Cooperation between Pik3ca and p53 Mutations in Mouse Mammary Tumor Formation

Jessica R. Adams1,2, Keli Xu1, Jeff C. Liu3, Natalia M. Ruiz Agamez4, Amanda J. Loch1, Ruth G. Wong1, Wei Wang1, Katherine L. Wright1,2, Timothy F. Lane4, Eldad Zacksenhaus3, and Sean E. Egan1,2

Abstract

PIK3CA, which codes for the p110α catalytic subunit of phosphatidylinositol 3-kinase, is one of the most frequently mutated genes in human breast cancer. Here, we describe a mouse model for PIK3CA-induced breast cancer by using the ROSA26 (R26) knock-in system, in which targeted Pik3ca alleles can be activated through transgenic expression of Cre recombinase. We mated Pik3caH1047R and Pik3caH1047R knock-in lines with MMTV-Cre transgenics, which express Cre in mammary epithelium. Starting at approximately 5 months of age, female R26-Pik3caH1047RMMTV-Cre mice, but not control R26-Pik3caH1047RMMTV-Cre mice, developed mammary tumors, as well as lymphoid and skin malignancies. R26-Pik3caH1047RMMTV-Cre mammary tumors were typically either adenosquamous carcinoma or adenomyoepithelium. As p53 is the most commonly mutated gene in breast cancer, we tested for genetic interaction between Pik3caH1047R and p53 loss-of-function mutations in R26-Pik3caH1047R;p53loxP/þ;MMTV-Cre mice. This led to decreased survival of double-mutant animals, which developed lymphoma and mammary tumors with rapid kinetics. Mammary tumors that formed in p53loxP/þ;MMTV-Cre conditional mutants were either poorly differentiated adenocarcinoma or spindle cell/EMT, whereas R26-Pik3caH1047R;p53loxP/þ;MMTV-Cre mammary tumors were mostly adenosquamous carcinoma or spindle cell/EMT indicating that double-mutant mice develop a distinct spectrum of mammary tumors. Thus, an oncogenic variant of PIK3CA implicated in multiple human breast cancer subtypes can induce a very diverse spectrum of mammary tumors in mice. Furthermore, Pik3caH1047R shows cooperation with p53, which altered the specific tumors that formed. Thus, the two most frequently mutated genes in human breast cancer show cooperation in mammary tumor formation. Cancer Res; 71(7); 2706–17. ©2011 AACR.

Introduction

The phosphoinositide 3-kinase (PI3K) signaling pathway is one of the most frequently mutated pathways in cancer. One common and direct mechanism by which the PI3K pathway is activated in breast, endometrial, colorectal, urinary tract, thyroid, and ovarian cancer is through gain-of-function mutations in PIK3CA (1–3). PIK3CA mutations are particularly important in human breast cancer, with activated alleles detected in 25% to 30% of tumors (4–7). These mutations have been found in ductal carcinoma in situ, suggesting that they play a role in breast tumor initiation (8–10). In addition, they are present at high frequency in estrogen receptor α (ERα)-positive, Her2/Neu+ and triple-negative breast tumors (TNT), suggesting an important role for mutant PIK3CA in etiology of multiple breast tumor subtypes (4). Metaplastic breast cancers have the highest percentage of PIK3CA mutations (11). Importantly, PIK3CA mutations can induce p53-dependent growth inhibition (12) and mutations in both genes occur together in some human breast tumors (13, 14).

PIK3CA point mutations occur most often within 2 hotspots: the helix and kinase domains (1). H1047R mutations in the kinase domain account for approximately 40% of breast cancer PIK3CA mutant alleles (4). This mutant shows elevated kinase activity (15–17) and is capable of transforming cells in culture (18), including mammary epithelial cells (19, 20). Despite the high frequency, and hence importance of this allele in breast cancer, there is no animal model to test for its role in transformation of mammary epithelium. Here, we describe generation of a Cre-mediated system to conditionally express Pik3ca alleles in mouse tissues, and use of this system to test for induction of mammary tumor formation by Pik3caH1047R. We report that ectopic expression of Pik3caH1047R, but not Pik3caH1047M, induced mammary tumors at high frequency. Furthermore, we have used the knock-in system to test for...
cooperation between Pik3ca and p53, showing that deletion of p53 selects tumor formation and alters the spectrum of mammary tumor subtypes that form in our Pik3caH1047R model.

Materials and Methods

Generation of transgenic mice

Shuttle vectors containing either wild-type or H1047R mutant Pik3ca were subcloned into the pROSA26PA gene-targeting vector (21, 22). R26-Pik3ca plasmids were linearized with KpnI (Fig. 1) and electroporated into mouse embryonic stem cells (mESC). G418-resistant clones were isolated, expanded, and screened by Southern blot (probing for the 3' recombination junction—see following text) and PCR (amplifying across the 5' recombination junction, forward primer (FP) 5'-CGCTTAAAGAAGGGCTTG-3', reverse primer (RP) 5'-GAAAGACGGCGAGAATGGTTG-3'). Targeted clones were transiently transfected (Fugen6, Roche 11815091001) with Cre or control vector (pCAGGS-Cre-IRESpuro; pCAGGS-Flip-E-IRESpuro; ref. 23). RNA was harvested (RNeasy, Qiagen 74104) and RT-PCR performed (SuperScript II, Invitrogen 18064022) by using FP in R26 exon 1 (5'-CTTGAAAGTGGAGTAACTAC to TCAGAAGCTTTGAACTAG-3') and a RP in Pik3ca cDNA (5'-ATTCTTCGATTGAGGATCTTTTCT-3'). Cre-inducible mESC lines were used for morula aggregation in the Toronto Centre for Phenogenomics (TCP).

Southern blot analysis

A standard Southern blot protocol was used. Genomic DNA (10 μg) was digested with EcoRI/XbaI. An approximately 940 bp probe on the 3' side of the targeted insertion site (CTTGAAGGTGAGTAATCT to TCAGAAGGTTGAAACTAGGA-3') was 35P-labeled, using the Random Primers DNA Labeling System (Invitrogen 18187013). This probe binds an 11 kb fragment in nontargeted R26, and a 9 kb fragment if the R26 locus is correctly targeted.

Mouse colony maintenance

Mice were housed at TCP according to guidelines from the Canadian Council on Animal Care (CCAC). Cre strains were genotyped by using FP 5'-TGGCAGATCTCTCTGACCC-3', RP 5'-GTCGGAGGATGATTCCG-3'. R26-Pik3ca mice were genotyped by using primers previously published (22). Multiparous females had 3 litters. p53loxP/loxP conditional mutant mice (FVB.129- Tp53'5'loxP/5'loxP-MMHCC 01XC2; for simplicity, this strain is labeled throughout as p53LoxP; ref. 24) were obtained from the Mouse Models of Human Cancer Consortium and genotyped by PCR (FP 5'-CACAAAAACAGTTAAACCAGC-3', RP 5'-AGCAGATAGGGAGGCAGAC-3'). p53 deletion was confirmed by using FP 5'-CACAACAAACAGTTAAACCAGC-3', RP 5'-GAGACAGAAAAAGGGAGGG-3' to amplify a 612 bp fragment (24).

RT-PCR on lin– mammary epithelium

Mammary glands from young virgin mice were minced and digested in collagenase/hyaluronidase (Stem Cell Technologies 07912) for 4 hours (37°C). Mammary epithelial (lineage-depleted; lin–) cells were isolated by using the EasySep kit (StemCell Technologies 19757). RT-PCR was performed as outlined earlier in the text.

Tumor collection

Mice were humanely sacrificed at endpoint, and mammary tumors dissected. Humane endpoint was based on criteria outlined by the CCAC (tumor volume = 1.7 cm3, tumor mass = 5% of body weight, or ulcerated tumor). Part of each tumor was fixed in 10% formalin and embedded in paraffin. The remaining tumor was divided into small samples and snap frozen.

Western blots

Tumors and control glands were lysed by using a TissueLyser homogenizer (Qiagen 9001271) in lysis buffer (ProteoJET Lysis Reagent, Fermentas K0301; PhosSTOP, Roche 04906837001; protease inhibitor, Roche 11836170001). Protein lysates (150 μg) were analyzed using a standard Western blot protocol. Primary antibodies were incubated overnight and secondary antibodies for 1 hour, both at room temperature. Western blots were quantified using Alphalmager software (Cell Biosciences) and normalized with respect to β-actin expression. Antibodies (used at recommended dilutions) are as follows: AktpT308 (Cell Signaling CS2965), AktpS473 (Cell Signaling CS927), PTEN (Cell Signaling CS9559), e-JunNterminal (Cell Signaling CS3270), SGK3pS486 (SantaCruz sc33044), ERα (SantaCruz sc71064), Atf3 (SantaCruz sc188), β-casein (BDTL 610154), carbonic anhydrase IX (CAIX; Roche Diagnostics AP2344), β-actin (abCam ab8226), goat anti-rabbit IgG-HRP (SantaCruz sc33004), goat anti-mouse IgG-HRP (SantaCruz sc2005), donkey anti-goat IgG-HRP (SantaCruz sc2020).

Histology and immunohistochemistry

Five-micrometer paraffin sections were either stained with hematoxylin and eosin, or subjected to antigen retrieval in a decloaking chamber (Biocare Medical; SetPoint1 = 125°C, 5 minutes; SetPoint2 = 90°C, 10 seconds), using epitope-retrieval solution (Biocare Medical BD1000G1 or RV1000G1). Slides were mounted into a Tecan FreedomEvo liquid-handling robot.

Immunohistochemistry: endogenous peroxidases were quenched in 3% H2O2/methanol (15 minutes, room temperature), then VectaStain ABC kits (Vector Laboratories) were used (PK-6101, PK-4002, PK-6105). Antibodies were incubated for 30 minutes at room temperature. 3,3′-Diaminobenzidine (DAB) substrate kit (Vector Laboratories SK-4100) was used for staining and slides were counterstained in hematoxylin (10 seconds).

Immunofluorescence: slides were blocked for 30 minutes (DakoCytomation X0909). Primary antibodies were incubated overnight (4°C), secondary antibodies were incubated for 1 hour (room temperature). Slides were mounted with fluorescent mounting medium (DakoCytomation S3023) containing 4′,6-diamidino-2-phenylindole (DAPI).

Antibodies (used at recommended dilutions) are as follows: Gata3 (ProteinTech 10417–1-AP), Cytokeratin 5 (Covance...
Figure 1. R26-Pik3caH1047R;MMTV-Cre mice develop tumors in the mammary gland, skin, and lymphocytic tissue. A, conditional knock-in mice were generated by recombining mouse Pik3ca cDNAs (preceded by a loxP-flanked transcriptional stop cassette) into the R26 locus (22). B, lin e mammary epithelium from R26-Pik3ca;MMTV-Cre mice expressed their respective transgene, whereas cells from Cre littermates did not. C, Kaplan–Meier survival curves for R26-Pik3caH1047R;MMTV-Cre female mice. D, cause of death data for R26-Pik3caH1047R;MMTV-CreNLST (left) and R26-Pik3caH1047R;MMTV-CreNLST (right) mice. 'Other' includes mice that died without obvious tumors (e.g., with dystocia) or mice that could not be examined because of decomposition. MGT, mammary gland tumors; L/T, lymphoma/thymoma; ST, skin tumors.
PRB-160P), ERα (SantaCruz sc542), Ki67 (BioCare Medical CRM326), Cytokeratin 8 (Fitzgerald 10R-C177AX), Cytokeratin 14 (Panomics E2624), Cytokeratin 10 (Covance PRB-159P), vimentin (Vim; SantaCruz sc32322), desmin (Des; DakoCyto- mation M0760), N-cadherin (N-Cad; Novus NB200-592), AlexaFluor488 anti-mouse (Invitrogen A21202), AlexaFluor594 anti-rabbit (Invitrogen A21442).

**TUNEL**

Five-micrometer paraffin sections were immersed in 2.5 μg/ml proteinaseK (2 minutes). Endogenous peroxidases were quenched via 3% H2O2 (15 minutes). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was performed by using terminal deoxynucleotidyl transferase (Fermentas EP0161) and biotin-16-UTP (Roche 11093070910). Signal was amplified (ABC, Vector Laboratories PK-6100) and β-galactosidase-mediated deletion of loxP-flanked transcriptional stop (FERmentas EP0161) was performed by using terminal deoxynucleotidyl transferase (Fermentas EP0161) and biotin-16-UTP (Roche 11093070910). Signal was amplified (ABC, Vector Laboratories PK-6100) and biotin-16-UTP (Roche 11093070910).

**Image capture**

Images were captured with an AxioCam HRm digital camera (Zeiss) by using AxioVision (release 4.6.3) software.

**Results**

**Generation of a Cre-inducible Pik3ca mammary tumor model**

To test for the ability of Pik3caH1047R or wild-type Pik3ca (Pik3cawt) to induce mammary tumors, we used the Cre-conditional R26S26 (R26) knock-in system, whereby Cre-mediated deletion of loxP-flanked transcriptional stop sequences allows for tissue-specific expression of either allele (Fig. 1A: refs. 21, 22). mESC were targeted and screened to identify clones with Cre-inducible transgene expression (Supplementary Fig. 1). Cre-responsive mESC clones were then used to generate chimeric mice, and germline transmission of each knock-in allele confirmed. R26-Pik3caH1047R and R26-Pik3caH1047R mice were mated with MMTV-Cre mice, which express Cre recombinase in mammary epithelium. Two different strains of MMTV-Cre were used: MMTV-Cre, which expresses Cre recombinase in mammary epithelium at high efficiency and also in skin and lymphocytes (25); MMTV-CreNLST, which is less efficient but more mammary restricted in its expression (Supplementary Fig. 2; refs. 26, 27). Mammary-inducible transgene expression was observed in R26-Pik3caH1047RMMTV-Cre mice but not in Cre-negative littermates (Fig. 1B).

Female R26-Pik3caH1047RMMTV-Cre mice from both Cre lines showed reduced survival (Fig. 1C; Supplementary Table 1; note, males were not studied). Some animals died abruptly, whereas most were sacrificed at humane endpoint due to lethargy and impaired breathing or solid tumor growth. Decreased survival was not because of Pik3ca overexpression as R26-Pik3caH1047RMMTV-Cre mice remained healthy (Fig. 1C). R26-Pik3caH1047RMMTV-Cre mice started developing mammary tumors at 5 months of age. Mammary tumors were observed in both virgin and multiparous mice using either MMTV-Cre strain. R26-Pik3caH1047RMMTV-CreNLST mice reached endpoint more rapidly than R26-Pik3caH1047RMMTV-CreNLST mice (Fig. 1C). In addition, R26-Pik3caH1047RMMTV-CreNLST mice reached endpoint with a more diverse set of tumor types. 69% of NLST mice and 42% of line A mice had palpable mammary tumors at endpoint (Fig. 1D). For this reason, we used MMTV-CreNLST transgenics for subsequent studies. Development of lymphoma/thymoma, skin, and other nonmammary tumors was attributed to Cre expression in other tissues (25). Interestingly, survival of R26-Pik3caH1047R mice, without Cre, was reduced in comparison with MMTV-Cre controls (Fig. 1C; Supplementary Table 1; P < 1 x 10^-6). This was due to development of blood vessel lesions in some R26-Pik3caH1047R animals (data not shown), potentially through spontaneous activation of Pik3caH1047R expression in endothelial cells (28).

**Pik3ca and p53 mutations cooperate in vivo**

To test for cooperation between Pik3ca and p53, we mated R26-Pik3caH1047RMMTV-CreNLST mice with Cre-conditional p53 mutants (24). Most R26-Pik3caH1047R;p53f/+MMTV-CreNLST mice died early with lymphoma/thymoma (Fig. 2A). On autopsy, some had small mammary tumors (Fig. 2C, left). Lymphoma/thymoma occurred at a much lower rate in R26-Pik3caH1047R;p53f/+MMTV-CreNLST mice. R26-Pik3caH1047R;p53f/+MMTV-CreNLST mice showed significantly reduced survival as compared with R26-Pik3caH1047RMMTV-CreNLST p53f/+, MMTV-CreNLST, and Cre-negative control mice (Fig. 2A; Supplementary Table 2). To test whether Pik3ca and p53 show an additive or synergistic interaction, we simulated a Kaplan–Meier survival curve by combining single-mutant cohorts, such that as any R26-Pik3caH1047RMMTV-CreNLST or p53f/+MMTV-CreNLST mouse reached endpoint, it was scored as an event within one cohort. R26-Pik3caH1047R;p53f/+MMTV-CreNLST double-mutant mice showed significantly decreased survival (P = 2.79 x 10^-5) compared with this hypothetical cohort indicating synergy between Pik3caH1047R and p53 gene loss. Synergistic interaction was also evident when considering only mice that reached endpoint from mammary tumors (Supplementary Fig. 3). p53 deletion was observed in R26-Pik3caH1047R;p53f/+MMTV-CreNLST and p53f/+MMTV-CreNLST tumors (Fig. 2B). Ninety-two percent of p53f/+MMTV-CreNLST and 80% of R26-Pik3caH1047R;p53f/+MMTV-CreNLST mice had mammary tumors (Fig. 2C). Many mice had mammary tumors in more than one gland. These were treated as distinct lesions.

Histologic analysis revealed that mammary tumors from R26-Pik3caH1047RMMTV-CreNLST mice were typically either adenosquamous carcinoma or adenomyoepithelioma (Fig. 3A; Supplementary Fig. 4). Adenosquamous tumors contained cystic regions composed of laminar keratin lined with squamous epithelium and adjacent glandular elements. Some tumors had squamous nodules or necrosis, and several had invasive margins. Expansile tumors were also observed. Adenomyoepitheliomas contained a mixture of glandular epithelium and interstitial fusiform cells with abundant polar cytoplasm. These myoepithelial stromal cells represented 10% to 80% of the tumor. Isolated lung metastasis were
observed in rare R26-Pik3caH1047R;MMTV-CreNLST mice (data not shown). Mammary tumors from p53f/+;MMTV-CreNLST mice were either spindle/epithelial-mesenchymal transition (EMT) type or poorly differentiated adenocarcinoma as previously reported (Fig. 3B; Supplementary Fig. 4; refs. 29–31). Spindle tumor cells had fusiform nuclei and polar cytoplasm. Swirling patterns and necrotic areas were observed. Poorly differentiated adenocarcinomas were composed of solid sheets of cells with little tissue architecture and some necrosis. Cells had large, pleomorphic nuclei and dark staining cytoplasm. Interestingly, mammary tumors that formed in R26-Pik3caH1047R;p53f/+;MMTV-CreNLST double-mutant mice were typically either spindle/EMT or adenosquamous carcinoma (Fig. 3C; Supplementary Fig. 4). Radial scar and poorly differentiated adenocarcinomas were also observed, although at a lower frequency (Fig. 3C; Supplementary Fig. 4). Radial scar tumors were small and had a stellate outline composed of dense connective tissue and a center with distorted neoplastic glands. Gross metastatic lesions were not observed in R26-Pik3caH1047R;p53f/f;MMTV-CreNLST mice (data not shown).
Akt activation in Pik3ca<sup>H1047R</sup> and p53 mutant mammary tumors

To test for PI3K pathway activation, lysates from representative tumors were analyzed for Akt phosphorylation, and for expression of PTEN and phospho-c-Jun (S73). Western blot signals were normalized to β-actin protein expression, and compared with levels in mammary lysates from a non–tumor-bearing 74-week-old R26-Pik3ca<sup>H1047R</sup> virgin female. Adenocystomas from R26-Pik3ca<sup>H1047R</sup>;MMTV-Cre<sup>NLST</sup> mice showed increased accumulation of phospho-Akt S473, PTEN, and phospho-c-Jun S73 (Fig. 4). Adenocystomas from R26-Pik3ca<sup>H1047R</sup>;p53<sup>f+/+</sup>;MMTV-Cre<sup>NLST</sup> mice showed increased PTEN and phospho-c-Jun S73 expression. Surprisingly, however, no change in phospho-Akt was observed in these tumors. The reason for this is not clear, perhaps representing a change in PI3K signaling such as a reduced dependence on Akt activation (32). Alternatively, the wild-type sample used for normalization in this experiment was whole mammary gland lysate. This tissue contains a large amount of insulin-responsive fat, and may show higher basal levels of Akt activation than mammary epithelium. We therefore tested additional adenocystomas from R26-Pik3ca<sup>H1047R</sup>;MMTV-Cre mice for phospho-Akt and used lin<sup>−</sup> mammary epithelium as control. Indeed, phospho-Akt<sup>T308</sup> and phospho-Akt<sup>S473</sup> were significantly elevated in 12 adenocystomas from R26-Pik3ca<sup>H1047R</sup>;p53<sup>f+/+</sup>;MMTV-Cre<sup>NLST</sup> mice (Supplementary Fig. 5).

Elevated levels of phospho-Akt<sup>T308</sup> and phospho-Akt<sup>S473</sup> were observed in lysates from adenomyoepithelioma lesions in R26-Pik3ca<sup>H1047R</sup>;MMTV-Cre<sup>NLST</sup> mice and in lysates from R26-Pik3ca<sup>H1047R</sup>;p53<sup>f+/+</sup>;MMTV-Cre<sup>NLST</sup> spindle/EMT tumors. Interestingly, elevated levels of phospho-Akt<sup>T308</sup> and phospho-Akt<sup>S473</sup> were also seen in lysates from spindle/EMT and in poorly differentiated adenocarcinomas from p53<sup>f+/+</sup>;MMTV-Cre<sup>NLST</sup> mice. Elevated levels of PTEN expression and of phospho-c-Jun accumulation were observed in each mammary tumor type studied. In the case of PTEN, this is likely a result of PI3K pathway–induced phosphorylation and stabilization of PTEN, a negative feedback loop which functions to decrease PIP<sub>3</sub> levels (33). Also, increased phospho-c-Jun is associated with elevated PI3K pathway activation (34). Finally, it has been suggested that activation of other PDK1 substrate kinases, such as SGK3, may play an important role downstream of PIK3CA gene mutation in breast cancer (32). We therefore analyzed SGK3 phosphorylation in these tumors. Phospho-SGK3<sup>S486</sup> was not elevated in any of the tumor types analyzed (Supplementary Fig. 6).

Pik3ca<sup>H1047R</sup> mice form multiple mammary tumor subtypes

PIK3CA gene mutations are found in ERα-positive, HER2-positive, and TNT cancers (4–7). To further define mammary tumor subtype in our Pik3ca model, we stained representative tumors for expression of luminal and basal cell cytokeratin, cytokeratin 8 (K8) and cytokeratin 14 (K14), respectively (Fig. 5; Supplementary Fig. 7). Glandular regions of R26-Pik3ca<sup>H1047R</sup>;MMTV-Cre<sup>NLST</sup> adenocystomas contained cells expressing either cytokeratin. Mammary architecture was maintained in glandular regions, in that K14<sup>+</sup> cells surrounded layers of K8<sup>+</sup> cells (Fig. 5A, middle).
K8⁺ cells in these regions also expressed Gata3, albeit at a level lower than in adjacent normal glands (data not shown; ref. 35). The glandular epithelium in R26-Pik3caH1047R;MMTV-CreNLST adenomyoepitheliomas also stained positive for both cytokeratin (Fig. 5A, right). K14⁺ expression was noted in interstitial fusiform cells surrounding glandular type structures (data not shown). Spindle tumors from p53f/+;MMTV-CreNLST mice had few K8- or K14-positive cells (Fig. 5B, left). In contrast, cells in poorly differentiated adenocarcinomas from p53f/+;MMTV-CreNLST mice were mostly either K14⁺ (Fig. 5B, middle) or K8⁺ (Fig. 5B, right). Some double-negative regions and regions with double-positive cells were also observed (data not shown).

Adenosquamous carcinomas from R26-Pik3caH1047R;MMTV-CreNLST and R26-Pik3caH1047R;p53f/+;MMTV-CreNLST mice showed the same staining pattern (Fig. 5C, left). Similarly, K14 and K8 staining in spindle/EMT (Fig. 5C, middle) and poorly differentiated adenocarcinomas (Fig. 5C, right) from R26-Pik3caH1047R;p53f/+;MMTV-CreNLST and p53f/+;MMTV-CreNLST mice was indistinguishable (note, right panel of Fig. 5C shows a tumor region with many K14⁺/K8⁺ double-positive cells; such regions were also frequently observed in poorly differentiated adenocarcinomas from p53f/+;MMTV-CreNLST mice as described earlier). K5 immunostaining closely matched with that observed for K14 in each case (data not shown).

ERα expression was noted in R26-Pik3caH1047R;MMTV-CreNLST (Fig. 5D) and R26-Pik3caH1047R;p53f/+;MMTV-CreNLST model mice. A, Western blot analysis of PI3K-pathway components and phospho-c-Jun. B, Western blot signals were quantified and normalized with respect to β-actin. Mean fold increase compared with mammary lysate from a control mouse was calculated after normalization. Bars, group mean values; error bars, ±SE; asterisks, significant increase in expression (1-sided 1-sample t test; α = 0.05; *, P < 0.05 and **, P < 0.1). Yellow dashed line, protein expression in a normal mammary gland.
(data not shown) adenosquamous carcinomas, although nuclear staining appeared reduced in most tumor cells as compared with that observed in normal ducts (Fig. 5D, inset). R26-Pik3caH1047R;MMTV-CreNLST adenomyoepitheliomas also contained nuclear ERα− cells (Fig. 5D, right). In contrast, less signal was observed in other tumor types. To better quantify ERα expression, Western blot analysis was performed on tumor lysates (Fig. 5D, bottom). Adenosquamous carcinomas (from R26-Pik3caH1047R;MMTV-CreNLST and R26-Pik3caH1047R; p53+/−;MMTV-CreNLST) as well as adenomyoepitheliomas from R26-Pik3caH1047R;MMTV-CreNLST mice expressed similar levels of ERα as in the control gland. In contrast, ERα expression was decreased in p53+/--;MMTV-CreNLST poorly differentiated carcinomas, R26-Pik3caH1047R; p53+/--;MMTV-CreNLST spindle/E MT tumors and trended toward decreased expression in p53+/--;MMTV-CreNLST spindle/E MT tumors (Fig. 5D).

Next, we stained for the EMT marker N-Cad and, in some tumors, for Des or Vim (Fig. 6; Supplementary Fig. 8; ref. 27, published online first April 12, 2017. © 2011 American Association for Cancer Research.). Adenosquamous carcinomas from R26-Pik3caH1047R;MMTV-CreNLST single or Pik3caH1047R; p53+/--;MMTV-CreNLST double-mutant mice also contained cells expressing a marker of squamous cell carcinoma (38) and accumulated to widely variable levels in our mammary tumors. For example, some R26-Pik3caH1047R;MMTV-CreNLST tumors (adenosquamous carcinomas and adenomyoepitheliomas) and some p53+/--;MMTV-CreNLST poorly differentiated carcinomas expressed Atf3. Expression was not observed in other tumor types (Supplementary Fig. 9). Mammary tumors of all genotypes appeared less bloody than MMTV-Wnt1 control tumors. This was typically associated with less vasculature and, in most cases, with elevated expression of carbonic anhydrase CAIX, an indirect marker of hypoxia (Supplementary Fig. 9; ref. 39).

Interestingly, elevated β-catenin levels, comparable with levels in MMTV-Wnt1 tumors, were observed in both major tumor types from R26-Pik3caH1047R;MMTV-CreNLST mice and in poorly differentiated adenocarcinomas in p53+/--;MMTV-CreNLST mice, suggesting that Wnt signaling may be activated in both. To quantify cell proliferation and apoptosis in each tumor type, we performed Ki67 immunohistochemistry and TUNEL analysis, respectively (Table 1; Supplementary Table 3). Each tumor type examined had very low levels of TUNEL-positive nuclei (<1% in all cases). This result is not surprising given that P13K pathway activation and p53 gene deletion are both associated with enhanced cell survival. In contrast, the percentage of Ki67-positive nuclei varied with genotype and tumor type. Mammary tumors from R26-Pik3caH1047R;MMTV-CreNLST mice had the highest proportion of Ki67-positive nuclei, whereas p53+/--;MMTV-CreNLST tumors had the lowest. In tumor type analysis, adenosquamous carcinomas had the highest Ki67 index whereas poorly differentiated carcinomas had the lowest.

**Discussion**

**PIK3CA**

**PIK3CA** is commonly mutated in human breast cancer. The PIK3CA locus is also amplified in some breast cancers (6, 40, 41). To test for initiation of mammary tumor formation by PIK3CA alleles we generated syngeneic R26-Pik3caH1047R and R26-Pik3caH1047R knock-in mice. Each strain carried a Cre-inducible allele of mouse Pik3ca, and each was crossed to MMTV-Cre mice to activate expression within mammary epithelium. Starting at 5 months, R26-Pik3caH1047R;MMTV-Cre females developed mammary tumors at a high frequency. Importantly, R26-Pik3caH1047R;MMTV-Cre mice did not. Thus, low-level ectopic expression of p110αH1047R, but not p110αW1, is sufficient to initiate mammary tumor formation in mice. This result is consistent with identification of H1047R mutations in premalignant lesions (8–10). We did not observe any gross abnormalities in glands of young R26-Pik3caH1047R;MMTV-CreNLST mice, likely because of inefficient Cre-deletion in this line. It will be important to define early responses to PIK3CAH1047R gene activation in our model, perhaps using line A or through *ex vivo*, Cre-induced, gene activation and transplantation.

**PIK3CA** mutations occur in all major subtypes of human breast cancer (4–7, 11). Our mouse model of Pik3caH1047R, induced breast cancer develops adenosquamous carcinoma and adenomyoepithelioma of the mammary gland at high frequency. Both tumor types were Erα−. Both also contained luminal and myoepithelial keratin-positive cells, suggesting that the cell of origin had bilineage potential. As PIK3CA and TP53 are the 2 most commonly mutated genes in human breast cancer, and mutations in both occur together in many tumors, we tested for cooperation between these genes in our model. Indeed, we observed reduced survival of R26-Pik3caH1047R;MMTV-CreNLST double-mutant animals. Cooperation between Pik3caH1047R and p53 loss-of-function mutation is in contrast to the situation observed in MMTV-myIPik3ca mice, in which mammary tumor formation is enhanced by expression of CDK4R24C but not by deletion of one p53 allele (42). In our model, apoptosis was very low in all of the tumors analyzed. In contrast, the proliferation rate varied significantly from one tumor type to another, tracking more closely with tumor type than with genotype. Thus, the observed cooperation between pik3caH1047R and p53 mutations cooperate in mammary tumor initiation and interact to control mammary tumor type. In total, on wild-type and p53 mutant backgrounds, we observed...
Figure 5. Expression of luminal and basal differentiation markers and ERα in Pik3caH1047R model mammary tumors. A, normal mammary ducts (left) express K14 and K8. Glandular regions of ASC (middle) or AME (right) from R26-Pik3caH1047R;MMTV-CreNLST mice. B, spindle cell tumor (left) and PDA (middle right) from p53f/+;MMTV-CreNLST mice. C, glandular region of ASC (left), spindle cell tumor (middle), and PDA (right) from R26-Pik3caH1047R;p53f/+;MMTV-CreNLST mice. D, immunohistochemistry for ERα in R26-Pik3caH1047R;MMTV-CreNLST ASCs (top left, top middle) and AMEs (top right) compared with normal mammary epithelium (inset). Western blot analysis for ERα (bottom left). Western signals were quantified as described earlier (bottom right). Bars, sample mean; error bars: ±SE; asterisks, significant increase (1-sided 1-sample t-test; \( \alpha = 0.05; * P < 0.05 \) and **, \( P < 0.1 \)). White scale bars, 40 μm; black scale bars, 20 μm. Yellow dashed line, protein expression in a normal mammary gland.
5 distinct types of mammary tumors in our PIK3CA model: adenosquamous carcinoma, adenomyoepithelialomas, spindle/EMT tumors, poorly differentiated adenocarcinomas, and radial scar type lesions. Although some of these tumor types are quite rare in humans, poorly differentiated adenocarcinomas and adenosquamous carcinomas are common, as are spindle/EMT tumors (Basal B tumors in humans). In addition, adenosquamous carcinomas are metaplastic, a tumor type in humans with a high frequency of PIK3CA mutations (11). This result is consistent with the wide spectrum of human breast cancers found to have PIK3CA mutations. In addition, our diverse collection of tumors includes ERα tumors and TNT-type tumors. This result contrasts with tumor models using Neu, Wnt, Myc, or Polyoma middle T, each of which induce a very specific mammary tumor type in mice (43).

We tested for PI3K pathway activation in major mammary tumor types that developed in our PIK3CAH1047R model mice. To this end we analyzed Akt phosphorylation, which was elevated in each tumor type. The modest level of Akt activation noted was somewhat surprising, although this was related to the use of whole mammary gland control tissue rather than lin− mammary epithelium for normalization (Supplementary Fig. 5). In any case, Akt-independent transformation has been noted in some breast tumors with a PIK3CA mutation (32), and other PDK1 substrates are thought to play a role in this case. Indeed, transgenic mice expressing activated alleles of Akt1 from the MMTV LTR do not form mammary tumors, highlighting the importance of other PI3K pathways in breast cancer (44, 45). Future studies will be required to define the importance of specific PIP3-responsive kinases and pathways in our Pik3caH1047R model.

By using the Cre-conditional knock-in system, we can compare mammary tumor formation in response to distinct alleles of Pik3ca, each expressed at precisely the same level and in the same cell types. For example, we can now directly compare mammary tumors in our R26-Pik3caH1047R model with tumor formation in mice expressing helical domain mutants of Pik3ca. As our system is Cre-inducible, we can...
also compare mammary tumor formation in R26-Pik3ca\textsuperscript{H1047R};MMTV-Cre\textsuperscript{NLST} mice and in Pten\textsuperscript{f/f};MMTV-Cre\textsuperscript{NLST} mice, in which PI3K pathway activation will occur in the same cell of origin but through a distinct mechanism. The importance of cell of origin can now be probed in our system as transgenic mice are available to activate \(Pik3ca\) alleles in luminal committed cells (WAP-Cre) and in bipotential cells expressing cytokeratin 14 (K14-Cre). Finally, this model can be used to identify genetic and cellular events associated with progression/metastasis, to screen for novel therapeutic targets in \(Pik3ca\textsuperscript{H1047R}\) mammary tumor cells \textit{ex vivo} and to develop preclinical data on PI3K pathway inhibitors for treatment of breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Table 1. Ki67 and TUNEL data for R26-Pik3ca\textsuperscript{H1047R};p53\textsuperscript{f/f};MMTV-Cre\textsuperscript{NLST} mouse mammary tumors

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Acknowledgments

We thank Neil Adams, Farrah Awan, Adrian Cozma, Zhe Jiang, Tao Deng, Hui Qin Li, Jodi Garner, Marina Gertsenstein, Alex Manno, Molly Ruggeri, Brenda Cohen, and Aaron Kucharczuk for advice and/or technical support; Drs. C-C. Hui, Andras Nagy, Janet Rossant, and Brian Ciruna for advice/reagents; and Dr. Jean Andrey for statistical advice. Finally, we thank Dr. Robert Cardiff for comments on mouse tumor pathology.

Grant Support

We thank Restracomp from the Hospital for Sick Children for J.R. Adam’s studentship and the Canadian Breast Cancer Foundation for funding of J.C. Liu. We thank the Komen Foundation for the Cure for funding to S.E. Egan and E. Zacksenhaus and the Canadian Breast Cancer Research Foundation—Ontario Division and Genome Canada for funding to S.E. Egan and the Canadian Breast Cancer Alliance for funding to E. Zacksenhaus.

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Received March 2, 2010; revised November 5, 2010; accepted January 25, 2011; published OnlineFirst February 15, 2011.

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Cooperation between *Pik3ca* and p53 Mutations in Mouse Mammary Tumor Formation


*Cancer Res* 2011;71:2706-2717. Published OnlineFirst February 15, 2011.

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