Original Paper

Synergistic Antitumor Effect of BKM120 with Prima-1^{Met} Via Inhibiting PI3K/AKT/mTOR and CPSF4/hTERT Signaling and Reactivating Mutant P53

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Key Words

BKM120 • Prima-1^{Met} • Akt • CPSF4 • hTERT • P53 • Antitumor

Abstract

Background/Aims: PI3KCA and mutant p53 are associated with tumorigenesis and the development of cancers. NVP-BKM120, a selective pan-PI3K inhibitor, exerts the antitumor activity by suppressing the PI3K signaling pathway. Prima-1^{Met}, a low molecular weight compound, can rescue the gain-of-function of mutant p53 by restoring its transcriptional function. In this study, we investigated whether PI3K inhibition combined with mutant p53 reactivation could enhance the antitumor effect in thyroid cancer cells. **Methods:** The effects of BKM120 and Prima-1^{Met} on the proliferation, apoptosis, migration and invasion of thyroid cancer cells were measured by MTT, colony formation, flow cytometry, wound-healing and transwell assays, respectively. Thyroid differentiation was assessed by detecting the expression levels of specific markers using RT-PCR and Western blot. The *in vivo* antitumor efficacy was analyzed in a mouse xenograft model. **Results:** The combinational treatment of BKM120 and Prima-1^{Met} significantly enhanced the inhibitions of cell viability, colony formation, migration and invasion, and the induction of apoptosis in thyroid cell lines, and synergistically suppressed tumor xenograft growth by inhibiting the PI3K/Akt/mTOR and EMT signaling pathways, up-regulating p53 targeted genes, and triggering the release of cytochrome c. Moreover, the combination of BKM120 and Prima-1^{Met} suppressed the stem-like traits of thyroid cancer cells and promoted their differentiation by upregulating the expression of thyroid-specific differentiation markers and repressing the expression of cancer

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stem cell markers. Furthermore, the mechanism study demonstrated that the combinational treatment synergistically abrogated the binding of CPSF4 at the promoter of hTERT and thus suppressed hTERT expression. Consistently, overexpression of hTERT rescued the inhibitions of cell viability, invasion and stem-like traits mediated by the combination of BKM120 and Prima-1^{Met}. **Conclusion:** Our results showed that the combination of BKM120 with Prima-1^{Met} synergistically suppressed the growth of thyroid cancer cells and tumor xenografts via inhibiting PI3K/Akt/mTOR and CPSF4/hTERT signaling and reactivating mutant p53.

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Introduction

Thyroid cancer is the most common endocrine malignancy with a rapidly increasing incidence [1]. According to histopathological characteristics, thyroid cancer can be classified into differentiated thyroid cancer (DTC) that includes papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC), and anaplastic thyroid cancer (ATC) [2-4]. In general, well-differentiated thyroid cancers have excellent prognosis and 10-year survival rates of 80%-95%, though 2-5% of the patients may develop recurrences and distant metastases with poor prognosis. In contrast, ATC, the most aggressive and undifferentiated subtype of thyroid cancer, is fatal and has poor prognosis. The median survival time of ATC patients is around four months, due to its rapid course, invasion to neighboring organs, and distant metastasis to lungs and bones [5-7]. Moreover, ATC is always resistant to conventional therapies, and has no effective therapy available so far. Hence, there is an urgent need to develop effective therapy for thyroid cancer.

Deepened understanding of the molecular changes that characterize thyroid cancer has given new hopes for the development of effective strategies to treat thyroid cancer [3, 8, 9]. p53, as “the guardian of the genome”, has a critical role in maintaining the genetic integrity of proliferating cells and thereby preventing malignant transformation. However, growing evidence shows that mutant p53 has not only lost its tumor suppressor activity but also promoted invasion, angiogenesis and cell differentiation blockades in cancers. These activities are referred to as “mutant p53 gain-of-function” [10-13]. The phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway plays a fundamental role in cell proliferation, differentiation, metabolism, motility and treatment resistance [13, 14]. Constitutive activation of PI3K signaling has been identified as a critical step in cancer development and drug resistance. PI3KCA encodes the p110 α catalytic subunit of PI3K, and its activation by mutation and gene amplification is frequently detected in thyroid cancer [15]. Compared with DTC, ATC has more PI3K and p53 mutations, which indicates that the level of PI3KCA and mutant p53 are related with the differentiation status of thyroid cancer. Lots of studies have shown that the combination of p53 inactivation and PI3K activation result in the tumorigenesis and development of ATC [16, 17]. Therefore, mutant p53 and PI3K/AKT/mTOR might be good candidate targets for thyroid cancer therapies.

NVP-BKM120, a selective pan-PI3K inhibitor, executes its antitumor effect through the inhibition of PI3K downstream signaling in multiple types of cancer [18, 19]. Prima-1^{Met}, a low molecular weight compound, could rescue mutant p53 gain-of-function through restoring its transcriptional function [20]. In this study, we investigated whether PI3K inhibition by BKM120 combined with mutant p53 reactivation by Prima-1^{Met} could have enhanced antitumor effect on the proliferation, apoptosis, migration, and differentiation of thyroid cancer cells. We also explored the underlying molecular mechanisms of this combination through analyzing the expression of key proteins and regulatory factors of p53 and PI3K/AKT/mTOR pathways. In addition, as human telomerase reverse transcriptase (hTERT) promoter mutations frequently exist in thyroid cancers, we also examined if BKM120 and Prima-1^{Met} affected the transcription of hTERT. The findings from this study suggested that the combinational treatment with BKM120 and Prima-1^{Met} holds great promise for future thyroid cancer treatment.

Materials and Methods

Reagents and antibodies

Prima-1^{Met} was purchased from Santa Cruz, and NVP-BKM120 was kindly provided by Novartis Pharmaceuticals Corporation. For *in vitro* studies, all reagents were first dissolved in DMSO for stocks and then diluted with medium before adding to the cells. The antibodies of β -actin, GAPDH, cleaved PARP, CPSF4, Bax, Bcl-2, NIS, ABCG2, Nanog, CD133, CD44 and OCT4 were purchased from Proteintech. The antibody of hTERT was ordered from Millipore. The antibody of mutant p53 was obtained from Abcam. The antibodies of p110 α , p-110 β , p-Akt (Ser473), Akt, p-PDK1 (Ser241), p-PTEN, p-c-Raf (Ser 259), E-cadherin, Vimentin, β -catenin and N-cadherin were purchased from Cell Signaling Technology.

Cell lines and cell culture

Six human thyroid cell lines were used in this study. Anaplastic thyroid cancer cell THJ-16T, THJ-21T and THJ-29T, and normal thyroid cell Nthy-ori-3-1 were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS). Follicular thyroid cancer cell FTC-133 and papillary thyroid cancer cell BCPAP were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% FBS. All the cells were maintained in a humidified incubator with 5% CO₂ at 37°C.

Cell viability assay

Cell viability was measured by MTT assay. Briefly, the cells were plated in 96-well plates (2,000 cells/well) and treated the next day with drugs in different concentrations. 48 hours after treatment, MTT was added to the cells, and four hours later, the absorbance value was detected at the wavelength of 490 nm. Data were presented as the mean $\pm$ SD of three independent experiments.

Colony formation assay

FTC-133 and THJ-29T cells were plated in 6-well plates and then treated with the indicated concentration of Prima-1^{Met}, BKM120, their combination or DMSO control. 48 hours later, cells were harvested, counted, and single cells were seeded (1,000 cells/well) into 6-well plates. After continuous incubation at 37°C with 5% CO₂ for 12–14 days, the cells were washed with phosphate buffered saline (PBS) solution, fixed with methanol:glacial:acetic (1:1:8) for 10 minutes, and stained with 0.1% crystal violet for 30 minutes. The clones with more than 50 cells were counted under an optical microscope.

Apoptosis assay

Apoptosis of thyroid cancer cells was analyzed with Annexin V-FITC Apoptosis Detection Kit (Dojindo Molecular Technologies) according to manufacturer's instructions. Briefly, the cultured cells were trypsinized with 0.25% trypsin without EDTA, washed twice with cold PBS, and stained with Annexin V-FITC and PI solution in the dark at room temperature for 15 minutes. Stained cells were then subjected to flow cytometry analysis (BD Biosciences, San Jose, CA).

Confocal immunofluorescence assay

Cells were cultured on coverslips in 6-well plates and treated with drugs for the desired time. Afterward, the cells were fixed with 4% paraformaldehyde for 30 minutes, permeabilized with 0.2% Triton X-100 in PBS for 5 minutes, and blocked with blocking buffer (10% BSA) for one hour. Then, cells were incubated with the primary antibody against Cyto-c overnight at 4°C. After washing with PBS, cells were incubated with the fluorescein isothiocyanate- and rhodamine-conjugated secondary antibodies and DAPI at room temperature. The images were taken with Leica confocal microscope and processed with Image-Pro Plus 5.1 software.

Immunoblotting and antibodies

Proteins from cell and tissue lysates were separated in 10% SDS-PAGE and electrophoretically transferred to Polyvinylidene Fluoride (PVDF) membranes. Immunoreactive protein bands were detected by enhanced chemiluminescence.

AO/EB staining

Acridine orange/Ethidium bromide (AO/EB) staining was performed to estimate the extent of apoptosis morphologically. The cells were harvested and suspended in PBS, and then stained with 5 mL of a cocktail mix containing AO (100 mg/mL) and EB (100 mg/mL) in PBS. Cell suspensions were incubated for 5 minutes at room temperature before being examined by fluorescence microscopy. Early apoptotic cells had bright green dots in nuclei with chromatin condensation, while late apoptotic cells had orange condensed and often fragmented nuclei. Necrotic cells had orange or red nuclei without condensed chromatin.

Wound-healing and transwell migration assay

Wound-healing assay was carried out to detect cell migration. Briefly, cells were plated in 6-well plates, grown to 70-80% confluence and treated with drugs accordingly. The cell monolayers were scratched by sterile tips, and the wound gaps were photographed using a Leica DM 14000B microscope. The mean width of each scratch was measured using Image-Pro Plus 5.1 software.

For the transwell migration assay, 4×10^4 cells were seeded in the upper chambers coated with Matrigel (BD, Biosciences). After treatment, invasive cells on the lower membrane surface were fixed and stained with 0.1% crystal violet. Cells on the underside of the filter were examined by light microscope and counted using a microscope at high-power fields.

Sphere formation

THJ-21T and THJ-29T cells were seeded in an ultralow attachment plate (Corning, Corning, NY) and suspended in serum-free DMEM/F12 with $1 \times B27$ (Life Technologies), 20 ng/mL epidermal growth factor and 10 ng/mL basic fibroblast growth factor (both from BD biosciences, Bedford, MA). The medium containing growth factors was replaced every 3 days. After 14 days of culture, spheres (>25 cells) were counted using a light microscope.

RT-PCR

Total RNA was prepared from cultured cells using Trizol Reagent (TaKaRa Bio.) following the procedures suggested by the manufacturer. cDNA synthesis was performed using PrimeScriptTM RT-PCR Kit (TaKaRa). The PCR primers corresponding to NIS, Tg and GAPDH functional gene sequences were synthesized by TaKaRa. The PCR products were visualized under ultraviolet light and the band density was measured for quantitative analysis.

Plasmid vector and transfection

A fragment of the hTERT promoter (-378 to +60) was amplified by PCR and inserted between the SacI and SmaI sites of the luciferase reporter vector pGL3-Basic (Promega Corp., Madison, WI) to generate the hTERT promoter-driven luciferase plasmid pGL3-hTERT-400 [21]. The hTERT overexpression plasmid pcDNA3.1-hTERT and the control vector pcDNA3.1 were designed and synthesized by Cyagen (Cyagen Biosciences Inc., United States).

DNA-protein binding by pulldown assay

The nuclear proteins (400 µg) were mixed with biotinylated double-stranded hTERT promoter probes (4 µg), streptavidin agarose beads (50 µl) in 500 µL PBSI buffer containing 0.5 mM PMSF, 10 mM NaF, 25 mM b-glycerophosphate and rotated overnight at room temperature. The DNA-protein complexes were collected by centrifugation, washed with PBSI buffer, resuspended with SDS-PAGE loading buffer and boiled at 95 °C. The supernatant was analyzed by Western blot.

Chromatin Immunoprecipitation (ChIP)

The ChIP assay was done as described previously [22, 23]. Briefly, cells were fixed with 1% formaldehyde for 10 minutes and cross-linking was quenched with 0.125 M glycine for 10 minutes. Cells were washed with PBS twice, scraped and collected, and resuspended in SDS lysis buffer (1% SDS, 10 mM Tris-HCl, pH 8.0). Lysates were sonicated on ice three times for 10 seconds each. One third of the lysates were used as the input control, and the rest were incubated with non-immune rabbit IgG or CPSF4-specific antibody at 4 °C overnight. Immunoprecipitated complexes were pulled down by protein A/G plus agarose beads (Proteintech Group, Inc., Chicago, USA). The protein/DNA complexes were washed with elution buffer

(20% SDS, 1M NaHCO₃), and then reverse-crosslinked at 65 °C. DNA was extracted with phenol/chloroform, precipitated with ethanol, resuspended and subjected to PCR amplification using specific hTERT promoter primers (Forward: 5'-TGGCCCCCTCCCTCGGGTTAC-3'; Reverse: 5'-CCAGGGCTTCCCACGTGC GC-3').

In vivo tumor model and tissue processing

All animal experiments were performed according to the Agreement for the Care and Use of Laboratory Animals of the Dalian Medical University. Female nude mice (4-5 weeks old) were obtained from Dalian Medical University and maintained in SPF laboratory animal central. All animal maintenance and experiment procedures were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, and approved by Animal Care and Ethics Committee of Dalian Medical University. THJ-29T thyroid cells (4×10⁶ in 100 µL PBS) were injected subcutaneously into the left flank of each mouse. When the formed tumor reached 50 mm³ after cell inoculation, mice were randomly divided into groups of six. BKM120 was dissolved in 0.5% methylcellulose solution and administered via oral gavage at 30 mg/kg/day. Prima-1^{Met} was dissolved in sterile water for intraperitoneal administration and dosed at 50 mg/kg/day. Tumor volume and body weight were measured twice a week. Tumor volume was calculated as $V = 1/2 (\text{width}^2 \times \text{length})$. At the end of the experiments, mice were humanely sacrificed by euthanasia. A portion of the tumors were fixed with 10% formalin for immunohistochemical staining, while the remainder of the tumors were used to prepare tumor tissue lysates for Western blot analysis.

Histology and immunohistochemistry (IHC)

Tumors were fixed in formalin overnight before paraffin embedding. Small tissues were embedded in paraffin for sectioning, incised to 6 µm thick, and stained with hematoxylin and eosin (H&E). Immunohistochemistry (IHC) was performed using the DAB (3, 3'-diaminobenzidine) Kit (Origene, China).

Statistical analysis

All the experiments were carried out three times. Means and standard deviations were calculated from at least three measurements. GraphPad Prism software was used for all statistical analysis. Analysis of variance and Student's *t*-test were used to compare the values of the test and control samples. The statistic significance was indicated by **P*<0.05, ***P*<0.01, and ****P*<0.001.

Results

BKM120 and Prima-1^{Met} had a synergistic inhibitory effect on the proliferation of thyroid cancer cells

To determine the combinational effects of NVP-BKM120 (Fig. 1a) and Prima-1^{Met} on cell proliferation, MTT assay was used to determine the viability of thyroid cancer cells with varied differentiation degrees after drug treatment. As is shown in Fig. 1b, the combination treatment markedly enhanced the suppression of cell viability in a dose-dependent manner in thyroid cancer cells compared with Prima-1^{Met} treatment alone. Notably, compared with the thyroid cancer cell lines, human normal thyroid cells (Nthy-ori-3-1) were significantly less sensitive to the combined drug treatment, and their viability was obviously inhibited only at higher drug concentrations. To evaluate the effectiveness of BKM120 in enhancing the anti-proliferative activity of Prima-1^{Met}, we measured the IC₅₀ values of Prima-1^{Met} in these six cell lines treated with or without BKM120. The combined treatment with BKM120 and Prima-1^{Met} resulted in a remarkable reduction of the IC₅₀ values of Prima-1^{Met} and the synergistic effect was most apparent in the ATC cell line THJ-16T (Fig. 1c). We next chose two thyroid cancer cell lines (THJ-29T, FTC-133) for colony formation assay. Consistent with the MTT assay results, lower colony-forming ability was seen in cells treated with both BKM120 and Prima-1^{Met} when compared to the cells treated with BKM120 or Prima-1^{Met} alone (Fig. 1d).

Furthermore, we explored the underlying molecular mechanisms of the combination treatment. We found that treatment with both BKM120 and Prima-1^{Met} dramatically suppressed the phosphorylation of AKT (Ser473), PDK1 and c-Raf, inhibited the expression

Fig. 1. Effect of the combination treatment of NVP-BKM120 and Prima-1^{Met} on cell proliferation in thyroid cancer cells. (a) Chemical structure of NVP-BKM120 and Prima-1^{Met}. (b) Thyroid cancer cells (THJ-16T, THJ-21T, THJ-29T, FTC-133, BCPAP) and normal thyroid cells (Nthy-ori-3-1) were treated with the indicated doses of Prima-1^{Met} alone or in combination with BKM120 (1 μM) for 48 hours, and cell viability was determined by MTT assay. (c) The IC₅₀ values of Prima-1^{Met} were determined for cell viability inhibition in cells treated with Prima-1^{Met} alone or in combination with BKM120 (1 μM). The data were presented as mean ± SD of three independent experiments. *P<0.05 and **P<0.01, significant differences compared to the control groups. (d) Colony formation was shown for two thyroid cancer cell lines (THJ-29T, FTC-133) treated with BKM120 (1 μM), Prima-1^{Met} (30 μM) or the two in combination for 48 hours. (e) THJ-29T and FTC-133 cells were treated with BKM120 (1 μM), Prima-1^{Met} (30 μM) or the two in combination for 48 hours. The protein levels of p110α, p110β, total and phosphorylated Akt, p-PDK1, p-c-Raf, p-PTEN, and mutant p53 were analyzed by Western blot. β-actin served as the loading control. All the experiments were repeated 3 times, and the data were shown as mean ± SD. P-values were calculated by student's t-test.

of p110α, p110β and mutant p53, but had little effect on total AKT protein level (Fig. 1e). PTEN, the most important negative regulator of the PI3K/Akt/mTOR signaling pathway, had dramatically increased phosphorylation in the combinational treatment group (Fig. 1e). These data suggested that BKM120 and Prima-1^{Met} could synergistically attenuate the dominant negative effect of mutant p53 and inhibit the activation of the PI3K/AKT/mTOR pathway.

BKM120 and Prima-1^{Met} synergistically inhibited the migration and invasion of thyroid cancer cells

Over one-third of the ATC patients have extra thyroidal and/or nodal invasion at diagnosis and over 40% have distant metastases. Also, recurrences and distant metastases were the main causes of the failure of differentiated thyroid cancer therapy [24, 25]. To examine the synergistic effect of BKM120 and Prima-1^{Met} on cell migration and invasion, wound-healing assay and transwell assay were performed in THJ-29T and FTC-133 cells. Combination treatment with BKM120 and Prima-1^{Met} significantly and synergistically inhibited cell migration (Fig. 2a, 2c left) and invasion capability (Fig. 2b, 2c right) in both thyroid cancer cell lines compared to the single-agent treatments. Epithelial-mesenchymal transition (EMT) was a critical biological process in tumor invasion and metastasis in various

Fig. 2. Effect of the combination treatment of NVP-BKM120 and Prima-1^{Met} on migration and invasion of thyroid cancer cells. (a) Cell migration was analyzed by wound healing assay. THJ-29T and FTC-133 cells were grown to 70–80% confluency. The cell monolayers were wounded with a sterile pipette tip, and washed with medium to remove detached cells from the plates. Then the cells were left either untreated or treated with BKM120 (1 μ M), Prima-1^{Met} (30 μ M), or the two in combination. After 48 hours, the wound gap was observed and photographed. * P <0.05, significant difference between the BKM120 + Prima-1^{Met} treated group and the BKM120 or Prima-1^{Met} treated groups. (b) FTC-133 and THJ-29T cancer cells were subjected to Matrigel invasion assay and photographed (magnification 20 $\times$, scale bar 100 μ m). (c) Left: Migration capability of cells in (a) was calculated; Right: invasion capability of cells in (b) was calculation. The data were presented as mean $\pm$ SD of three independent experiments. *** P <0.005, significant difference between the treatment and control groups. (d) Protein levels of Vimentin, β -catenin, E-Cadherin and N-Cadherin were detected after treatment with BKM120 (1 μ M), Prima-1^{Met} (30 μ M) or the two in combination. * P <0.05, significant difference between treatment group and DMSO control group. ** P <0.05, significant difference between combination treatment group and single-agent treatment group. All the experiments were repeated 3 times, and the data were shown as mean $\pm$ SD.

malignancies including thyroid cancers [26–28]. We checked the levels of E-Cadherin, β -Catenin, N-Cadherin and Vimentin, the key proteins involved in EMT in the different treatment groups, and found that BKM120 and Prima-1^{Met} in combination could block the EMT process effectively (Fig. 2d). The results above collectively proved that combination treatment of BKM120 and Prima-1^{Met} strongly inhibited the migration and invasion of thyroid cancer cells.

BKM120 synergized with Prima-1^{Met} to induce apoptosis in thyroid cancer cells

We examined apoptosis induction by BKM120 and Prima-1^{Met} as single treatments and in combination by Annexin V-FITC/PI staining-based FACS analysis. As is shown in Fig. 3a, the combination treatment greatly enhanced cell apoptosis compared to single-agent treatment and control groups. Consistent with FACS analysis, acridine orange/ethidium bromide (AO/EB) staining showed that more early apoptotic cells (yellow), late apoptotic cells (orange), and even dead cells (red) were seen in the combinational treatment group (Fig. 3b). To explore whether the enhanced apoptosis was attributed to the activation of p53 and inhibition of PI3K signaling pathways, we detected the effect of BKM120 or Prima-1^{Met} alone, and in combination on the expression of p21, Bax, Bcl-2, and cleaved PARP in THJ-29T and FTC-133 cells. As expected, BKM120 and Prima-1^{Met} in combination markedly increased

Fig. 3. Effect of the combination treatment of NVP-BKM120 and Prima-1^{Met} on apoptosis of thyroid cancer cells. (a) THJ-29T and FTC-133 cells were treated with BKM120 (1 μ M) and Prima-1^{Met} (30 μ M). 48 hours after treatment, apoptosis was determined by FACS analysis and presented as percentages of apoptotic cells. (b) Acridine orange/ethidium bromide fluorescence staining was performed in THJ-29T and FTC-133 cells. (c) The levels of cleaved PARP, Bax, Bcl-2 and p21 proteins were analyzed by Western blot. * $P < 0.05$, significant difference between the BKM120/Prima-1^{Met}-treated group and the BKM120 or Prima-1^{Met}-treated group. (d) The release of cytochrome-c from the inter-mitochondrial space into the cytosol was analyzed by immunofluorescence analysis.

the expression of p21, Bax and cleaved PARP, but down-regulated the expression of Bcl-2 (Fig. 3c).

As the downstream effector of Bax activation, cytochrome-c (cyto-c) plays a crucial role in the caspase-dependent apoptosis. Immunofluorescence analysis was performed to monitor the subcellular localization of cyto-c in thyroid cancer cells. Compared with the single agent treatment groups, the combination of BKM120 and Prima-1^{Met} triggered more cyto-c released from mitochondria to cytosol (Fig. 3d). These results demonstrated that BKM120 and Prima-1^{Met} synergistically enhanced the p53/cytochrome-c/caspase-mediated apoptosis signaling pathway.

BKM120 synergized with Prima-1^{Met} to suppress the stem-like traits and increase the expression of thyroid-specific differentiation markers in thyroid cancer cells

Amplification of PI3KCA and mutation of p53 occur more frequently in poorly differentiated thyroid cancers than in differentiated thyroid cancers. Therefore, we tested the synergistic effect of BKM120 and Prima-1^{Met} on the expression of some cancer stem cell (CSC) specific markers as well as the thyroid differentiation specific markers, NIS and Tg, in ATC cell lines THJ-29T and THJ-21T [29]. As is shown in Fig. 4a, combination treatment with BKM120 and Prima-1^{Met} dramatically inhibited sphere formation in THJ-29T and THJ-21T cells. Sphere formation efficiency (SFE) of cells treated with both agents was significantly lower, with about 43% decrease in THJ-29T cells and 32% decrease in THJ-21T cells (Fig. 4b). Next, we measured the expression of several recognized CSC markers including CD133, CD44, OCT4, Nanog, and ABCG2. The combination treatment groups had decreased expression of these CSC markers compared with the single-agent treatment groups in both ATC cell lines (Fig. 4c). RT-PCR was used to examine the transcription of the thyroid-specific markers NIS and Tg. Consistent with the results above, combinational use of BKM120 and Prima-1^{Met} effectively increased transcription and expression of the thyroid-specific markers

Fig. 4. Effect of the combination treatment of NVP-BKM120 and Prima-1^{Met} on stem-like traits of thyroid cancer cells and the expression of thyroid-specific differentiation markers. (a) THJ-29T and THJ-21T cells were grown in 3D, treated with drugs as indicated for 14 days, and quantitated for structural integrity. (b) Sphere formation efficiency of the cells in (a) was calculated. (c) Protein level of CD44, CD133, OCT4, ABCG2 and Nanog were determined in THJ-29T and THJ-21T cells treated with BKM120, Prima-1^{Met} or the two in combination. (d) mRNA level of NIS and Tg were determined in THJ-29T and THJ-21T cells treated with BKM120, Prima-1^{Met} or the two in combination. (e) The expression level of NIS in THJ-29T and THJ-21T cells treated with BKM120, Prima-1^{Met} or the two in combination were analyzed by Western blot.

(Fig. 4d, 4e). Taken together, these results indicated that inhibition of PI3K by BKM120 and restoration of p53 function by Prima-1^{Met} synergistically repressed stem-like traits and promoted re-differentiation in thyroid cancer cells.

BKM120 and Prima-1^{Met} synergistically regulated the transcription and expression of hTERT via CPSF4

According to the TCGA study, 51.3% of 117 cases of poorly differentiated and anaplastic thyroid cancer patients had mutations in hTERT promoter, which was the highest among various types of cancer (Fig. 5a). Studies have suggested that high prevalence of hTERT promoter mutation was associated with aggressiveness and metastatic potential of thyroid cancer [30, 31]. We then evaluated the synergetic effect of BKM120 and Prima-1^{Met} on hTERT transcription and protein expression. THJ-29T and THJ-21T cells were transfected with hTERT promoter-driven luciferase reporter plasmids. Luciferase reporter assay showed that BKM120 and Prima-1^{Met} synergistically suppressed hTERT promoter activity (Fig. 5b), and Western blot analysis showed that the combination treatment inhibited protein expression of hTERT (Fig. 5c). Based on our previous research, we conjectured that the combination treatment could influence the binding of cleavage and polyadenylation specific factor 4 (CPSF4) at hTERT promoter and thereby regulate the transcription and expression of hTERT [23, 32, 33]. Pulldown assay indicated that the binding of CPSF4 to the hTERT promoter was almost undetectable after combination treatment with BKM120 and Prima-1^{Met} (Fig. 5d). To further confirm that, ChIP assay was performed. As is shown in Fig. 5e, CPSF4 protein bound on the endogenous hTERT promoter weakly in the combination treatment group. These results indicated that combined use of BKM120 and Prima-1^{Met} inhibited the transcription and expression of hTERT via blocking the binding of CPSF4 on the hTERT promoter.

Fig. 5. Effect of the combination treatment of NVP-BKM120 and Prima-1^{Met} on the CPSF4/hTERT signaling. (a) The data were downloaded from TCGA database. (b) THJ-29T and THJ-21T cells were treated with BKM120, Prima-1^{Met} or the two in combination after transfection with hTERT promoter-driven luciferase plasmids. The luciferase activity was detected by luciferase reporter assay kit. The data were presented as the mean $\pm$ SD of three independent experiments. *** $P < 0.005$, significant difference between treatment group and DMSO control group. (c) Protein level of hTERT and CPSF4 were determined in THJ-29T and THJ-21T cells treated with BKM120, Prima-1^{Met} or the two in combination. (d) 48 hours after treatment, the binding of CPSF4 to the hTERT promoter probe was analyzed by a streptavidin-agarose pull down assay in THJ-29T and THJ-21T cells. (e) 48 hours after treatment, chromatin in cells was immunoprecipitated with antibodies against CPSF4. The hTERT promoter region in the precipitated chromatin was amplified by PCR in THJ-29T and THJ-21T cells.

The synergetic effect of BKM120 and Prima-1^{Met} was weakened when hTERT was overexpressed

To further validate the role of hTERT in thyroid cancer proliferation, invasion and differentiation, we rescued the expression of hTERT using an hTERT overexpressing plasmid. As is shown in Fig. 6a, the proliferation of THJ-29T and THJ-21T cells was partially rescued by the ectopic expression of hTERT. Consistently, the invasion and sphere formation ability of the cells were enhanced in the ectopic hTERT expression groups (Fig. 6b, 6c and 6d). These results indicated that the expression of hTERT protein could reverse the synergetic effect of p53 reactivation and PI3K inhibition, and promote the proliferation, invasion, and stem-like traits of thyroid cancer cells. These findings suggested that hTERT was associated with drug resistance and played an important role in the development and progression of thyroid cancer.

BKM120 and Prima-1^{Met} synergistically inhibited tumor growth in xenograft mouse models

We also evaluated the effect of combination treatment with BKM120 and Prima-1^{Met} on tumor growth *in vivo* in immunodeficient BALB/c mice. The combinational therapy dramatically suppressed anaplastic thyroid tumor growth in size, volume and weight by comparison to the control and single-agent groups (Fig. 7a, 7b and 7c). Notably, the combination treatment had little effect on mice body weight. In addition, H&E staining showed that the BKM120 and Prima-1^{Met} co-treated tumor cells had large and more deformed nuclei with high nucleocytoplasmic ratio (Fig. 7d). Consistently, Ki67 staining displayed that cells co-treated with BKM120 and Prima-1^{Met} had remarkably decreased proliferative indexes (Fig. 7e). To explore the inhibition of signaling pathways by combination treatment

Fig. 6. Effect of hTERT overexpression on synergistic effect of BKM120 and Prima-1^{Met} in thyroid cancer cells. (a) MTT assay was performed in THJ-29T and THJ-21T cells treated with BKM120 (1 μ M) and Prima-1^{Met} (30 μ M) after transfection with hTERT overexpression or lac Z control plasmid. (b) Invasion assay was performed in THJ-29T and THJ-21T cells treated with BKM120 (1 μ M) and Prima-1^{Met} (30 μ M) after transfection with hTERT overexpression or lac Z control plasmid. (c) THJ-29T and THJ-21T cells were grown in 3D model and treated with BKM120 (1 μ M) and Prima-1^{Met} (30 μ M) after transfection with hTERT overexpression or lac Z plasmids. (d) The sphere formation efficiency of the cells in (c) was calculated.

Fig. 7. Effect of the combination treatment of NVP-BKM120 and Prima-1^{Met} on tumor growth in xenograft mouse models. An orthotopic mouse model of human thyroid cancer with THJ-29T cells was used to evaluate the effect of the combination of BKM120 (30 mg/kg/day) and Prima-1^{Met} (50 mg/kg/day). The xenografts were harvested 18 days after treatment, and the pictures of the xenografts were obtained (a). Tumor volume (b) and tumor weight (c) were measured. (d) H&E staining. (e) Immunohistochemical analysis of Ki67, p-AKT, CD133, h-TERT, E-cadherin and p-PTEN protein expression in tumor samples. (f) Protein level of p-AKT, CD133, hTERT, p110 α , E-cadherin and p-PTEN from tumor xenografts were determined.

of BKM120 and Prima-1^{Met}, we analyzed the xenograft tumors by immunohistochemical assay. Consistent with *in vitro* study, expression of p-Akt, CD133 and hTERT was markedly reduced, while E-Cadherin and p-PTEN had increased expression (Fig. 7e). Furthermore, Western blot of the cell lysates from the xenograft tumors supported such changes in the expression of these proteins (Fig. 7f). All these results proved that p53 activation and PI3K inhibition synergistically exerted antitumor effects in thyroid cancer, which were at least in part mediated by regulating hTERT expression.

Discussion

Overactivation of PI3K signaling plays a crucial role in the development, progression and dedifferentiation of thyroid cancer. Mutation of p53 protein occurs with higher frequency in the more aggressive forms of thyroid cancer, and ATC has the highest frequency of p53 mutation. Previous studies have reported that PI3K activation and p53 loss of function mutation in combination accelerated the development of drug resistance and the deterioration of thyroid cancer [34, 35]. Prima-1^{Met} could reactive mutant p53 through covalent binding to the core domain and pharmacological reactivation of mutant p53, thereby activating multiple signaling pathways [36, 37]. BKM120 is a potent pan-PI3K inhibitor causing suppression of PI3K downstream signaling. However, single-agent treatments might not be effective enough [38], and combinational treatment of BKM120 and Prima-1^{Met} might be a more effective strategy. To our knowledge, this is the first study to report the combinational effects of BKM120 and Prima-1^{Met} on thyroid cancer and to reveal the potential underlying mechanisms.

In our study, BKM120 and Prima-1^{Met} synergistically inhibited the proliferation, migration and invasion, and induced apoptosis and redifferentiation in thyroid cancer cells. The enhanced inhibition of p-AKT and the decrease of mutant p53 in cells co-treated by the two drugs were observed. Of note, PTEN, the most important negative regulator of the PI3K/Akt/mTOR signaling pathway, had remarkably increased phosphorylation in the combinational treatment group. According to previous researches, although FTC-133 cells have one PTEN allele deleted and a splice variant in the remaining allele, it still expresses PTEN at comparatively low levels [39], and it still had elevated phosphorylation under co-treatment with BKM120 and Prima-1^{Met} compared to single drug treatment alone in our study. Our results suggested improved inactivation of PI3K/Akt signaling pathway in thyroid cancer cells under synergized effect of BKM120 and Prima-1^{Met}. It deserves our further investigation whether reactivation of mutant p53 and inhibition of PI3K pathway could induce the PTEN gene repair or enhance the function of the remaining PTEN gene through some complex mechanisms. Consistent with the *in vitro* data, the combined treatment with BKM120 and Prima-1^{Met} significantly enhanced the inhibition of tumor growth in xenograft mouse models. The effective therapeutic strategy by simultaneously targeting PI3K/AKT and p53 signal pathways, and the discovery of their related key signaling molecules are expected to provide important clinical implications in thyroid cancer treatment.

The differentiation degree of thyroid cancer is closely related to its sensitivity to anti-cancer drugs. The dedifferentiated thyroid cancer tends to be significantly more aggressive and associated with worse prognosis. In comparison with the differentiated thyroid cancer and normal thyroid cells, anaplastic thyroid cancer cells show significantly decreased expression of thyroid specific functional proteins such as sodium iodide transporter (NIS) and thyroid globulin (Tg). Accordingly, as is the case in other cancers, certain cells in thyroid cancer tissues also have stem-like properties [40-42]. The combined treatment of BKM120 and Prima-1^{Met} *in vitro* and *in vivo* obviously inhibited the stem-like traits of thyroid cancer cells and induced their differentiation. It has been previously reported that inhibition of PI3K/AKT/mTOR signaling pathway or reactivation of mutant p53 inhibited the stemness and induced differentiation in cancer cells. Here, our study suggested that simultaneous inhibition of PI3K/AKT/mTOR signaling and reactivation of mutant p53 led to more potent

stemness blockade and differentiation induction in thyroid cancer cells, which resulted in suppression of tumor growth and metastasis. However, it is unclear which pathway is more dominant during the whole progress, and whether they take place in parallel or successively. All of these questions deserve further study in the future.

hTERT activation, a hallmark of tumor aggressiveness clinically, is considered to be correlated with distant metastases, treatment resistance, and poor prognosis. Its activation seems to be more frequent in thyroid cancer types with poor differentiation, suggesting that telomerase activation may contribute to (or be associated with) more aggressive behavior of thyroid cancer [43]. Given the regulation of hTERT expression by p53 and other mechanisms [44, 45], we further examined the synergistic effect of p53 reactivation and PI3K/Akt pathway inhibition on hTERT expression. Our results not only showed that BKM120 and Prima-1^{Met} synergistically down-regulated hTERT promoter activity and its expression, but also demonstrated such down-regulation was mediated by decreased CPSF4 expression and its binding to the promoter region of hTERT. Additionally, rescue experiment was conducted and overexpression of hTERT was found to reverse the synergetic inhibitory effect of BKM120 and Prima-1^{Met} on cell proliferation, invasion and sphere formation, suggesting again the key role of CPSF4/hTERT signaling in sensitizing thyroid cancer cells to the co-treatment of BKM120 and Prima-1^{Met}.

Conclusion

In summary, we found that PI3K inhibition by BKM120 and mutant p53 reactivation by Prima-1^{Met} had synergetic antitumor effect. Furthermore, we elucidated that the combined effects of BKM120 and Prima-1^{Met} on thyroid cancer cells might be achieved through activating cytochrome-c and apoptotic signaling, and inhibiting PI3K/Akt/mTOR and CPSF4/hTERT pathways (Fig. 8). Our data indicated that the combinational use BKM120 and Prima-1^{Met} has the potential to become a novel approach for thyroid cancer treatment.

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Disclosure Statement

The authors declare to have no conflicts of interests.

Fig. 8. Synergistic antitumor-effect of BKM120 and Prima-1^{Met} in thyroid cancer by targeting multiple signaling pathways. BKM120 in combination with Prima-1^{Met} significantly enhanced the inhibition of proliferation and induced more apoptosis by inactivating PI3K signaling and triggering cytochrome-c release. Combination with Prima-1^{Met} and BKM120 inhibited cell invasion and migration by suppressed the expression of β-catenin. Such enhanced effect was also found in improving the inhibition of stem-like traits and differentiation promotion through several pathways. The combinational treatment synergistically enhanced the inhibition of hTERT expression.

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