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

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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 oncoproteins 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 PI3KCA10472A, 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 ERα− 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 (ERα). Transcriptional profiling has been used to identify common breast cancer subtypes, including luminal A and luminal B (both of which express ERα), 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
signature" (BPMIS) 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 ERα+ 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 adenosquamous carcinomas or adenomyoepitheliomas. 

Mice were housed in the National Cancer Institute (NCI) laboratory. Mice were genotyped using primers listed below. Males were not studied. Genotyping primers used in this work are as follows: (i) for Cre recombinase we used forward primer (FP): 5'-TCCGGATACTTCTATATCCTCATGTTACTCC-3' and reverse primer (RP): 5'-GCTGACGACCAGTTAGTTACC-3' (13), (ii) for R26-Stat3 we used FP: 5'-AACGTCGCTCGTCTATTGATT-3' and RP: 5'-GAACGCGAGAGAAATTGATGGTCTTGTCAACC-3' (22), (iii) for R26-Pik3ca we used FP: 5'-AACTGAAGGTTTGTCAACC-3' and RP: 5'-GCGAAGAGTTTGTCCTCAACC-3' (21).

Aim 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 we used forward primer (FP): 5'-TCCGGATACTTCTATATCCTCATGTTACTCC-3' and reverse primer (RP): 5'-GCTGACGACCAGTTAGTTACC-3' (13), (ii) for R26-Stat3 we used FP: 5'-AACGTCGCTCGTCTATTGATT-3' and RP: 5'-GAACGCGAGAGAAATTGATGGTCTTGTCAACC-3' (22), (iii) for R26-Pik3ca we used FP: 5'-AACTGAAGGTTTGTCAACC-3' and RP: 5'-GCGAAGAGTTTGTCCTCAACC-3' (21).

Tumor collection

Mice were monitored for onset/progression of neoplastic disease, and humanely sacrificed at endpoint. For mammary tumor bearing mice, part of each tumor (with adjacent normal or hyperplastic mammary gland if possible) was fixed in 10% formalin/PBS (Fisher Scientific HC200-20) and then paraffin-embedded. The rest of each tumor was divided into smaller samples with a sterile razor blade, frozen, and transferred to a −80°C freezer for storage.

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 predetermined 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 "|") 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 < 0.05 and tests were run one-sided at a 95% confidence interval.

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 RV1000G1). Slides were rinsed in running tap water for 5 minutes and then mounted onto a Tecan FreedomEvo liquid-handling robot. Staining was performed according to standard procedures (13, 27). Images were captured with an AxioCam HRm 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 is available online. The survival information in these datasets 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 Pik3caH1047R or Stat3C. A–C, Kaplan–Meier mammary tumor-free survival curves for control (MMTV-Cre and Cre-negative R26-Pik3caH1047R, R26-Stat3C, and K-RasG12D) and experimental mice. Six cohorts of MMTV-Cre–positive experimental mice were used: R26-Pik3caH1047R, R26-Stat3C, both Pik3caH1047R and Stat3C at the R26 locus, K-RasG12D, R26-Pik3caH1047R with K-RasG12D, and R26-Stat3C with K-RasG12D. D, number of mammary tumors that developed per mouse. (Continued on the following page.)
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 mammary tumors

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 (18–20), we tested for cooperation between Pik3ca $^{H1047R}$ and Stat3$^{W664C/N663C}$ (31). This allele, commonly known as Stat3C, produces a mutant protein with prolonged activity in response to p70S6K phosphorylation, and induces elevated expression of Stat3 target genes. Both effects are linked to a slower rate of Stat3C dephosphorylation in the nucleus, as well as to increased DNA binding affinity (32–34). The R26 Cre-conditional transgenic system has been used to express Pik3ca $^{H1047R}$ (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-Pik3ca$^{[H1047R,MMTV-Cre]}$;Stat3C$^{+/+}$, (ii) R26-Pik3ca$^{[H1047R,Stat3C]}$;MMTV-Cre$^{+/+}$, (iii) R26-Stat3C$^{+/-}$;MMTV-Cre$^{+/-}$, and (iv) negative controls (including transgenics without Cre as well as MMTV-Cre$^{NLS}$ only mice). No statistically significant difference in tumor formation was seen between mice in double and triple transgenics (cohorts i and ii, respectively; Fig. 1A; Supplementary Table S1). Also, mammary tumors that formed in R26-Pik3ca$^{[H1047R,MMTV-Cre]}$ and R26-Pik3ca$^{[H1047R,Stat3C]}$;MMTV-Cre$^{+/-}$ mice were mostly either adenomyoepitheliomas or adenosquamous carcinomas, and the ratio of major tumor types was not affected by expression of Stat3C (Fig. 1E and F). 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 B and Supplementary Table S1).

Continued.) Analysis was based on observational data collected at necropsy. E–I, mammary tumors from R26-Pik3ca$^{[H1047R,MMTV-Cre]}$;Stat3C$^{+/+}$, (ii) 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 mice did not develop mammary tumors. R26-Pik3ca$^{[H1047R,MMTV-Cre]}$;Stat3C$^{+/+}$, and MMTV-Cre$^{NLS}$ female mice (F) also developed mostly adenocarcinomas and adenomyoepitheliomas (Fig. 1E). Ras model mice (K-RasG12D;MMTV-CreNLST, (2)) developed adenocarcinomas such as poorly differentiated adenocarcinoma (PDA) or complex adenocarcinoma, which contain multiple histologies (CAC) at a high frequency (Figs. 1G and H, and 2A and B; ref. 17).

We next tested for cooperation between K-RasG12D and activated Stat3 (Stat3C). As noted above, R26-Stat3C$^{+/-}$;MMTV-Cre$^{NLS}$ mice did not develop mammary tumors (Fig. 1A/C). However, when K-RasG12D and Stat3C were expressed together, overall survival and mammary tumor-free survival were significantly reduced in comparison with mice expressing K-RasG12D by itself.
Once again, cooperation was not seen at the level of mammary tumors per mouse (Fig. 1D). As with other Ras models (K-RasG12D alone, or K-RasG12D with Pik3caH1047R), K-RasG12D;R26-Stat3C;MMTV-CreNLST 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 (ERα) expression in these lesions. Indeed, the majority of tumors that formed in K-RasG12D alone, K-RasG12D plus Pik3caH1047R and K-RasG12D plus Stat3C cohorts were ERα⁺ (Fig. 2A–D; Supplementary Fig 2). Interestingly, these tumors stained positive for Stat3pY705 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, Pik3caH1047R, 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-Pik3caH1047R;MMTV-CreNLST 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 (4, 38), with similarity to luminal A breast cancer in humans and with features related to normal alveolar function. Mammary tumors from K-RasG12D;MMTV-CreNLST mice formed adenocarcinomas with some squamous differentiation. These clustered near Pik3caH1047R tumors with a Squamous-likeEx signature.

Figure 2.
Experimental mice developed mostly adenocarcinoma that expressed ERα. A, K-RasG12D;MMTV-Cre mice developed poorly differentiated adenocarcinomas (PDA, top), complex adenocarcinomas (CAC, not shown), and spindle cell tumors (SCT, third panel), some of which were negative for ERα expression and some of which were positive (e.g., second panel from top and bottom panel). The first and third rows show hematoxylin and eosin–stained sections. B, R26-Pik3caH1047R;K-RasG12D;MMTV-Cre mice developed similar tumor types, which were ERα⁺-positive. C, R26-Stat3C;K-RasG12D;MMTV-Cre mice also developed similar ERα⁺ tumor types, as well as squamous cell carcinomas (SCC). Scale bar, 500 μm in each image. The second and forth rows show tumor sections stained with antibodies against ERα. D, overall, most tumors examined were positive for ERα expression (n = number of tumors stained).
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 Pik3ca\textsuperscript{H1047R} 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 Pik3ca\textsuperscript{H1047R};Stat3\textsuperscript{c} transgensics for disseminated disease. Metastases occurred in a small percentage of tumor bearing
Both cohorts had mice with less than ten lung metastases (as in the bottom panel). R26-Pik3ca\(^{H1047R}\);MMTV-Cre mice were mostly nonmetastatic, while 24% of R26-Pik3ca\(^{H1047R}\);Stat3\(^{C}\);MMTV-Cre mice had 1 to 2 lung metastases. In contrast, about half of the mice that expressed K-RasG12D and developed mammary tumors also had lung metastases. B, an example of one of the mice from each Ras-expressing/double-oncogene cohort (K-Ras\(^{G12D}\);Pik3ca\(^{H1047R}\) and K-Ras\(^{G12D}\);Stat3\(^{C}\), 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\(^{G12D}\);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\(^{H1047R}\);K-Ras\(^{G12D}\);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\(^{C}\);K-Ras\(^{G12D}\);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; \(P = 2.3 \times 10^{-5}\); 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; \(P = 1.9 \times 10^{-7}\); 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; \(P = 8.4 \times 10^{-5}\); for survival: HR, 22.4; 95% CI, 5.4–93; \(P = 1.8 \times 10^{-5}\)).

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\(^{+}\) subtypes (28). Consistent with these findings, we saw a negative correlation between ER pathway activity and Ras signaling (activity correlation \(-0.44\), Wilcoxon test \(P = 9.3 \times 10^{-11}\)). 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\(^{+}\), 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\(^{-}\) 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.
Table 1. Statistical analysis shows that Ras pathway activity is associated with relapse and survival of patients with luminal breast cancer

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with relapse of basal-like breast cancer patients (HR, 6.7; 95% CI, 0.95 – 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 – 12,000; 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 extravasation 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 and adapted to grow under very specific conditions. However, if these cells enter the circulation and travel to another tissue, many of the signals required for growth and/or survival may not be present at the new site. Strikingly, elevated Ras signaling can, in the right context, promote all of these steps. For example, Ras can activate EMT, cellular invasion, motility, survival, angiogenesis, and even altered metabolism (50–52). In the context of ER+ breast tumor metastasis, it is unclear which of these are enhanced by Ras.

Advanced breast tumors can metastasize to the lung, liver, bone, and brain. Additional genetic or epigenetic alterations may be required for breast cancer cells to home to these sites and then to grow within a new environment. Over the past decade, the Massagué lab have used mouse models to investigate these phenomena and subsequently reported on a number of molecular properties associated with dissemination of breast cancer to specific organs (53–55). For example, metastasis to bone is associated with elevated Src tyrosine kinase activity (56, 57). Other studies have highlighted the importance of an IL6/IL11 signal to Stat3 in metastatic dissemination of breast cancer to the bone, as well as Jagged1/Notch, Rankl/Rank signaling, and suppression of interferon signaling (58). Many of the screens for signaling pathways involved in site-specific metastatic dissemination have been performed on MDA-MB-231 cells, a high metastatic cell line with activated K-Ras (59). Thus, the importance of Ras signaling in metastatic dissemination to many tissues has not been tested directly.

The connection between Ras signaling and metastasis was discovered over 25 years ago (3). While Ras genes are not commonly mutated in human breast cancer, this pathway is activated downstream of tyrosine kinase receptors (28, 60). In addition, GTP loading of p21RAS occurs in response to loss-of-function mutations or reduced expression of Ras GTPase activating protein (GAP), like NF1 or Rasal2 (1, 2). Ras is activated downstream of Polyoma Middle T and Her2/Neu in mouse models of metastatic breast cancer (15, 16). In each case, Ras is activated together with PI3K and Stat3. We therefore studied these pathways 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 dissemination. PIK3CA is the most commonly mutated gene in human luminal-subtype breast cancers, but Pik3ca models...
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 Class<sup>–</sup> mammary tumors developed in MMTV-Hi-Ras mice (4, 38). These signatures have similarity to luminal A and normal-like breast cancer in humans, respectively. The K-Ras model study here formed mammary tumors with a more squamous gene expression profile (Fig. 3), and similarity to HER2<sup>+</sup> subtype tumors in humans. Remarkably, when activated Ras and Pik3ca 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, Pik3ca, 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 Gatta 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 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>WAF1/CIP1</sup>–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 Pik3ca/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 ER<sub>a</sub> pathways could be targeted sequentially.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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

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