PEAK1 attenuation sensitizes anaplastic thyroid carcinoma cells in vitro to BRAF\textsuperscript{V600E} inhibitor Vemurafenib

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Abstract

Pseudopodium-enriched atypical kinase 1 (PEAK1) has been demonstrated to be upregulated in human malignancies and cells. Enhanced PEAK1 expression facilitates tumor cell survival and chemoresistance, and vice versa. However, the role of PEAK1 in ATC and vemurafenib resistance in ATC cells is still unknown. Here, we observed that vemurafenib could effectively and quickly inhibit ERK phosphorylation (pERK1/2), but quickly reactivate pERK1/2 in ATC cell line 8505C and SW1736 cells harboring BRAF\textsuperscript{V600E} in vitro. Vemurafenib could also quickly activate AKT phosphorylation (pAKT). Furthermore, vemurafenib transiently upregulated pro-apoptotic PUMA and Bim expression, which quickly disappeared with long time vemurafenib treatment in both of the cells in vitro. Vemurafenib treatment upregulated PEAK1 expression. And PEAP1 depletion inhibited the cell viability, colony formation and cell cycle, but not cell apoptosis, and vice versa in both of the cells in vitro. Furthermore, PEAP1 depletion sensitized 8505C and SW1736 cells to vemurafenib by decreasing cell viability, colony formation and inducing cell apoptosis. Mechanistically, combined PEAK1 depletion and Vemurafenib treatment inhibited reactivation of ERK1/2 and upregulated JNK1/2-dependant Bim as well as inhibited the activation of AKT and upregulated AKT-dependant PUMA. In conclusion, vemurafenib resistance in ATC cells harboring BRAF\textsuperscript{V600E} is associated with PEAK1 activation, reactivation of ERK1/2 and activation of AKT, resulting in the inhibition of pro-apoptotic PUMA and Bim protein. PEAK1 depletion could inhibit both ERK1/2 and AKT signal and released PUMA and Bim from vemurafenib-treated cells, resulting in enhancing cell apoptosis. Therefore, targeting PEAK1 may be an effective strategy to sensitize BRAF-mutant ATC to vemurafenib.

Key words: Anaplastic thyroid carcinoma; BRAF inhibitors; vemurafenib; Apoptosis; chemoresistance; Pseudopodium-enriched atypical kinase 1

Introduction

Anaplastic thyroid carcinoma (ATC) is a rare form of undifferentiated thyroid carcinoma, which represents about 1-2% of all thyroidal malignancies with an overall survival from 3 to 5 months and a one year survival rate of 20% in most of the cases [1, 2]. More than 80% of ATC patients have already experienced organ invasion or distant lymph node metastasis when they are first diagnosed, and it is commonly aggressive or metastatic at the initial presentation [3]. This disease progresses rapidly, and precludes the initiation of therapeutic attempts. In addition, ATC tends to develop in older persons who often have immune and/or organ dysfunction. Therefore, the patients may not tolerate aggressive anti-cancer treatment, and difficult to achieve successfully ATC treatment. It is refractory to radioiodine, and traditional chemotherapy and radiotherapy are of limited benefit, response rates to standard systemic therapies are < 15%, despite the comprehensive treatment in many aspects, the survival of the advanced ATC patients has not been significantly improved [4]. Therefore, effective therapies are urgently needed for the disease.

ATC is commonly derived from a differentiated tumor such as papillary thyroid cancer (PTC) or follicular thyroid cancer (FTC), which precedes or coexists with approximately 50% of ATCs. ATC typically harbors several oncogenic mutations, most commonly in the MAPK pathway [5]. The harbor activating BRAF\textsuperscript{V600E} mutation is the most prevalent
genetic alteration in PTC (36-86%) and ATC (20-25%). BRAFV600E mutations are an early and common driver mutation in PTC. The activated MAPK pathway resulting from the BRAFV600E has been shown to drive uncontrolled cell proliferation and growth and is implicated in poor therapeutic outcome in ATC cells [6]. Thus effective treatment of ATC through BRAFV600E would meet an urgent clinical need.

Vemurafenib is the BRAFV600E inhibitor by targeting ERK1/2 pathway for the treatment of BRAFV600E-positive metastatic melanoma [7]. It is also an effective and well-tolerated treatment strategy in advanced PTC patients harboring the BRAFV600E mutation [8, 9]. BRAF inhibitors may yield short-term clinical benefits in these patients because of the reactivation of ERK1/2, resulting in the resistance to Vemurafenib [10-13]. However, resistance to Vemurafenib inhibition can be achieved by combined therapeutic modalities for MAPK pathway inhibition [14-16]. In a mouse model, Vemurafenib inhibited growth of human ATC cells with BRAFV600E mutation [17]. In a case with BRAF-mutated ATC, Vemurafenib treatment resulted in nearly total eradication of metastatic disease in a 51-year-old man [18]. A further study demonstrated there were anecdotal responses with Vemurafenib treatment among 7 patients with BRAF-mutated ATC [19]. In 8505C, an ATC cell line model, 8505C had poor treatment response with Vemurafenib alone, but increased effect with Vemurafenib in combination with MAPK inhibitors [20].

The PI3K/AKT pathway plays an important role in thyroid tumorigenesis, proliferation, growth, apoptosis and invasion. AKT was found to be robustly activated in thyroid cancer. Activation of the PI3K/AKT pathway also resulted in acquired BRAF inhibitor resistance in thyroid cancer [21]. Montero-Conde et al. reported that Vemurafenib treatment in ATC cells transiently activated AKT [22], thereby contributing to the acquired resistance of BRAF inhibition in BRAF mutant ATC cells [21-23]. Dual blocking AKT and MAPK pathways can effectively inhibit ATC [63], and sensitized ATC cells to Vemurafenib treatment [23-25].

Except for the re-activation of the MAPK signal transduction pathway and activation/reactivation of the AKT pathway in BRAFV600E-mutant ATC with the targeting BRAF treatment, resistance to drugs that target the BRAF also relies on modulation of the caspase-dependent apoptotic cascade [10, 26]. In addition, Vemurafenib inhibited c-Jun N-terminal kinase (JNK) signal and blocked Vemurafenib-induced cell apoptosis [27]. Vemurafenib also induces apoptotic cell death mediated by caspase-3, suggesting that addition of a procaspase-3 activator could enhance anticancer effects [28].

Inhibition of MEK resulted in the up-regulation of the BH3-only proteins PUMA and Bim and down-regulation of the anti-apoptotic protein Mcl-1 [29]. While Vemurafenib increased ERK phosphorylation, enhanced anti-apoptotic Mcl-1 and suppressed pro-apoptotic PUMA and Bim [30]. In addition, vemurafenib suppressed apoptosis also through the inhibition of multiple off-target kinases upstream of c-Jun N-terminal kinase (JNK) [27]. While Bim expression was increased by JNK/c-Jun dependent, and the Noxa and Puma expression was entirely JNK/c-Jun independent but 50% of Puma/Noxa expression was p53 dependent [31]. As for the PUMA, AKT inhibition upregulated p53-independent PUMA transcription and Bax-mediated intrinsic mitochondrial apoptosis [32]. Therefore, simultaneous inhibition of ERK and AKT signals or activating JNK/c-Jun signals and pro-apoptotic protein would improve the efficacy of vemurafenib and counteract the resistance to vemurafenib.

Pseudopodium enriched atypical kinase 1 (PEAK1 or Sgk269) is a non-receptor tyrosine kinase which is enriched in the pseudopodia of migrating cells and played an important role in regulating cell migration and proliferation [33]. PEAK1 was reported to be overexpressed in human breast cancer [34], colon cancer [35], lung cancer [36] and pancreatic cancer [37]. High PEAK1 expression was significantly associated with advanced clinical stage and poor prognosis in colon cancer [35] and tumor growth and development in breast cancer [33] and pancreatic cancer [37]. However, the expression and role of PEAK1 in human anaplastic thyroid carcinoma is unknown. Enforced PEAK1 expression was reported to promote invasion in pancreatic ductal epithelial cells through enhanced JAK1/Stat3 signaling [38]. In basal breast cancer cells, PEAK1 promoted ERK and Stat3 activation and decrease anchorage-independent growth, and vice versa [39]. In colon cancer cells, PEAK1 regulates the p130Cas-Crk-paxillin and ERK signaling pathways to control cell spreading, migration, and proliferation [35]. In PEAK1-depleted HMVECs and HUVECs, phosphorylation of MEK/ERK, SRC, and AKT were all inhibited, resulted in impaired cell proliferation, migration, and survival [40]. A recent study found that collagen-mediated activation of DDR1 induced PEAK1, SRC, and PYK2 signals in pancreatic cancer cells, resulting in collagen-induced chemoresistance and tumor progression [41]. These accumulating data indicate that PEAK1 regulates MEK/ERK and AKT signals, and chemoresistance. Therefore, PEAK1 is possibly a new therapeutic target for cancer. However, the role and mechanisms of PEAK1 on BRAFV600E inhibitor
resistance in ATC cells is unknown. In the present study, we examined the role and mechanisms of PEAK1 on ATC cells and on the sensitivity of ATC cells to BRAFV600E inhibitor Vemurafenib. Here we found that Vemurafenib transiently inhibits ERK1/2 signals then lastly re-activates ERK1/2 signals, resulting in JNK signals and JNK-dependent pro-apoptotic Bim inhibition and Vemurafenib resistance. Vemurafenib also activates AKT signal, resulting in AKT-dependent pro-apoptotic PUMA inhibition and Vemurafenib resistance. PEAK1 is upregulated in ATC cells with Vemurafenib treatment. Targeting PEAK1 alone has shown less anti-proliferation in ATC cell lines. PEAK1 depletion simultaneously blocks Vemurafenib -induced ERK1/2 re-activation and AKT activation, and reverses pro-apoptotic Bim and PUMA expression, resulting in the sensitivity of ATC cells to Vemurafenib treatment.

Materials and Methods

Cell culture

The BRAFV600E mutant ATC cell line 8505C and SW1736 were obtained from European Collection of Cell Cultures. The two cell lines were cultured in complete RPMI 1640 medium containing 10% fetal bovine serum, and authenticated and detected for Mycoplasma before use, and the identity of the cell lines was verified by short tandem repeat (STR) analysis. A monolayer of 50-70% confluent cells was used in all of the assays.

siRNA (Small interfering RNA) transfection

One siRNA oligonucleotides targeting Pseudopodium Enriched Atypical Kinase 1 (PEAK1 siRNA) was from Sigma (SASI_Hs02_00357289). siRNA guide sequences targeting BIM (Bim siRNA) and PUMA (PUMA siRNA) was obtained from CST (Cell Signaling Technology, Shanghai, China). Nontargeting siRNA was purchased from Dharmacon and served as a negative control. Cells were seeded 24 h before transfection at 50-60% confluence in growth medium without antibiotics. 100 nM siRNAs were then transfected into culture’s 8505C and SW1736 cells using Lipofectamine 2000 reagent (Invitrogen, Shanghai, China) as the manufacturer's instruction. The cells were harvested 48 h after transfection for Western blot analyses.

Construction of PEAK1 shRNA expression vector and transfection

The short-hairpin RNA direct against human PEAK1 gene (PEAK1 shRNA) by shRNA design tools (http://rnadesigner.thermofisher.com/rnaiexpress/) was synthesized and constructed into the pcDNA3.1 expression vector (Shanghai, China) as the manufacture’s instruction. The constructed vectors (PEAK1 shRNA or NC shRNA) were transfected into the 8505C and SW1736 cells using Lipofectamine 3000 reagent (Invitrogen, Shanghai, China) as the manufacturer's instruction. The PEAK1 shRNA or NC shRNA transfected 8505C and SW1736 cells were selected by puromycin (10 mg/ml) for 5 days. The puromycin-resistant colonies were then picked and expanded. The relative protein was detected by Western blot assay.

Plasmid constructs and transfection

The full-length human PEAK1 adenovirus was constructed as to the manufacturer’s instructions using the AdEasy Adenoviral Vector System. Viral particles were produced by GenScript Biotechnology, China. The virus particles containing PEAK1 or control vector were used to infect 8505C and SW1736 cells. Transfected cells were selected with G418 (400 μg/ml, Gibco) for 10-12 days. The expression of PEAK1 in stable PEAK1 transfected colonies was detected by western blot analysis.

Drug treatments

Vemurafenib was dissolved in absolute dimethyl sulfoxide (DMSO, vehicle) (Sigma, USA) to achieve a stock concentration of 10 mM for in vitro assays. 8505C or SW1736 cells were treated for 72h in the presence of 0.2% FBS DMEM at the presence of 0.2% FBS DMEM with: 0.1, 0.5, 1, 2.5, 5 or 10 μM of vemurafenib. Vehicle was used as untreated control (2% DMSO diluted in 0.2% FBS DMEM). PD98059 (a MEK inhibitor) and JNK1/2 inhibitor SP600125 were purchased from Selleckchem (Houston, TX). The cells were pretreated with 30 μM of PD98059 for 24 h, or 10 μM SP600125 for 24 h, then treated with 2 μM of Vemurafenib for 72 h. The non-ATP competitive pan Akt inhibitor MK-2206 was purchased from ChemieTek (Indianapolis, IN). It was dissolved in dimethylsulfoxide and perifosine in PBS in 10 mM stock. The cells were pretreated with 1 μM of MK-2206 for 24 h, then treated with 2 μM of Vemurafenib for 72 h. Cells were transfected with PUMA siRNA or Bim siRNA or N5 siRNA (100 nmol/L) for 16 h, then treated with a series of concentrations of vemurafenib or vehicle (DMSO) for 3 d.

Western blot assay

Cells were lysed with 100μL Triton X-100 lysis buffer and clarified by centrifugation at 12,500 rpm for 30 minutes. Protein concentrations were measured according to the manufacturer’s protocol (Bio-Rad). Cell lysates containing 30 μg total protein were analyzed using the standard Western blotting methods. The primary antibodies were obtained from
Cell Signaling Technologies: PEAK1, WT-P53, PUMA, Bim, total AKT, phosphorylated AKT (Ser473), phosphorylated ERK1/2 (Thr202/Tyr204), JNK1/2, pJNK1/2 (Thr183/Tyr185) and Tubulin. The secondary antibody (anti-rabbit IgG) was from Santa Cruz Biotechnology. Equal protein sample loading was monitored using an β-actin antibody. Protein bands were detected using chemiluminescence reagents.

**MTT assay**

The 8505C or SW1736 cells were plated at a density of 5000 cells/well in a 96-well plate and treated the next day with PEAK1siRNA or NS siRNA (100 nmol/L) for 1 d-5 d. To explore the effect of vemurafenib (Selleck Chemicals, Houston, TX) on the cell viability, 8505C or SW1736 cells were plated on a 96-well plate (5000 cells/well) to determine the dose-response after 3 d of vemurafenib or vehicle (DMSO) treatment. To explore the effect of PEAK1siRNA or NS siRNA on vemurafenib sensitivity, 8505C or SW1736 cells were plated at a density of 5000 cells/well in a 96-well plate and treated the next day with PEAK1siRNA or NS siRNA (100 nmol/L) for 16 h, then treated with a series of concentrations of vemurafenib or vehicle (DMSO) for 3 d. To explore the effect of PUMA or Bim on vemurafenib sensitivity, 8505C or SW1736 cells were plated at a density of 5000 cells/well in a 96-well plate and treated the next day with PUMA siRNA or Bim siRNA or NS siRNA (100 nmol/L) for 16 h, then treated with a series of concentrations of vemurafenib or vehicle (DMSO) for 3 d. MTT was added at the end point to determine the cell viability.

**Colony formation assay**

The stable PEAK1shRNA or NS shRNA transfected 8505C or SW1736 cells (1×10^5) were seeded into 6-well plates and cultured for 10 days. To explore the effect of vemurafenib on colony formation, stable PEAK1shRNA or NS shRNA transfected 8505C or SW1736 cells were seeded (1×10^5) in six well plates and treated with vehicle (DMSO) or vemurafenib for 5-7 days. Fresh media without vemurafenib was then added to the cells and cultured for another 3 days. To explore the effect of PUMA or Bim on vemurafenib sensitivity, the stable PEAK1shRNA or NS shRNA transfected 8505C or SW1736 cells were plated at a density of 1000 cells/well in a 6-well plate and treated the next day with PUMA siRNA or Bim siRNA or NS siRNA (100 nmol/L) for 16 h, then treated with 2 μM of vemurafenib or vehicle (DMSO) for 5-7 d. Fresh media without vemurafenib was then added to the cells and cultured for another 3 days. After 10 days, cells were fixed and stained with MayGrunwald-Giemsa. The number of colonies were counted and reported in graphs.

**Apoptosis and cell cycle analyses**

Cells apoptosis in early and late stages was detected using an Annexin V-FITC apoptosis detection kit from Bio-Vision (Mountain View, USA) according to the manufacturer's protocol. Briefly, 3×10^4 8505C or SW1736 cells were seeded into 6-well plates and treated the next day with PEAK1siRNA or NS siRNA (100 nmol/L) for 3 d. Furthermore, 8505C or SW1736 cells (3×10^5) were seeded into 6-well plates and treated the next day with PEAK1siRNA or PUMA siRNA or Bim siRNA or NS siRNA (100 nmol/L) for 16 h, then exposed to vemurafenib for 3 d. In addition, the stable PEAK1shRNA or NS shRNA transfected 8505C or SW1736 cells (3×10^5) were seeded into 6-well plates and treated the next day with PUMA siRNA or Bim siRNA or NS siRNA (100 nmol/L) for 16 h, then exposed to vemurafenib for 3 d. Then the cells were collected and flow cytometry was performed. All samples were assayed in triplicate. For cell cycle analysis, 1×10^5 cells were stained with 10 μg/ml PI after fixation in 70% ice cold ethanol. Cell cycles were determined by FACStation (BD Biosciences) and analyzed by using CellQuest software.

**Statistical analysis**

The Chi-square test or Student’s t test was used for analysis of the significance of each corresponding group. p < 0.05 was considered statistically significant. All data were analyzed using the SPSS22.0 software.

**Results**

**PEAK1 inhibition prevents ATC cell growth, whereas overexpression of PEAK1 enhances ATC cell growth in vitro**

PEAK1 siRNA (PEAK1 si)1-3 (100 nM) or NC siRNA (NC) (100 nM) was transfected into 8505C and SW1736 cells for 48 h, the knockdown efficiency was determined by western blot assay (Fig. 1a). We confirmed that PEAK1 si3 has the highest knockdown efficiency, the PEAK1 protein expression showed 90% decrease in the two cells, respectively. No significant change was showed in NC siRNA transfected cells. Therefore, we used PEAK1 si3 (PEAK1 si) for further study.

To determine the biologic effect of targeting PEAK1 on 8505C and SW1736 cells, we measured the effects on cell viability of 8505C and SW1736 cells after transfection by MTT assay. PEAK1 knockdown significantly inhibited cell viability of 8505C cells, which was similar to the previous research results [34,
No significant affect was found in SW1736 cells (Fig. 1b).

We next determined whether PEAK1 overexpression promoted cell growth in 8505C and SW1736 cells. The results showed that enforced PEAK1 expression did not affect cell viability in 8505C cells (Fig. 1b), but promoted cell viability in SW1736 cells (Fig. 1b). It was most likely that 8505C cell expressed PEAK1 at high levels and elevated endogenous PEAK1 levels may not be sufficient to promote 8505C cell growth. However, SW1736 cell expressed PEAK1 at less levels and elevated endogenous levels may be sufficient to promote SW1736 cell growth. Enforced PEAK1 promoted colony formation in human MDA-MB-435 cancer cells, and vice versa [42]. We then performed colony formation assays to evaluate the effectiveness of targeting PEAK1 or PEAK1 overexpression on 8505C and SW1736 cells. PEAK1 protein showed completely decrease in both PEAK1 shRNA transfected 8505C and SW1736 cells compared the scrambled shRNA transfected 8505C and SW1736 cells (Fig. 1c). PEAK1

Figure 1. Effect of PEAK1 on cell growth in vitro. a. PEAK1 siRNA transfection blocked PEAK1 expression by western blot assay in 8505C and SW1736 cells. b. Targeting PEAK1 expression suppresses 8505C cell proliferation in vitro. PEAK1 overexpression promotes SW1736 cell proliferation in vitro. c. Western blot analysis of PEAK1 in whole cell lysates from PEAK1 shRNA and PEAK1 transfected 8505C and SW1736 cells. d. The cell growth of 8505C and SW1736 cells by colony formation assays. Vs control; *P<0.05.
protein increased less in PEAK1 transfected 8505C cells, but much increased in PEAK1 transfected SW1736 cells compared with pcDNA3 transfected 8505C and SW1736 cells (Fig. 1c). We found that 8505C cells transfected with PEAK1 shRNA produced fewer colonies compared with scrambled shRNA-transfected cells (Fig. 1d). PEAK1 shRNA did not significantly affect colony number in SW1736 cells (Fig. 1d). However, the enforced PEAK1 expression did not affect the number colonies in 8505C cells, but increased the number colonies in SW1736 cells (Fig. 1d).

Silence of PEAK1 promotes cell cycle arrest, but not affect cell apoptosis in ATC cells

To determine the mechanism by which targeting PEAK1 reduced viability, we first evaluated the PEAK1 siRNA-induced apoptosis in 8505C and SW1736 cells by Annexin V/PI staining and evaluated by flow cytometric analysis. The results showed that PEAK1 inhibition for 72 h increased apoptotic cells compared to the scrambled siRNA transfected cells in 8505C and SW1736 cells, but did not reach statistical significance (Fig. 2a). PEAK1 inhibition was reported to reduce cell cycle and cell cycle associated proteins. We next determined whether PEAK1 inhibition promotes cell cycle arressin ATC cells. As showed in Fig. 2b, the cell cycles were evidently arrested at the G2/M phase in the PEAK1 siRNA transfected in 8505C, but not in SW1736 cells (Fig. 2b) in comparison with NC siRNA transfected cells. However, PEAK1 overexpression reduced cell cycle progression in both of the cells, but significant difference was only shown in SW1736 cells (Fig. 2c).

**PEAK1 inhibition increased the sensitivity of ATC cells to vemurafenib in vitro**

We first determined the sensitivity of the 8505C and SW1736 cells to vemurafenib. 8505C and SW1736 cells were exposure to 0.1, 0.5, 1, 2.5, 5 or 10 μM of vemurafenib for 72 h. Both of the cells are resistant to vemurafenib with IC50 of 2.4 μM and 1.5 μM by MTT assay, respectively (Fig. 3a), which is consistent with the previous reports [12]. To assess the effect of PEAK1 inhibition on vemurafenib response, the 8505C and SW1736 cells were transfected with PEAK1 siRNA or NC siRNA, then treated with 2 μM of vemurafenib for 72 h. The results showed that the combined PEAK1 siRNA and vemurafenib treatment induced significant cell death in 8505C and SW1736 cells by MTT assay, respectively (Fig. 3b). In addition, the number of total colonies decreased in stable PEAK1 shRNA transfected SW1736 and 8505C cells in presence of vemurafenib (2.0 μM) (Fig. 3c).
**PEAK1 overexpression prevents the sensitivity of ATC cells to vemurafenib in vitro**

To determine whether enforced PEAK1 increased the vemurafenib resistance, 8505C and SW1736 cells stably expressing exogenous PEAK1 were exposed to 2 μM vemurafenib for 72 h. We confirmed that the PEAK1 protein expression showed increase in the stable PEAK1 transfected cells in the two cells, respectively (Fig. 1c). No significant change was showed in scrambled transfected cells (Fig. 1c). Exogenous expression of PEAK1 did not affect vemurafenib resistance in 8505C cells, but significantly increased vemurafenib resistance in SW1736 cells compared to the scrambled transfected cells (Fig. 3d). In addition, the number of total colonies increased in stable PEAK1 transfected SW1736 cells in presence of vemurafenib (2.0 μM), but did not affect 8505C cells in presence of vemurafenib (2.0 μM) (Fig. 3e).

**PEAK1 inhibition improves cell response to vemurafenib-induced apoptosis**

8505C and SW1736 cells is relatively resistant to vemurafenib treatment by MTT and colony formation assay, we further investigated whether vemurafenib treatment induces PEAK1, which increases vemurafenib resistance in both cells. For these experiments, 8505C and SW1736 cells were exposure to 2.0 μM vemurafenib for 1h, 2h, 6h, 8h, 12h, 24h, 48h and 72h. PEAK1 protein was detected by Western blot assay. PEAK1 expression was gradually upregulated in SW1736 cells from 6h, and reached the peak at 12h and reached the basal level at 48h (Fig. 4a). PEAK1 expression was slightly enhanced from 6h-12h in 8505C cells (Fig. 4a). This data indicated that PEAK1 upregulation may be related to the acquired resistance to vemurafenib in 8505C and SW1736 cells.

**Targeting PEAK1 sensitizes both cells to vemurafenib by inhibiting cell viability and colony formation**

We next determined whether PEAK1 inhibition improves vemurafenib-induced apoptosis. 8505C and SW1736 cells were depleted of PEAK1 by siRNA treatment and allowed to be exposure to vemurafenib (2.0μM) for 72 h. We observed that vemurafenib treatment induced fewer apoptotic cells in 8505C and SW1736 cells, but PEAK1 inhibition markedly enhanced vemurafenib-induced cell apoptosis in 8505C and SW1736 cells evaluated by flow cytometric analysis (Fig. 4b). These data suggest that targeting PEAK1 sensitizes BRAF-mutated cells to vemurafenib treatment by inducing cell apoptosis.

**PEAK1 deletion is critical for vemurafenib-induced apoptosis by upregulating PUMA and Bim**

Vemurafenib upregulated PUMA and Bim, which contributed to vemurafenib-induced apoptosis in BRAFV600E mutant melanoma cells [13]. In our study, no significant affect was found in Bim and
PUMA protein expression induced by vemurafenib (2.0 μM) for 72 h in 8505C cells (Fig. 5a). In the SW1736 cells, PUMA and Bim protein gradually enhanced at 2 h-12 h (Fig. 5b). Then, both of the protein of PUMA and Bim decreased (Fig. 5b). PEAK1 deletion alone slightly upregulated PUMA and Bim expression in 8505C cells (Fig. 5c), but not in SW1736 cells (Fig. 5d). Transfection with PEAK1 siRNA significantly promoted vemurafenib-induced PUMA protein and Bim protein expression in both cells, and reached a maximum at 48 h and maintained until 72 h (Fig. 5e-5f). PEAK1 deletion alone and vemurafenib (2.0 μM) alone has less extent of an effect on cell apoptosis in both of the cells. And combined PEAK1 deletion and vemurafenib systematically increased the pro-apoptotic effect. We therefore determined whether PUMA and Bim upregulation was related to the systematic effect. Transfection with PUMA siRNA or Bim siRNA significantly inhibited the upregulation of PUMA and Bim in both of the cells with combined PEAK1 deletion and vemurafenib (2.0 μM) treatment for 72 h (Fig. 5g). We next examined the effects of the deletion of PUMA or Bim alone or the combined deletion on cell apoptosis. Deletion of PUMA or Bim alone partly attenuated PEAK1 deletion in combination with vemurafenib (2.0 μM) induced cell apoptosis in both of the cells by flow cytometric assay (Fig. 5h). However, deletion of PUMA and Bim together much significantly inhibited PEAK1 deletion/vemurafenib (2.0 μM) induced cell apoptosis in both of the cells by flow cytometric assay (Fig. 5h).

**Vemurafenib re-activates ERK phosphorylation in vitro**

Since these data suggest that 8505C and SW1736 cells are not fully responsive to vemurafenib, the signal pathway of vemurafenib resistance was further evaluated. Vemurafenib is a potent inhibitor of MAPK signaling, by which triggers therapeutic responses in BRAF V600E mutant melanoma [43] and thyroid cancer cells [44, 12], but not of cancer cell lines with wild-type BRAF [12]. Transient inhibition of ERK1/2 pathway by vemurafenib was reported in several cancers, resistance develops invariably because of the ERK1/2 pathway re-activation [28, 43, 8, 10, 45]. In the present study, 8505C and SW1736 cells were exposure to 2 μM vemurafenib for 72 h. The phospho-ERK1/2 (pERK1/2) activities in 8505C and SW1736 cells were examined by western blotting. As shown in Fig. 6a, pERK1/2 activity was completely blocked after vemurafenib exposure for 4 h in 8505C cells, then

![Figure 4. Targeting PEAK1 improves vemurafenib-induced apoptosis.](https://www.medsci.org)
elevated gradually from 6 h and reached the higher level in 24 h and maintained the levels until 72 h. In SW1736 cells, 2 μM vemurafenib exposure for 72 h, pERK1/2 activity was slightly elevated after 24 h exposure, although pERK1/2 activity was almost completely inhibited 24 h before (data not shown). This incomplete suppression of ERK may underlie the relative insensitivity of 8505C and SW1736 cells to vemurafenib. The reactivation in ERK seen after vemurafenib treatment likely contributes to the resistance of 8505C and SW1736 cells to vemurafenib.

PEAK1 deletion activates JNK1/2 and JNK1/2-dependent Bim and partly sensitizes ATC cells to vemurafenib

The c-Jun N-terminal kinases (JNKs) have three isoforms: JNK1, -2, and -3. They are the subfamily of (MAPK) mitogen-activated protein kinases [46]. JNK plays paradoxical roles in cell survival and apoptosis. One of the effects is that activation of JNK promotes cell apoptosis. Our results showed that vemurafenib (2 μM) transiently activated JNK1/2 signals in both cells, then JNK1/2 activity gradually recovered to the basal level in 8505C (Fig. 6a) and SW1736 cells (data not shown). PEAK1 deletion continuously activated and maintained JNK1/2 phosphorylation at a high level in the presence of vemurafenib in 8505C cells (Fig. 6b) and SW1736 cells (data not shown). However, the pretreatment with 10 μM JNK1/2 inhibitor SP600125 followed by vemurafenib treatment completely inhibited in PEAK1 deletion-induced JNK1/2 phosphorylation and Bim in 8505C cells (Fig. 6c) and SW1736 cells (data not shown). Furthermore, pretreatment with 30 μM of PD98059 followed by vemurafenib treatment inhibited ERK signal and induced JNK1/2 phosphorylation and Bim in 8505C cells (Fig. 6d) and SW1736 cells (data not shown). P53 is also regulated by JNK in apoptosis [47], PUMA was regulated by p53. We observed the change of PUMA in presence of vemurafenib or/and PEAK1 deletion. Vin et al. reported that PUMA expression was unchanged by vemurafenib exposure [32]. In the present study, the pretreatment with 10 μM JNK1/2 inhibitor SP600125 followed by vemurafenib treatment in the PEAK1 deletion cells did not affect PUMA in 8505C cells (Fig. 6c) and SW1736 cells (data not shown), but downregulated Bim expression in 8505C cells (Fig. 6c) and SW1736 cells (data not shown).
cells (data not shown), although vemurafenib treatment in PEAK1 deletion cells significantly upregulated PUMA expression in 8505C cells (Fig. 6b) and SW1736 cells (data not shown). Vemurafenib treatment in the presence of PD98059 also did not affect PUMA expression, but upregulated Bim expression in 8505C cells (Fig. 6d) and SW1736 cells (data not shown). In addition, P53 was not affected with PEAK1 deletion or/and in presence of vemurafenib exposure in 8505C cells (Fig. 6a-b) and SW1736 cells (data not shown). Therefore, PUMA upregulation is P53-independent. This data indicated that PEAK1 deletion activates JNK1/2-dependent Bim through inactivation of ERK, but PEAK1 deletion inactivates ERK and ERK-independent PUMA expression.

**Vemurafenib transiently induces AKT phosphorylation by vemurafenib in vitro**

It has reported that reactivation of PI3K/AKT pathway following vemurafenib treatment was as the key mechanism to mediate the resistance to BRAF inhibition in ATC cells [21]. In addition, PI3K/AKT itself is hyperphosphorylated in PTC and ATC cells, leading to the resistance to BRAF inhibition [21, 23]. We detected AKT phosphorylation in 8505C and SW1736 cells, both of which exhibited significant levels of AKT phosphorylation (Fig. 7a-b). Byeon et al. [21] reported that vemurafenib treatment for 6 h increased the p-AKT levels in 8505C cells, but p-AKT was unchanged in SW1736 cells. Montero-Conde et al [22] reported that, in 8505C cells, vemurafenib (2.0 μM) treatment for 48 h induced AKT activation in a time-dependent way and reached the peak at 8 h and restored to the lowest level at 24 h. In SW1736 cells, vemurafenib (2.0 μM) treatment induced AKT activation and reached the peak at 10 h and restored to the basal level at 48 h. In our study, we treated 8505C and SW1736 cells with vemurafenib (2.0μM) for 72 h in both 8505C and SW1736 cells and also observed the robust activation of AKT in early time (Fig. 7a-b), the results of which is similar to previous research results [22]. These data indicated AKT could be transiently and early activated by vemurafenib treatment in ATC cells. Extending action time beyond 48 h of VEMURAFENIB has no effect on the reactivation of AKT.

![Figure 6. PEAK1 deletion activates JNK1/2 and JNK1/2-dependent Bim through inactivation of ERK signal. a, 8505C cells were treated with vemurafenib (2.0 μM) for 72 h, the different protein expression was detected by western blot assay; b, the PEAK1 siRNA transfected 8505C cells were treated with vemurafenib (2.0 μM) for 72 h, the different protein expression was detected by western blot assay; c, the PEAK1 siRNA transfected 8505C cells were co-treated with vemurafenib (2.0 μM) and 10 μM JNK1/2 inhibitor SP600125, the different protein expression was detected by western blot assay; d, 8505C cells were treated with 30 μM of PD98059 followed by vemurafenib treatment, the different protein expression was detected by western blot assay. *P<0.05; **P<0.01.](https://www.medsci.org)
PEAK1 deletion inactivates AKT and activates PUMA and partly sensitizes ATC cells to vemurafenib

PUMA is a key downstream apoptotic effector that mediate PI3K-AKT inhibition-induced cell death [23,48]. Shao et al. [49] reported that BH3-only protein silencing contributes to acquired resistance to PLX4720 in human melanoma. PLX4720 and vemurafenib has reported to share structural features and have similar activities [13]. We found that vemurafenib treatment affected less PUMA expression in 8505C and SW1736 cells. We first determined whether inactivation of the re-activated AKT signal in presence of vemurafenib exposure upregulated PUMA expression. 8505C and SW1736 cells were treated with MK2206 at 1 μm for 24 h for inhibition of phosphorylated (p)-Akt, then exposure to vemurafenib for 72 h. We found that pAKT was almost completely inhibited and PUMA protein was enhanced in presence of VEMURAFENIB in both of the cells (Fig. 7c-7d). In addition, almost complete suppression of AKT signal in presence of vemurafenib exposure in 8505C and SW1736 cells transfected with siRNA directed against PEAK1 followed by significant upregulation of PUMA (Fig. 7c-7d), suggesting that PEAK1 deletion was AKT-dependend in presence of vemurafenib exposure in 8505C and SW1736 cells.

PEAK1 was also activated phosphorylation of Stat3 at Y705 in breast cancer [35]. We detected phosphorylation of Stat3 in the PEAK1-down-expressing 8505C and SW1736 cells. The results showed that PEAK1 detection or overexpression did not affect phosphorylation of Stat3 in both of the cells (data not shown).

Discussion

Gene mutations, gene amplifications, gene translocations and altered signalling pathways are closely related to the occurrence and progression of thyroid cancer [50]. Aberrant MAPK kinase driven by the mutant BRAFV600E and activation of PI3K/AKT pathways plays an important role in thyroid tumorigenesis. These pathways often in close connection and cooperation constitute the primary oncogenic mechanism that promotes the development and progression of thyroid cancer [50]. BRAFV600E mutation is a frequent event in ATC, with the 20-25% mutation rate. Vemurafenib is highly selective inhibitors of BRAFV600E to block the MAPK pathway tested in clinical trials for BRAFV600E-positive cancers with promising results, but primary resistance and development of secondary resistance to vemurafenib resulted in only a transient initial response [43], the reasons of which for this disparity remains unclear. Several studies have tried to unravel the molecular basis of chemoresistance to vemurafenib. Interest has centered on the role of reactivation of cell proliferation and anti-apoptotic pathways [12,27,27, 51]. However, the precise mechanism involved...
vemurafenib resistance by apoptotic signaling inhibition needs to be elucidated for ATC.

PEAK1 is overexpressed in many human malignancies [35-38]. Previously, enhanced PEAP1 expression was reported to promote tumor cell proliferation and invasiveness in pancreatic ductal epithelial cell [39], HMVECs and HUVECs [40] and MEFs [52]. In our study, we used 8505C and SW1736 cells to investigate the role of PEAP1 in vitro and in vivo. The results demonstrated that enforced PEAP1 expression enhanced growth in SW1736 cells with low basal PEAP1 expression in vitro, but no affect was observed in 8505C cells with high basal PEAP1 expression. Whereas targeting PEAP1 expression inhibited cell growth and reduced size of individual colonies in 8505C cells in vitro, but no affect was observed in SW1736 cells in vitro. We further studied the mechanism of targeting PEAP1 on cell growth inhibition. We observed that targeting PEAP1 did not significantly affect cell apoptosis in both cells, but reduced cell cycle progression in 8505C cells, indicating that targeting PEAP1 expression inhibited cell growth through reducing cell cycle progression.

PEAK1 overexpression has reported to be related with collagen-mediated chemoresistance in pancreatic cancer cells [41]. Kelber et al. [53] and Fujimura et al. [42] reported that targeting PEAK1 sensitized the PDAC cells to the current first-line chemotherapy gemcitabine, and gemcitabine treatment robustly increased PEAK1 protein expression in resistant PDAC cells, but not sensitive PDAC cells [42], suggesting that required PEAK1 expression may be related to gemcitabine resistance. In our study, high PEAP1 expression was observed in 8505C cells, which was resistant to vemurafenib treatment, and low PEAP1 expression was observed in SW1736 cells, which was sensitive to vemurafenib treatment. Whereas targeting PEAP1 restored sensitivity to vemurafenib in 8505C cells, and PEAP1 overexpression contributed to vemurafenib resistance in SW1736 cells. These data indicated that PEAK1 expression may have utility as a biomarker to identify vemurafenib sensitivity and PEAK1 may be as the target to reduce vemurafenib resistance in ATC cells. Importantly, targeting PEAP1 expression also increased the vemurafenib sensitivity in SW1736 cells with low PEAP1 expression. We found that vemurafenib treatment increased PEAK1 protein expression in SW1736 cells, but not in 8505C cells, which was contrary to the previous report [42]. Our study indicated that PEAK1 upregulation with vemurafenib treatment rendered sensitive SW1736 cells to vemurafenib resistance.

While we demonstrated that targeting PEAP1 expression reduced vemurafenib resistance, and vice versa, however the underlying mechanisms is unknown. In our study, targeting PEAK1 alone or vemurafenib alone inhibiting cell viability and colony formation, but not induce apoptosis, however the combined PEAK1 inhibition and vemurafenib markedly enhanced cell apoptosis in 8505C and SW1736 cells, suggesting that targeting PEAK1 sensitizes ATC cells to vemurafenib treatment by inducing cell apoptosis. The proapoptotic BH3-only Bim and Pum have play a key role in the control of apoptosis and in the initiation of the apoptotic pathways [54, 55]. In the present study in vitro, targeting PEAK1 alone or vemurafenib treatment alone did not significantly upregulate PUMA and Bim expression in both of the cells. But the combined PEAK1 inhibition and vemurafenib markedly upregulated PUMA and Bim expression in both of the cells. Our findings reported here demonstrate that targeting PUMA or Bim alone by siRNA transfection partly inhibited vemurafenib -induced cell apoptosis in presence of PEAK1 inhibition in vitro, respectively. Combined PUMA or Bim depletion significantly inhibited vemurafenib -induced cell apoptosis in presence of PEAK1 inhibition in vitro. These results indicate that targeting PEAP1 sensitized ATC cells to vemurafenib partially through upregulation PUMA and Bim.

Re-activation of MEK/ERK signals have proven to cause the vemurafenib resistance in thyroid cancer cells after transient MEK/ERK signal inhibition by vemurafenib treatment [10-13]. However, MEK/ERK signal inhibition could overcome the resistance [14-16]. In addition, JNK can phosphorylate Bim and induce Bax:Bax interactions, resulting in apoptosis [56]. In the study, targeting PEAK1 inhibited ERK signal and activated JNK signal, resulting in Bim and PUMA upregulation in vitro. However, JNK signal inhibition reduced Bim expression, and PUMA was not affected. Therefore, targeting PEAK1 upregulated Bim expression through ERK/ JNK pathways, and upregulated PUMA expression through other pathways in ATC cells.

Elevated levels of endogenous pAKT or acquired pAKT will increase the resistance of malignant tumor cells to chemotherapy [57-60]. pAKT affects cell apoptosis caused by chemotherapeutic drugs by regulating cell apoptosis signals and affects the sensitivity of cells to chemotherapeutic drugs [61-62]. Previous study reported that targeting PI3K/AKT signals impedes the growth of colorectal cancer cells by inducing apoptosis via PUMA independent of p53 [63], whereas targeting Puma led to an increased drug resistance in leukemic cells [64]. In our study, vemurafenib treatment activated AKT in ATC cells, the AKT specific inhibitor MK2206 can inhibit AKT,
resulting in AKT-dependent PUMA upregulation and increasing vemurafenib-inducing cell apoptosis in vitro. However, MK2206 treatment did not affect Bim expression, suggesting that targeting AKT sensitized ATC cells to vemurafenib by PUMA-dependent signal. We further observed that PEAK1 depletion inhibited activated AKT and ERK-independent PUMA in presence of vemurafenib in vitro. We therefore concluded that PEAK1 regulated ERK-JNK dependent Bim and AKT-dependent PUMA signal to affect vemurafenib-induced cell apoptosis. However, we do not rule out the possibility that PEAK1 regulates other pathways to affect vemurafenib resistance/sensitivity in ATC cells.

In summary, our study demonstrates that reactivation of ERK signal and activation of AKT signal, resulting in the inhibition of pro-apoptotic Bim and PUMA signal, is the mechanism of vemurafenib resistance. PEAK1 overexpression or PEAK1 upregulation with vemurafenib treatment also rendered ATC cells to vemurafenib resistance. Our results confirm that targeting PEAK1 can inhibit ERK reactivation and AKT activation and upregulate JNK-dependent Bim expression and AKT-dependent PUMA expression to neutralize vemurafenib resistance. Therefore, targeting PEAK1 using small molecule inhibitors is a new strategy to treat BRAF-mutated advanced thyroid cancer patients that are either primarily insensitive to vemurafenib or those have developed resistance against BRAF inhibition.

Competing Interests

The authors have declared that no competing interest exists.

References


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