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Phosphatase and Tensin Homologue Deleted on Chromosome 10 Deficiency Accelerates Tumor Induction in a Mouse Model of ErbB-2 Mammary Tumorigenesis

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Abstract

Loss of the tumor suppressor phosphatase and tensin homologue deleted on chromosome 10 (PTEN) and amplification or elevated expression of ErbB-2 are both involved in human breast cancer. To directly test the importance of these genetic events in mammary tumorigenesis, we have assessed whether mammary-specific disruption of PTEN could cooperate with activation of ErbB-2.

Transgenic mice expressing ErbB-2 under the transcriptional control of its endogenous promoter (ErbB-2^{KI}) were interbred with mice carrying conditional PTEN alleles and an MMTV/Cre transgene. Loss of one or both PTEN alleles resulted in a dramatic acceleration of mammary tumor onset and an increased occurrence of lung metastases in the ErbB-2^{KI} strain. Tumor progression in PTEN-deficient/ErbB-2^{KI} strains was associated with elevated ErbB-2 protein levels, which were not due to ErbB-2 amplification or to a dramatic increase in ErbB-2 transcripts. Moreover, the PTEN-deficient/ErbB-2^{KI}-derived mouse mammary tumors display striking morphologic heterogeneity in comparison with the homogeneous pathology of the ErbB-2^{KI} parental strain. Therefore, inactivation of PTEN would not only have a dramatic effect on ErbB-2-induced mammary tumorigenesis but would also lead to the formation of mammary tumors that, in part, display pathologic and molecular features associated with the basal-like subtype of primary human breast cancer.

Introduction

The progression of a normal mammary epithelial cell to a malignant phenotype is thought to involve multiple genetic events including the activation of dominant acting oncogenes and the loss of specific tumor suppressor genes. ErbB-2 (Neu, HER2) is a receptor tyrosine kinase and a member of the epidermal growth factor receptor (EGFR) family of receptors, which also consist of EGFR, ErbB-3 (HER3), and ErbB-4 (HER4; ref. 1). Amplification and elevated expression of the *erbB-2* proto-oncogene is observed in 20% to 30% of human breast cancers and is inversely correlated with the survival of the patients (2,3). In addition, ErbB-3 protein levels have been shown to be dramatically overexpressed in ErbB-2-induced mammary tumors (4). Whereas ErbB-2 is known to recruit adapter proteins that primarily function through the Ras signaling pathway (5), ErbB-3 is thought to be involved in the recruitment of the p85

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adapter for the phosphatidylinositol 3' kinase (PI3K) signaling pathway to the other members of the EGFR family (6,7). Moreover, the ErbB-2/ErbB-3 heterodimer complex is believed to be the most biologically active and protumorigenic form of these receptor complexes (8,9). Taken together, these observations suggest that mammary tumorigenesis in the transgenic mouse models used in the studies mentioned above requires the concerted activation of both the Ras and PI3K signaling pathways through the formation of ErbB-2/ErbB-3 heterodimers (8,9).

Although these transgenic studies suggest that *erbB-2* is critical in mammary tumor progression, the fact that *erbB-2* is driven by a strong viral promoter may be a confounding issue. In an attempt to more closely mimic the events involved in ErbB-2-induced mammary tumorigenesis, we have recently derived transgenic mice carrying a Cre-inducible activated *erbB-2* under the transcriptional control of the endogenous *erbB-2* promoter (herein referred to as the ErbB-2^{KI} strain; refs. 10,11). In contrast to the rapid tumor progression observed in the mouse mammary tumor virus (MMTV)-activated *erbB-2* mouse models, poorly metastatic unifocal mammary tumors arose only after an extended latency (11). Furthermore, elevated expression of both ErbB-2 protein and transcript was observed during tumor progression in the ErbB-2^{KI} strain. Remarkably, elevated ErbB-2 expression was correlated with selective genomic amplification of the *erbB-2* transgene (10). A detailed characterization of these mammary tumors further revealed a number of other chromosomal alterations such as centrosome abnormalities and recurrent deletions of chromosome 4 (12). High-resolution mapping of the *erbB-2* region revealed coamplification of 10 genes identical to those observed in HER2-amplified primary human breast cancer (13). Therefore, this unique transgenic mouse model recapitulates the human disease and indicates that ErbB-2 amplification is a critical event in HER2-positive breast carcinomas.

Another frequent alteration observed in human breast carcinomas is the loss of the tumor suppressor phosphatase and tensin homologue deleted on chromosome 10 (PTEN) through mutation, loss of heterozygosity (LOH), and epigenetic down-modulation, and has been reported in nearly 50% of human cancers including breast cancers (14). PTEN is a key tumor suppressor and a lipid phosphatase (15,16) that normally opposes the proto-oncogenic PI3K/Akt pathway by removing a phosphate group from phosphatidylinositol 3,4,5-trisphosphate to generate phosphatidylinositol-4,5-bisphosphate (17,18). The PTEN-controlled PI3K/Akt signaling pathway regulates key biological events related to normal development, including cell proliferation and survival (15,19), cell migration (20,21), cellular senescence (22), and stem cell self-renewal (23–25). Recently, it has been suggested that, in addition to antagonizing tumorigenesis, PTEN would improve the efficacy of Herceptin, a humanized monoclonal antibody against ErbB-2 (26). The increased sensitivity to Herceptin treatment conferred by PTEN, the association between ErbB-2 and ErbB-3 overexpression, and the involvement of PTEN and ErbB-3 in PI3K regulation together suggest a potential cross talk between ErbB-2 and PTEN during mammary tumor formation.

To directly explore the role of PTEN in ErbB-2-induced mammary tumorigenesis, we interbred the Cre-inducible ErbB-2^{KI} strain with mice harboring conditional PTEN alleles. Remarkably, mammary-specific deletion of PTEN alleles resulted in a dramatic acceleration of ErbB-2-induced mammary tumor progression and increased occurrence of lung metastases. Moreover, although elevated ErbB-2 protein levels were observed in the mammary tumors, *erbB-2* DNA amplification was not present and *erbB-2* transcript levels were similar to those observed in nonamplified ErbB-2^{KI} mammary tumors (10). These observations suggest that loss of PTEN function in the mammary gland can lead to elevated ErbB-2 protein levels through a mechanism independent of *erbB-2* amplification. Interestingly, detailed histologic analyses revealed that unlike the typical ErbB-2-induced comedo-adenocarcinoma, the PTEN-deficient mammary tumors acquire some pathologic characteristics of the basal-like subtype of human breast cancer

(27). These findings have important implications in understanding the molecular determinants in breast cancer progression and the pathologic heterogeneity observed in breast cancers, as well as in improving the evaluation of clinical outcomes.

Materials and Methods

Animal husbandry and genotyping

Generation of ErbB-2^{KI} and MMTV-Cre transgenic mice has previously been described (10). Flox-PTEN mice were purchased from The Jackson Laboratory. ErbB-2^{KI} (FVB/N), MMTV-Cre mice (FVB/N) and Flox-PTEN mice (129/J) were interbred to generate two bigenic transgenic mouse models, PTEN^{+/-}/ErbB-2^{KI} and PTEN^{-/-}/ErbB-2^{KI}, as well as PTEN^{+/-} and PTEN^{-/-} control mouse strains. Genotyping of conditional PTEN-knockout and ErbB-2^{KI} transgenic mice was determined by PCR as previously described (10,28). Nulliparous female mice were monitored weekly for tumor formation by physical palpation. All procedures involving mice were conducted in accordance with McGill University Animal Care guidelines.

Histologic analysis

Mammary tumors and lung tissue were harvested from mice that were tumor-bearing for 4 to 8 wk. Tissue was fixed and paraffin embedded as previously described (5). Paraffin sections of 5 µm were stained with H&E (Histology Services, McGill University). Lung metastases were identified by microscopic analysis of lung step sections. Immunohistochemical staining was done as previously described (29). Sections were incubated first with one of the following primary antibodies: Neu (Homemade, 1:400), PTEN (Cell Signaling; 1:100), CK6 (Covance; 1:800), CK8 (Fitzgerald; 1:1,000), smooth muscle actin (SMA; Sigma; 1:1,000), EGFR (Cell Signaling; 1:50), Ki67 (Abcam; 1:1,000), and CK5 (AF 138-Covance, 1:200), followed by incubation with the Elite antimouse, antirabbit, or anti-guinea pig IgG Vectastain kit (Vector Laboratories) according to the manufacturer's instructions.

Immunoprecipitation and immunoblotting

Tissue samples were prepared as previously described (4). Neu immunoprecipitations were done with 500 µg of cell lysate using the Neu Ab4 mouse monoclonal antibody (Oncogene Research Products, Inc.). Immunoblot analyses were done on 20 µg of total cell lysate as described (4,30) using the following antibodies: Neu (Santa Cruz 1:1,000), ErbB-3 (Santa Cruz; 1:1,000), EGFR (Cell Signaling; 1:1,000), PTEN (Cell Signaling; 1:1,000), Akt (Cell Signaling; 1:1,000), p-Akt (Cell Signaling; 1:1,000), mitogen-activated protein kinase (MAPK; Cell Signaling; 1:1,000), p-MAPK (Cell Signaling; 1:1,000), and actin (Sigma; 1:10,000). Horseradish peroxidase-conjugated secondary antibodies (1:10,000) were obtained from The Jackson Laboratory.

RNA extraction and real-time reverse transcription-PCR

Total RNA was isolated from individual flash frozen mammary tumor samples derived from PTEN^{+/-}/ErbB-2^{KI}, PTEN^{-/-}/ErbB-2^{KI}, PTEN^{+/-}, and ErbB-2^{KI} mouse strains using the RNEasy Midi Kit (Qiagen). RNA quantity and quality were determined with a spectrophotometer (Nanodrop) and BioAnalyzer 2100 and RNA 6000 Pico Assay Kit (Agilent Technologies). RNAs were analyzed by reverse transcription-PCR (RT-PCR) using the LightCycler and SYBR Green I RNA Amplification Kit (Roche). RT-PCR reactions were done in triplicate and transcript levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer sequences were as follows: PTEN, sense 5'-CCCAGTCAGAGGCGCTATGTATAT-3' and antisense 5'-GTTCCGCCACTGAACATTGG-3'; activated ErbB-2, sense 5'-GTGCTAGACAACCGAGATCCTCAGG-3' and antisense 5'-CCCTTCAG-

GATCTCTGTGAGACTTCG-3'; ErbB-3, sense 5'-GGAGCTTGTCTGGAT-TCTG-3' and antisense 5'-GGGAGTAAGCAGGCTGTGTC-3'; GAPDH, sense 5'-GCAAAGTGGAGATTGTTGCC-3' and antisense 5'-ATTCTCGGCCTTG-ACTGTGC-3'.

DNA extraction and Southern blot

DNA was obtained from tumors and examined for amplification of the *erbB-2* transgene and LOH at the PTEN locus as previously described (10). To determine PTEN LOH status, DNA was digested by *SacI* and the probe was generated by PCR on mouse genomic DNA with the following primers: sense 5'-CGTAGCCACAGG-GACTCCTA-3' and antisense 5'-ATTCGTGACGGTGTCAATCA-3'.

Gene expression data acquisition

Data analysis was carried out in R⁴ with Bioconductor (31). Gene expression data for 119 genes were obtained for mouse models and human breast cancers from Herschkowitz et al., from seven PTEN^{+/-}/ErbB-2^{KI}-derived mammary tumors, and from mammary glands of two 10-mo-old virgin FVB females (Supplementary Table S1). These data were analyzed using the human/mouse cross-species intrinsic gene list developed by Herschkowitz et al. The mean log-expression values of the normal mammary gland tissue was independently subtracted from the tumor samples within each data set, followed by transformation of each sample to $\sim N(0, 1)$ over all possible overlapping genes. Samples were combined and genes were median centered across data sets. Unsupervised hierarchical clustering was done using Pearson correlation as a distance measure and with Ward's minimum variance agglomeration method. Centroids were calculated as the mean expression of each gene across all samples of a particular human breast cancer subtype.

Results

Heterozygous or homozygous deletion of PTEN accelerates ErbB-2-induced mammary tumorigenesis

To determine the influence of a homozygous or heterozygous loss of PTEN on ErbB-2-induced mammary tumorigenesis, we interbred a transgenic mouse model expressing an activated rat ErbB-2 allele under the control of its endogenous promoter specifically in mammary epithelial cells (ErbB-2^{KI}) with a mouse model harboring a homozygous or heterozygous conditional PTEN allele (PTEN^{+/-} or PTEN^{-/-}). To direct the excision of the PTEN alleles to the mammary epithelium, we also introduced an MMTV/Cre transgene (10). The advantage of this genetic approach is that inactivation of PTEN by Cre recombinase is coupled to the simultaneous Cre-mediated activation of ErbB-2. Thus, every mammary epithelial cell expressing the activated *erbB-2* allele will have one or both deleted PTEN alleles. Using this approach, several cohorts of female mice have been generated with various genotypes: PTEN^{-/-}/ErbB-2^{KI}, PTEN^{+/-}/ErbB-2^{KI}, PTEN^{+/-}, and PTEN^{-/-}. The onset of mammary tumor development was monitored by weekly physical palpation. As shown in Fig. 1A, the tumor onset is dramatically accelerated in PTEN^{-/-}/ErbB-2^{KI} and PTEN^{+/-}/ErbB-2^{KI} virgin females as compared with the PTEN^{+/-} or ErbB-2^{KI} (11) parental strains, with tumor latencies of 2.4 and 6.5 compared with 15.5 and 15.3 months, respectively. Note that no mammary tumors were generated from the PTEN^{-/-} mouse model over a period of 16 months (Fig. 1A). Sixty-eight percent ($n = 25$) of the PTEN-heterozygous or PTEN-homozygous deficient/ErbB-2^{KI} mouse cohorts developed unifocal mammary tumors whereas the rest of them developed tumors in two mammary glands. In contrast with either of the parental strains (conditional PTEN, ErbB-2^{KI}), the tumors occurred in every transgenic female generated (Table 1). Collectively, these data argue that deletion of

⁴<http://www.R-project.org>

one or both PTEN alleles can dramatically accelerate mammary tumor progression in the ErbB-2^{KI} mouse model. In addition to the dramatic acceleration of tumor onset observed in PTEN-deficient/ErbB-2^{KI} mice, the incidence of lung metastases is also significantly increased in PTEN^{+/-}/ErbB-2^{KI} animals (35%) in comparison with ErbB-2^{KI} parental strain (5%; Table 1).

Histologic examination of PTEN-deficient/ErbB-2^{KI}-derived mammary tumors revealed an even distribution of four recognizable phenotypes: as might be expected, ~25% of the tumors were adenomyoepitheliomas (Fig. 1B-c), consistent with PTEN knockout. Another 25% of the tumors could be identified as consistent with the ErbB-2 type neoplasm (Fig. 1B-a), with more glandular differentiation than expected in this group (10, 11). About 25% of the tumors exhibited squamous metaplasia and retention of myoepithelium (Fig. 1B-d), which are characteristics of the Wnt pathway of neoplasms (32). Most intriguing was the last group of tumors that tended to be glandular and be composed of a relatively distinctive large cell population (Fig. 1B-b). These neoplasms seem to be the “signature” phenotype for the PTEN-deficient/ErbB-2^{KI} mammary tumors and comprise a phenotype that has not been previously described.

Taken together, these observations suggest that loss of PTEN has a dramatic effect on ErbB-2-induced tumorigenesis, including accelerated tumor progression, heterogeneous tumor morphology, and a higher incidence of lung metastasis for the heterozygous deletion of PTEN.

Tumor progression is associated with LOH at the PTEN locus in 50% of PTEN^{+/-}/ErbB-2^{KI}-derived mammary tumors

One interesting observation in this study is that ErbB-2^{KI} animals lacking only one copy of PTEN exhibit a dramatic acceleration of tumor induction. Because LOH at the PTEN locus is a common event in numerous types of human cancers (16,33), we assessed whether the mammary tumors induced in this genotype lost the remaining wild-type PTEN allele. To accomplish this, we performed Southern blot analyses using a probe that distinguishes between the conditional and wild-type alleles (Fig. 2A-a). Inspection of 16 PTEN^{+/-}/ErbB-2^{KI} tumors revealed that 56% displayed loss of the PTEN wild-type allele. Consistent with the LOH data, examination of PTEN protein levels by either immunoblot or immunohistochemical analysis on a larger set of tumors ($n = 16$) revealed low expression levels of PTEN in ~50% of PTEN^{+/-}/ErbB-2^{KI} mammary tumors in comparison with the parental ErbB-2^{KI} tumors (Fig. 2A-b; Table 1; Supplementary Fig. S1). Taken together, these observations establish that tumor induction in the PTEN^{+/-}/ErbB-2^{KI} mouse model can occur either through haploid PTEN insufficiency or LOH of the remaining PTEN allele.

Elevated ErbB-2 expression in PTEN-deficient tumors occurs in the absence of ErbB-2 amplification

Our previous studies had shown that tumor progression in the ErbB-2^{KI} mouse model was associated with selective amplification of the transgene (10,11,13). Like the ErbB-2^{KI} parental strain-derived tumors, the PTEN-deficient/ErbB-2^{KI} mammary tumors expressed high ErbB-2 protein levels that were constitutively tyrosine phosphorylated (Fig. 2B-a, lanes 1 and 3-5). To assess whether elevated ErbB-2 expression in PTEN-deficient tumors was associated with *erbB-2* amplification, we determined the extent of *erbB-2* amplification in PTEN^{+/-}/ErbB-2^{KI} and PTEN^{-/-}/ErbB-2^{KI}-derived mammary tumors. The analysis identified that the activated *erbB-2* transgene was present at the single copy level associated with the wild-type ErbB-2 allele (Fig. 2B-b, lanes 1-5 compared with lane 8). Given the apparent absence of gene amplification in PTEN-deficient tumors, we next assessed whether the elevated amount of ErbB-2 protein was due to increased ErbB-2 transcript expression. Using real-time RT-PCR, we showed that the increase in ErbB-2 protein was not associated with high levels of ErbB-2

transcript (Fig. 2B–c), in sharp contrast to amplified ErbB-2^{KI} tumors. These observations suggest that the elevated ErbB-2 protein levels observed in the PTEN-deficient/ErbB-2^{KI} tumors may reflect other molecular mechanisms such as increased ErbB-2 stability or translation.

PTEN-deficient/ErbB-2^{KI} mammary tumors display characteristics of the basal-like subtype of human breast cancer

Another striking feature of the PTEN-deficient/ErbB-2^{KI}-derived mammary tumors is their unique pathology (Fig. 1B). To elucidate their nature and cellular origin, we evaluated whether the tumors obtained from the various PTEN-deficient backgrounds were composed of luminal or myoepithelial cells. We performed immunohistochemistry with either basal/myoepithelial markers such as SMA and cytokeratin 6 (CK6; ref. 34) or luminal markers such as CK8 (35). Unlike the parental ErbB-2^{KI}-derived tumors expressing primarily CK8 (Supplementary Fig. S2A) with few scattered CK6-positive cells (Fig. 3A–a), PTEN-deficient tumors, which also abundantly express CK8 (Supplementary Fig. S2), display an enrichment in relatively large clusters of CK6-positive cells (Fig. 3A, b–d). The anti-CK6 antibody intensely stained a subpopulation of large abnormal cells in relatively normal ducts and acini (Supplementary Fig. S3, IIC). The cells in the early mammary intraepithelial neoplasm also had intense staining for CK6 (Supplementary Fig. S3). However, at the end stage, palpable tumors had less staining for CK6 but had the scattered clusters of CK6-positive cells (Fig. 3A). Consistent with these findings, we observed a corresponding increase in the SMA-positive cell population in the PTEN-deficient/ErbB-2^{KI} compared with ErbB-2^{KI} mammary tumors (Fig. 3B, compare b–d with a). These data suggest that the pathologic heterogeneity observed in the PTEN-deficient/ErbB-2^{KI} mammary tumors may reflect a contribution from multiple cell types.

The remarkable heterogeneous pathology of the PTEN-deficient/ErbB-2^{KI} tumors is reminiscent of a similar heterogeneity observed in primary human breast carcinomas with poor prognosis that belong to the basal-like subtype according to the classification of Sorlie et al. Basal-like breast cancers are also defined by the expression of cytokeratin markers of basal cell differentiation in normal mammary gland epithelia such as CK5, CK14, and CK17 (36, 37). To further explore this observation, we examined the PTEN-deficient/ErbB-2^{KI} mammary tumors by immunohistochemistry for expression of numerous markers of this subtype of human breast cancer [CK5, CK14, EGFR, estrogen receptor (ER)- α , and Ki67; refs. 38,39]. Consistent with our primary observation, these studies revealed that the PTEN-deficient/ErbB-2^{KI} tumors were positive for CK5 and CK14 (Fig. 4A;Supplementary Fig. S4B) and EGFR (Supplementary Fig. S4A) and exhibited a high Ki67 index (Supplementary Fig. S5), whereas they were negative for ER α (ESR1; data not shown). In addition, a set of 106 genes has recently been established and used by Herschkowitz et al. to identify the similarities and differences between human breast cancers and mouse mammary tumors. The gene set consists of the most conserved elements from each of the intrinsic lists developed independently for each species. When applied to combined human and mouse data, this set of genes classified the tumors into three primary groups, with the human samples separated into luminal, HER2, and basal-like subtypes (40). Interestingly, our PTEN^{+/-}/ErbB-2^{KI}-derived mammary tumors cluster primarily with the HER2 group and secondarily with the human basal-like subtype (Fig. 5A). Consistent with previously obtained immunohistochemical staining, we observed that the PTEN-deficient/ErbB-2^{KI} mouse model shares important similarities at the transcriptional level to the ER-negative human subtypes, including overexpression of ErbB-2 and Grb7 (HER2), Kit (basal), and several basal cytokeratins (basal; Fig. 5B). Interestingly, the PTEN^{+/-}/ErbB-2^{KI}-derived tumors are capable of displaying some luminal features such as overexpression of Xbp1 (Fig. 5A and B). To further clarify the association between our murine tumors and the human subtypes, we next quantified the level of cohesiveness between mouse and human tumors. The correlation between the centroids of each human subtype and individual PTEN^{+/-}/ErbB-2^{KI}-

derived mammary tumors revealed that approximately half of the PTEN-deficient/ErbB-2^{KI} tumors were most closely associated with the human basal-like subtype, whereas the other half displayed the highest correlation with the human HER2 subtype (Fig. 5C). These findings support our previous observations that PTEN^{+/-}/ErbB-2^{KI}-derived mammary tumors have characteristics of both human HER2 and basal-like breast cancers (Fig. 5A and B). When comparing the PTEN-deficient/ErbB-2^{KI} transcriptional profile to numerous transgenic mouse models including basal-like (Wnt1, Brca1-deficient, and p53 models) and luminal-like models (Neu, Myc, and PyMT models), similar results were obtained (Supplementary Figs. S7 and S8; ref. 40). PTEN-deficient/ErbB-2^{KI}-derived tumors clustered with several basal-like mouse models, yet shared some molecular features with both basal-like and luminal-like models. Collectively, these data support our histopathologic results, suggesting that loss of PTEN in ErbB-2-induced mammary tumorigenesis gives rise to tumors that acquire some human basal-like characteristics.

Discussion

Two events are consistently observed in some human breast cancers. First, the overexpression of ErbB-2 is found in 20% to 30% of human breast cancers and many other cancer types (2, 3,41). Second, the loss of the tumor suppressor PTEN through mutations or epigenetic down-modulation has been reported in 5% to 10% of human breast cancers as well (42). Here, we have characterized mouse models that express an activated ErbB-2 under the control of its endogenous promoter in the context of a heterozygous or homozygous conditional loss of PTEN in the mammary epithelium. We show that ErbB-2-induced mammary tumorigenesis is dramatically accelerated in the absence of PTEN. Indeed, the latency period is reduced to 2.4 and 6.5 months for homozygous and heterozygous loss of PTEN, respectively, as opposed to 15.5 and 15.3 months for the parental strains, PTEN^{+/-} and ErbB-2^{KI}, respectively. Moreover, unlike the parental mouse models, our PTEN-deficient/ErbB-2^{KI} transgenic strains developed mammary tumors with 100% penetrance (Table 1; ref. 11). It should be noted that ErbB-2 overexpression along with homozygous deletion of PTEN also led to the early development of lymphoma at a high frequency, thus preventing mammary tumor formation (data not shown). This phenomenon can be explained through the previously shown role of PTEN in B-cell and T-cell homeostasis (21,43) and the documented leakiness of the MMTV-long terminal repeat promoter (44) that drives Cre expression in these mouse models. The same phenomenon was observed in PTEN^{-/-} female mice, although to a lesser extent than in the PTEN^{+/-}/ErbB-2^{KI} strain (data not shown). Among the cohort of PTEN^{-/-} females that did not develop lymphomas, none grew mammary tumors in the 16-month time period. The marked difference between our findings and those of Li et al. (28) might be explained by the different genetic backgrounds of the cohorts of mice used in both studies as well as the source of the Cre-recombinase. However, the “escape” mechanism observed in our study would be in accordance with the recent observations of Chen et al. (22) showing that complete loss of PTEN leads to a p53-induced senescence in prostate cells. Together, these observations suggest that a similar mechanism might occur in the mammary gland and therefore may prevent mammary tumor formation in the absence of ectopic expression of activated ErbB-2.

Immunohistochemical and biochemical studies on the mammary tumors confirmed the overexpression of the ErbB-2^{KI} allele along with the loss of PTEN expression. The variation in the extent of PTEN loss between the tumors is most likely due to the extent of LOH at the PTEN locus, the efficiency of Cre recombination, and the proportion of stromal tissue in the tumors. The majority of the PTEN-deficient/ErbB-2^{KI}-derived mammary tumors ($n = 22$) exhibited high levels of expression and phosphorylation of ErbB-2 in comparison with PTEN^{+/-} mammary tumors, as expected (Fig. 2B-a). Strikingly, this increase in ErbB-2 protein was not due to amplification (Fig. 2B-b) as previously shown for the parental ErbB-2^{KI} strain (10). These results suggest that PTEN deletion in the mammary epithelium can lead to elevated

ErbB-2 protein levels through a mechanism that is independent of ErbB-2 amplification. However, because ErbB-2 transcript levels in these mammary tumors are similar to those in nonamplified ErbB-2^{KI} tumors, the phenomenon is possibly due to posttranscriptional regulation. In this regard, it has previously been reported that a small number of HER2-expressing human breast carcinomas do not show evidence of HER2 amplification (45).

Examination of known upstream and downstream targets of PTEN and ErbB-2, such as ErbB-3, EGFR, Akt, and mammalian target of rapamycin, revealed no significant differences in abundance and/or activation of these molecules between the PTEN-deficient/ErbB-2^{KI} mouse models and the parental strains (Supplementary Fig. S6). Activation of Akt by EGFR or ErbB-2 is known to be mediated through recruitment of the p85 subunit of PI3K, predominantly to phosphorylated residues on ErbB-3 (6, 7). Collectively, these data indicate that sustained activation of the PI3K/Akt signaling pathway is not the mechanism responsible for the accelerated mammary tumor formation in our PTEN-deficient/ErbB-2^{KI} mouse models.

Histologically, loss of PTEN in the mammary gland is associated with relatively benign tumors that are composed of irregular glands with an intervening stroma composed of smooth muscles (adenomyoepitheliomas). The earliest PTEN-deficient mammary tumors observed were small intra-alveolar tumors with identical cell types (46). The typical mammary neoplasm found in our PTEN^{+/-} mice represents the first evidence that the adenomyoepithelioma is a signature tumor in the PTEN-deficient mammary gland.

Interestingly, mammary tumors derived from our PTEN-deficient/ErbB-2^{KI} mouse models are remarkable for their histologic heterogeneity with the presence of four distinct phenotypes: adenomyoepitheliomas typical of PTEN-knockout mammary tumors, ErbB-2 type neoplasms, Wnt-type mammary tumors, and a unique, not previously described, PTEN-deficient/ErbB-2^{KI}-specific phenotype (Fig. 1B). Examination of mammary glands from PTEN-deficient/ErbB-2^{KI} mice without palpable tumors gave some insight into the origin of the unique phenotype of the mammary tumors observed in the PTEN-deficient/ErbB-2^{KI} mice. Indeed, a number of focal atypias that can be considered mammary intraepithelial neoplasms were present. The mammary intra-epithelial neoplasm exhibited unique microscopic patterns not previously observed in mouse mammary neoplasms. Most mammary intraepithelial neoplasm lesions had complex proliferations of epithelium and myoepithelium, some of which are papillary (Supplementary Fig. S3). These patterns could conceivably lead to the Wnt and myoepithelial patterns. Another pattern included peripheral myoepithelium with a solid nodular proliferation that has the ErbB-2 type organization. However, both of these patterns contain a very unique large cell subpopulation composed of sheets of cells with large, somewhat pleomorphic nuclei with an open chromatin pattern and abundant pale cytoplasm, which seems to be the characteristic and unique signature of the PTEN-deficient/ErbB-2^{KI} mammary tumors.

Transcriptional profiling of primary human breast tumors revealed distinct subtypes of breast carcinomas associated with different outcomes (27): luminal A, luminal B, HER2⁺/ER⁻, basal-like, and normal breast-like. The basal-like subtype is typically negative for ER (ESR1) and HER2, shows some characteristics of breast myoepithelial cells, and strongly correlates with poor prognosis. Interestingly, one of the typical features of the basal-like subtype of human breast cancers lies in the fact that they display a high histopathologic variability, suggesting the contribution of multiple cell lineages. Mammary tumors derived from PTEN-deficient/ErbB-2^{KI} mouse models, although predominantly composed of luminal epithelial cells as shown by CK8 immunostaining (Supplementary Fig. S2), were enriched with basal/myoepithelial (CK6⁺, SMA⁺) cells as compared with the ErbB-2^{KI} parental strain (Fig. 3). These findings suggest that these mammary tumors may derive from a bipotent cell population

and could represent a subgroup of HER2-positive human breast cancers sharing histopathologic features with the basal-like subtype.

Consistent with this hypothesis, we showed that the PTEN-deficient/ErbB-2^{KI} mammary tumors were ER α negative; CK5, CK14, and EGFR positive; and with a high Ki67 index. All these features have been defined as characteristics of the basal-like subtype of human breast cancer (refs. 39,47;Fig. 4;Supplementary Figs. S4 and S5). In support of our data, recent studies showed that loss of PTEN results in the formation of mouse mammary tumors and human breast cancers with basal-like characteristics (48,49). The comparison of the transcription of PTEN^{+/-}/ErbB-2^{KI}-derived tumors with primary human breast cancers showed that our mouse model displays features of both the basal-like and HER2 human breast cancer subtypes (Fig. 5). In addition, the PTEN-deficient/ErbB-2^{KI} system shares similarities not only histologically but also transcriptionally with various other mouse models of mammary tumors (Supplementary Figs. S7 and S8), such as the basal-like Wnt1, Brca1-deficient, and p53 mouse models (32,40).

Taken together, our results suggest that loss of PTEN would confer some basal-like characteristics to HER2-positive human breast carcinomas. Therefore, our PTEN^{+/-}/ErbB-2^{KI} strain could represent a new and unique model system to study the molecular mechanisms of a subclass of HER2-positive primary human breast cancers with basal-like features and could be a useful tool for the development of novel approaches to targeted treatments of this human disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. PTEN-deficient/ErbB-2^{K1} mammary tumors exhibit accelerated tumor onset and histologic heterogeneity. *A*, tumor kinetics. Percentages of mice that were tumor-free were plotted against the age when tumors were first palpable. *T*₅₀, age at which 50% of the females first developed a tumor. *n*, number of mice analyzed for each strain as indicated. *B*, four H&E-stained images illustrating the signature histopathology observed in the bigenic animals: *a*, ErbB-2 type tumor showing the solid nodular growth of intermediate cells surrounded by a peripheral palisade of cells; *b*, PTEN-type tumor showing a mixture of gland-like spaces and sheets of cells. The cells have larger nuclei with prominent nucleoli and more abundant cytoplasm that is either pink or red; *c*, adenomyoepithelial tumor with well-formed, widely spaced glands embedded in a

cellular matrix; *d*, adenosquamous carcinoma. The field illustrates a prominent keratin pearl with concentric lamellae of a cellular keratin surrounded by a poorly organized squamous epithelium.

Figure 2. Determination of PTEN and ErbB-2 status in the PTEN-deficient/ErbB-2^{KI} mouse models. *A, a*, PTEN LOH. Southern blot analysis of independent tumor DNAs from the indicated mouse strains, which were digested with *SacI* and probed with a ³²P-labeled PTEN probe. The two bands represent the wild-type and mutant alleles as indicated. *b*, total protein lysates from independent mammary tumors (20 µg) were subjected to Western blot analyses with a polyclonal anti-PTEN antibody and a monoclonal anti-β-actin antibody. *B, a*, ErbB-2 was immunoprecipitated from 1 mg of total tumor lysates. Half of the immunoprecipitate was subjected to two immunoblot analyses with a phosphotyrosine antibody (*P-Tyr*; *top*) and the other half with an ErbB-2-specific antibody (*middle*). Equivalent volumes of total lysates (20

μg) were subjected to immunoblot analysis with a monoclonal anti-β-actin antibody for loading controls (*bottom*). *b*, Southern blot analysis showing the presence or absence of activated *erbB-2* amplification (4.5 kb) with respect to the *erbB2* wild-type allele (7.5 kb). *c*, relative ErbB-2 mRNA levels were quantified by real-time RT-PCR with primers specific to activated ErbB-2 and normalized against GAPDH. **, $P < 0.001$, versus FVB and ErbB-2^{KI} (A). A, amplified transgene; NA, nonamplified; IP, immunoprecipitation; IB, immunoblotting.

Figure 3. PTEN-deficient/ErbB-2^{KI} mammary tumors are enriched in CK6 and SMA. A, immunohistochemical stains illustrating the distribution of CK6 in ErbB-2 type tumors (a), PTEN-type tumors (b), adenomyoepitheliomas (c), and squamous cell carcinoma (d). Note that the cells overlap with CK5 but do not stain the myoepithelial cells identified by anti-SMA and anti-CK5. B, immunohistochemical stains illustrating the distribution of SMA. a to d, same as in A. Note the marked differences in the staining for SMA and CK6.

Figure 4. PTEN^{+/-}/ErbbB-2^{KI} mammary tumors display histologic features of the basal-like subtype. PTEN-deficient/ErbbB-2^{KI} mammary tumors express the basal marker CK5. Immunohistochemical stains illustrating the distribution of CK5 in ErbbB-2 type tumors (A), PTEN-type tumors (B), adenomyoepitheliomas (C), and three squamous cell carcinomas (D). Note that the distribution of CK5-positive cells is very similar to SMA (Fig. 3B).

Figure 5. PTEN^{+/-}/Erbb-2^{KI} mammary tumors exhibit molecular characteristics of the human basal-like subtype. **A**, unsupervised cluster analysis of seven PTEN^{+/-}/Erbb-2^{KI} mammary tumors and 181 human breast cancers using 104 genes from the intersection of human and mouse intrinsic gene lists (40). The horizontal dendrogram represents the distance between the tumor samples. *Red*, human basal-like; *pink*, human HER2; *blue*, human luminal; *purple*, PTEN^{+/-}/Erbb-2^{KI}. **B**, expression of genes well defined as being expressed in luminal, basal-like, or HER2 samples. **C**, correlation between the individual PTEN^{+/-}/Erbb-2^{KI}-derived mammary tumors and the centroid of each human breast cancer subtype, using the cross-species intrinsic gene set. Approximately half of the PTEN-deficient/Erbb-2^{KI} tumors are closely correlated

with the human basal subtype, whereas the other half is more closely correlated with the human HER2 subtype. *, PTEN-deficient/ErbB-2^{KI} mouse model.

Table 1

Phenotypic characteristics of the different PTEN-deficient mouse models

Strain	Average age of mammary tumor formation (mo) [*]	Penetrance of tumors (%) [†]	% Animals with lung lesions [‡]	% Mammary tumors with PTEN loss [§]
PTEN ^{-/-} /ErbB-2 ^{KI}	2.4	100	0 (0/6)	100 (6/6)
PTEN ^{+/-} /ErbB-2 ^{KI}	6.5	100	35 (6/17)	56 (9/16)
PTEN ^{-/-}	NA	0	NA	NA
PTEN ^{+/-}	15.5	75	18 (2/11)	17 (1/6)
ErbB-2 ^{KI} (11)	15.3	83	5.7 (2/35)	ND

Abbreviations: NA, not applicable; ND, not determined.

^{*} Values represent the time in months when 50% of the mice had palpable mammary tumors.

[†] Penetrance of mammary tumors in percent, determined in virgin female mice.

[‡] Percentage of virgin females possessing lung lesions that were tumor-bearing for 30 to 60 d; data in parentheses indicate the number of animals with lung lesions over the number of examined animals.

[§] Percentage of animals with mammary tumor showing a reduced amount of PTEN by Western blot analysis; data in parentheses indicate the number of tumors with reduced PTEN protein level over the number of analyzed tumors.