Tumor inhibitory effect of epigenetic agents（Zebularine，Valproic　acid）on oral squamous cell carcinoma

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[Purpose]

Oral cancer has the sixth highest incidence rate among all cancers (Rivera, 2015), and oral squamous cell carcinoma (OSCC) accounts for about 90% of oral cancer (Khurshid et al., 2018). Although surgical excision and nonsurgical therapies such as radiation and chemotherapy have been used to treat the cancers, the 5-year survival rate is about 60% . The development of new approaches to the cancer will be needed to increase the survival rate.

In this study, we examined the effect of Zebularine (Zebu), an epigenetic DNA methyltransferase inhibitor (DNMTi), and histone deacetylase inhibitor (HDACi) Vebularine inhibitors (HDACi) Valproic acid (Vpa) on human oral squamous cell carcinoma cell lines *in vitro* and *in vivo.*

[Methods]

1. Cytotoxicity test of Zebu and Vpa

In this study, two types of oral squamous cell carcinoma cell lines, SAS and HSC4 were used. To determine the optimal concentrations of Zebu and Vpa for SAS and HSC4, Zebu, and Vpa were added at the concentration of 1.0, 10, 100, 200, and 400 M and of 0.1, 1.0, 2.0, 5.0, and 10 mM, respectively. Combination of Zebu・Vpa was added at the concentration of Zebu 10 μM・Vpa 2 mM, Zebu 100 μM・Vpa 1 mM, Zebu 100 μM・Vpa 2 mM, Zebu 100 μM・Vpa 5 mM, Zebu 200μM・Vpa 2mM. The cell numbers were counted after they stained with 0.5% Trypan Blue solution at 1, 3 and 7days.

2. RNA sequence

SAS and HSC4 were treated with Zebu at 100 μM and Vpa at 2mM for 1 week. Total RNA was extracted for comprehensive analysis (RNA sequence, RNA-seq) using Next Generation Sequencer (NGS).

3. mRNA expression analysis by qRT-PCR

From the RNA-seq results, *CNTN4* was a common gene in the elevated expressions of SAS and HSC4 genes. Quantitative real-time PCR (qRT-PCR) analyses using *p16, p21, RASSF1, NPY, CNTN4* and *Gapdh* were performed to observe the expression levels of mRNAs.

4. DNA methylation analysis by qMSP

To confirm the effects of Zebu alone and a combination of Zebu and Vpa on DNA methylation, we performed methylation analysis of *CNTN4, p16, p21, RASSF1,* and *NPY* by quantitative methylation-specific PCR (qMSP) method.

5. *in situ HDAC activity assay*

The HDAC activity of the cells in the experiments was confirmed with the In Situ HDAC Activity Fluorometric Assay Kit.

6. Xenograft tumor formation assay in nude mice

SAS and HSC4 cells（1.0×10^6 cells per mouse）were injected into 6-week-old male nude mice. Once tumors reached an average volume of 80 mm3, A combination of 1000mg/kg of Zebu and 400mg/kg of Vpa was injected in each mouse every day for 3

weeks. The tumor volumes were calculated using the formula tumor volume = (length × width^2) × 0.5.

7. mRNA expression analysis of in vivo

qRT-PCR analyses were performed to observe the expression levels of *CNTN4, p16, p21, RASSF1, NPY,* and *Gapdh.*

8. DNA methylation analysis by qMSP of in vivo

Methylation analysis using qMSP was performed for *CNTN4, p16, p21, RASSF1,* and *NPY* genes.

【Results and discussion】

1. Cytotoxicity test of Zebu and Vpa

The Zebu significantly decreased the number of SAS cells at concentrations of 200 μM and 400 μM on day 3 and 7 compared to the control (*$p < 0.05$; Mann-Whitney U test, n=4). The Zebu significantly decreased the number of HSC4 cells at concentrations of 200 μM and 400 μM on days 1, 3 and 7 (*$p < 0.05$; Mann-Whitney U test, n=4). The Vpa significantly decreased the number of SAS cells at concentrations of 10 mM on day 1 and of 5 mM and 10 mM on day 3, 7 compared to control (*$p < 0.05$; Mann-Whitney U test, n=4). No inhibition of cell proliferation was observed for both SAS and HSC4 in the combination of Zebu 10 μM and Vap 2 mM or of Zebu 100 μM and Vap 1 mM.

The number of the cells was significantly reduced in the combination of Zebu 100 μM and Vpa 2 mM, and of Zebu 100 μM and Vap 1 mM on days 3 and 7 compared to the controls (*$p < 0.05$; Mann-Whitney U test, n=4). The numbers of the cells were

significantly reduced in the combination of Vap 2 mM and Zebu 100 M, and of Vap 2 or 4 mM and Zebu 200 μM on days 3 and 7 compared to the controls (*$p < 0.05$; Mann-Whitney U test, n=4).

These results suggest that the combination of Zebu and Vpa enhances the effects of each drug on OSCC.

2. RNA sequence

CNTN4 was found to be a common gene in up-regulated genes in SAS and HSC4. *CNTN4* has been shown to suppress proliferation, migration, and invasion of lung adenocarcinoma cells, while its high level expression leads to a poor prognosis in gastric cancer. *CNTN*4 may act as a tumor suppressor gene in oral squamous cell carcinoma.

3. Epigenetic Analysis of in vivo

The combination of Zebu and Vpa significantly increased mRNA expression and decreased methylation levels in *CNTN4, p16, p21, RASSF1,* and *NPY* in both SAS and HSC4 transplanted into nude mice (*$p < 0.05$; Mann-Whitney U test, n=4, *$p < 0.05$; chi-square test, n=3). These results suggest that changes in their DNA methylation levels may be involved in their mRNA expressions.

The *in situ* HDAC activity assay showed a significant decrease in HDAC activity in the Vpa alone and the combination of Zebu and Vpa compared to the controls (**$p < 0.01$; Turkey's test, n=4). In addition, a significant decrease in HDAC activity was observed in the combination of Zebu and Vpa compared to the Vpa alone. The combination therapy of Zebu and Vpa may enhance the effect of each drug on OSCC.

4. Tumor suppressive effect of Zebu,Vpa in tumor forming mice

The tumor size of SAS transplanted into nude mice was 3056 mm^3 and 2409 mm^3 in the control and the combination of Zebu and Vpa groups, respectively. There was no statistical significance in size between two groups. Significant differences were observed in the mRNA expressions of *p16, p21, RASFF1, NPY,* and *CNTN4* in both SAS and HSC4 between the combination and the control groups. DNA methylation analysis showed a significant decrease in methylation levels only in *RASFF1, NPY* and *CNTN4* in SAS, and a significant decrease in methylation levels in all genes in HSC4. The tumor growth is usually consistent with their grade of differentiations. Therefore, the effect of combination of Zebu and Vpa may be depend on the grade of tumor differentiation.

【Conclusion】

The combination of DNMTi (Zebu) and HDACi (Vpa), may be an effective treatment for several types of squamous cell carcinoma.

【References】

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