( P = 0.02 \)). It was also associated with death of patients with luminal A (HR, 294; 95% CI, 2.5–34,000; \( P = 0.019 \)) or luminal B tumors (HR, 20; 95% CI, 2.4–170; \( P = 0.006 \)). The reason why Ras activation was not associated with prognosis for patients with basal and HER2 \(^+\) subtype tumors could be related to the small sample size of these cohorts and/or to Ras signaling levels being above a critical threshold in most if not all cases.

### Discussion

The metastatic process involves many steps, starting with primary tumor cell invasion, followed by neoangiogenesis and intravasation into small blood or lymphatic vessels. Alternatively, access to blood vessels can be associated with vascular mimicry (40). Within the circulation, a metastatic tumor cell or clump of cells must survive as it travels to another organ or tissue (41, 42). At secondary sites, a tumor cell must extravasate out of the vasculature and into the surrounding tissue. These events often involve an epithelial to mesenchymal transition, which may have to be reversed as cells establish secondary lesions (43). Finally, after a variable period of dormancy, micrometastases must survive and grow to form secondary lesions (44–49). Cells within a primary tumor have evolved individually and in pairs, in an effort to test for cooperation as well as for the role of each pathway in mammary tumor type and specific breast cancer subtypes as determined by pathway-specific gene signatures; D, relationship between survival and pathway activity for patients with distinct breast cancer subtypes. Bold, significant \( P \) values.

develop mostly keratinized tumors (such as adenosquamous carcinomas) and adenomyoepitheliomas with similarity to "normal-like" tumors in humans (38). Also, as noted above, Neu<sup>+</sup> and Class8<sup>+</sup> mammary tumors developed in MMTV-H-Ras mice (4, 38). These signatures have similarity to luminal A and normal-like breast cancers in humans, respectively. The K-Ras model studied here formed mammary tumors with a more squamous expression gene profile (Fig. 3), and similarity to HER2<sup>+</sup> subtype tumors in humans. Remarkably, when activated Ras and Plk3ca were expressed together, metastatic luminal B-like mammary tumors formed. Thus, elevated Ras signaling promotes formation of metastatic mammary tumors in mice and, when expressed with the luminal-associated oncogene, Plk3ca<sup>p21<sup>FlI</sup>1047R</sup>, establishes a reproducible model for ER<sup>+</sup>/luminal B breast cancer.

To study the importance of PI3K, Stat3, and Ras signaling on relapse and survival in humans with breast cancer, we used pathway-specific signatures to analyze publicly available gene expression from cohorts with linked outcome data (28). Importantly, the Ras signature described by Gatza and colleagues was developed in human mammary epithelial cells infected with an H-Ras–expressing adenovirus and validated against colon cancer samples with known K-Ras mutational status (28). This signature is activated in many human breast cancers particularly in basal-like and HER2<sup>+</sup> tumors (28, 61). Importantly, then, the signature identifies breast tumors with high Ras signaling, despite the absence of Ras gene mutations in this disease. In addition, this signature does not discriminate between H-Ras<sup>+</sup> and K-Ras signaling and it is biased towards genes that are expressed in mammary epithelium. Using this signature, we found a strong relationship between Ras pathway activation and relapse, as well as reduced survival for patients with luminal A and B subtype disease. This effect was not enhanced when we tested for tumors with high levels of signaling through Ras and PI3K pathways, or through Ras and Stat3 pathways. Importantly, the effect of Ras was particularly strong when pathway activity was considered as a continuous variable, suggesting that p21<sup>FlI</sup>1047-GTP may well promote metastasis in a concentration-dependent manner. Such a dose-dependent effect would also explain why mutation of Rasal2 enhanced metastatic dissemination and outgrowth in MMTV-Neu mice, a model that is already metastatic (2).

On the basis of published work with MCF7 cells (62, 63), where oncogenic Ras makes this line estrogen independent, as well as on the fact that high Ras signaling as a binary variable is associated with poor prognosis for ER<sup>+</sup> breast cancer patients treated with tamoxifen (39), it seems likely that hormonal therapy would prove ineffective in our Plk3ca/Ras mouse model of luminal B-like breast cancer. However, this model could be exploited to screen for novel therapeutics to treat hormone therapy–resistant tumors. Also, given that Ras signaling functions as a continuous variable, it may be worth combining Ras pathway–targeted therapeutics, like Mek inhibitors, together with hormone therapy, even for luminal breast cancer patients with relatively low pathway signaling. Finally, as Ras pathway signaling can sensitize cells to chemotherapy, perhaps Ras and ERT pathways could be targeted sequentially.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: K.L. Wright, J.R. Adams, S.E. Egan

Development of methodology: K.L. Wright, J.R. Adams, P. Hu

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.L. Wright, J.R. Adams, J.C. Liu, A.J. Loch, R.G. Wong, C.E.B. Jo, L. Weiss, X. Mei, T.F. Lane, S.B. Korolov, P. Hu


Writing, review, and/or revision of the manuscript: K.L. Wright, J.R. Adams, J.C. Liu, R.G. Wong, X. Mei, J.R. Woodgett, E. Zacksenhaus, P. Hu, S.E. Egan

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K.L. Wright, J.R. Adams, J.C. Liu, R.G. Wong, L.A. Beck, L. Weiss, X. Mei, P. Hu

Study supervision: E. Zacksenhaus, S.E. Egan

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**References**


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