Original Article
YM155 down-regulates survivin and induces PUMA-dependent apoptosis in oral squamous cell carcinoma cells

Xinghua Yang¹, Lin Peng², Yanli Shi³

¹School of Dentistry and Oral Health, Shandong University, Shandong, China; ²Clinical Laboratory, Laiwu Hospital Affiliated to Taishan Medical College, Shandong, China; ³Stomatology, Shandong Provincial Hospital Affiliated to Shandong University, Shandong, China

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Abstract: Objective: YM155, which inhibits the anti-apoptotic protein survivin, is known to exert anti-tumor effects in various cancers. However, there were few reports describing the inhibitory effect of YM155 on human oral squamous cell carcinoma cells that highly express survivin. In this study, we investigated the anti-tumor effects of YM155 on oral squamous cell carcinoma cells and explored its molecular mechanisms. Methods: Oral squamous cell carcinoma SCC9 cells was treated with series of concentration of YM155 (0.01, 0.1, 1 and 10 ng/ml) for 6, 12 and 24 h. The effect of YM155 on growth of SCC9 cells was detected by MTT and colony formation assay. Cell apoptosis was detected by flow cytometric analysis and the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assays. Western blot was used to detect the expression of survivin, p53 and PUMA protein. Caspase-3 activity was measured by cleavage of the caspase-3 substrate. To test the role of PUMA and caspase-3 on YM155-induced apoptosis and growth inhibition, the SCC9 cells was transfected with PUMA siRNA or caspase-3 siRNA or control siRNA for 16 hs before YM155 (1 and 10 ng/ml) treatment for 24 h. In addition, we also investigated the effect of YM155 in an in vivo xenograft model. Results: Treatment of YM155 efficiently reduced survivin expression in the SCC9 cells. The downregulation of survivin induced remarkable apoptosis and growth inhibition of SCC9 cells and resulted in significant upregulation of PUMA expression and caspase-3 activation. However, the induction of cell apoptosis of growth inhibition was reversed by PUMA siRNA or caspase-3 transfection. In addition, YM155 efficiently retarded tumor growth in established tumors of human SCC9 cell xenografted mice. Conclusion: YM155 is a potent inhibitor of progression of SCC9 cells, which could be due to attenuation of survivin, and activation of PUMA/caspase-3 cellular signaling processes. This study suggests that YM155 may be a potential molecular target with therapeutic relevance for the treatment of oral squamous cell carcinoma.

Keywords: Oral squamous cell carcinoma, survivin, YM155, p53, PUMA, apoptosis

Introduction

Oral cancer is one of the 10 most common cancers in the world. Its high mortality rate and the disfigurement that survivors may suffer gives rise to a considerable global public health burden [1]. About 90% of malignant oral neoplasms are oral squamous cell carcinomas (OSCC), followed by adenocarcinoma and, rarely, other types of tumor [2]. Despite advances in treatment for OSCC, the 5-year survival rate remains poor [3]. As an important hallmark of OSCC, apoptosis resistance restricts the efficacy of traditional therapies [4]. Survivin (BI-RC5), a member of the inhibitor of apoptosis protein (IAP) gene family, has been shown to inhibit apoptosis, enhance proliferation and promote angiogenesis [5-7]. Survivin is selectively expressed in fetal and proliferating tissues and in various solid tumors, such as OSCC [8], and elevated survivin expression in OSCC has been associated with poor prognosis, high recurrence rate and chemotherapy and radiation resistance [8]. Therefore, targeting survivin is promisingly beneficial for OSCC therapies.

Sepantronium bromide (YM155) is the first-in-class survivin inhibitor [9]. YM155 has shown potent antiproliferative effects on a variety of human cancer cell lines [10]. Numerous studies
have demonstrated that YM155 alone or in combination decreases tumor growth, induces apoptosis, sensitizes resistant cells to apoptosis, and prolongs survival of tumor-bearing mice [11-13]. However, its molecular mechanism of action is still unclear.

In vitro studies revealed that YM155 triggered apoptosis of head neck squamous cell carcinoma (HNSCC) cells in mitochondria and death receptor-dependent manner. In addition, YM155 not only downregulated the expression of survivin but also remarkably suppressed the activation of the mTOR signaling pathway in vitro and in vivo [14]. In human oral cancer cell lines, YM155 inhibited the growth and caused caspase-dependent apoptosis in the MC3 and HN22 cells, the mechanism that YM155 causes apoptosis of human oral cancer cell lines was through downregulation of Sp1 and Mcl-1 [15]. Tang et al. has showed YM155 exhibited its anti-tumor activities in oral cancer cell lines by downregulation of Mcl-1 [16]. In adenoid cystic carcinoma (ACC) cells, YM155 caused significant autophagy-dependent cell death. In addition, YM155-induced autophagy and cell death in vivo was correlated with the suppression of Erk1/2 and S6 activation as well as increased TFEB nuclear translocation [17].

PUMA (p53 upregulated modulator of apoptosis) is a pro-apoptotic member of the BH3-only subgroup of the Bcl-2 family. It is a key mediator of p53-dependent and p53-independent apoptosis [18, 19]. PUMA transduces death signals primarily to the mitochondria, where it acts indirectly on the Bcl-2 family members Bax and/or Bak by relieving the inhibition imposed by antiapoptotic members. It directly binds and antagonizes all known antiapoptotic Bcl-2 family members to induce mitochondrial dysfunction and caspase activation [20].

It has been shown that survivin inhibits Fas (CD95)-mediated apoptosis by supporting caspase3/p21 formation as a result of interaction with cdk4 [21]. In addition, survivin was shown to suppress the cell death induced by Bax [22]. A recent study has reported that targeting survivin resulted in increased transcription of p53 targets, such as BAX, PUMA, NOXA and p21, and increased p53-dependent breast cancer cells apoptosis [23], suggesting PUMA signals may be regulated by survivin.

In this study, we evaluated the anticancer effects of YM155 in oral squamous cell carcinomas cell line in vitro and xenografts in vivo. In addition, we investigated the effect of YM155 on PUMA signals expression to determine whether YM155 affects the PUMA pathways.

Materials and methods

Cell culture and reagents

The study was conducted in accordance with the guidelines in the Declaration of Helsinki. The human oral squamous cell carcinomas (OSCC) SCC9 cell was purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China). It was maintained in DMEM-F12 (Gibco), and supplemented with 10% fetal bovine serum and maintained at 37°C in a 5% CO₂ humidified incubator. Antibodies against Survivin, PUMA, activated-caspase-3 and β-actin was from Santa Cruz (Shanghai, China). YM155 were purchased from Selleck Chemicals (Shanghai, China). PUMA siRNA, caspase-3 siRNA or control siRNA were purchased from Santa (Shanghai, China).

siRNA transfection

PUMA siRNA or caspase-3 siRNA or mismatched siRNA (control siRNA) were transiently transfected into SCC9 cells using Lipofectamine 2000 reagent (Invitrogen, Inc., Carlsbad, CA, USA) as the manufacturer’s instruction. Briefly, SCC9 cells (2 × 10⁵) were plated in each well of a 96-well plate. Experimental conditions were set in quadruplicate. After cells were attached, the culture medium was replaced with serum-free medium plus 3 μl of siRNA (20 μM) and mixed with 1 μl transfection reagent and 100 μl Lipofectamine medium supplied by the kit. Then, the siRNA-transfection reagent complex was incubated with 500 μl of diluted cells (5 × 10⁴ cells/well) for 24 hours at 37°C and 5% CO₂. The cells without siRNA transfection were used as the control. The knockdown effect was verified by Western blot analysis. Stable SCC9 cell line transfected with siRNA were screened by administration of G₄₁₈ (Invitrogen, Carlsbad, CA).

Western blot analysis

SCC9 cells were treated with 0.01, 0.1, 1 and 10 ng/ml YM155 for 6, 12 and 24 hs, respectively, or transfected with PUMA/caspase-3 siRNA or control siRNA for 16 h before YM155 (1 and 10 ng/ml) treatment for 24 h, then the
cells were lysed and protein was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The following antibodies were applied: monoclonal human anti-survivin, anti-p53 and anti-PUMA, anti-activated caspase-3 and anti-β-actin. Secondary antibodies (dilution 1: 20,000) were horseradish peroxidase-conjugated (Hangzhou, China).

Caspase-3 activity assay

Caspase-3 activity was measured by cleavage of the caspase-3 substrate. Briefly, SCC9 cells (2 × 10⁴) were treated as the methods above. Reactions were spiked with DMSO or the Caspase-3 specific inhibitor Ac-DMQD-CHO at a final concentration of 2 mM. Measurements were done in triplicate. The mean of three biological replicates is shown.

Cell viability assay

Cell viability was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) according to the manufacturer’s instruction. Briefly, SCC9 cells (1 × 10⁴) were plated in each well of a 96-well plate. The following day, the cells were incubated with increasing concentrations of YM155 (ranging from 0.01 to 10 ng/ml) and incubated at 37°C for 6-24 hs. To assess the role of PUMA/caspase-3 on YM155-induced growth of SCC9 cells, the SCC9 cells (1 × 10⁴) were transfected PUMA siRNA or caspase-3 siRNA or control siRNA for 16 h before the incubation with the concentrations of YM155 (1 and 10 ng/ml) for 24 h. Then the cells (1 × 10⁵) were suspended in 500 ml of Annexin V binding buffer. 5 ml of Annexin V-FITC and 5 mL of PI were added and incubated with for 15 min in dark. 400 mL binding buffer was added to each sample. The stained cells were analyzed directly by flow cytometry using the Cell Quest program (Becton Dickinson, San Jose, CA, USA).

TUNEL staining

For in vitro assay, SCC9 cells, PUMA siRNA or caspase-3 siRNA transfected SCC9 cells were treated with YM155 (1 and 10 ng/ml) for 24 h. Cell apoptosis was detected using Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) in situ cell death detection kit according to the manufacturer’s instructions. The apoptotic index was determined by dividing the number of apoptotic cells by the total number of cells in the cells of at least 20 randomly selected fields (× 200).

In vivo experiments

Animal experiments were approved by the ethics committee of the Taishan Medical University.
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and were carried out according to the China guidelines for animal care and protection in order to minimize distress for the animals. 4 to 6 week-old severe combined immunodeficient (SCID) female mice were utilized for all experiments. SCC9 (1 × 10^7) cells were injected into the flank. Tumor growth was monitored by measuring tumor volume (length × width^2/2/ mm^3).

When subcutaneous tumors reached a size of 50 mm^3 (day 0), xenografted animals were randomly allocated into vehicle (saline) and YM155 (50 mg/kg) groups. YM155 was subcutaneously administered as a 3-day continuous infusion per week for 2 weeks using the Alzet Osmotic Pump® (Model 1003D). At experiment termination, mice were dissected and tumor tissue was processed for immunohistochemistry (IHC) and TUNEL assay. For statistical analysis of tumor growth, two-way ANOVA and Bonferroni post correction were applied.

Immunohistochemistry (IHC) was performed using standard procedures. Briefly, 4 μm sections from xenograft tumor blocks were deparaffinized and rehydrated, heated for 10 min in 10 mM citrate buffer (pH 6.0) in a pressure cooker for epitope retrieval, and then incubated for 60 min at room temperature with anti-survivin and anti-PUMA antibodies. Antibody binding was detected by means of the UltraVision LP detection system according to the manufacturer’s recommendations.

Statistical analysis

The significance of the results was determined by the Student’s t test). Values are expressed as mean ± SD from at least three separate experiments and a P value less than 0.05 was considered to be statistically significant.

Results

YM155 inhibits growth and colony formation, and induces apoptosis of SCC9 cells

SCC9 cells were first treated with 0.01, 0.1, 1 and 10 ng/ml YM155 for 24 hs. YM155 treatment resulted in 18-86% decrease in cell viability (P<0.05 and P<0.01, Figure 1A), suggesting that YM155 treatment reduced viability of SCC9 cells in a dose-dependent manner. To confirm cell growth inhibition, we have also conducted the cell colony formation assay. We found similar results as MTT assay using this method (P<0.05 and P<0.01, Figure 1B).

Next, we examined whether the inhibition of cell growth was also accompanied by the induc-
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Figure 2. Effect of YM155 on survivin, p53 and PUMA expression and caspase-3 activity. SCC9 cells were treated with 0.01, 0.1, 1 and 10 ng/ml YM155 for 6, 12 and 24 h. A: Survivin, p53 and PUMA protein expression was detected by western blot assay. B: Caspase-3 activity was detected by cleavage of the caspase-3 substrate. vs control, *P<0.05, **P<0.01 and ***P<0.001.

YM155 inhibits survivin and induces PUMA/caspase-3 upregulation

SCC9 cells were treated with 0.01, 0.1, 1 and 10 ng/ml YM155 for 6, 12 and 24 h. Survivin and PUMA expression was detected by western blot assay. Caspase-3 activity was measured by cleavage of the caspase-3 substrate. The results showed that survivin was overexpressed in the SCC9 cells, and YM155 treatment caused survivin inhibition and PUMA upregulation in dose- and time-dependent manner (Figure 2A). Activation of caspase-3 was also in dose- and time-dependent manner (Figure 2B), indicating the specificity of the apoptotic effect of YM155 by caspase activation in SCC9 cells.

PUMA was regulated through p53-dependent and p53-independent pathway [18-20]. We
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Figure 3. Effect of PUMA/caspase-3 on YM155-induced apoptosis and growth inhibition of SCC9 cells. SCC9 cells were transfected with PUMA siRNA or caspase-3 siRNA for 16 h, then treated the transfected cells with YM155 (1 and 10 ng/ml) for 24 h. A: PUMA protein expression was detected by western blot assay; B: Caspase-3 activity was detected by cleavage of the caspase-3 substrate. C: Cell survival rate was detected by MTT assay; D: Colony formation assay; E: Apoptosis was determined by FACS analysis. F: Apoptosis was determined by TUNEL assay. vs control, *P<0.05.
next investigated whether p53 was regulated by YM155/Survivin. As shown in Figure 2A, treatment with 0.01, 0.1, 1 and 10 ng/ml YM155 for 6, 12 and 24 h in the SCC9 cells did not affect wt-p53 expression, suggesting that PUMA was p53-independent regulation by YM155 treatment.

We also found that treatment with YM155 (10 and 1 ng/ml) for 24 h resulted in completely survivin inhibition. In addition, treatment with YM155 rapidly increased the PUMA expression in the SCC9 cells at 6 h, and reached the highest level at 12 h, and significantly decreased at 24 h (Figure 2A). In addition, caspase-3 activity was also at the highest level at 12 h, significantly decreased at 24 h (Figure 2B). The reason of why PUMA/caspase-3 expression was downregulated after 24 h is not clear.

YM155 induces apoptosis and inhibits growth of SCC9 cells through PUMA/caspase-3 activation

As shown in Figure 2A, treatment with 1 and 10 ng/ml of YM155 for 24 h could completely inhibit survivin expression. So we selected 1 and 10 ng/ml of YM155 for further study. To confirm the mechanism responsible for YM155-mediated apoptosis and growth inhibition, we transfected PUMA siRNA or caspase-3 siRNA or control siRNA into SCC9 cells, then treated the transfected cells with YM155 (1 and 10 ng/ml) for 24 h. As shown in Figure 3A, targeting PUMA by PUMA siRNA transfection inhibited YM155-induced PUMA expression and caspase-3 activation (Figure 3B). In addition, targeting caspase-3 by caspase-3 siRNA transfection also inhibited YM155-induced caspase-3 activation (Figure 3B). Transfection of SCC9 cells with PUMA siRNA or caspase-3 siRNA significantly reduced the ability of YM155 to induce cell apoptosis and inhibit growth in SCC9 cells (Figure 3C-F). The control siRNA did not cause any significant change in cell viability and cell apoptosis (data not shown). Although PUMA siRNA or caspase-3 siRNA significantly reduced the ability of YM155 to induce cell apoptosis and growth inhibition, PUMA siRNA or caspase-3 siRNA could not completely rever the effect of YM155, suggesting that the apoptosis-inducing effect by YM155 is partly mediated through the activation of PUMA/caspase-3 pathway.

YM155 treatment inhibited growth of SCC9 xenograft tumor in nude mice

To confirm the anti-tumor effects of YM155 in vivo, we used a SCID mouse xenograft model. Animals bearing SCC9 tumors were administered subcutaneously as a 3-day per week continuous infusion for 2 weeks using the Alzet Osmotic Pump® (Model 1003D). Animals treated with YM155 showed more than 60% tumor growth inhibition compared to the controls (Figure 4A). TUNEL assay showed the more apoptotic cells was found in YM155 treated tumors (P<0.01, Figure 4B). In addition, Survivin expression was inhibited and PUMA expression was increased with YM155 treatment by immunohistochemical assay (Figure 4C, 4D).

Discussion

Survivin is one of the most frequently overexpressed genes in all types of cancer. Increased survivin expression in cancer patients is an unfavorable prognostic marker correlating with decreased overall survival in several malignancies, including pancreatic cancer [24], breast carcinomas [25], non-small cell lung [26], colorectal, hepatocellular carcinoma [27] and neuroblastoma [28]. Increased survivin expression was also associated with increased risk of recurrence, lymph node invasion and metastasis. Survivin has been shown to inhibit cell apoptosis and promote cell proliferation and angiogenesis, all of which make survivin a potentially attractive target for therapy [29]. In OSCC, survivin was found to be strikingly increased in malignant cells, and increased survivin expression was also associated with bad prognosis [30]. Therefore, targeting survivin is promisingly beneficial for OSCC therapies.

Several strategies have been used to suppress survivin expression [31]. One approach is to use antisense oligonucleotides or siRNA to knock down survivin expression, resulting in cell growth arrest and increased apoptosis in a broad range of tumor cell lines [32]. YM155 is a survivin suppressant identified in a high-throughput screen of compounds that selectively inhibited survivin promoter activity [9].

Our current data show that YM155 treatment induced apoptosis and decreased cell proliferation in human SCC9 cells in vitro, and that
YM155 suppressed *in vivo* SCC9 cell growth by inducing apoptosis without apparent body weight loss (data not shown). Our results strongly suggest that survivin expression contributes to human SCC9 tumor progression, and that survivin inhibition by YM155, a novel survivin suppressant, provides an antitumor effect on human SCC9 cells via induction of apoptosis.

To further explore the mechanism of YM155-induced cell apoptosis and growth inhibition, possible proteins involved were detected in vitro and in vivo by Western blot. Previous data suggest that survivin depletion triggers p53 activation and sensitizes cancer cells to PARP inhibition [33]. In our study, YM155 treatment did not affect wt-p53 expression in the SCC9 cells, suggesting that there was no relation between p53 and YM155/survivin-induced apoptosis.

PUMA as a BH3-only Bcl-2 family protein that plays an essential role in p53-dependent and

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**Figure 4.** YM155 inhibits xenograft growth of SCC9 cells. A: Tumor xenografts were established by s.c. injection of SCC9 cells into the flanks of the mice. Animals bearing SCC9 tumors were administered subcutaneously as a 3-day per week continuous infusion for 2 weeks. Tumor size was measured every two days. The tumor growth curve was shown. *p*<0.05 compared with the control group. B: Apoptotic cells was detected by TUNEL assay, *p*<0.01. C: Survivin was detected by immunohistochemical staining assay. D: PUMA was detected by immunohistochemical staining assay.
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- independent apoptosis [18, 19]. In the present study, the expression of PUMA was up-regulated in SCC9 cells when treated with YM155 for 6-24 h. Therefore, PUMA upregulation was p53-independent. To assess whether YM155-induced apoptosis and growth inhibition was PUMA-dependent, SCC9 cells was transfected with PUMA siRNA to knockdown of PUMA expression, only to find YM155-induced apoptosis and growth inhibition was partly reversed with PUMA inhibition. We propose that the mechanism of YM155-induced apoptosis may be attributable to the down-regulation of survivin as well as the upregulation of PUMA.

Because survivin inhibits apoptosis through both direct and indirect inhibitions of caspase-3, a downstream gene of PUMA. We next investigated whether YM155/survivin inhibition could upregulate caspase-3 activity. In the present study, increases of caspase-3 activity by YM155 treatment were observed, which was consistent with the results of PUMA, demonstrating YM155-induced apoptosis and growth inhibition may be partly due to inhibition of survivin/PUMA/caspase-3 pathway.

Although apoptosis provoked by YM155/survivin depended on the activation of PUMA/caspase-3 signal, but how survivin regulated PUMA/caspase-3 has remained unknown. Studies have reported that targeting the Ras/Raf/MEK/ERK pathway induces PUMA-dependent apoptosis in cancer cells irrespective of p53 status [34, 35], suggesting that PUMA was negatively regulated by ERK signals. Wang et al. has reported that YM155-induced autophagy and cell death in vivo was correlated with the suppression of survivin/Erk1/2 and S6 activation [36]. We supposed in the present study that YM155/survivin might downregulate ERK1/2 signal, resulting in upregulation of PUMA/caspase-3 expression and activation. The hypothesis need further investigation in the further. We also demonstrated in our study although YM155 induced apoptosis and growth inhibition, targeting PUMA or caspase-3 could partly reverse the function of YM155, suggesting that some other proapoptotic signals may take part in YM155/survivin-induced apoptosis and growth inhibition of SCC9 cells.

It is interesting that in our study PUMA/caspase-3 expression reached the peak at 12 h with YM155 treatment, and was dramatically decreased at 24 h. The cause of why PUMA/caspase-3 expression was downregulated at 24 h was not clear. A previous study has reported that activation of ERK/Slug signal by Cytarabine contributed to the PUMA downregulation in HL-60 cells after 48 h [37]. Whether some anti-apoptotic signals which inhibited PUMA upregulation were induced by YM155 or YM155/survivin in our study need further investigation.

Conclusions

In summary, we presented experimental evidence, which strongly supports the antitumor effects of YM155 in SCC9 cells in vitro and in vivo. Thus, we believe that YM155 could potentially be an effective therapeutic agent for the inactivation of survivin and activation of PUMA/caspase-3 signal, resulting in the induction of cell apoptosis and inhibition of cell growth. Our study suggests that YM155 represent a promising novel agent that should be developed for the treatment of OSCC.

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Disclosure of conflict of interest

None.

Address correspondence to: Xinghua Yang, School of Dentistry and Oral Health, Shandong University, Shandong, China. E-mail: yangxinghuakq@gmail.com

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