Mutually exclusive mutations of the *Pten* and *ras* pathways in skin tumor progression

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***Pten* heterozygous (*Pten*$^{+/-}$) mice develop increased papilloma numbers and show decreased carcinoma latency time in comparison with controls after skin treatment with dimethyl benzanthracene (DMBA) and tetradecanoyl-phorbol acetate (TPA). *H-ras* mutation is normally a hallmark of DMBA-TPA-induced skin tumors, but 70% of carcinomas from *Pten*$^{+/-}$ mice do not exhibit this mutation, and in all cases have lost the wild-type *Pten* allele. Tumors that retain the *Pten* wild-type allele also have *H-ras* mutations, indicating that activation of *H-ras* and complete loss of *Pten* are mutually exclusive events in skin carcinomas. Mitogen-activated protein kinase (MAPK) is consistently activated in the tumors with *H-ras* mutations, but is strongly down-regulated in *Pten*$^{-/-}$ tumors, suggesting that this pathway is dispensable for skin carcinoma formation. These data have important implications in designing individual therapeutic strategies for the treatment of cancer.**

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The *Pten* tumor suppressor gene encodes a dual protein and lipid phosphatase that antagonizes PI3K/Akt signaling, a pathway that triggers a cascade of responses important for tumor development (Vivanco and Sawyers 2002). Somatic alterations that negate *Pten* function are commonly detected in a number of human tumor types (Li et al. 1997; Steck et al. 1997). In addition, germ-line mutations in *Pten* have been described in heritable cancer risk syndromes, Cowden disease (CD) and Bannayan-Zonana syndrome (Liaw et al. 1997; Marsh et al. 1997). Homozygous deletion of *Pten* results in embryonic lethality (Di Cristofano et al. 1998; Stambolic et al. 1998; Podsypanina et al. 1999), indicating that it is a developmentally essential gene. Mice that are heterozygous for *Pten* are viable, but they show abnormal pathological features and are susceptible to developing tumors in a number of tissues (Di Cristofano et al. 1998; Podsypanina et al. 1999). The skin of these mice shows focally increased epidermal thickness due to hyperkeratosis (Di Cristofano et al. 1998), a feature commonly observed with CD patients. The association of squamous cell carcinomas with CD has also been reported (Camisa et al. 1984; Hildenbrand et al. 2001), but these lesions were not observed in *Pten*$^{+/-}$ animals. On the other hand, 100% of mice with complete *Pten* deficiency, and a proportion with *Pten* heterozygosity, in their keratinocytes spontaneously develop papillomas and squamous cell carcinomas (Suzuki et al. 2003), indicating that *Pten* is an important suppressor of skin tumorigenesis.

We have used dimethyl benzanthracene (DMBA)-tetradecanoyl-phorbol acetate (TPA)-induced skin carcinogenesis to study the molecular mechanisms that underlie the development of this malignancy. A signature of DMBA treatment is an activating mutation in *H-ras*, which is the most common initiating event for tumor formation (Balmain et al. 1984; Quintanilla et al. 1986). We demonstrate here that most skin carcinomas from *Pten*$^{+/-}$ mice do not have this signature mutation, but instead show complete loss of *Pten* by deletion of the wild-type allele. These genetic changes are correlated with major differences in signaling through the mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways.

Results and Discussion

To investigate the role of *Pten* in skin carcinogenesis, we treated an equal number of *Pten*$^{+/-}$ mice and wild-type littermates with DMBA and TPA. We used germ-line *Pten*$^{+/-}$ mice (Di Cristofano et al. 1998), as opposed to tissue-specific knockout (KO) models (Suzuki et al. 2003) to better recapitulate the condition of CD. The basal level of Pten protein is clearly reduced in the skin of these heterozygous animals (Fig. 1A), and this is relevant given that *Pten* expression is rapidly up-regulated, both at the RNA and protein levels, in response to TPA treatment (Fig. 1B). The increase in levels of a tumor suppressor protein such as Pten is intriguing in view of the fact that TPA treatment strongly induces proliferation in the epidermis. A similar situation is, however, seen with other negative growth regulators such as TGFβ (Akhurst et al. 1988), which are also induced in response to TPA treatment, possibly as part of a mechanism that ensures a regulated return to homeostatic equilibrium within the epidermis. In light of these observations, we predicted *Pten*$^{+/-}$ mice to be more susceptible to DMBA-TPA-induced skin carcinogenesis, although the risk of spontaneously developing skin cancer was not noticeably altered (Di Cristofano et al. 1998; Podsypanina et al. 1999). Both *Pten*$^{+/-}$ and wild-type mice developed papillomas after 7–8 wk of DMBA-TPA treatment. However, the papilloma number was significantly higher in *Pten*$^{+/-}$ animals in comparison with wild type in both males and females (Fig. 1C). By 20 wk posttreatment, the mean number of papillomas per mouse was more than threefold higher in *Pten*$^{+/-}$ mice ($p < 0.0001$), indicating that loss of one *Pten* allele renders animals more susceptible to DMBA-TPA-induced skin carcinogenesis.

Papillomas are generally benign, but a small number eventually progresses to the carcinoma stage. Mice were monitored for carcinoma development for up to 60 wk post-DMBA treatment. We observed carcinomas on *Pten*$^{+/-}$ mice as early as 20 wk post-DMBA treatment (Fig. 1D). By 44 wk posttreatment, carcinomas were found on all *Pten*$^{+/-}$ animals, whereas >70% of the wild-type littermates remained carcinoma free. The decrease

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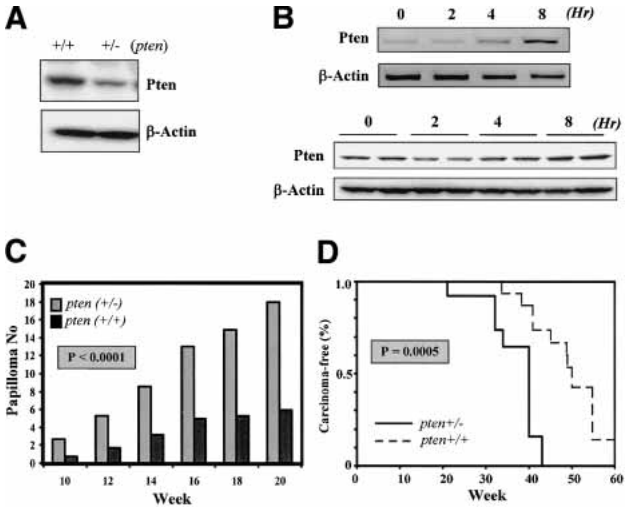

Figure 1. Pten heterozygosity increases skin cancer susceptibility. (*A*) *Pten* protein expression in the skin of wild-type and *Pten*$^{+/-}$ mice. (*B*) Expression of *Pten* is induced in wild-type mice at RNA (*top*) and protein (*bottom*) levels at indicated time points after TPA treatment. Pten protein levels in skin of two different mice are shown at each time point. (*C*) Average papilloma number per mouse at the indicated time points after DMBA-TPA treatment. (*D*) Latency of carcinoma onset.

in carcinoma latency period in *Pten*$^{+/-}$ versus wild-type mice was highly significant ($p < 0.005$), indicating that an impartial loss of *Pten* function not only increased susceptibility to tumor initiation, but also accelerated tumor progression.

A two-hit mechanism to inactivate tumor suppressor genes has been well documented, and *Pten* appears to follow this paradigm in many tumor types. We examined the status of *Pten* in skin tumors using microsatellite markers at, and also proximal and distal to, the *Pten* genomic locus. In 15 papillomas and 12 carcinomas from wild-type mice, we did not detect any deletions at the *Pten* locus. In contrast, loss of heterozygosity (LOH) was observed in papillomas and a high number of carcinomas from *Pten*$^{+/-}$ mice (Fig. 2A). In many instances, LOH extends to markers as far as 20 cM from the *Pten* locus, and may reflect a loss of the entire chromosome 19. However, LOH at adjacent markers did not occur independently of LOH at the *Pten* locus, indicating that *Pten* is the primary target for LOH in these tumors.

Loss of expression due to regulatory mutations or epigenetic events is one mechanism of inactivating gene activity. However, RT–PCR analysis indicated that the *Pten* transcript is present in all papillomas and carcinomas from wild-type animals (Fig. 2B). Pten protein is also clearly detectable in these papillomas and carcinomas (Fig. 3A). We sequenced the entire coding region of *Pten* in these tumors and did not detect any mutation. In papillomas and carcinomas from *Pten*$^{+/-}$ animals, the presence or absence of the *Pten* wild-type transcript correlated with Pten protein status, and these were in agreement with the microsatellite results. In the majority of carcinoma samples, the wild-type *Pten* transcript is clearly absent (Fig. 2B). On the other hand, the transcript corresponding to the KO allele is retained in all papillomas and carcinomas, indicating that LOH selectively targets the chromosome that carries the wild-type *Pten* allele.

The incidence of *Pten* LOH in papillomas was 25% (5/20), which is significantly lower than the 66% (6/9) we observed for carcinomas ($p < 0.05$), indicating that a complete loss of *Pten* efficiently drives the conversion of benign papillomas to malignant carcinomas. The concept of subtypes of papillomas with high or low malignant potential has been previously described (Hennings et al. 1985), with those having the higher potential for malignant conversion originating from hair follicle stem cells (Perez-Losada and Balmain 2003). One interpretation of our data is that *Pten* may be important in regulating epidermal stem cell proliferation, similar to that described for neural stem cells (Groszer et al. 2001). In this respect, a complete loss of *Pten* could result in an increase in the number of hair follicle stem cells and thus the number of potential targets for malignant skin tumor development. In support of this idea, animals with keratinocyte-specific *Pten* deficiency do indeed have increased hair follicles and decreased interfollicular epidermis (Suzuki et al. 2003). Alternatively, complete loss of *Pten* may be able to drive progression regardless of the cell of origin.

A lack of *Pten* alterations in tumors from wild-type mice was a surprising finding because homozygous inactivation of *Pten* in keratinocytes can clearly cause skin cancer (Suzuki et al. 2003). On the other hand, our data

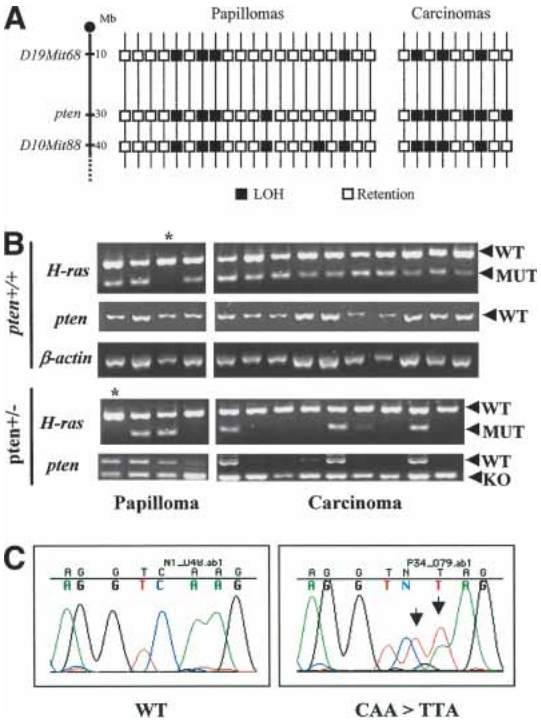

Figure 2. *H-ras* and *Pten* alterations are mutually exclusive. (*A*) LOH analysis of tumors from *Pten*$^{+/-}$ mice using the indicated microsatellite markers on chromosome 19, as obtained from the Celera database. Papilloma #16 (fifth from the *right*) shows loss of distal markers on chromosome 19, but is wild type at *Pten*, and has a mutation in *H-ras* (Table 1). (*B*) Papillomas and carcinomas from wild type (*top* panel) and *Pten*$^{+/-}$ (*bottom* panel) were analyzed for *H-ras* mutation by digestion of PCR products as described in text, and for *Pten* expression by RT–PCR. Asterisk indicates samples with TTA alteration, as opposed to the more common CTA, at codon 61 of *H-ras*, and hence was not digested by XbaI. (*C*) Chromatogram showing the CAA → TTA alteration at codon 61 of *H-ras* that occurred in a small number of papillomas.

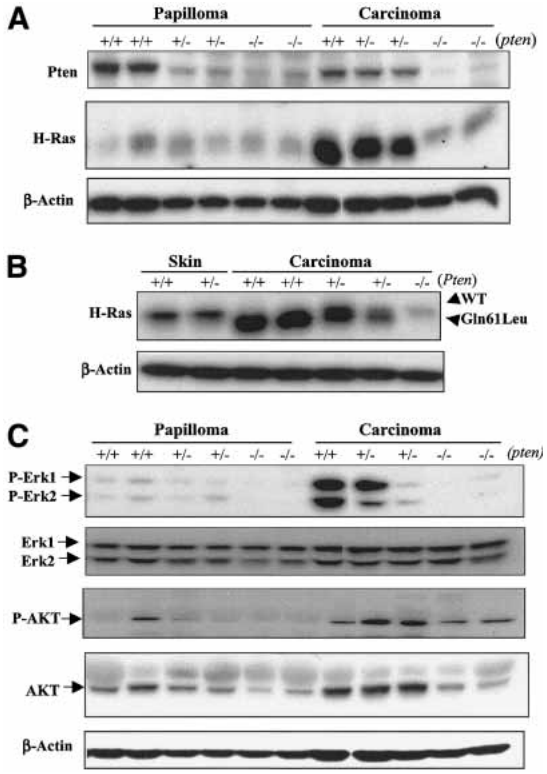

Figure 3. *H-ras* activation and *Pten* inactivation elicit differential biochemical responses. The status of pten for each tumor, as determined by microsatellite and RT–PCR analysis, is indicated. (*A*) Pten and H-Ras protein levels in papillomas and carcinomas. (*B*) Separation of wild-type and mutant (Gln61Leu) H-Ras protein in carcinomas by high-percentage SDS-PAGE. (*C*) Analysis of Erk and Akt activation in papillomas and carcinomas using antibodies to detect phosphorylated and total protein, as indicated.

are consistent with findings in human skin tumors, where a lack of somatic *Pten* alterations has also been reported (Kubo et al. 1999). It is possible that inactivation of both *Pten* alleles is required during skin carcinogenesis, a situation that is more favorable when the first event has already occurred through germ-line transmission, as is the case with CD and $Pten^{+/-}$ mice. In this respect, genetic alterations at other loci may substitute for *Pten* inactivation in tumors from wild-type mice and may even preclude the need for *Pten* LOH in some tumors from $Pten^{+/-}$ animals.

Activating mutations in *H-ras*, usually a CAA-to-CTA change at codon 61, occur at a high frequency in DMBA-initiated skin tumors (Balmain et al. 1984; Quintanilla et al. 1986). *H-Ras* has a stimulatory effect on PI3K/Akt signaling; thus its activation could occur in place of *Pten* inactivation. We analyzed skin tumor DNA for *H-ras*-activating mutations, taking advantage of the XbaI restriction site created by the CTA mutation (Nagase et al. 2003). Activating *H-ras* mutations were detected in all 10 carcinomas from wild-type animals (Fig. 2B). In two papillomas where the mutant product was not detected, DNA sequencing revealed a TTA alteration at codon 61 in place of the more common CTA (Fig. 2C). Interestingly, both CTA and TTA alterations resulted in the same Gln61Leu amino acid change, pointing to importance and selectivity of this residue in activated H-Ras protein. Taken together, our data showed that the codon 61-activating *H-ras* mutation occurs in every DMBA-TPA-induced papilloma and carcinoma from wild-type mice.

In contrast, the majority of skin carcinomas derived from $Pten^{+/-}$ mice did not carry this *H-ras* mutation, and all of the tumors in this category had undergone *Pten* LOH. We did not detect any mutations in *H-ras* despite sequencing the entire coding region. Because mutations in *K-ras* have been found in skin tumors from DMBA-TPA treated *H-ras* KO mice (Ise et al. 2000), we also sequenced the entire coding region of *K-ras* in these carcinomas and did not detect any mutation. There is therefore a highly significant correlation between the absence of *ras* mutations in carcinomas and the loss of the wild-type *Pten* allele. Table 1 summarizes results of *Pten* and *H-ras* status in papillomas and carcinomas from wild-type and $Pten^{+/-}$ mice. It can be seen that although the majority of the benign papillomas in $Pten^{+/-}$ mice still had activated *H-ras* in the usual manner, ~30% had lost the *Pten* allele. Of these, two had both activated *H-ras* and loss of *Pten*, in contrast to the situation seen in the carcinomas. This may indicate that papillomas carrying both mutations are less likely to progress to malignancy than those showing complete loss of *Pten*.

Table 1. *Summary of* H-ras *and* pten *alterations*

Pten(+/−)	*H-ras*	*Pten*	*Pten*(+/+)	*H-ras*	*Pten*
Papilloma			Papilloma		
1	MUT[a]	WT	1	MUT	WT
2	MUT	WT	2	MUT[a]	WT
3	MUT	WT	3	MUT	WT
4	MUT	WT	4	MUT	WT
5	MUT	LOSS	5	MUT	WT
6	MUT	WT	6	MUT	WT
7	WT	LOSS	7	MUT	WT
8	MUT	LOSS	8	MUT	WT
9	MUT	WT	9	MUT	WT
10	MUT	WT	10	MUT	WT
11	MUT	WT	11	MUT	WT
12	WT	LOSS	12	MUT	WT
13	MUT	WT	13	MUT	WT
14	MUT	WT	14	MUT[a]	WT
15	MUT	WT	15	MUT	WT
16	MUT	WT			
17	MUT	WT	Carcinoma		
18	WT	LOSS	1	MUT	WT
19	MUT	WT	2	MUT	WT
20	MUT	WT	3	MUT	WT
			4	MUT	WT
Carcinoma			5	MUT	WT
1	MUT	WT	6	MUT	WT
2	WT	LOSS	7	MUT	WT
3	WT	LOSS	8	MUT	WT
4	WT	LOSS	9	MUT	WT
5	MUT	WT	10	MUT	WT
6	WT	LOSS			
7	WT	LOSS			
8	MUT	WT			
9	WT	LOSS			

[a]Codon 61 mutation CAA → TTA.
(WT) Wild type; (MUT) mutant.

The strong up-regulation in H-ras protein level in carcinoma demonstrates the importance of this gene in papilloma-to-carcinoma conversion (Fig. 3A). The mechanism underlying this increase in expression appears to be at the level of gene copy number (Nagase et al. 2003). By using high-percentage polyacrylamide SDS gels to discriminate between mutant and wild-type H-Ras protein by mobility, we could show that it is the oncogenic form of H-Ras protein that is selectively up-regulated in carcinomas (Fig. 3B). In addition, the extent of up-regulation is stronger in tumors with intact *Pten* than in those that are *Pten* heterozygous. In carcinomas with complete *Pten* inactivation, not only is there a lack of *H-ras*-activating mutation, but there is also no up-regulation at the expression level. On the basis of these results, it appears that *Pten* inactivation confers similar advantages to activated *H-ras* and its subsequent overexpression, at least at the stage of carcinomas. This could account for the decreased carcinoma latency time in $Pten^{+/-}$ mice because LOH involving the wild-type *Pten* allele would be the single limiting event, whereas with *H-ras* there is a requirement for mutagenesis as well as allele-specific duplication or amplification (Nagase et al. 2003).

We analyzed the status of downstream targets of H-Ras and Pten in order to better understand the biochemical consequences of their alterations. Several lines of evidence suggest that the activation of Erk-1 and Erk-2 MAPK proteins, which are important components of the H-Ras network, can also be dependent on the status of *Pten* (Shan et al. 2000; Suzuki et al. 2001, 2003). Despite relatively similar levels of total Erk proteins, the phosphorylated or active forms of both Erk proteins (P-Erk) were only detectable in papillomas or carcinomas that do not have complete *Pten* inactivation (Fig. 3C) and are most likely a direct consequence of the oncogenic H-Ras protein present in these tumors (see earlier). Even in these cases, the level of P-Erk proteins was barely detectable in papillomas. The Erk proteins are more strongly activated in carcinomas, which are consistent with the up-regulated mutant H-Ras expression in these malignant tumors, but the extent of activation can be variable. The almost complete lack of P-Erk in papillomas and carcinomas with complete *Pten* inactivation may be a signature of these tumors, and suggests that Erk activation is dispensable in skin carcinogenesis or at least during papilloma-to-carcinoma conversion.

Central to the tumor-suppressing function of Pten is the inhibition of Akt signaling (Stambolic et al. 1998). In contrast, H-Ras activates PI3K activity, which in turn stimulates the Akt pathway (Cox and Der 2003). We observed an increasing trend in levels of phosphorylated Akt (P-Akt) from papillomas to carcinomas as previously reported (Fig. 3C; Segrelles et al. 2002), suggesting that Akt activation is important for carcinoma development and consistent with the role of Akt signaling in promoting cell proliferation and survival (Vivanco and Sawyers 2002). Interestingly, whereas many studies with cultured cells have shown regulation of Akt signaling in response to *Pten* alterations to be largely at the level of phosphorylation (Stambolic et al. 1998; Suzuki et al. 2003), we found that in papilloma and particularly carcinoma specimens the regulation appears to occur at the level of total Akt protein as well. Tumors with retention of the wild-type *Pten* allele and carrying *H-ras* mutations in general had higher levels of total Akt protein. It is possible that these higher levels may be a consequence of the activity of Ras/MAP in inducing cell death (Deng et al. 2004), causing a feedback response leading to up-regulation of Akt-mediated survival signals. A dynamic balance between these opposing pathways may explain why no tumors carrying both genetic alterations have progressed to malignancy, whereas those with only *Pten* loss, and no MAPK activity, have high malignant potential. These results may be relevant to prognosis of human cancer carrying both kinds of genetic changes, which may be expected to have a more indolent growth pattern.

A lack of concurrent *Pten* alteration and *N-ras* or *K-ras* mutation has been reported for melanoma cell lines (Tsao et al. 2000) and endometrial cancer (Ikeda et al. 2000), respectively. However, the relationship between these mutational events is still unclear, and some melanomas or uncultured cells have been reported to have both mutations in *B-raf* and loss of *Pten* (Tsao et al. 2004). In this study, we have excluded the possibility that the common sites of activation of *K-ras* or *B-raf* (Davies et al. 2002) are mutated in the tumors that show complete loss of *Pten* (data not shown). We conclude that *Pten* inactivation and *H-ras* activation are mutually exclusive in malignant skin carcinomas, suggesting that these events are genetically redundant, and that alteration in both genes does not confer a further advantage. It is also possible that *H-ras* activation and *Pten* loss is in fact disadvantageous when these events occur together in the same cell. We detected two papillomas with both *H-ras* mutation and *Pten* inactivation, but no carcinomas with both changes. Although these numbers are small, papillomas with both genetic alterations are possibly at a relative disadvantage for tumor progression to carcinomas, possibly due to reported effects of activated *ras* in induction of apoptosis (Chang et al. 2003) or growth arrest (Serrano et al. 1997).

These observations of discrete pathways leading to malignancy in this model system have important implications for the design of therapeutic strategies for the treatment of skin cancer as well as other cancers with frequent *Pten* and/or *ras* alterations. Tumors with complete loss of *Pten* and low levels of signaling through the Ras/Raf/Erk pathway will obviously not respond to small molecule drugs that inhibit these targets. Such treatments may even be detrimental, because they may alter the balance between cell death and survival pathways in cancers carrying both mutations.

Materials and methods

Mice and chemical carcinogenesis

$Pten^{+/-}$ mice (C57BL/6), provided by Dr. P.P. Pandolfi (Memorial Sloan-Kettering Cancer Center, New York; Di Cristofano et al. 1998), were crossed with FVB/N to generate F1 wild-type and $Pten^{+/-}$ mice. To induce skin carcinogenesis, we used a single dose of DMBA treatment followed by twice-weekly application of TPA for 20 wk as described (Balmain et al. 1984; Quintanilla et al. 1986). Papilloma number was recorded up to 20 wk and carcinoma development monitored up to 60 wk post-DMBA treatment. Mice were killed if moribund, if the tumor load was excessive, if any individual tumor exceeded 1.5 cm in diameter, or at the termination of the experiment. Surgically removed tumors were immediately snap-frozen in liquid nitrogen, and stored at –70°C. We scored tumors as carcinomas or papillomas on the basis of their morphological appearance and histological features.

DNA/RNA and protein preparation

Tumors were ground into a fine powder in liquid nitrogen and aliquotted for DNA and RNA extraction and protein lysate preparation. DNA was isolated using standard phenol/chloroform extraction following an over-

night 55°C incubation with Proteinase K. Total RNA was purified using TRIzol Reagent (Invitrogen). For total protein lysates, powdered tumors were incubated in ice-cold lysis buffer (50 mM Tris-Cl at pH 7.4, 2 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% Triton X-100, 0.1% SDS) supplemented with Complete Mini Protease Inhibitor Cocktail (Roche). Soluble protein lysates were quantified using BCA Protein Assay kit (Pierce).

LOH analysis

Tumor and corresponding normal DNA was used for LOH studies with microsatellite markers at the *Pten* locus and flanking regions chosen from the Celera database. PCR amplification was performed in 20-µL volume containing 1× PCR buffer (Bioline), 200 µM of each dNTP (Pharmacia), 6.6 µM of each primer (Qiagen Operon), 1.5 mM $MgCl_2$, 2 units of Taq polymerase (Bioline), and 80 ng of DNA. Amplifications were initially denatured for 3 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 55°C or 52°C, and 30 sec at 72°C. PCR products were electrophoresed in 4% (3% NuSieve/1% normal) agarose gel and visualized by ethidium bromide staining.

Pten expression analysis

RT–PCR was performed using Thermoscript RT–PCR (GIBCO-BRL). Mutation analysis of the *Pten* coding sequence was performed by direct sequencing of gel-purified PCR products of tumor cDNA. Primer pairs used in the analysis were Pten1F (5′-CATCTCTCTCCTCCTTTTTCT TCA-3′) and Pten1R (5′-CAAACATCATCTTGTGAAACAGC-3′), Pten2F (5′-GGCAAATTTTTAAAGGCACAAG-3′) and Pten2R (5′-AAGTTGA ACTGCTAGCCTCTGG-3′), and Pten3F (5′-ACTTTGAGTTCCCTCA GCCA-3′) and Pten3R (5′-TTCATGGTATTTTATCCCTCTTGA-3′).

Immunoblotting

Equal amounts of proteins were resolved by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane using standard protocol. We used 18% polyacrylamide gel in order to distinguish between wild-type and codon 61 H-Ras mutant protein. Rabbit polyclonal antibody to phospho-Ser 473–PKB/AKT was a gift from Dr. David Stokoe (University of California at San Francisco). We purchased Pten (A2B1), H-Ras (C-20), and ERK1 (C-16) antibodies from Santa Cruz Biotechnology, and P-Erk (#9101) and Akt (#9272) antibodies from Cell Signaling Technology.

Statistical analysis

SPSS statistical package was used for all statistical analysis. We used the nonparametric test to compare papilloma number and the Kaplan-Meire method to compare latency in progression to carcinoma.

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