European Review for Medical and Pharmacological Sciences 2016; 20: 2581-2587

Targeting TGF-β1 and AKT signal on growth and metastasis of anaplastic thyroid cancer cell *in vivo*

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Abstract. – OBJECTIVE: We have recently reported that therapies targeting TGF-β1 signaling were effective to prevent the anaplastic thyroid cancer (ATC) cell growth, but not the invasion. Phosphatidylinositol 3-kinase (PI3K)/AKT signaling are activated in ATC and play a major role in ATC invasion. Herein, we examined the effects of targeting TGF-β1 by shRNA in combination with pan-AKT inhibitor, MK-2206 on growth and metastasis of ATC xenografts implanted in severe combined immunodeficient mice.

MATERIALS AND METHODS: 8505C cells or 8505C/shRNA cells or 8505C/TGF-β1 shRNA cells were implanted sc in 5-week-old female nude mice. Upon establishment of palpable tumours, MK-2206 was administered at 60 mg/kg, orally, three times a week for 6 weeks.

RESULTS: The results showed that TGF-β1/shRNA alone only prevents anaplastic thyroid cancer (ATC) tumor formation, but not lung metastasis. MK-2206 alone only inhibits lung metas is, but not tumor formation. The combined ment with TGF-β1/shRNA and MK-2206 led an approximately 71% growth inhibition comp d with TGF-β1/shRNA (44%) and MK-2 (15%). combined treatment with TGF-β1 and 2206 significantly inhibits lung tasta s

CONCLUSIONS: These fi s trat ed that targeting TGF-β n c nation with MK-2206 was the effec method treatment of ATC.

Key Words:
Anaplastic th d cancer, D, Phosphatidylinositol 3-kinas 3K)/AKT.

Introduction

id carcinoma (ATC) is one of the m thal of all human malignancies. Although i an 2% of all thyroid cancer patients occurs, it contributes to (14-50)% of the deaths for thyroid cancer[1]. Because of its highly malignant, all the ATC patients are classified as having stage IV disease by the American Joint Committee on Cancer[2]. Rare morbidity and short survival time (median survival of 3 to 5 m de it difficult for the scientists to fi n effe d widely accepted methods for th tment o TC[3]. Given our poor ability co ATC gression with conventiona modalitie tigation of novel anti-proli tive i-met asis and gene therapies are bee treati this disease.

TGF-β ansfor wth factor-β) has three i TGF-β GF-β 2 and TGF-β3[3]. TGF-β signa nction through binding to TGF-β t H recepto t dimerize with TGF-β type eceptors and tivates the TGF-β dependent nonical si nal transducers SMADs[4]. TGF-β is ultifun nal cytokine. It is involved in the f cell proliferation, differentiation and survival/or apoptosis of many cells[5-6]. TGFβ1 is rexpressed in many cancers, and high TGF-β1 pression has a poor prognosis for these patients[7-9]. High expression of TGFβ1 was found to closely related with the occurrence of thyroid cancers[10]. We have recently found that knockdown of TGF-β1 by siRNAs transfection decreased proliferation and invasion, and increased apoptosis in ATC cells *in vitro*, but not prevented ATC primary tumor organ metastasis *in vivo*[11,12]. These data support the hypothesis that targeting a single constitutively activated signaling pathway is not sufficient for the treatment of ATC. An effective treatment strategy must take into account more than one deregulated signaling pathway. We, therefore, suggested that TGF-β1 inhibition in combination with other metastasis-targeted therapies may have a better effect.

The phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway plays a central role in the regulation of tumor cell proliferation, migration, survival and angio-

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genesis[13]. The PI3K/AKT pathway is frequently activated in thyroid cancer, and activated AKT is correlated with increased cell motility *in vitro* and metastasis *in vivo*[14-19]. In our previous study[11], we found AKT was activated in the anaplastic thyroid cancer 8505C cell line, and TGF-β1 did not affect the pAKT levels. We, therefore, suggested that knockdown of TGF-β1 combined with AKT inhibition would appear to be a promising strategy for the effective treatment of ATC.

MK-2206 is a selective, potent, oral allosteric inhibitor of all AKT isoforms with antitumor activity in preclinical models[20]. It inhibits the phosphorylation of Thr[308] and Ser [473] of Akt. *In vivo* and *in vitro*, MK-2206 or/ and in combination with other agents could markedly block tumor growth and metastasis[21,22]. Phase I study of the MK-2206 alone or in combination with other agents also shown significant tumor growth and metastasis inhibition[23-25].

In this study, we investigated whether targeting TGF-β1 in combination with MK-2206 has better therapeutic effects for ATC *in vivo*.

Materials and Methods

Cell Line and Culture

The human anaplastic thyroid cancer cell line 8505C was purchased from DSMZ (Beijing, China). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal calf serum (FCS), at 37°C in 5% carbon dioxide and 95% air.

Plasmids

Short chain oligonucleotides were designed according to the TGF-β1 mRNA sequence provided by Genebank. The two oligonucleotides were selected as: forward, 5′-GATCCTCGCTGCTACCTTCAAGAGAGGTAGCA GCGCTGCATTTTTGGAAA-3′; reverse, 3′-AGCTTTCGCAAAAATGCTGCTCGCTGCTGCTACCTCTCTTGAAGGTAGCGCGGCAGGG-5′. It was chemosynthesized by Sangon (Shanghai, China). It was ligated into the insert of two oligonucleotides above into the pGPU6 plasmid (which encodes GFP as reporter protein). The recombinant TGF-β1 shRNA expression vector was evaluated by using restriction enzyme digestion. A negative control plasmid had the same place using the following oligonucleotides: 5′-GCTACGCCTTCATACGTGCTTCAAACGGGCATGCGCCATAGTCTTTTTTGTCGACA-3′; reverse, 3′-GGCTAAGATTTCCGCGGACGAAGCCTTG CCGTACCCCGACGAAAAACAGCTG CGAGA-5′. The combined TGF-β1-shRNA plasmid was confirmed by digestion and gene sequencing. Plasmid pcDNA3.1 was as the control plasmid. For TGF-β1 shRNA transfection, 24 hours after shRNA or control shRNA transfection, the 8505C cells were split into 96-well plates and subjected to the G418 (1 mg/ml) selection for 3 weeks. The transcriptional silencing TGF-β1 was screened using Western blot as described below.

Orthotopic Model

Human anaplastic thyroid cancer cells 8505C were purchased DSMZ (Deutsche Sammlung von Mikroorganismen Zellkulturen GmbH). Its genetic characteristics have been described previously. All animal experiments were done in accordance with institutional guidelines for animal welfare. 8505C cells, 8505C/shRNA cells and 8505C/TGF-β1 shRNA cells were implanted sc (0.1 ml of a 5 × 10[6] cells/PBS) in 5-week-old female nude mice. After establishment of palpable tumours after 4 weeks, and treatment was started. MK-2206 (Selleck, Shanghai, China) was administered at 60 mg/kg three times a week for 6 weeks[21]. Tumor volumes were measured at least 3 times weekly. For lung metastasis assay (n = 5 for each group), mice were sacrificed, the lungs were fixed, paraffin-embedded, cut, and stained with H&E staining after six weeks. The primary tumors were divided into three portions for cell lysate production, and for making paraffin blocks for Ki-67 immunohistochemistry and TUNEL staining.

Western Blot Assay

Protocols were used as previously described[11]. Protein expression was quantified by densitometry relative to the loading control protein b-actin using ImageJ.

Ki67 Immunohistochemistry

The tissue section was deparaffinized, blocked and incubated with rabbit anti-human Ki67 antibody (1:200) for 1 h at room temperature. Then, the tissue section was stained and examined and photographed with a fluorescence microscope (Olympus BX51, Shinjuku, Japan). The average number of fluorescence dots of three images from each treatment group was calculated.

TUNEL Assay

Five serial sections (5 um thick) were obtained for each frozen tumor, mounted on glass slides,

and then fixed in 4% paraformaldehyde. TUNEL assay was performed on the sections using the ApopTag Red kit according to the manufacturer's instructions (Intergen Co., Shanghai, China). Tissue sections processed in the absence of terminal deoxynucleotidyl transferase served as negative controls. Slides were observed under a fluorescence microscope (Canon, Tokyo, Japan).

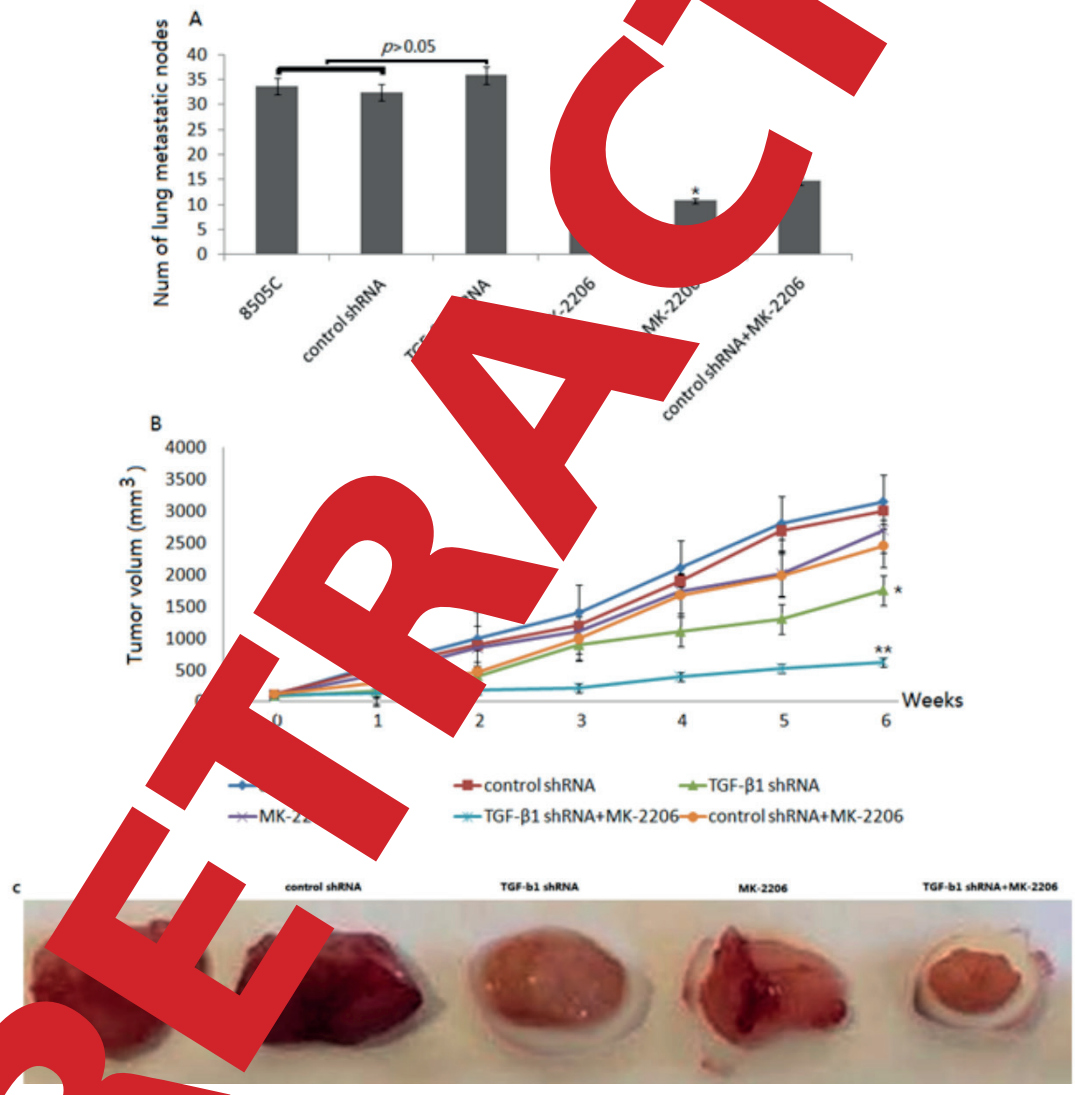
Statistical Analysis

Statistical analyses were performed with a two-tailed unpaired *t*-test. *p*-values <0.05 were considered to be statistically significant.

Results

Characterization of 85 Xenogra

All SCID mice deve palpable t rs (100%) in the s.c. after 4 w tion. ei- ght loss was observed mice b mors at the end of the riment. Lu astatic nodes were evide 3.6±3 (Figure A). The xenografts were fas owth tes, and the tumor volume r ±480) at the end of the experi ent (B-1 Phosphorylated Akt and F-β1was t much higher level by w rn blot assay re 2A). The im-



Fi Eff hRNA and MK-2206 on tumor growth and lung metastasis of 8505C cells *in vivo*. After 6 weeks of t mice were sacrificed and the tumors were isolated. The number of lung metastasis was determined by counting the metastatic nodules on the lung surface. A, The number of lung surface metastases formed by 8505C cells in each grou initiation and growth of tumors were determined by measuring the average tumor volume. C, Tumors in different grou e data were presented as the mean ± SD (n = 8). The control group represents normal mice without treatment. $^{*}p<0.05$; $p<0.01$, compared to the control group.

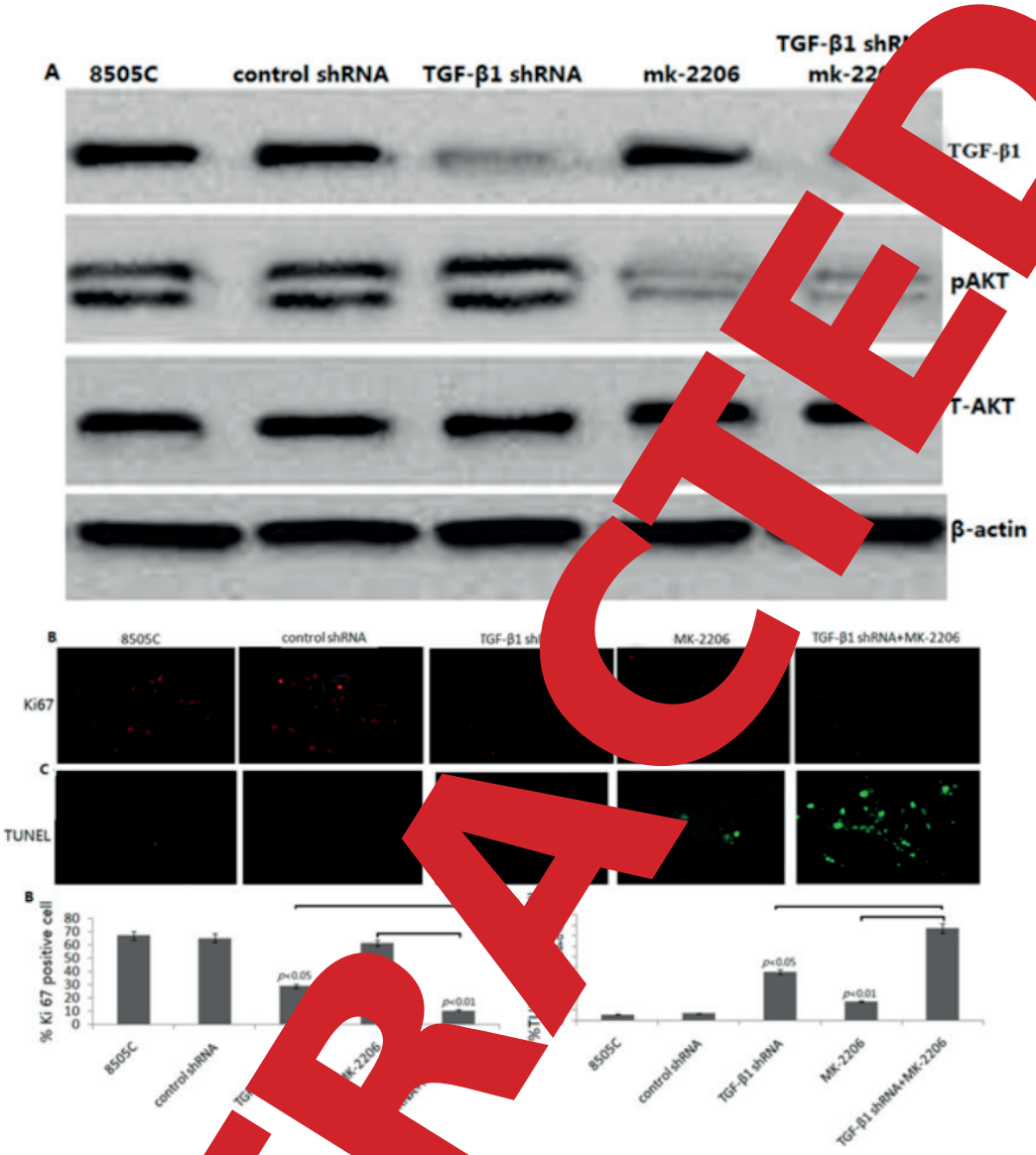


Figure 2. Effect of TGF-β[illegible]d MK-2206 on apoptosis and proliferation of 8505C cells in vivo. ***A,*** Western blot analysis showed the effect of d[illegible] the protein expression of TGF-β1, total AKT and pAKT. Ki67 immunohistochemical staining of the tumo[illegible]ues. T[illegible]howed the average number of fluorescence dots of images from each treatment group. Values are me[illegible]as ± SD of three p[illegible]hs. ***C,*** TUNEL staining of the tumor tissues. The graph showed the average number of fluoresce[illegible]ots of images from[illegible]n treatment group. Values are means ± SD of three photographs.

munohistoc[illegible]ing r[illegible]led high levels of Ki67 expre[illegible]ure 2[illegible]nd low TUNEL positive cells (Fig[illegible]

Ch[illegible]cter[illegible]TGF-β1 shRNA t[illegible]d X[illegible]g.

C[illegible]0%) mice developed palpable tumors in the s[illegible] weeks injection. Lung metastatic nodes we[illegible]dent (33.4±4.3), but there was no significant d[illegible]ference compares with control shRNA/8505C or untreated 8505C cells groups ($p>0.05$, Figure 1A). TGF-β1 shRNA/8505C clones showed much slower growth compared with control shRNA/8505C and untreated 8505C clones (Figure 1B; $p<0.05$), and the tumor volume was (1750±230) mm^3 at the end of the experiment, which was more than 44% growth inhibition. Western blot assay showed that TGF-β1 was less detectable in the 8505C/TGF-β1 shRNA xenografts, and phosphorylated Akt was not changed compared to the control (Figure 2A). The immunohi-

stochemical staining revealed low levels of Ki67 expression (Figure 2B) and high TUNEL positive cells (Figure 2C).

Characterization of MK-2206 treated Xenografts

Upon establishment of palpable tumors after 4 weeks, MK-2206 was administered at 60 mg/kg, orally, three times a week for 6 weeks. Lung metastatic nodes were 12.3±2.8, which were significantly decreased compared with the control shRNA/8505C or untreated 8505C cells groups (Figure 1A and Figure 3). In the MK-2206 treated 8505C clones, the tumor volume was (2690±320) mm^3 at the end of the experiment, which was only 15% growth inhibition (Figure 1B). Therefore, MK-2206 alone did not significantly inhibit 8505C cells growth.

The levels of phosphorylated Akt in sections of vehicle control and treated tumors were determined by Western blot assay. Akt phosphorylation in 8505C xenografts tumors was inhibited by MK-2206 treatment (Figure 2A). Total Akt levels were not different between control and treated tumors (Figure 2A).

Although immunohistochemical staining revealed low levels of Ki67 expression and [illegible] TUNEL positive cells in MK-2206 groups [illegible], there was no significant differences compar[illegible] the control (Figure 2A-2B, $p>0.05$).

Characterization of TGF-β1 s[illegible] combined with MK-2206 t[illegible]ed Xenografts

After 6-weeks treatment, [illegible]tic nodes were 9.67±2.4 in TGF[illegible]1 sh[illegible]-2206 groups, which was significant[illegible] com-pared with TGF-β1 shRNA [illegible]ups (p [illegible] Figure 1A), but there was no [illegible]ificant diff[illegible]es compares with MK-220[illegible] groups (p [illegible]05, Figure 1A).

In the TGF-β1 [illegible]hRNA/[illegible]ated groups, the tumor v[illegible]ne was (62[illegible] mm^3 at the end of the exp[illegible]ent, [illegible]ch was [illegible]ore than 71% growth inh[illegible]on. [illegible]as sig[illegible]ificant high than the TGF-β [illegible]<0.00[illegible] MK-2206 ($p< 0.05$) alo[illegible] (Fig[illegible] su[illegible]sting that the combining [illegible]tment sig[illegible] inhibited tumor grow[illegible]otal Akt leve[illegible]ere not different between [illegible]d treated tumors (Figure 2A). The le[illegible]of p[illegible]ylated Akt were decreased, but there was no sig[illegible] differences compared wi[illegible]-2206 alone g[illegible]ps (Figure 2A). The im-[illegible]histochemical staining revealed low levels [illegible]i67 expressio[illegible] (Figure 2C) and high TUNEL [illegible]tive cells (Fig[illegible]2B).

Discussion

During early carcinogenesis, the TGF-β1 signaling is a major tumor suppressor, however, during late tumor stage, its growth-suppressive activity is commonly lost. We have previously demonstrated that targeting TGF-β1 decreased proliferation and colony formation, and induced apoptosis of 8505C cells *in vitro*; however, it did not inhibit invasion of 8505C cells *in vivo*[11,12].

In this study, we found that TGF-β1 shRNA alone significantly inhibited the growth of the 8505C xenografts tumors. The apoptotic cells were signi-

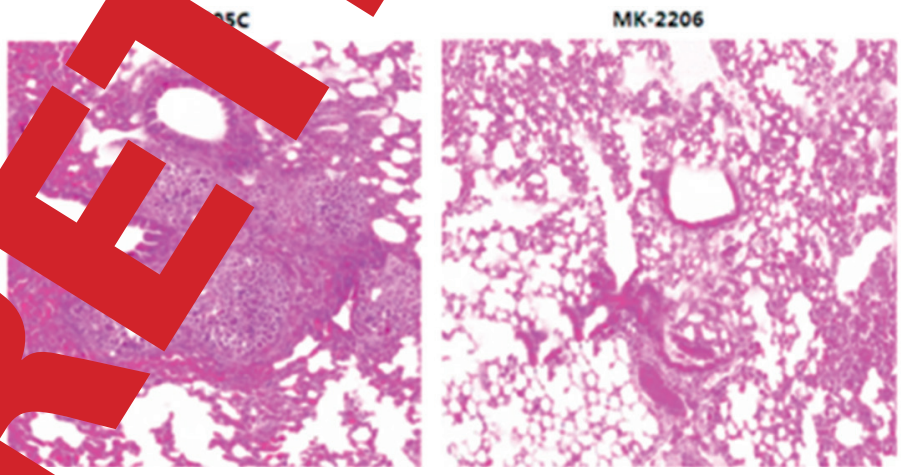


Figure 3. MK[illegible]nhibits lung metastasis of 8505C cells. Representative lung tissue sections were stained with H&E and photographed at 150[illegible] magnification. Evident metastatic node was found in the lung of the 8505C tumor.

ficantly increased and cell proliferation was decreased in the TGF-β1 shRNA transfected tumors, suggesting that TGF-β1 shRNA inhibited the tumor growth by inducing cell apoptosis. However, in the TGF-β1 shRNA transfected groups, the lung metastatic nodes were not decreased compared with the control shRNA/8505C or untreated 8505C cells groups, suggesting that the targeting TGF-β1 alone did not inhibit organ metastasis.

Accumulating evidence is emerging that PI3K/Akt signaling axis actively engages with the migratory process in the motile cells, including metastatic cancer cells. The interference with the role of PI3K/Akt-mediated cell motility impairs cellular development and attenuates malignant progression of cancer metastasis[27]. The broad roles of this enzyme in cancer have established Akt as an attractive therapeutic candidate in cancer. Small molecule inhibitors of the PI3K/Akt pathway are being developed for clinical use. Several Akt inhibitors have been synthesized, including MK-2206, a novel allosteric kinase inhibitor of Akt[28,29]. MK-2206 has shown promising preclinical activity and is currently undergoing phase II clinical evaluation[23,30-32].

In the present study, the effects of MK-2206 on phosphorylated Akt levels in 8505C xenograft tumors were studied using Western blot. Phosphorylated Akt levels were significantly reduced in tumors treated with MK-2206.

Treatment with MK-2206 significantly inhibited lung metastasis of mice. However, in the MK-2206 treated 8505C xenograft tumors, tumor inhibition was only 15%, suggesting that MK-2206 alone mainly inhibits tumor metastasis, but partly inhibits tumor growth.

Strikingly, the treatment of 8505C xenografts with the TGF-β1 shRNA in combination with the MK-2206 significantly reduced its growth. The combined treatment led to more than 70% growth inhibition compared with TGF-β1/shRNA (44%) and MK-2206 (15%). The fluorescence image analysis of TUNEL-labeled tumor sections revealed that the combined treatment produces significant increase in apoptotic cells compared with TGF-β1 shRNA or MK-2206 alone. Also, Ki67 labeled the sections revealed that combined treatment group showed significant cell proliferation compared with TGF-β1 shRNA or MK-2206 alone.

To further evaluate the effect of combination treatment on metastasis, we administered MK-2206 at 120 mg/kg orally, three times a week for 6 weeks. We found that the treatment group MK-2206 or combined group MK-2206+ TGF-β1 shRNA has fewer lung metastasis compared to the TGF-β1 shRNA alone group. However, significant differences were found between MK-2206 and MK-2206+ TGF-β1 shRNA groups.

Conclusions

Our findings demonstrate that targeting TGF-β1 signaling was effective against anaplastic thyroid cancer (ATC) cell growth and metastasis *in vivo*. Particularly, MK-2206 significantly inhibits Akt phosphorylation and 8505C lung metastasis *in vivo*. We therefore, suggested that TGF-β1 inhibition in combination with PI3K-Akt signaling inhibition may have a synergistic effect.

Conflicts of interest

The authors declare no conflicts of interest.

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