Inhibition of phosphatidylinositol 3-kinase delays tumor progression and blocks metastatic spread in a mouse model of thyroid cancer

Fumihiko Furuya, Changxue Lu, Mark C.Willingham and Sheue-yann Cheng

Laboratory of Molecular Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA and Department of Pathology, Wake Forest University, Winston-Salem, NC 27109, USA

To whom correspondence should be addressed. Tel: +1 301 496 4280; Fax: +1 301 402 1344; Email: chengs@mail.nih.gov

Aberrant activation of the phosphatidylinositol 3-kinase (PI3K)–AKT/protein kinase B-signaling pathway has been associated with multiple human cancers, including thyroid cancer. Recently, we showed that, similar to human thyroid cancer, the PI3K–AKT pathway is overactivated in both the thyroid and metastatic lesions of a mouse model of follicular thyroid carcinoma (TRβPV/PV mice). This TRβPV/PV mouse harbors a knockout mutant thyroid hormone receptor β gene (TRβPV mutant) that spontaneously develops thyroid cancer and distant metastasis similar to human follicular thyroid cancer. That the activation of the PI3K–AKT signaling contributes to thyroid carcinogenesis raised the possibility that this pathway could be a potential therapeutic target in follicular thyroid carcinoma. The present study tested this possibility by treating TRβPV/PV mice with LY294002 (LY), a potent and specific PI3K inhibitor, and evaluating the effect of LY on the spontaneous development of thyroid cancer. LY treatment inhibited the AKT–mammalian target of rapamycin (mTOR)–p70S6K signaling, and it decreased cyclin D1 and increased p27Kip1 expression to inhibit thyroid tumor growth and reduce tumor cell proliferation. LY treatment increased caspase 3 and decreased phosphorylated-BAD to induce apoptosis. In addition, LY treatment reduced the AKT–matrix metalloproteinase 2 signaling to decrease cell motility to block metastatic spread of thyroid tumors. Thus, these altered signaling pathways converged effectively to prolong survival of TRβPV/PV mice treated with LY. No significant adverse effects were observed for wild-type mice treated similarly with LY. The present study provides the first preclinical evidence for the in vivo efficacy for LY in the treatment of follicular thyroid cancer.

Introduction

The incidence of thyroid cancer is increasing by 5–6% per year in the USA (1). The differentiated thyroid cancers, papillary and follicular thyroid carcinomas, constitute ~90% of thyroid cancers (2–4). The majority of patients with differentiated thyroid cancer have a good prognosis; however, recurrence could develop in 20–40% of patients, and with occurrence of distance metastases and extensive local invasion, the prognosis is significantly poorer. Patients with distant metastases at the time of diagnosis, especially with follicular thyroid carcinoma, have a 5-year survival rate of ~50% (3,5). The molecular mechanisms underlying the initiation and progression of thyroid cancer are not fully understood, but it is generally believed that deregulation of cell growth and cell death is involved.

The creation of a mouse model of follicular thyroid cancer (TRβPV/PV mice) has provided a valuable tool to elucidate the molecular basis underlying thyroid tumor progression and metastatic spread.

Abbreviations: AKT, v-akt murine thymoma viral oncogene homolog; Bad, Bcl-associated death promoter; FoxO, Forkhead; GSK-3β, glycogen synthase kinase-3β; LY, LY294002; MMP2, matrix metalloproteinase 2; mTOR, mammalian target of rapamycin; p-AKT, phosphorylated AKT; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog; SDS, sodium dodecyl sulfate.

Materials and methods

Experimental animals

All animal experiments were performed according to the protocols approved by the Animal Care and Use Committee at the National Cancer Institute. The

Published by Oxford University Press 2007.

2451

Carcinogenesis vol.28 no.12 pp.2451–2458, 2007
doi:10.1093/carcin/bgm174
Advance Access publication July 28, 2007
mice harboring the TRPV gene were prepared and genotyped as described previously (8). LY, a PI3K-specific inhibitor, was a generous gift from Dr. J. Starling, Eli Lilly and Company (Indianapolis, IN). The treatment protocol including the effective dosages was determined from those reported in previous publications (23–25). LY (25 mg/kg/day) or vehicle (dimethyl sulfoxide) was injected intra-peritoneally into TRPV mice (n = 24) and wild-type mice (n = 23) twice per week beginning at the age of 2 months for >10 months. Moribund mutant mice and controlled wild-type littermates were euthanized to harvest thyroids for weighing, histological analysis and the biochemical studies shown below.

Primary cell culture
Primary thyroid cell lines were grown at 37°C, 5% CO2 atmosphere in primary cell media as described previously (21). Cells were then used for either western blot studies or cell cycle analysis.

Western blot analysis
Thyroids dissected from TRPV mice and wild-type siblings were washed with phosphate-buffered saline (PBS) and the thyroid extracts were prepared in a manner similar to what has been described previously (21). Extracts (50 μg) were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and the western blot analysis was carried out as described by Furumoto et al. (26). Primary antibodies for phosphorylated-S473 AKT (#9271), total AKT (#9272), phosphorylated-T205/Y202 GSK-3β (#9313), total GSK-3β (#9315), phosphorylated-p70S6K (#4873), total p70S6K (#4874), phosphorylated-glycogen synthase kinase-3β (GSK-3β) (#9331), and phospho-Akt S473 (#9272) antibody were purchased from Cell Signaling Technology, Santa Cruz, CA. Anti-Bim antibody (OPA-01201) was purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Anti-Bim antibody (OPA-01021) was purchased from Affinity BioReagents, Golden, CO. The total AKT and phospho-S473 AKT antibodies were purchased at a 1:1 dilution. The others were used at a 1:500 dilution. For control of protein loading, the blots were stripped and re-reacted with the antibodies against α-tubulin (T6199; Sigma, St. Louis, MO).

Histological analysis
Thyroid gland, lung, heart and lymph nodes were dissected and embedded in paraffin. Five micrometer thick sections were prepared and stained with hematoxylin and eosin. For each animal, single random sections through the thyroid (usually both lobes), through the lung and through the heart were examined. For thyroids, morphological evidence in that single section of hyperplasia, capsular invasion, vascular invasion and anaplasia were routinely counted. Hyperplasia was generally diffuse throughout the gland. Evidence of any of these changes in any section was counted as positive for that change. On average, in those cases with capsular invasion and/or vascular invasion, these morphological changes were seen in multiple locations (usually two or three) in any one single thyroid section. The presence of a single microscopic focus of metastatic follicular carcinoma in the lung was counted as positive for metastasis in that animal.

Immunohistochemistry
Ki-67 staining was performed as previously described (27). Cleaved caspase 3 was detected via immunohistochemistry in paraffin sections by standard methods. Briefly, sections were processed by antigen retrieval using Anti-Ki-67 or cleaved caspase 3 using peroxidase and were then reacted with diaminobenzidine–peroxide substrate in PBS, and the sections were then counterstained with hematoxylin. The morphometric quantitation of immunoperoxidase labeling of Ki-67 and cleaved caspase 3 was determined by capturing bright-field digital images from sections of thyroids labeled with Ki-67 or cleaved caspase 3 using peroxidase. Using histogram functions of Adobe Photoshop 7.0, a defined threshold was chosen and the 'magic wand' tool was used to select all similar areas with the same color characteristic of peroxidase reaction product. Five determinations were made, each in a total area of 1.55 × 10^6 μm² of section. The data are expressed in square microns occupied by labeled structures.

Zymography of MMP2 activity
Gelatin zymography was performed to determine gelatinolytic activities of MMP2. Thyroid extracts (40 μg) were treated with sampling buffer (0.5 M Tris–HCl, 10% glycerol, 10% SDS and 0.1% bromphenol blue) in a final solution of 20 μl. SDS–polyacrylamide gel electrophoresis was performed using 10% polyacrylamide gel containing 0.1% gelatin (Invitrogen, Carlsbad, CA, EC6175) at 125 V for 90 min. SDS was removed with re-naturing buffer (Invitrogen, LC2670) for 30 min, and the gel was incubated in a developing buffer (Invitrogen, LC2671) overnight at 37°C. Gels were stained for 1 h with 0.5% Coomassie G250 and destained for 60 min in 7% acetic acid and 35% methanol. The gelatinolytic activities were detected as a clear band against a dark background.

Flow cytometric analysis of thyroid tissue
Preparation of cells from frozen thyroid tissue for cell cycle analysis was adapted from a protocol described previously (27,28). Briefly, thyroids were dissected from mice and frozen in liquid nitrogen. About 30 mg of thyroid tissue was thawed on ice. Afterward, the thyroid sample was placed in a 40 μm cell strainer on the top of a 50 ml tube (BD Falcon, Bedford, MA) and gently pushed against the strainer in about 0.5 ml of PBS, and the strainer was rinsed with ~4 ml of additional PBS. Cell numbers in the suspension were determined by counting the cells in a cell counter (Beckman Coulter, Fullerton, CA). The cell suspension was centrifuged at ~400g and re-suspended in PBS such that the cell concentration was 1 × 10^7–2 × 10^7 cell per ml in PBS. Approximately 1 × 10^6 cells were aliquoted into a 12 × 75 mm Falcon tube (BD Falcon) and incubated in an ice bath for 15 min. Cells were fixed with 70% ethanol and kept at 4°C overnight. The next day, cells were centrifuged and the ethanol was decanted. The samples were incubated for 20 min at room temperature with 100 U DNAase activated RNase (Sigma). Propidium iodide was added at a concentration of 40 μg/ml (Invitrogen) and incubated at 4°C for 30 min. Cell cycle analysis was performed using a FACSCalibur system (BD). Data from 20,000 single-cell events per sample were collected. Cell cycle histograms were analyzed using the ModFit LT program (Verity Software House, Topsham, ME). All samples had a low coefficient variation of G1/G0 peak (coefficient variation between 5 and 6%).

Statistical analysis
All data are expressed as means ± standard deviations. Statistical analysis was performed with the use of analysis of variance, and P < 0.05 was considered significant unless otherwise specified. StatView 5.0 was used to perform Kaplan–Meier cumulative survival analysis, and Student’s t-test using odds ratios and Fisher’s exact probability test were used to analyze the data of pathological progression. GraphPad PRISM 4.0a (GraphPad Software, San Diego, CA) was used for log-rank testing for statistical significance.

Results
LY blocks metastatic spread of thyroid tumors in TRPV mice
We have previously shown that PI3K is markedly activated in thyroid tumors of TRPV mice, leading to the increased p-AKT. The over-activated p-AKT results in increased cell motility that could contribute to the invasive potential of tumor cells (20). These findings suggested that PI3K is a potential molecular target for prevention of metastasis. We therefore tested this hypothesis by treating TRPV mice with LY, a potent and selective PI3K inhibitor (20), to determine the effect on pathological progression by inhibiting the PI3K signaling. TRPV mice spontaneously develop follicular thyroid carcinoma as they age (6,12). Therefore, TRPV mice were treated with LY or vehicle only (dimethyl sulfoxide; controls), beginning at the age of 2 months, until they became moribund with dehydration and laboratory breathing. They were then euthanized. Figure 1 compares Kaplan–Meier cumulative survival curves for TRPV mice treated with and without LY. The 50% survival age for TRPV mice treated with LY or vehicle was 329 ± 64.5 days (n = 24) or 244 ± 63.4 days (n = 23), respectively, indicating that the LY-treated mice survived significantly longer. No death was observed for wild-type mice similarly treated with LY during the same observation period (n = 10; data not shown). These results indicate that LY was effective in prolonging the survival of TRPV mice.

The above findings predicted that inhibition of the PI3K-signaling pathway by LY could delay the progression of follicular thyroid carcinoma. We therefore first analyzed the effect of LY on thyroid growth. Indeed, LY treatment led to a significant decrease (2-fold reduction) in thyroid weight of TRPV mice as compared with mice treated only with vehicle (controls; compare bar 2 with bar 1, Figure 1B). TRPV mice exhibit growth retardation as compared with wild-type mice (8). LY treatment had no significant effect on weight gain...
of TRβPV/PV mice (data not shown). Interestingly, although LY treatment significantly reduced weight gain in wild-type mice by ~20% (data not shown), the thyroid growth was not affected by LY treatment as indicated by no significant changes in the thyroid weight of wild-type mice (compare bar 1 with bar 2, Figure 1C). These findings indicate that LY selectively inhibited the thyroid growth of TRβPV/PV mice, but not that of wild-type mice.

We next evaluated the tumor progression by histopathological evaluation of primary lesions in the thyroid and metastatic spread in the lung of age-matched TRβPV/PV mice with or without LY treatment. Representative examples are shown in Figure 2. Whereas the untreated mice exhibited advanced hyperplasia (panel a, Figure 2A), the treated mice showed only early hyperplasia (panel b, Figure 2A). While vascular invasion was evident in untreated mice (arrow in panel c, Figure 2), vascular invasion was rare with frequent appearance of apoptotic bodies in the treated mice (arrows in panel d, Figure 2A). Panel e shows the occurrence of lung metastasis of untreated mice, but no metastasis was observed in the lung of treated mice (panel f, Figure 2A). These histopathological findings are summarized in Figure 2B. There was a significant reduction (~90%) in the percentage occurrence of vascular invasion in LY-treated mice as compared with untreated mice (P < 0.001). Remarkably, while 20% of untreated mice manifested distant metastasis to the lung, no metastasis occurred in the treated mice (Figure 2B). Thus, treatment of LY clearly delayed the thyroid tumor progression and blocked metastatic spread to the lung.

**LY delays tumor progression by inhibiting tumor growth and reducing cell proliferation**

Signaling through the PI3K–AKT–mTOR pathway leads to an increase in translation, particularly of proteins regulating cell cycle progression and metabolism. To understand how LY treatment delayed thyroid tumor progression, we evaluated whether the AKT–mTOR–p70S6K pathway involved in cell growth regulation was affected. Previously, we have shown that, during thyroid carcinogenesis, this PI3K downstream pathway is activated (21). As shown in lanes 3 and 4 in panels a, c and e, there were consistently increases in p-AKT, p-mTOR and p-p70S6K in thyroid tumors of TRβPV/PV mice as compared with those effectors in wild-type mice (Figure 3). Remarkably, treatment of TRβPV/PV mice led to a significant deactivation of the AKT–mTOR–p70S6K pathway evidenced by the reduced p-AKT, p-mTOR and p-p70S6K shown in lanes 5 and 6 in panels a, c and e, respectively (Figure 3). The total cellular levels of total AKT, total
mTOR and total p70S6K shown in lanes 5 and 6 in panels b, d and f, respectively (Figure 3), were not significantly altered, indicating that the deactivation of these effectors was mediated by the PI3K downstream phosphorylation cascade. Panel g of Figure 3 shows the loading controls. These results indicate that blocking this pathway accounted, in part, for the reduced thyroid growth mediated by the inhibition of PI3K with LY.

We further examined whether LY treatment could inhibit cell proliferation by analyzing cell cycle progression. Figure 4A shows that the G0/G1 to S phase progression was significantly delayed in thyroid tumors treated with LY as compared with thyroid tumors of untreated TRβPV/PV mice (Figure 4A-a and -b). The cyclin-dependent kinase inhibitor p27kip1 was concomitantly activated in thyroid tumors of LY-treated TRβPV/PV mice (Figure 4B-c, compare lanes 3 and 4 with lanes 1 and 2) to further block the progression of cells from the G0/G1 to the S phase (29). To understand how the inhibition of PI3K by LY led to the reduced cyclin D1, we analyzed the protein levels of the phosphorylated-GSK-3β, a downstream effector of the PI3K–AKT pathway that is reported to regulate the stability of cyclin D1 (30). Phosphorylation of GSK-3β by AKT inhibits the kinase activity of GSK-3β to phosphorylate cyclin D1, thereby preventing the degradation of cyclin D1 by the proteasome machinery (30). In LY-treated TRβPV/PV mice, consistent with reduced p-AKT (Figure 3a), a decreased phosphorylated-GSK-3β was apparent (compare lanes 3 and 4 with lanes 1 and 2, Figure 4B-d). No apparent changes in total GSK-3β were observed (Figure 4B-e).

mTOR and total p70S6K shown in lanes 5 and 6 in panels b, d and f, respectively (Figure 3), were not significantly altered, indicating that the deactivation of these effectors was mediated by the PI3K downstream phosphorylation cascade. Panel g of Figure 3 shows the loading controls. These results indicate that blocking this pathway accounted, in part, for the reduced thyroid growth mediated by the inhibition of PI3K with LY.

We further examined whether LY treatment could inhibit cell proliferation by analyzing cell cycle progression. Figure 4A shows that the G0/G1 to S phase progression was significantly delayed in thyroid tumors treated with LY as compared with thyroid tumors of untreated TRβPV/PV mice (Figure 4A-a and -b). The cyclin-dependent kinase inhibitor p27kip1 was concomitantly activated in thyroid tumors of LY-treated TRβPV/PV mice (Figure 4B-c, compare lanes 3 and 4 with lanes 1 and 2) to further block the progression of cells from the G0/G1 to the S phase (29). To understand how the inhibition of PI3K by LY led to the reduced cyclin D1, we analyzed the protein levels of the phosphorylated-GSK-3β, a downstream effector of the PI3K–AKT pathway that is reported to regulate the stability of cyclin D1 (30). Phosphorylation of GSK-3β by AKT inhibits the kinase activity of GSK-3β to phosphorylate cyclin D1, thereby preventing the degradation of cyclin D1 by the proteasome machinery (30). In LY-treated TRβPV/PV mice, consistent with reduced p-AKT (Figure 3a), a decreased phosphorylated-GSK-3β was apparent (compare lanes 3 and 4 with lanes 1 and 2, Figure 4B-d). No apparent changes in total GSK-3β were observed (Figure 4B-e).
Inhibition of PI3K delays thyroid carcinogenesis

Fig. 3. Expression of key regulators of the PI3K–AKT–mTOR–p70S6K pathway in the thyroid of wild-type and TRβPV/PV mice, treated with or without LY. Thyroid extract (50 μg) of mice at the age of 7.5 months was used for determination of the expressions of p-(S473)AKT (a), total AKT (b), p-mTOR (c), total mTOR (d), p-p70S6K (e), total p70S6K (f) and α-Tubulin (g) as described in Materials and methods. Total mice used were four and 10 for wild-type and TRβPV/PV mice, respectively. But only the representative results are shown.

Taken together, these results indicate that inhibition of the PI3K activity by LY decreased tumor cell proliferation by reducing cyclin D1 via decreased phosphorylated-GSK-3β.

These biochemical findings were further supported by immunohistochemical staining of tumor cells with the proliferation marker Ki-67. The arrows in Figure 4C-a show a significant nuclear staining of Ki-67 of untreated thyroid, whereas in the treated thyroid of TRβPV/PV mice (Figure 4C-b), the Ki-67 staining was less apparent. The staining intensities of Ki-67 were quantified to indicate the values for controls were 2.07 ± 0.42 units (area in square microns) and the LY-treated thyroid cells were 0.19 ± 0.07 units (area in square microns; n = 5, each in an area of 1.55 × 10^4 μm^2 of section), representing a significant 88% reduction in the Ki-67 staining of LY-treated thyroid cells (P < 0.05). These results further indicate that treatment of TRβPV/PV mice with LY resulted in decreased cell proliferation.

LY delays tumor progression by inducing apoptosis

The decreased thyroid weight could be due to increased apoptosis, in addition to the inhibition of thyrocyte proliferation (see Figure 1B). We therefore first used immunohistochemical analysis to determine whether cleaved caspase 3 protein abundance was increased in thyroid tumors of TRβPV/PV mice treated with LY. As shown by the arrows in Figure 5A-b, cleaved caspase 3 protein abundance was significantly increased. We further quantified the staining intensities of cleaved caspase 3. For the control, the values were 0.08 ± 0.04 units (area in square microns) and 0.55 ± 0.12 units (area in square microns; n = 5, each in an area of 1.55 × 10^4 μm^2 of section). These data represented a 6.9-fold increase in the protein abundance of cleaved caspase 3 in the LY-treated cells as compared with the controls (P < 0.01), indicating an increased apoptotic activity in the thyroid tumors of TRβPV/PV mice treated with LY as compared with untreated mice (compare Figure 5A-b with Figure 5A-a).

We further analyzed the expression profiles of apoptosis key regulators that are downstream of PI3K–AKT. The pro-apoptotic activity of BAD is blocked by AKT phosphorylation (31). Therefore, the inhibition of phosphorylation of BAD by AKT would lead to increased apoptosis and reduced survival. Indeed, Figure 5B-a shows that the phosphorylated-BAD protein level was lower in tumor cells of TRβPV/PV mice treated with LY than in cells of untreated mice (two mice for each treatment; compare lanes 3 and 4 with lanes 1 and 2). The total BAD protein abundance was not significantly altered between LY-treated and untreated TRβPV/PV mice (Figure 5B-b), indicating that the effect of LY treatment was mediated by AKT phosphorylation. Another PI3K–AKT downstream target involved in regulating apoptosis is the Forkhead (FoxO) transcription factor, FoxO3a. When phosphorylated by AKT, p-FoxO3a associates with 14-3-3 proteins and translocates to the cytoplasm. Conversely, when dephosphorylated, FoxO3a undergoes nuclear translocation to activate its target genes involved pro-apoptotic genes such as Bim in cancer cells (32,33), thereby inducing apoptosis. Figure 5B-c shows that in LY-treated TRβPV/PV mice virtually no p-FoxO3a was observed (compare lanes 3 and 4 with lanes 1 and 2, Figure 5B-c) while no significant changes in total FoxO3a were found between LY-treated and untreated TRβPV/PV mice (Figure 5B-d). In response to the reduction of p-FoxO3a, Bim, a pro-apoptotic regulator (34) whose expression is positively regulated by FoxO3a (35), was increased in the thyroid of TRβPV/PV mice (Figure 5B-e, compare lanes 3 and 4 with lanes 1 and 2) to activate apoptotic activity. Thus, collectively, these results indicate that the inhibition of PI3K by LY led to an induction of apoptosis that contributed to decreased tumor growth and delayed carcinogenesis.

LY blocks tumor invasion and metastasis

To understand how LY treatment inhibited invasion and metastasis of tumor cells, we examined the PI3K–AKT downstream effect involved in cell motility and invasion of TRβPV/PV mice. Previously, we found that activation of the PI3K–AKT signaling in the thyroids of PV/PV mice leads to increased expression of MMP2 (21) and cell motility (19). Elevated levels of matrix MMP2 have been found in many tumors and are known to play an important role in cellular invasion and metastasis (36). We therefore evaluated whether this pathway could be deactivated by LY treatment, thereby reducing cell motility and invasion. Indeed, Figure 6A shows that MMP2 protein abundance was significantly reduced (two mice for each condition; compare lanes 3 and 4 with lanes 1 and 2). Consistently, its proteolytic activity was reduced as shown by zymogen assays (Figure 6B), indicating that the pro-MMP2 as well as the active MMP2 was significantly reduced in a concentration-dependent manner (compare lanes 3 and 4 with 1 and 2; lanes 7 and 8 with lanes 5 and 6; Figure 6B). These results suggest that inhibition of PI3K–AKT pathway by LY could result in the blocking of cell motility/invasion via inhibiting the MMP2 activity.

Discussion

Since the first report of LY as a specific inhibitor of PI3K (22), interest in using this inhibitor as a potential anticancer agent has been intense as evidenced by increasing numbers of publications in this area. Most studies, however, have centered on using cultured cell systems to demonstrate the anti-proliferative effects of LY. Several recent studies have used tumor cell xenograft models to test its efficacy, either alone or in combination with other modalities, in treating ovarian cancer (23,37), bladder cancer (24) and glioma (25). The present study evaluated the efficacy of LY in vivo in a mouse model that spontaneously develops thyroid cancer. LY effectively delayed tumor progression, blocked the metastatic spread and prolonged survival. In the wild-type mice, except for some weight loss (about ~20%) and scaly skin in ~80% of treated mice, no apparent severe symptoms were observed.
and no deaths occurred during the treatment. These results indicate that the dosages effective in treating thyroid cancer did not cause severe adverse effects in a relatively long-term treatment.

Extensive biochemical analysis indicates that the beneficial effects of LY in delaying thyroid carcinogenesis of TRβPV/PV mice were a result of alterations in multiple pathways downstream of the PI3K–AKT signaling. The AKT–mTOR–p70S6K pathway that regulates cell growth was attenuated and the activity of key regulators in G0/G1 phase was altered to inhibit cell cycle progression to decrease cell proliferation. Moreover, the apoptotic pathway was activated to decrease cell survival. The expression and the activity of MMP2 were reduced to decrease cell invasion. These findings indicate that these diverse pathways acted in concert to delay tumor progression, to block the metastatic spread and to prolong survival.

However, inhibition of the PI3K–AKT signaling by LY did not prevent the development of thyroid cancer in TRβPV/PV mice. While the development of tumors was significantly delayed in TRβPV/PV mice treated with LY (Figure 1), all treated mice eventually developed thyroid cancer. This observation suggests that the activation of the PI3K–AKT signaling most likely is not the triggering event leading to thyroid carcinogenesis, but instead its activation promotes progression and metastatic spread. This notion is consistent with the findings in a recent study in which the PTEN gene was specifically deleted in mouse thyrocytes via Cre-mediated recombination (38). The PTEN tumor suppressor opposes PI3K activity by dephosphorylation of phosphatidylinositol-3,4,5-triphosphate to phosphatidylinositol-4,5-bisphosphate (39). Its deletion leads to constitutive activation of the PI3K–AKT signaling. In spite of normal circulating levels of thyroid hormones and thyroid stimulating hormone, the PTEN mutant mice develop follicular adenomas, further supporting our findings that activation of the PI3K signaling contributes to thyrocyte proliferation. However, it is important to note that no neoplastic transformation of follicular adenomas was observed up to 10 months of age (38), suggesting that the constitutive activation of PI3K alone via the loss of PTEN is not sufficient to cause thyroid carcinoma. Taken together, these findings suggest that the activated PI3K–AKT signaling collab-
arates with the other genetic changes to promote thyroid tumor progression.

![Fig. 4.](https://academic.oup.com/carcin/article-abstract/28/12/2451/2476249)
Recent studies of human thyroid cancer specimens by several groups show AKT over-expression and overactivation in primary thyroid cancers (17,18). In addition, analysis and sequencing of the genomic DNA from primary thyroid tumors indicate that amplification of the \textit{PIK3CA} gene and consequent AKT activation are relatively common in follicular thyroid cancer (40). These observations suggest that the PI3K–AKT pathway could be an effective therapeutic target in thyroid cancer. The \textit{Tr}^{PV/PV} mice presented an opportunity to test this possibility \textit{in vivo}, and, indeed, the blocking of this pathway by LY delayed the tumor progression and importantly prevented the metastatic spread that is the leading cause of death in thyroid cancer. While the current treatment of \textit{Tr}^{PV/PV} mice by LY did not lead to complete remission, the present study has provided a strong rationale to seek a second generation of PI3K inhibitors that could be more effective than LY to treat thyroid cancer. Alternatively, LY treatment in combination with other specific inhibitors of downstream effectors such as mTOR could provide additional therapeutic effectiveness in treating thyroid cancer. Sirolimus (rapamycin) and its derivatives are known to block mTOR and yield potential anti-proliferative activity in a variety of malignancies (41). The availability of \textit{Tr}^{PV/PV} mice as a preclinical mouse model will allow these possibilities to be tested in due course.

**Funding**

Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research.

**Acknowledgements**

We thank Dr J.Starling, Eli Lilly and Company, for the generous supply of LY used in the study.

**Conflict of Interest Statement:** None declared.

**References**
