SAHA-induced loss of tumor suppressor Pten gene promotes thyroid carcinogenesis in a mouse model

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Abstract

Thyroid cancer is on the rise. Novel approaches are needed to improve the outcome of patients with recurrent and advanced metastatic thyroid cancers. FDA approval of suberoylanilide hydroxamic acid (SAHA; vorinostat), an inhibitor of histone deacetylase, for the treatment of hematological malignancies led to the clinical trials of vorinostat for advanced thyroid cancer. However, patients were resistant to vorinostat treatment. To understand the molecular basis of resistance, we tested the efficacy of SAHA in two mouse models of metastatic follicular thyroid cancer: ThrpbPV/PV and ThrpbPV/PVPten+/− mice. In both, thyroid cancer is driven by overactivation of PI3K-AKT signaling. However, the latter exhibit more aggressive cancer progression due to haplodeficiency of the tumor suppressor, the Pten gene. SAHA had no effects on thyroid cancer progression in ThrpbPV/PV mice, indicative of resistance to SAHA. Unexpectedly, thyroid cancer progressed in SAHA-treated ThrpbPV/PVPten+/− mice with accelerated occurrence of vascular invasion, anaplastic foci, and lung metastasis. Molecular analyses showed further activated PI3K-AKT in thyroid tumors of SAHA-treated ThrpbPV/PVPten+/− mice, resulting in the activated effectors, p-Rb, CDK6, p21 Cip1, p-cSrc, ezrin, and matrix metalloproteinases, to increase proliferation and invasion of tumor cells. Single-molecule DNA analysis indicated that the wild-type allele of the Pten gene was progressively lost, whereas carcinogenesis progressed in SAHA-treated ThrpbPV/PVPten+/− mice. Thus, this study has uncovered a novel mechanism by which SAHA-induced loss of the tumor suppressor Pten gene to promote thyroid cancer progression. Effectors downstream of the Pten loss-induced signaling may be potential targets to overcome resistance of thyroid cancer to SAHA.

Introduction

Thyroid cancer is the most common malignancy of the endocrine organs. It consists of an array of several different histological and biological types; however, the majority of clinically important human thyroid cancers are the differentiated papillary and follicular types. Papillary thyroid cancer commonly metastasizes to lymph nodes, whereas follicular thyroid cancer shows blood-borne metastases. Although the overall survival of patients with
these types of tumor is generally better than many other cancers, approximately 30% of patients do not survive beyond 20 years, even with successful primary surgical therapy. The treatment of recurrent and metastatic thyroid carcinoma is still a major challenge. Radioactive iodine therapy is currently the treatment of choice for patients whose thyroid cancer has spread to distant sites. However, an ominous histological sign in these tumors is the appearance of anaplastic foci (areas of spindle cell or embryonic dedifferentiation), and once these anaplastic foci have developed, the prognosis is considerably worse.

One consequence of dedifferentiation of tumor cells is the loss of the ability to uptake radioiodine, thus severely limiting treatment options (Reiners et al. 2011). Intensive efforts have been made to search for effective ways to re-differentiate the dedifferentiated tumor cells to regain iodine uptake ability to improve patients' outcome.

One approach that has offered great promise is via modulation of epigenetic events by inhibition of histone deacetylation. Histone acetylation by histone acetyl transferases results in open chromatin structures to facilitate transcription activation. The reverse process of histone deacetylation by histone deacetylases (HDACs) leads to compact chromatin structures, resulting in transcription repression (Grunstein 1997, Khan & La Thangue 2012, Marks et al. 2001). Because aberrant HDAC activity is implicated in a variety of cancers, HDAC inhibitors have been developed as potential anticancer therapies. Suberoylanilide hydroxamic acid (SAHA; vorinostat) is one of the HDAC inhibitors approved for the treatment of cutaneous manifestations of cutaneous T-cell lymphoma (Mann et al. 2007, Olsen et al. 2007). Several preclinical studies have demonstrated the efficacy of SAHA treatment to induce cell cycle arrest and apoptosis in thyroid cancer cell lines (Mitsiades et al. 2005, Luong et al. 2006, Borbone et al. 2010). SAHA also restores thyroid-specific gene expression such as sodium/iodide symporter as well as radioiodine uptake (Hou et al. 2010). These promising preclinical findings led to clinical trials of SAHA and another inhibitor in patients with radioactive iodine refractory thyroid carcinoma (Woyach et al. 2009, Sherman et al. 2013); however, the patients showed no major responses (Sherman et al. 2013) and the cancer of a few even progressed during SAHA treatment (Woyach et al. 2009). The outcome of these clinical trials, although very limited, suggested that in contrast to the positive findings from cell-based studies, patients are resistant to such treatment. At present, how patients’ thyroid cancer is resistant and even progresses further during SAHA treatment is not clear.

The availability of mouse models of follicular thyroid cancer (FTC) allowed us to dissect the molecular basis in the resistance of thyroid cancer to SAHA treatment. The Thrbβ+/PV+/ mice, harboring a dominant negative thyroid hormone receptor β (TRβPV), spontaneously develop FTC similar to human thyroid cancer with a pathological progression from hyperplasia to capsular invasion, vascular invasion, and eventually metastasis (Suzuki et al. 2002). Extensive molecular analyses of altered signaling pathways during thyroid carcinogenesis further confirmed that the ThrbPV+/PV− mice are a preclinical mouse model of FTC. As found in human FTC, ThrbPV+/PV− mice exhibit aberrant signaling pathways that include constitutive activation of phosphatidylinositol 3-kinase (PI3K)-AKT (Funuya et al. 2006, 2007) and integrin-cSrc-MAPK signaling (Beronoukhim et al. 2010) and aberrant accumulation of the oncogenic pituitary tumor transforming gene protein (Ying et al. 2006) and β-catenin (Guigon et al. 2008). Another mutant mouse model that spontaneously develops FTC is the ThrbPV+/PV− Pten−/− (Guigon et al. 2009). PTEN (phosphatase and tensin homologue deleted from chromosome 10) functions as a tumor suppressor by opposing the PI3K-AKT signaling pathway (Li et al. 1997). PTEN haplodeficiency further exacerbates the overactivated PI3K-AKT signaling, leading to a more aggressive cancer phenotype with decreased survival and increased distant metastasis (Guigon et al. 2009). The use of ThrbPV+/PV− and ThrbPV+/PV− Pten−/− mice allowed us to understand the effect of SAHA on thyroid cancer progression with different genetic changes. Using these two mouse models, we found that thyroid cancer progression in ThrbPV+/PV− mice was resistant to SAHA treatment. Unexpectedly, SAHA treatment significantly increased thyroid tumor growth of ThrbPV+/PV− Pten−/− mice. In addition, SAHA promoted carcinogenesis by increasing the occurrence of vascular invasion, anaplastic foci, and distant lung metastasis. Molecular analysis showed that PI3K-AKT signaling was further exacerbated in SAHA-treated ThrbPV+/PV− Pten−/− mice. Moreover, the extent of thyroid tumor growth was correlated with the progressive loss of the wild-type Pten allele in the ThrbPV+/PV− Pten−/− mice. Thus, this study uncovered that the loss of the Pten gene is one mechanism by which SAHA induced more aggressive thyroid cancer in ThrbPV+/PV− Pten−/− mice.

Materials and methods

Animals and treatment of SAHA

The National Cancer Institute Animal Care and Use Committee approved the protocols for animal care and...
handling in this study. Mice harboring the ThrβPV gene (ThrβPV+/− mice) were previously described (Kaneshige et al. 2000). Pten+/− mice were kindly provided by Dr. Ramon Parsons (Columbia University, New York, NY, USA). ThrβPV+/−Pten+/− mice were obtained by crossing Pten+/− mice with ThrβPV+/− mice, followed by crossing ThrβPV+/−Pten+/− with ThrβPV+/−Pten+/− mice. Vorinostat (SAHA) (Selleckchem, Cat #: S1047) was dissolved in DMSO to make a 10 mg/mL stock and administered by oral gavage daily at a dose of 50 mg/kg body weight/day starting at the age of 6 weeks for 8 weeks. The thyroids and lungs were dissected after mice were killed for weighing, histological analysis, and biochemical studies.

Western blot analysis

The Western blot analysis was carried out as described by Zhu et al. (2014). Primary antibodies for p-AKT (#9271), total-AKT (#9272), PTEN (#9552), CDK4 (#2906), CDK6 (#3136), p-RB (#9307), MMP7 (#3801), and GAPDH (#2118) were purchased from Cell Signaling Technology. The p21 primary antibody (sc-6246), Rb (sc-50), and MMP2 (sc-10736) were purchased from Santa Cruz Biotechnology. Primary antibodies against ERBB2 (RB-103-P0) was purchased from Neomarkers (Fremont, CA, USA). Antibodies were used at a concentration recommended by the manufacturers. For control of protein loading, the blot was probed with the antibody against GAPDH.

Histological analysis and immunohistochemistry

Thyroid glands, heart, and lung were dissected and embedded in paraffin. Sections of 5 µm thickness were prepared and stained with hematoxylin and eosin (H&E). For each mouse, single random sections through the thyroid, lung, and heart were examined. Immunohistochemistry was performed with paraffin sections by standard methods. Primary antibodies for p-AKT (S473) (#4060, 1:120 dilution) and PTEN (#9552, 1:120 dilution) were purchased from Cell Signaling Technology.

Hormone assays

The serum levels of total T4 (TT4) and T3 (TT3) were determined by using a Gamma Coat T4 and T3 assay RIA kit. TSH levels in the serum were measured as described in Zhao et al. (2012).

RNA extraction and real-time RT-PCR

Total RNA from thyroids was isolated using TRIzol (Invitrogen), as indicated by the protocol of the manufacturer. Real-time RT-PCR was performed using a QuantiTect SYBR green RT-PCR kit from Qiagen, following the instructions of the manufacturer (Qiagen). Primers were as follows: for mouse Pten, forward, 5′-TGGGGGAGTAAGGCCGAGAG-3′; reverse, 5′-TCACCTTTAGCTGGCAGACC-3′; for the endogenous control gene mouse glyceraldehyde-3-phosphate dehydrogenase (Gapdh), forward, 5′-cgctccgtagacaaatgtg-3′; reverse, 5′-gaatttgcctgtagttggt-3′.

Single-molecule DNA analysis by droplet digital PCR reaction

In single-molecule DNA analysis, by using the genomic DNA instead of mRNA as templates, the droplet digital PCR (ddPCR) enables us to quantify the loss of the Pten gene in mouse thyroid tumors. Genomic DNA from fresh-frozen thyroid tissue samples was extracted using the Qiagen blood and tissue kit according to the manufacturer’s protocol (Qiagen). DNA concentration was measured with the NanoDrop 2000c spectrophotometer (Thermo Scientific). For EvaGreen-based ddPCR, reaction mixtures contained 1X ddPCR EvaGreen Supermix (Bio-Rad), 100nM primers (Integrated DNA Technologies, Coralville, IA, USA), and mouse thyroid genomic DNA template in a final volume of 22 µL. Droplet digital PCR were performed in the CCR Genomics Core using QX200 autoDG droplet digital PCR system. Primers were as follows: for mouse Pten, forward, 5′-TGGGGGAGTAAGGCCGAGAG-3′; reverse, 5′-CGCACACAGGTCCACTGAT-3′; for the control mouse telomerase reverse transcriptase (Tert) gene, forward, 5′-TGCTTGATGGACACTAC-3′; reverse, 5′-TGCTTCACACTCTTACGG-3′. The numbers of positive droplets for the wild-type Pten gene and the control telomerase reverse transcriptase (Tert) gene were read with the QX200 droplet reader. The number of copies of the wild-type Pten gene per genome was calculated by dividing the number of the wild-type Pten copies by the number of the Tert gene copies. The number of copies of the wild-type Pten gene per diploid genome was calculated by multiplying the number of copies per genome by 2.

Statistical analysis

All data are expressed as mean ± s.e.m. Statistical analysis was performed and P<0.05 was considered significant.
All statistical tests were two sided. GraphPad Prism version 5.0 for Mac OS X was used to perform analyses of variances.

**Results**

**SAHA increases thyroid tumor growth in**

\( \text{Thrb}^{\text{PV/PVPten}}_{+/−} \) **mice**

We treated wild-type mice, \( \text{Pten}^{+/−} \) mice, \( \text{Thrb}^{\text{PV/PV}} \) mice, and \( \text{Thrb}^{\text{PV/PVPten}}_{/+} \) mice with SAHA for 2 months and evaluated its effect on thyroid carcinogenesis. SAHA had no effect on the thyroid growth of wild-type mice (3.2 ± 0.4 mg (\( n=14 \)) vs 4.5 ± 0.5 mg (\( n=14 \)) without or with SAHA, respectively, Fig. 1). SAHA treatment increased the weight of thyroid from 2.6 ± 0.1 mg (\( n=6 \)) to 4.3 ± 0.4 mg (\( n=8 \)) in \( \text{Pten}^{+/−} \) mice (data set 3 vs 4). No significant differences in tumor weight were detected in SAHA-treated (108.4 ± 10.8 mg, \( n=10 \)) or untreated \( \text{ThrbPV/PV} \) mice (96.4 ± 14.8 mg, \( n=10 \)). However, \( \text{ThrbPV/PVPten}^{+/−} \) mice treated with SAHA had a 1.6-fold increase in thyroid tumor weight compared with no SAHA treatment (203.0 ± 30.0 mg (\( n=12 \)) vs 125.5 ± 12.2 mg (\( n=17 \)), data set 8 vs 7, Fig. 1). These results indicate that, although SAHA had no effects on thyroid tumor growth of \( \text{ThrbPV/PV} \) mice, it increased thyroid tumor growth in \( \text{ThrbPV/PVPten}^{+/−} \) mice.

**Increased thyroid growth in** \( \text{Thrb}^{\text{PV/PVPten}}_{+/−} \) **mice is not mediated by altered TSH levels**

TSH is the primary growth stimulator of thyroid epithelial cells (Rivas & Santisteban 2003). To evaluate whether TSH could contribute to the increased thyroid growth in \( \text{Thrb}^{\text{PV/PVPten}}_{+/−} \) mice treated with SAHA, we compared serum TSH of the mice without or with SAHA treatment (Fig. 2A). There were no significant differences in serum TSH levels of wild-type, \( \text{Pten}^{+/−} \), and \( \text{ThrbPV/PV} \) mice between the mice without and those with SAHA treatment (wild type: 43.8 ± 3.3 ng/mL (\( n=15 \)) vs 43.5 ± 6.5 ng/mL (\( n=14 \)); \( \text{Pten}^{+/−} \): 38.9 ± 4.9 ng/mL (\( n=8 \)) vs 45.4 ± 4.6 ng/mL (\( n=6 \)); \( \text{ThrbPV/PV} \): 19,529 ± 2700 ng/mL (\( n=11 \)) vs 16,662 ± 2349 ng/mL (\( n=11 \)). In \( \text{ThrbPV/PVPten}^{+/−} \) mice, the serum level of TSH in mice with SAHA treatment (12,899 ± 1446 ng/mL, \( n=12 \)) was significantly lower than that in those without SAHA treatment (26,276 ± 4382 ng/mL, \( n=11 \)) (Fig. 2A). There were no significant differences in serum total T4 (Fig. 2B) and total T3 (Fig. 2C) levels between the mice treated with SAHA or without SAHA. The findings that SAHA treatment significantly decreased TSH levels in \( \text{Thrb}^{\text{PV/PVPten}}_{+/−} \) mice indicated that increased thyroid tumor growth in \( \text{Thrb}^{\text{PV/PVPten}}_{+/−} \) mice was not an effect of TSH (Fig. 2A).

**SAHA promotes thyroid tumor progression in**

\( \text{Thrb}^{\text{PV/PVPten}}_{+/−} \) **mice**

We treated wild-type mice, \( \text{Pten}^{+/−} \), \( \text{ThrbPV/PV} \), and \( \text{ThrbPV/PVPten}^{+/−} \) mice for 8 weeks after they were weaned at 6 weeks. Pathohistological analysis was performed to determine the effects of SAHA on thyroid carcinogenesis in mice at the same age (Fig. 3). Thyroids and lungs of the wild-type mice treated with SAHA exhibited no apparent abnormalities (Fig. 3A-a, e, i, and m). Although the thyroid weight of the \( \text{Pten}^{+/−} \) mice treated with SAHA increased, morphology of the thyroid and lung after
SAHA treatment was similar to that of the mice without SAHA treatment (Fig. 3A-b, f, j, and n). Thyroids of ThrbPV/PVPten+/− mice displayed extensive hyperplasia. Only a few of the ThrbPV/PV mice displayed capsular invasion (Fig. 3A-c), but was not affected by SAHA treatment (Fig. 3A-g). No lung metastasis was detected in ThrbPV/PV mice (panels k and o). In thyroids of ThrbPV/PVPten+/- mice with no SAHA treatment, capsular

Figure 2
Serum TSH, total T4, and total T3 levels in wild-type and mutant mice treated with or without SAHA. Serum total TSH (A), TT4 (B), and TT3 (C) of wild-type, Pten+/−, ThrbPV/PV, and ThrbPV/PVPten−/− mice with or without SAHA treatment were determined as described in the Materials and methods section. The P values are marked (n=5–14); NS, not significant.

Figure 3
Histopathological features of thyroids of wild-type mice and mutant mice treated with or without SAHA. (A) After mice were treated with SAHA for 8 weeks as described in the Materials and Methods section, thyroids (panels a–h) and lungs (panels i–p) were dissected and stained with H&E: wild-type (a, e, i, and m), Pten−/− (b, f, j, and n), ThrbPV/PV (c, g, k, and o), and ThrbPV/PVPten−/− (d, h, l, and p) mice. Panels show hyperplasia (c, d, g, h, arrow), capsular invasion (d, g, h, arrow), vascular invasion (h-ii, arrow), anaplastic foci (h-iii, arrow), and microscopic lung metastases (p, arrow). The anaplastic focus is shown at a higher magnification in panel l. (B) Quantitative analysis of occurrence frequency (%) of (a) capsular invasion, (b) vascular invasion, (c) anaplastic foci, and (d) lung metastasis of ThrbPV/PVPten−/− mice with or without SAHA treatment. Sections of thyroids and lungs were stained with H&E and analyzed for pathological progression. The data are expressed as the percentage of occurrence frequency of the mice examined. The # indicates zero occurrence frequency (%). The P values are indicated. "&" in panel d shows that statistical analysis could not be analyzed as there was no occurrence (zero) of lung metastasis in the absence of SAHA. A full color version of this figure is available at http://dx.doi.org/10.1530/ERC-16-0103.
invasion was frequently observed (Fig. 3A-d). None of the ThrbPV/PVPten+/− mice was found to have lung metastasis (Fig. 3A-i). After SAHA treatment, the thyroids of ThrbPV/PVPten−/− mice showed capsular invasion (Fig. 3A-h-i), vascular invasion (Fig. 3A-h-ii), and anaplastic foci (Fig. 3A-h-iii). Moreover, lung metastases were frequently detected in the SAHA-treated ThrbPV/PVPten−/− mice (Fig. 3A-p).

The effect of SAHA treatment on the occurrence frequency of pathohistological features shown above (Fig. 3A) in ThrbPV/PVPten−/− mice is shown in Fig. 3B. All ThrbPV/PVPten+/− mice, that were of the same age, had developed capsular invasion for which the frequency of occurrence was not affected by SAHA treatment (Fig. 3B-a). In untreated ThrbPV/PVPten−/− mice, 17% of thyroid tumors had developed vascular invasion. SAHA treatment accelerated the occurrence of vascular invasion from 17 to 50% (Fig. 3B-b). Although only 8% of the untreated ThrbPV/PVPten−/− mice had developed anaplasia, the frequency of occurrence of anaplasia increased to 25% by SAHA treatment (Fig. 3B-c). In the absence of SAHA, no lung metastasis was developed in ThrbPV/PVPten+/− mice. However, lung metastasis was detected in 25% of SAHA-treated ThrbPV/PVPten+/− mice (Fig. 3B-d). These results show that SAHA promotes thyroid cancer progression in ThrbPV/PVPten+/− mice.

**SAHA activates PI3K-AKT signaling in thyroid tumors of ThrbPV/PVPten+/− mice**

To dissect the molecular events responsible for SAHA-induced aggressive thyroid cancer progression in ThrbPV/PVPten+/− mice, we examined altered cell signaling pathways involved in tumor growth and progression. We have previously shown that Pten haploinsufficiency further overactivates the PI3K-AKT signaling in ThrbPV/PVPten+/− mice (Guigon et al. 2009). We therefore first evaluated whether protein levels of key regulators in the PI3K-AKT signaling pathway were detected in vehicle-treated ThrbPV/PVPten+/− mice. After SAHA treatment, the Pten protein signals were barely detectable (panel iv) of Fig. 4B-I-a; also see the quantitative data shown in Fig. 4B-II-a). These immunohistochemical findings are consistent with the Western blot analysis that SAHA treatment lowered Pten protein levels to activate PI3K-AKT signaling in thyroid tumors of ThrbPV/PVPten+/− mice.

To understand how activation of p-AKT led to increased tumor growth, we examined the expression of the PI3K-AKT signaling downstream effectors. Cross talk between AKT signaling and the Rb pathway is critical in cell cycle regulation (Imai et al. 2014). Phosphorylation of Rb drives cell cycle progression from the G1 to the S phase. Panel d (Fig. 4A-I) shows that p-Rb(S748) protein levels were elevated in SAHA-treated ThrbPV/PVPten+/− mice (lanes 11 and 12 vs 9 and 10) without apparent changes in the total Rb protein level (panel e, Fig. 4A-I). The ratio of p-Rb(S748) to total Rb was increased (Fig. 4A-II, panel c). No marked effects of SAHA on the p-Rb(S748) levels in the wild-type mice (lane 2 vs 1), Pten+/− mice (lane 3 vs 4), and thyroid tumors of ThrbPV/PVPten+/− mice (lanes 5 and 6 vs 7 and 8) were observed. CDK6 protein levels were higher in SAHA-treated ThrbPV/PVPten+/− mice than in untreated mice (lanes 11 and 12 vs 9 and 10, Fig. 4A-I-f; see also quantitative data shown in Fig. 4A-II-c); however, no SAHA-induced changes were detected in CDK6 levels in wild-type (lane 2 vs 1), Pten+/− mice (lane 3 vs 4), and thyroid tumors of ThrbPV/PVPten+/− mice (lanes 5 and 6 vs 7 and 8).
Figure 4
SAHA altered the protein abundance of key cell cycle regulators in thyroid tumors of ThrβPV/Pten−/− mice (A-I) Total protein extracts were prepared from thyroids of wild-type, Pten+/−, ThrβPV+/−, and ThrβPV/Pten−/− mice treated with or without SAHA. Western blot analysis was carried out for PTEN (a), p-AKT (S473) (b), total-AKT (c), p-Rb (S748) (d), total Rb (e), CDK6 (f), p21Cip1 (g), and GAPDH (h) as described in the Materials and methods section. Representative examples from thyroids of two mice for each genotype are shown. (A-II) Quantitative analysis of relative protein expression levels of PTEN (a), ratios of p-AKT (S473) to total AKT (b), ratios of p-Rb (S748) to total Rb (c), CDK6 (d), and p21Cip1 (e) on the basis of the intensities of the bands are shown in (A-I), the p values are marked. The statistical analysis was carried out using Western blot data from three to five thyroids of vehicle-treated or SAHA-treated wild-type mice, Pten+/− mice, and ThrβPV+/− mice. For the analysis of Western blot data of tumors of ThrβPV/Pten−/− mice, we used three to four thyroid tumors of vehicle-treated ThrβPV/Pten−/− mice and six to eight thyroid tumors of SAHA-treated ThrβPV/Pten−/− mice. The error bars show the standard error of the means. (B-I) Immunohistochemical analysis was carried out using antibody against PTEN (a) or p-AKT (b) for thyroid tumor tissues. Antibody IgG were used as a negative control. (B-II) PEN-positive cells (a) or p-AKT-positive cells (b) were quantified. The thyroid tumors treated with SAHA had significantly lower cell numbers stained for PTEN and higher cell numbers stained for p-AKT compared with those treated with vehicle (*P<0.0001). A full color version of this figure is available at http://dx.doi.org/10.1530/ERC-16-0103.
We also examined the effect of SAHA on the protein abundance of p21Cip1, which is another effector downstream of the PI3K-AKT pathway (Warfel & El-Deiry 2013). p21Cip1 is a potent cyclin-dependent kinase inhibitor (CKI). It binds to and inhibits the activity of cyclin–CDK6 complexes and thus functions as a negative regulator of cell cycle progression from the G1 phase to the S phase (Gartel & Radhakrishnan 2005). The abundance of p21Cip1 protein was higher in the thyroid tumors of ThrbPv/PvPten−/− mice than in wild-type and Pten+/− mice; however, the p21Cip1 protein levels were not affected by SAHA treatment (lanes 5-8). The abundance of p21Cip1 protein was further elevated in the ThrbPv/PvPten−/− mice (lanes 9 and 10); however, SAHA treatment lowered the p21Cip1 protein levels by 45% (lanes 11 and 12, Fig. 4A-I-g; see also Fig. 4A-II-e). The quantitative analyses of the band intensities are shown in Fig. 4A-II (panel a for PTEN, panel b for the ratios of p–AKT vs total AKT; panel c, ratios of p-Rb vs total Rb; panel d, CDK6 and panel e, p21Cip1 proteins). The analysis was normalized to the loading controls using GAPDH (Fig. 4A-I-h). These findings indicate that the decreased PTEN level induced by SAHA led to activation of PI3K-AKT to drive cell cycle progression to promote thyroid tumor cell proliferation in ThrbPv/PvPten−/− mice.

It is known that AKT complexes with cSrc to link to cytoskeletal proteins such as ezrin to alter cell migration (Srivastava et al. 2005, Heiska et al. 2011). Moreover, AKT signaling mediates activation and secretion of matrix metalloproteinases (MMPs), which play key roles in tumor metastasis (Cho et al. 2008). We found a higher level of activated p-cSrc (panel a, lanes 11 and 12, Fig. 5A-a) without apparent effect of total c-Src (panel b, lanes 11 and 12; Fig. 5A-b) in SAHA-treated ThrbPv/PvPten+/− mice. Moreover, ezrin protein levels were also higher in SAHA-treated ThrbPv/PvPten+/− mice (lanes 11 and 12, panel c, Fig. 5A-c). SAHA had no apparent changes in the ezrin levels in wild-type (lanes 1 and 2, Fig. 5A-c), Pten+/− mice (lanes 3 and 4), and ThrbPv/PvPten−/− mice (lanes 7 and 8 vs 5 and 6; Fig. 5A-c).

MMP2 was increased in ThrbPv/PvPten−/− mice (lanes 5-8; Fig. 5A-d) than in wild-type (lanes 1 and 2, Fig. 5A-d) and Pten+/− mice (lanes 3 and 4). However, SAHA had no detectable effects on the MMP2 levels in thyroid tumors of ThrbPv/PvPten−/− mice. However, MMP2 protein levels were clearly elevated in the thyroid tumors of SAHA-treated ThrbPv/PvPten+/− mice by 1.9-fold compared with untreated mice (lanes 11 and 12 vs 9 and 10, see also Fig. 5A-d and Fig. 5B-c). Similar patterns of changes were also found for MMP7. As shown in lanes 11 and 12 (Fig. 5A-e), SAHA treatment led to markedly increased MMP7 in thyroid tumors of ThrbPv/PvPten−/− mice (4.2-fold), whereas no apparent effects were detected on the relatively low levels of MMP7 in wild-type (lanes 1 and 2, Fig. 5A-e).
Pten\(^{-/-}\) mice (lanes 3 and 4), and Thrb\(^{PV/PV}\) mice (lanes 7 and 8 vs 5 and 6; Fig. 5A-e). The SAHA-induced changes in p-cSrc, ezrin, MMP2, and MMP7 can be seen more clearly in the quantitative analysis shown in Fig. 5B-a, b, c, and d respectively.

**Loss of the wild-type Pten allele is responsible for reduced Pten expression in thyroid tumors of SAHA-treated Thrb\(^{PV/PV}\)Pten\(^{-/-}\) mice**

We further looked into the mechanism responsible for SAHA-induced PTEN down-regulation in thyroid tumors of Thrb\(^{PV/PV}\)Pten\(^{-/-}\) mice (Fig. 4A-I-a and A-II-a). We ascertained the effect of SAHA on the Pten mRNA expression in thyroid tumors of Thrb\(^{PV/PV}\)Pten\(^{-/-}\) mice. Quantitative real-time PCR analysis indicated that Pten mRNA abundance was significantly reduced by 81% in thyroid tumors of SAHA-treated Thrb\(^{PV/PV}\)Pten\(^{-/-}\) mice (Fig. 6A). The results indicated that SAHA suppressed the Pten gene expression at the transcription level.

Although there are multiple potential regulatory mechanisms that could lead to the decreased expression of the Pten mRNA, we focused on testing the possibility of the loss of the Pten gene copy due to selection pressure by the therapeutic agent SAHA. This phenomenon was recently observed in a patient with metastatic breast cancer carrying an activated PIK3CA mutation, resulting in constitutive overactivation of AKT signaling (Juric et al. 2015). Treatment of the patient with the PI3K\(^{\alpha}\) inhibitor BYL719 led to the copy loss of the PTEN gene. The patient developed resistance to the treatment and eventually died (Juric et al. 2015). The Thrb\(^{PV/PV}\)Pten\(^{-/-}\) mice used in our studies have only one copy of the functional wild-type Pten allele. We therefore examined whether SAHA treatment led to the loss of the wild-type Pten allele by using single-molecule DNA analysis to determine the relative number of copies of the wild-type Pten allele. By using the genomic DNA isolated from thyroid tumors as PCR template, single-molecule DNA ddPCR analysis was used to assess the genetic loss of the Pten gene. Compared with Thrb\(^{PV/PV}\) mice without SAHA treatment, Thrb\(^{PV/PV}\)Pten\(^{-/-}\) mice had only one copy of the Pten allele (bar 1 vs bar 3, Fig. 6B). With SAHA treatment, no reduction in the relative copy number was observed in the thyroid of Thrb\(^{PV/PV}\) mice (bar 2 vs 1). In contrast, 50% of thyroid tumor cells had lost one copy of the Pten allele (bar 4 vs 3). Interestingly, the extent of the Pten copy loss was positively correlated with the increased tumor growth. Figure 6C shows that in thyroid tumors with weight under 200 mg (average normal mouse thyroid weight = 3.24 (3.24 \(\pm\) 1.40; n = 14)), 30% of tumor cells had lost one copy of the wild-type Pten allele; with weight over 400 mg, 70% of tumor cells had lost one wild-type Pten allele. These results indicate that one mechanism by which the decreased expression of the Pten mRNA is due to the loss of the Pten allele in the thyroid tumor of SAHA-treated Thrb\(^{PV/PV}\)Pten\(^{-/-}\) mice. The loss of the Pten gene, a PI3K-AKT-negative regulator, resulted in the constitutive activation of AKT signaling, rendering that SAHA is therapeutically ineffective to treat thyroid cancer.
Discussion

As SAHA was approved to treat cutaneous T-cell lymphoma by the U.S. Food and Drug Administration (Duvic et al. 2007, Xu et al. 2007, Grant et al. 2010), clinical trials have been carried out to treat many types of solid tumors such as lung, prostate, and colon cancers (Mann et al. 2007, Olsen et al. 2007). However, clinical trials in solid tumors have not had success because of the resistance of solid tumors to the treatment (Ramalingam et al. 2010). Thyroid tumors often progress further even during the SAHA treatment (Woyach et al. 2009). The molecular basis of the resistance of SAHA treatment, however, has not been elucidated. Using two preclinical mouse models of thyroid cancer, we found that SAHA was ineffective as a therapeutic to treat thyroid cancer in ThrbPV/PV mice. In ThrbPV/PVPten+− mice, SAHA is not only ineffective therapeutically but paradoxically promotes thyroid cancer progression. These observations are reminiscent of the outcome of the clinical trial of 19 patients with advanced thyroid cancer (Woyach et al. 2009) in that no patient achieved a partial or complete response. Trials for some patients were terminated owing to clinical progression of the disease. The present findings are in contrast to the early observations from cell-based studies in which SAHA was effective in the inhibition of tumor cell proliferation and induction of apoptosis (Mitsiades et al. 2005, Luong et al. 2006, Borbone et al. 2010). The present results support the usefulness of these mouse models in testing potential therapeutic agents in vivo for the treatment of thyroid cancer.

Therapeutic resistance is a major obstacle for effective treatment of cancers by HDAC inhibitors. HDAC inhibitors induce acetylation of histones and nonhistone proteins involved in the regulation of gene expression and in various cellular pathways including cell growth arrest, differentiation, DNA damage and repair, redox signaling, and apoptosis. Depending on the cellular context and genetic makeup of the cancer cells, the mechanisms of resistance to HDAC inhibitors vary. Resistance to HDAC inhibitors may involve aberrant expression and modifications of signaling molecules, causing intrinsic resistance to HDAC inhibitors in cancer cells, such as high-level expression of thioredoxin, overactivation of pro-survival BCL-2, or constitutive activation of STAT proteins (Fedier et al. 2007, Fantin et al. 2008, Shao et al. 2010, Lee et al. 2012). In the aforementioned clinical trial of SAHA in 19 patients (Woyach et al. 2009), it was not clear what regulators were aberrantly induced and/or signaling molecules were aberrantly activated by SAHA treatment, leading to resistance and/or progression of the disease. In the study, we showed that SAHA treatment of ThrbPV/PVPten+− mice led to the aberrant loss of the Pten gene, a negative regulator of the PI3K-AKT pathway. The loss of the PTEN tumor suppressor led to constitutive activation of the AKT and its downstream signaling to stimulate tumor growth and to promote cancer invasion and metastasis (Fig. 7). Thus, this study uncovered a novel mechanism by which SAHA not only lacks effectiveness as a therapeutic agent but also detrimentally promotes cancer progression.

It is important to point out that ThrbPV/PV mice treated with SAHA only exhibited resistance without the progressive disease found in ThrbPV/PVPten+− mice. These findings further highlight the importance of different sensitivities to SAHA treatment due to cellular context and genetic makeup of the cancer cells. The ThrbPV/PV mouse is a model of FTC harboring a powerful dominant negative TRβPV mutant (Suzuki et al. 2002). The development and progression is partly driven by overactivation of PI3K-AKT signaling (Furuya et al. 2006, 2007). The ThrbPV/PVPten+− mouse is also a model of FTC; however, in addition to harboring the TRβPV mutation, it has lost one allele of the Pten gene (Guigon et al. 2009). Thyroid carcinogenesis is driven further by PI3K-AKT signaling. At present, it is not clear how SAHA treatment led to the selective loss of the remaining Pten gene in the thyroid tumors of ThrbPV/PVPten+− mouse, but not in the thyroid tumors of the ThrbPV/PV mouse. Still, it is known that a reduced level of the PTEN gene due to methylation or other epigenetic silencing occurs in close association with activating
genetic alterations of the PI3K-AKT pathway, constituting a unique self-enhancement mechanism in this pathway (Xing 2010). It is likely that loss of one Pten allele may render thyroid tumor cells more susceptible to further loss of the remaining functional allele through a PI3K-AKT self-enhancement mechanism. As a result, convergent bi-allelic loss of the Pten gene could cause enhanced AKT signaling to overcome antitumor effects of SAHA, resulting in elevated activity of the downstream effectors to drive aggressive thyroid carcinogenesis (Fig. 7).

That cancer cells could lose a tumor suppressor gene through selective pressure of anticancer agents is not without precedent. A recent report documented that treatment of a patient with metastatic breast cancer expressing an activating PIK3CA mutation with a PI3Ka inhibitor, BYL719, led to loss of the PTEN gene (Juric et al. 2015). Although initially achieving a positive clinical response, the patient eventually progressed during treatment and died shortly thereafter. It is important to point out that in this study, although the patient had no loss of the PTEN gene in the primary lesions, the metastatic tumors resistant to the inhibitor treatment have a copy loss of the PTEN gene, suggesting that the loss of the PTEN gene is coupled to the resistance to inhibitor treatment. This clinical case together with our findings from the thyroid cancer mouse model highlighted the susceptibility of the loss of the PTEN gene to the selection pressure by anticancer agents targeting the PI3K-AKT pathway. On the basis of these findings, when cancer patients develop resistance to anticancer agents targeting the PI3K-AKT pathway, the loss of the PTEN gene should be suspected and explored such that other strategies and treatment modalities could be developed to improve patients’ outcome.

The above findings indicated that the selective pressure by a PI3Ka inhibitor could drive tumor cells to evolve mechanisms, resulting in the PTEN gene loss to overcome inhibitory effects. These studies also suggested that the loss of the PTEN gene could also be susceptible to other selective pressure such as SAHA as shown in this study. Although it was not clear in the nature of genetic alterations in the thyroid cancer patients for whom vorinostat (SAHA) treatment was ineffective or even progressed (Woyach et al. 2009), on the basis of the findings by Juric et al. (2015) and this study, it is tempted to speculate that the selective pressure by SAHA could also lead to the PTEN gene loss in those thyroid cancer patients to counteract the potential therapeutic effect of SAHA. The validation of this conjecture would await future studies.

Consistent with the clinical trial, the present findings clearly show that SAHA treatment was ineffective to improve the outcome of thyroid cancer in the two mouse models. Even so, our studies uncovered that SAHA led to the loss of the Pten gene, which resulted in the activation of the PI3K-AKT signaling. These findings raised the possibility that a combination treatment of SAHA with anticancer agents targeting PI3K-AKT downstream effectors could be beneficial. Moreover, as the U.S. Food and Drug Administration has approved two HDAC inhibitors, vorinostat and romidepsin, for the treatment of cutaneous T-cell lymphoma (Duvic et al. 2007, Marks & Breslow 2007, Grant et al. 2010), more than 20 chemically different HDAC inhibitors are in clinical trials for hematological cancer and solid tumors. In addition to the trial of vorinostat (SAHA), a phase I trial of romidepsin in patients with advanced thyroid cancer has been completed. The dosages used were tolerable by patients; however, no objective responses were detected (Amiri-Kordestani et al. 2013). Thus, it would be possible to use the same schedule to combine with other anticancer agents for the treatment of patients with advanced cancers. One of the possible treatments is to combine SAHA treatment with radioiodine as SAHA may enhance thyroid gene expression (Cheng et al. 2016). The Thrb<sup>PV/PV</sup> and Thrb<sup>PV/PV</sup>Pten<sup>+/−</sup> mice would be useful models to test the possibility of combined treatments of anticancer agents with SAHA or romidepsin in the future studies.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
Sheue-yann Cheng conceived the project, analyzed the data, and wrote the paper. Xuguang Zhu designed and performed the experiments, analyzed the data, and wrote the paper. Dong Wook Kim initiated SAHA treatment of the mice and performed the experiments. Mark C Willingham analyzed pathological features of tumors. Li Zhao performed IHC experiments.

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