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의학석사 학위논문

Synergistic effect of cytokine-induced killer  
cell with valproate inhibits growth of  
hepatocellular carcinoma cell in a mouse model

Mouse 간세포암 모델에서 Cytokine-induced killer  
cell과 valproate 병합 시 나타나는 항암 시너지  
효과에 대한 연구

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A thesis of the Master's degree

Mouse 간세포암 모델에서 Cytokine-induced  
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Synergistic effect of cytokine-induced killer cell  
with valproate inhibits growth of hepatocellular  
carcinoma cell in a mouse model

February 2017

Seoul National University

Internal Medicine

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## **Abstract**

# **Synergistic effect of cytokine-induced killer cell with valproate inhibits growth of hepatocellular carcinoma cell in a mouse model**

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**Objective:** Long-term prognosis of hepatocellular carcinoma (HCC) remains poor owing to the lack of treatment options for advanced HCC. Cytokine-induced killer (CIK) cells are ex vivo expanded T lymphocytes expressing both NK- and T-cell markers. CIK cell therapy alone is insufficient for treating advanced HCC. Thus, this study aimed to determine whether treatment with CIK cells combined with valproic

acid (VPA) could provide a synergistic effect to inhibit tumor growth in a mouse model of HCC.

**Methods:** Upregulation of natural killer group 2D (NKG2D) ligands (retinoic acid early inducible 1 [RAE-1], mouse; major histocompatibility complex class I polypeptide-related sequence A [MIC-A], human) were evaluated by FACS. VPA concentrations that did not reduce tumor volume were calculated to avoid VPA cytotoxicity in a C3H mouse model of HCC. CIK cells were generated from mouse splenocytes using interferon gamma, a CD3 monoclonal antibody, and interleukin 2. The potential synergistic effect of CIK cells combined with VPA was evaluated in the mouse model and tissue pathology was investigated.

**Results:** After 24 h of incubation with VPA, RAE-1 and MIC-A expression were increased in four HCC cell lines compared with that in control (2.3-fold in MH-134, 2.4-fold in Huh-7, 3.7-fold in SNU-761, and 6.5-fold in SNU-475). The maximal *in vivo* VPA dosage that showed no significant cytotoxicity compared with control was 10 mg/kg/day. CIK cells were well generated from C3H mouse splenocytes. After 7 days of treatment with CIK cells plus VPA, a

synergistic effect was observed on relative tumor volume in the mouse model of HCC. While the relative tumor volume in untreated control mice increased to 11.25, that in the combination treatment group increased to only 5.20 ( $P= 0.047$ ).

**Conclusions:** The VPA-induced increase in NKG2D ligands expression significantly enhanced the effects of CIK cell therapy in a mouse model of HCC.

**Student Number:** 2015-21968

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## Introduction

Hepatocellular carcinoma (HCC) is a common and fatal cancer, with increasing incidence worldwide.<sup>1, 2</sup> Even though many curative therapies have been developed, the long-term prognosis for patients with HCC remains poor because of its high recurrence rates.<sup>3</sup> We recently determined that adjuvant immunotherapy using cytokine-induced killer (CIK) cells appeared to reduce the recurrence of HCC.<sup>4</sup> Although adjuvant CIK cell-based immunotherapy is a promising method, it is not enough to treat primary HCC and block HCC recurrence.

CIK cells are *ex vivo* expanded T lymphocytes expressing both NK- and T-cell markers. CIK cell therapy alone is insufficient for treating primary and advanced HCC. CIK cells detect major histocompatibility complex class I polypeptide-related sequence A (MIC-A) and sequence B (MIC-B) through the natural killer group 2D (NKG2D) receptor.<sup>5</sup> Adjuvant therapy with CIK cells offered better prognosis in patients with gastric cancer who overexpressed MIC-A than in those with low MIC-A-expressing tumors. Thus, MIC-A status is associated with

outcome and may be a predictive factor in CIK cell therapy.<sup>6</sup>

Valproic acid (VPA) is reported to reduce tumor growth *in vivo* with several mechanisms. One of these mechanisms is a inhibition of histone deacetylase (HDAC) and this can modulate the biology of diverse tumor cells.<sup>7</sup> In previous *in vitro* and *in vivo* studies, the antitumor effect of VPA alone was insufficient for many kinds of tumors, including HCC. However, a synergistic effect of VPA with other agents has been reported in some promising results.<sup>8-10</sup> VPA upregulates MIC-A and MIC-B,<sup>11</sup> and this upregulation on the tumor surface enhances CIK cells effects. Moreover, VPA has been safely used as an antiepileptic drug for decades, it is relatively inexpensive, and its adverse effects have been regarded as tolerable. In the present study, we determined whether treatment with CIK cells combined with VPA may produce a synergistic effect to inhibit the growth of HCC cells in mice.

## **Materials and Methods**

### ***Chemicals***

VPA and vorinostat were purchased from Sigma-Aldrich Co., LLC. (Seoul, South Korea) and were dissolved in distilled water before each experiment. All other chemicals and reagents were from standard commercial sources and of the highest purity available.

### ***Cell lines, cell culture, and reagents***

The following four HCC cell lines were used in this study: MH-134 (a mouse HCC cell line), Huh-7 (a well-differentiated HCC cell line), SNU-475 (a poorly differentiated HCC cell line), and SNU-761 (a poorly differentiated HCC cell line). MH-134, SNU-475, and SNU-761 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100,000 U/L penicillin and 100 mg/L streptomycin.<sup>12</sup> Huh-7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 100,000 U/L penicillin, and 100 mg/L streptomycin, with or without 100 nM insulin. In all experiments performed in this study, cells were serum-starved

overnight prior to the experiments to avoid the effects of serum-induced signaling. The cells were incubated under standard culture conditions (20% O<sub>2</sub> and 5% CO<sub>2</sub>, at 37°C).

***Estimation of RAE-1 or MIC-A expression in HCC cell lines treated with VPA in vitro***

MH-134, Huh-7, SNU-475, and SNU-761 cell lines were detached from culture dishes using 0.25% Trypsin/EDTA and incubated with VPA (5 mM). Cell line was harvested 40 hours after drug treatment, as well as at 8 hours intervals after the medium was replaced with a drug-free medium. After being incubated with a specific monoclonal antibody for 30 min at 4°C, the cells were washed three times with cold phosphate-buffered saline (PBS). Finally, fluorescence-activated cell sorting (FACS) was conducted and the results analyzed. SFI of retinoic acid early inducible-1 (RAE-1) labeling in MH-134 cells and MIC-A labeling in Huh-7, SNU-475, and SNU-761 cells were calculated by dividing the median fluorescence level that was obtained with the specific monoclonal antibody by the corresponding isotype control.

### ***Real-time PCR analysis***

The mRNA expression of PD-L1 and PD-L2 were evaluated by a real-time reverse-transcriptase polymerase chain reaction (PCR) assay. Total RNA was extracted by Trizol Reagent (Invitrogen; Carlsbad, CA, USA). Complementary DNA (cDNA) templates were synthesized using oligo-dT random primers and Moloney murine leukemia virus reverse transcriptase. The primer sequences were as follows: PD-L1, forward: 5'-aatggaacctggcgaaagc;-3' and reverse: 5'-gatgagcccctcaggcattt-3'; PD-L2, forward: 5'-gtcttgggagccagggtgac;-3' and reverse: 5'-tgaaaagtgcaaatggcaagc;-3'.<sup>13</sup> After the reverse transcription reaction, the cDNA template was amplified by Taq polymerase (Invitrogen; Carlsbad, CA, USA). Relative quantification was calculated using the  $2^{-\Delta\Delta CT}$  method, with GAPDH as the internal control. All PCR experiments were performed in triplicate.

### ***Immunoblot analysis***

Antibodies against PD-L1 and PD-L2 were purchased from Invitrogen (Carlsbad, CA, USA). Cells were lysed for 20 min on ice in lysis buffer

(50 mM Tris-HCl, pH 7.4; 1% Nonidet P-40, 0.25% sodium deoxycholate; 150 mM NaCl; 1 mmol/L EDTA; 1 mM phenylmethylsulfonyl-fluoride; 1 mM Na<sub>3</sub>VO<sub>4</sub>; 1 mM NaF; and 1 µg/mL each of aprotinin, pepstatin, and leupeptin) and then centrifuged at 14,000 g for 15 min at 4°C. Samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, blotted with appropriate primary antibodies, and incubated with peroxidase-conjugated secondary antibodies (Biosource International; Camarillo, CA, USA). Bound antibodies were visualized by a chemiluminescent substrate (ECL; Amersham, Arlington Heights, IL, USA) and exposed to Kodak X-OMAT film.

### ***In vitro cytotoxicity assay***

SNU-761 cells were incubated with VPA (5 mM) or vorinostat (2 µM) for 40 hours. The cells were then mixed with washing media (RPMI 1640 with FBS 1%), centrifuged (300g, 4 minutes), and the cell pellet was resuspended at concentrations of  $2 \times 10^5$  cell/mL in RPMI 1640 with FBS 1%. Following IRB approval (Seoul National University Hospital IRB No. 1605-143-765) and informed consents, human CIK

cells were obtained from human mononuclear cells cultured at 37°C for 12–21 days with interleukin-2 and immobilized monoclonal antibody to CD3, according to a modified protocol.<sup>14, 15</sup> Washing medium was added to separated CIK cells (effector cells) to a final concentration of  $3 \times 10^6$  cells/mL. Each well consisted of 50  $\mu$ L of target cells and 100  $\mu$ L of CIK cells. After a 4-hour incubation period, treated cells were centrifuged (300g, 4 minutes), and 75  $\mu$ L of supernatant were added to wells of a Black plate (SPL Life Sciences, Cat No. 31496). Optical density was estimated at 485 $\pm$ 9 nm.

#### ***Identification of maximum VPA dose without cytotoxicity in vivo***

Animal experiments were conducted to determine the maximum dose of VPA that did not affect HCC cell growth. MH-134 cells were cultured for 2 weeks to a density of  $2.5 \times 10^6$ /mL. MH-134 cells ( $2.5 \times 10^6$ /mL in 100  $\mu$ L of RPMI-1640) were subcutaneously injected into 15 male C3H mice (6 weeks old). In previous studies, 10 mg/kg and 30 mg/kg were selected as minimum VPA doses.<sup>16, 17</sup> VPA treatment was started when tumors had grown to a palpable size (0.2 cm<sup>3</sup>). VPA was dissolved in distilled water and injected into the peritoneum of five mice once daily at a dose of 10 mg/kg/day for 7 days. The control

group was injected with solvent (n = 5). The tumor size was measured with calipers. Tumor volumes were calculated by standard formula:  $\pi/6 \times (\text{length}) \times (\text{width}^2)$ .<sup>18</sup> Relative tumor volume (relative to the volume of first treatment day) was also calculated. Body weights were recorded continuously to determine the maximum dose of VPA that did not inhibit the growth of the HCC cells.

### ***Generation of mouse CIK cells***

We acquired four spleens from 5-week-old C3H mice and maintained the splenocytes in RPMI at a density of  $5 \times 10^6$  cells/ml. These cells were incubated with interferon gamma (1000 U/mL) for 24 h. After 24 h, the cells were transferred to CD3 monoclonal antibody-coated dishes and exposed to interleukin 2 (750 U/mL). When the cell density reached over  $2 \times 10^6$  cells/mL, the cells were split and transferred to new CD3-free culture dishes with additional interleukin 2 exposure (750 U/mL). To identify the differentiation to CIK cells, flow cytometry was performed using CD3, CD8, CD161 markers (Figure 1).

### ***Synergistic effect of CIK and VPA in vivo***

MH-134 cells ( $2.5 \times 10^6$ /mL in 100  $\mu$ L of RPMI-1640) were injected

subcutaneously into the back side of 6-week-old C3H mice (n = 24). Once the implanted MH-134 tumor bud grew to 0.2 cm<sup>3</sup> in more than 80% of mice, the mice were divided into the following four treatment groups with five mice each group: control, VPA, CIK cells, CIK cells + VPA. The mice in the VPA group were injected with 10 mg/kg of VPA intraperitoneally once daily between D0 and D6 for 7 days. The mice in the CIK cell group were injected once at D0 with 1 × 10<sup>7</sup> CIK cells around the tumor bud. The mice in the CIK cell + VPA group were treated with both the VPA and CIK cell injections as described above. All mice were sacrificed on day 7 to acquire their tumors for analysis at D7 (Figure 1). Tumor volumes and relative tumor volumes were calculated by standard formula:  $\pi/6 \times (\text{length}) \times (\text{width}^2)$ .<sup>18</sup> The pathologic analysis was performed by using Aperio image analysis (Aperio Technologies, Inc., Vista, CA, USA). Staining positivity was defined as strong positive and positive results in Aperio image analysis.

### ***Statistical analysis***

Results from at least three independent *in vitro* experiments and at least five mice *in vivo* experiments are expressed as the mean ± SEM and analyzed using Spearman's rank order correlation analysis, Mann

Whitney U-test, and student T test. P values  $<0.05$  are considered statistically significant.

***Ethics statement***

All animal experiment protocols were approved by the Seoul National University Institutional Animal Care and Use Committee (approval number, 12-2014-00-22).

## Results

### *VPA increases NKG2D ligands expression in vitro*

FACS was performed to confirm the molecular mechanism of VPA *in vitro*. The RAE-1 in MH-134 cell lines and MIC-A expression levels in three human HCC cell lines (Huh-7, SNU-761, and SNU-475) were quantified by calculating the Specific fluorescence indices (SFI). In all HCC cell lines, the expression of RAE-1 or MIC-A was markedly increased after VPA treatment. After the cells were incubated for 24 h with VPA, the expression of RAE-1 was increased 2.3-fold in MH-134 cells (control group:  $130.3 \pm 38.7$ ; VPA exposed group:  $301 \pm 5.54$ ,  $P = 0.050$ ) and MIC-A was increased 2.4-fold in Huh-7 cells, 3.7-fold in SNU-761 cells, and 6.5-fold in SNU-475 cells compared with control (Figure 2a, 2b). As a positive control for HDAC inhibition, we also evaluated whether vorinostat increases MIC-A expression in HCC cells. As shown in Figure 2c, the expression of MIC-A was increased 3.4-fold by 1  $\mu$ M vorinostat and 19.4-fold by 2  $\mu$ M vorinostat treatment. These results are consistent with the assertion that inhibition of HDAC by

VPA or vorinostat increased the expression of RAE-1 or MIC-A protein by accelerating the transcription of its mRNA. We additionally evaluated if VPA and/or vorinostat alters the expression levels of immunosuppressive molecules such as PD-L1 (programmed cell death ligand 1) and PD-L2. As shown in Figure 3a, VPA and vorinostat decreased the transcription of PD-L1 (control:  $1.00\pm 0.01$ ; vorinostat:  $0.95\pm 0.01$ ,  $P=0.047$ ; VPA:  $0.56\pm 0.03$ ,  $P=0.017$ ) and PD-L2 (control:  $1.04\pm 0.10$ ; vorinostat:  $0.71\pm 0.04$ ,  $P=0.005$ ; VPA:  $0.43\pm 0.01$ ,  $P<0.001$ ). Protein expression levels were also decreased by VPA and vorinostat (Figure 3b). Altogether, these results indicate that HDAC inhibitors may potentiate the efficacy of immunotherapies against HCC.

### ***In vitro cytotoxicity assay***

Since VPA and vorinostat increased MIC-A expression in HCC cells, we next evaluated whether VPA and vorinostat indeed enhance CIK cell cytotoxicity against HCC cells. Human CIK cells were obtained for this purpose, and *in vitro* cytotoxicity assays were performed. As shown in Figure 4, VPA and vorinostat significantly increased CIK cell cytotoxicity against SNU-761 cells (control:  $22.2\pm 7.6$ ; VPA:

60.40±10.16, P<0.001; vorinostat: 56.54±9.73, P<0.001).

### ***Determination of the appropriate VPA dose in vivo***

We next determined the appropriate VPA dosage for examining effects that were synergistic but not cytotoxic when VPA treatment was combined with other agents in a mouse model of HCC. Based on previous studies, we estimated that dosage to be 10 or 30 mg/kg/day administered once daily for 7 days. After 7 days of VPA treatment, the changes in the tumor volume in the control group (saline) and in the group of mice receiving 10 mg/kg/day of VPA were not different ( $P= 0.100$ ). However, the tumor volume was significantly reduced in the group receiving 30 mg/kg/day of VPA compared with that in the control group ( $P= 0.008$ ). Therefore, the maximal VPA dose was determined to be 10 mg/kg/day because it did not have significant cytotoxicity (Figure 5).

### ***CIK cell culture***

CIK cells were generated according to the protocol described in **Materials and Methods section**. Murine splenocytes obtained by splenectomy for manufacturing the CIK cells were collected from the

spleens of 5-week-old C3H mice. Mononuclear cells derived from the spleen were separated and cultured for 14 days with interleukin 2 and a monoclonal antibody to CD3. During cell culture, the absolute cell count was increased and the proportions of cells that were positive for the T-cell marker CD3 (D0,  $3370.3 \pm 763.4$ ; D7,  $7826.7 \pm 930.8$ ; D14,  $8868.0 \pm 446.5$ ;  $P = 0.050$ , D7 vs. D0;  $P = 0.050$ , D14 vs. D0) and the cytotoxic T-cell marker CD8 $\alpha$  (D0,  $1452.3 \pm 474.5$ ; D7,  $2539.0 \pm 631.2$ ; D14,  $3738.3 \pm 1178.7$ ;  $P = 0.127$ , D7 vs. D0;  $P = 0.127$ , D14 vs. D0) were also increased. The proportion of cells positive for the natural killer cell marker CD161 was maintained during the 2 weeks of culture (D0,  $499.3 \pm 174.0$ ; D7,  $409.7 \pm 91.5$ ; D14,  $477.3 \pm 152.4$ ;  $P = 0.127$ , D7 vs. D0;  $P = 0.827$ , D14 vs. D0). These results suggest that CIK cells, which have a mixed natural killer cell- and cytotoxic T cell-like phenotype, proliferated well compared to isotype IgG control (Figure 6).

### ***Synergistic effect of CIK with VPA***

Compared with that in the control group, the relative tumor volume in the group treated with CIK cells alone was slightly but not significantly reduced ( $P = 0.597$ ) and that in the group treated with VPA only was

also unchanged ( $P= 0.754$ ). This result confirms that this dose of VPA (10 mg/kg/day) did not have a cytotoxic effect. However, the combination therapy of CIK cells and VPA produced a significant synergistic effect. Following 7 days of combined CIK cells and VPA treatment, the relative tumor volume increased to 5.20 while that in the control group increased to 11.25 ( $P= 0.047$ ; Figure 7).

### ***CIK cells infiltration and apoptosis in tumor tissue***

CIK cell infiltrations were observed in tumor tissue of CIK cell treated mice and CIK cells combined with VPA treated mice. More CIK cell infiltrations and apoptosis were found in tumor tissue of combination treatment mice than that of CIK cell single treatment mice. Tumor tissue of VPA single treatment mice showed some apoptosis in TUNEL assay without lymphocyte infiltration in H and E stain. In TUNEL assay, ratio of stain positive cells to total stain cell are  $24.31\pm 6.28\%$  in control,  $28.31\pm 2.30\%$  in VPA treated mice,  $28.77\pm 4.50\%$  in CIK cell treated mice, and  $38.30\pm 2.79\%$  in combination treatment mice. Single treatment with VPA or CIK cells represented a mild increase of apoptosis in each tumor tissue (Figure 8).

## Discussion

We attempted to prove the synergism of CIK cells and VPA in an HCC mouse model, and obtained results that supported our hypothesis. To determine whether VPA and/or vorinostat alters the expression levels of MIC-A, which is a stress-inducible surface glycoprotein for the NKG2D<sup>19</sup> in HCC cells, we used FACS and found that the MIC-A expression level was increased in VPA- and vorinostat-treated compared with untreated HCC cell lines. We also found that VPA and vorinostat decreased the expression levels of PD-L1 and PD-L2 in HCC cells. As a next step, we investigated whether VPA treatment-induced expression of the NKG2D ligand on the surface of tumor cells enhances the therapeutic efficacy of CIK cells. In our study, the synergistic effect of CIK cells and VPA treatment was investigated in a subcutaneous HCC model. Treatment with either therapy alone was ineffective, whereas combination therapy with the same dosages of CIK cells and VPA showed significant cytotoxic effects. These results suggest that VPA has significant activity as a helper for CIK cells, and

the molecular biology background for these molecules supports this suggestion.

Many previous studies have reported antitumor effects of CIK cells in diverse tumors. CIK cells show a potent MHC unrestricted tumor killing ability against various tumors, including HCC, leukemia, gastric, ovarian, renal, and gastric cancers, mainly through the use of the innate immune system.<sup>9, 20-22</sup> Those CIK cells are mixtures of T lymphocytes. Among them, the CD3+CD56+ subset is the main effectors against tumor cells. Unfortunately, this subset comprises less than 5% of fresh peripheral blood mononuclear cells, suggesting that the human immune system alone is insufficient to cope with tumor cells. However, this CD3+CD56+ subset of T lymphocytes can be expanded *ex vivo* through the use of molecular biology.<sup>5</sup>

CIK cells kill tumor cells by recognizing NKG2D ligands on the tumor cell surface.<sup>16</sup> Although CIK cells show clear antitumor effects, these effects are insufficient for application in clinical treatment. A satisfactory antitumor effect of CIK cell therapy may be achieved once the ratio of CIK:tumor cells is greater than 30:1.<sup>20</sup> However, this high level of CIK cells causes adverse effects. Thus, combinations with

novel agents have been investigated in an attempt to reduce the CIK cell concentration to that which does not induce adverse toxicity. Moreover, owing to molecular biology development, the antitumor mechanisms of CIK cells are now better understood and indicate that CIK cell-based antitumor therapy is a promising novel strategy.

VPA has been used as an antipsychotic agent and in the treatment of epilepsy, bipolar disorder, migraine, and other disorders. Even though VPA has been widely used for decades and its antitumor effects have been gradually identified, its diverse antitumor mechanisms have not yet been completely elucidated. However, the HDAC inhibitory mechanism of VPA is relatively well known.<sup>8-10</sup> HDAC is reported to be aberrantly expressed in diverse cancers. HDAC inhibition can increase the expression of several genes by accelerating their transcription, which can enhance the innate immune system and lead to suppression of tumor growth.<sup>9,24</sup>

VPA alters the expression of various genes involved in the interaction with the cellular immune system, which is known as NKG2D ligand in tumor cells. The observed NKG2D-mediated activation of cytotoxic lymphocytes critically relies on NKG2D ligand

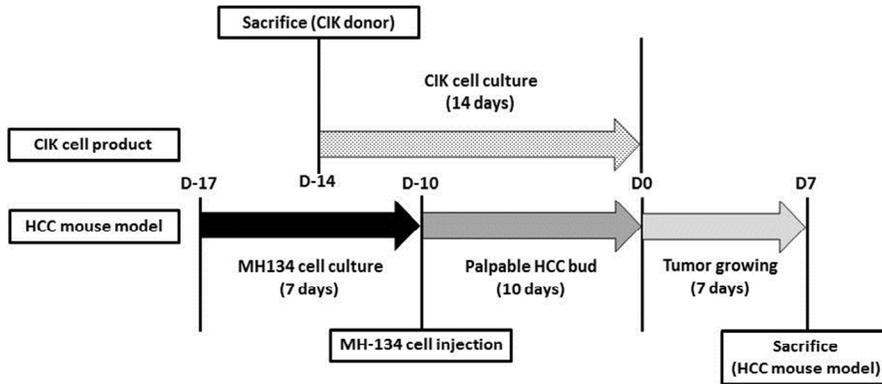
expression levels at the tumor cell surface.<sup>25</sup> Among NKG2D ligands, MIC-A and MIC-B are constitutively expressed at low levels compared with transcript levels of a housekeeping gene in the HCC cell line. Treatment with VPA increases MIC-A and MIC-B messenger-RNA (mRNA) without significantly altering the signal for the housekeeping gene.<sup>26</sup> These results suggest that VPA as an HDAC inhibitor may induce HCC cell death through cytotoxic NK cells by increasing NKG2D ligand expression. The mechanism of tumor immune-escape is achieved by degradation of NKG2D ligand on the tumor cell surface,<sup>27</sup> reducing the host's innate immunity against tumor cells. As already mentioned, VPA increases mRNA levels of NKG2D ligand, which can activate the innate immune system through cytotoxic NK cells.<sup>26</sup> This cytotoxicity through the innate immune system has been shown to be critically dependent on the NKG2D/NKG2D ligands interaction, because it is blocked by addition of anti-NKG2D.<sup>27</sup> NKG2D ligands make tumor cells susceptible to the innate immune system. Like cytotoxic NK cells, CIK cells can enhance antitumor effect under NKG2D ligand overexpression condition which can be induced by VPA in innate immune system.

Although CIK cell therapy has shown efficacy as an adjuvant therapy for HCC recurrence after curative treatment, it has limited applicability in primary HCC therapy. Here, we showed that the antitumor effect of CIK cells could be enhanced by co-treatment with VPA. These results suggest that combination therapy of CIK cells and VPA may be applied in primary HCC or more severe conditions, such as advanced stage HCC or metastatic HCC. The adverse effects of CIK cell therapy can be decreased by using a lower dosage in conjunction with treatment of VPA, which is readily available, inexpensive, and tolerable.

In conclusion, this study determined that a VPA-induced increase in NKG2D ligand expression enhanced the effects of CIK cell therapy in the treatment of HCC. We also determined the appropriate VPA concentration for enhancing MIC-A expression without causing cytotoxicity for use in future studies utilizing this mouse model of HCC.

**Financial Support:** Funding: This study was supported by grants from Ministry for Trade, Industry, and Energy, Republic of Korea and from the Liver Research Foundation of Korea.

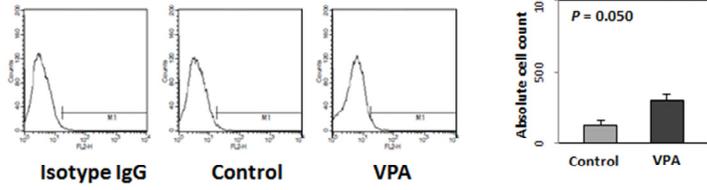
## Figures.



**Figure 1. Schedule of CIK cells differentiation and a HCC mouse model generation.** Schedule of CIK cells differentiation and a HCC mouse model generation. MH-134 cells were cultured for 7 days. 4 days before MH-134 cell injection to C3H mice, splenocytes were obtained by sacrificing other 5-week-old C3H mice. With these splenocytes, CIK cells were cultured for 14 days. Interventions (control, VPA, CIK cells, CIK cells + VPA) were performed at D0. After then, each tumor volume was observed for 7 days. All mice were sacrificed at D7 to acquire their tumors for analysis.

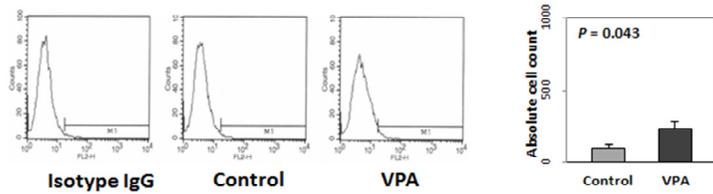
2a.

MH-134 (RAE-1)

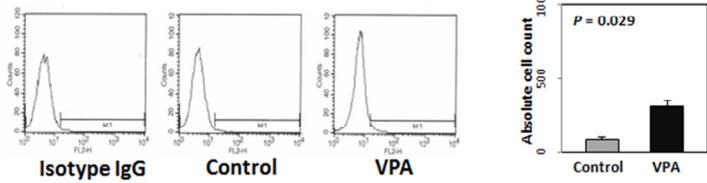


2b.

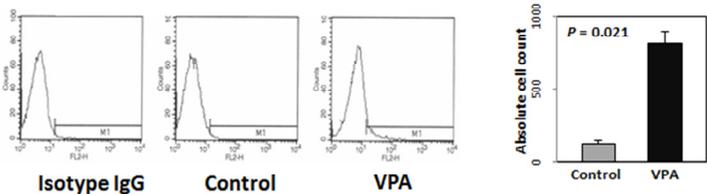
Huh-7 (MIC-A)



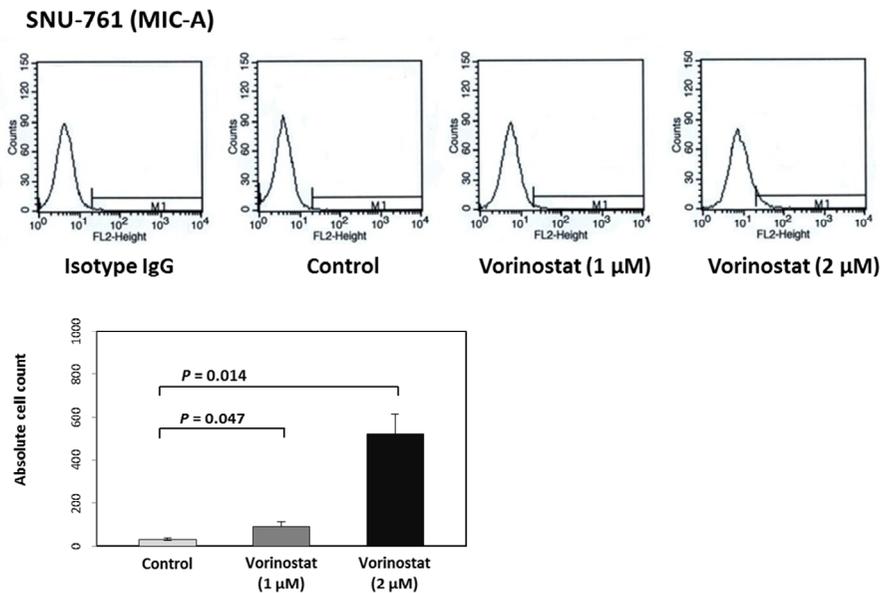
SNU-761 (MIC-A)



SNU-475 (MIC-A)



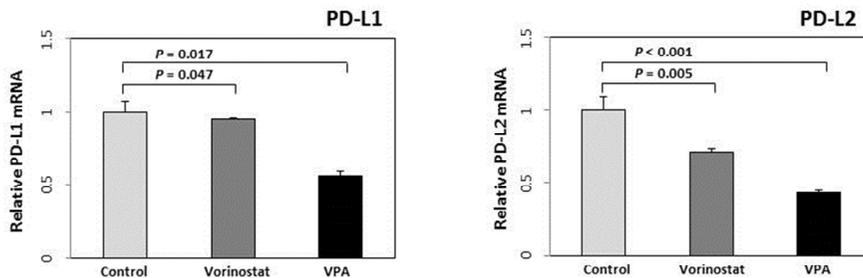
2c.



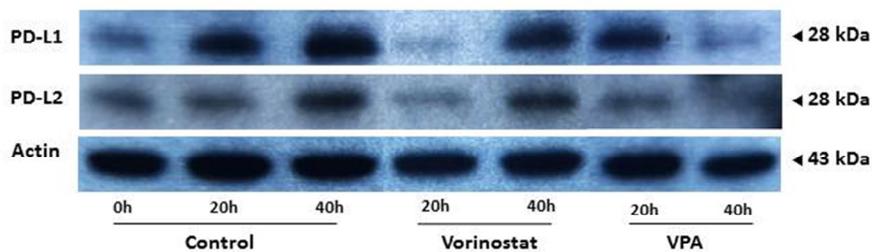
**Figure 2. Effect of VPA or vorinostat on NKG2D ligand (RAE-1 or MIC-A) expression in four HCC cell lines. a.** MH-134 cell lines were evaluated for identifying RAE-1 expression. After 40 hours of incubation with VPA (5mM), the expression of RAE-1 was increased 2.3 fold in MH-134. **b.** Huh-7, SNU-475, and SNU-761 cell lines were evaluated for identifying MIC-A expression. After 40 hours of incubation with VPA (5 mM), the expression of MIC-A was increased 2.4 fold in Huh-7, 3.7 fold in SNU-761, and 6.5 fold in SNU-475 compared to control. **c.** SNU-761 cell lines were evaluated for

identifying MIC-A expression following vorinostat treatment. After 40 hours of incubation with vorinostat (1  $\mu$ M and 2  $\mu$ M), the expression of MIC-A was increased 3.4 fold with 1  $\mu$ M, and 19.4 fold with 2  $\mu$ M, respectively.

3a.



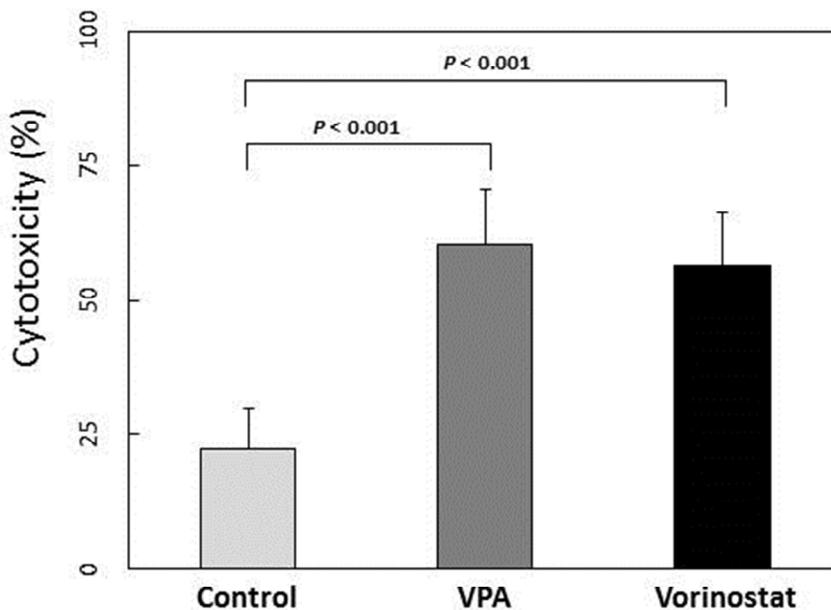
3b.



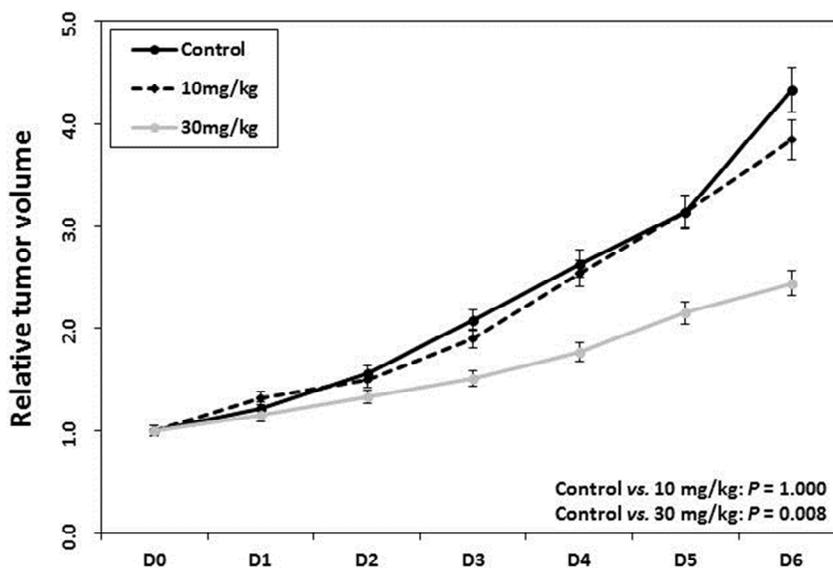
**Figure 3. Effect of VPA or vorinostat on PD-L1 and PD-L2 expression in HCC cells.**

a. The mRNA expressions of PD-L1 and PD-L2 in SNU-761 cells were quantified by real-time PCR and normalized to GAPDH expression levels. VPA (5 mM) and vorinostat (2  $\mu$ M) decreased transcription of PD-L1 (control:  $1.00\pm 0.01$ ; vorinostat:  $0.95\pm 0.01$ ,  $P=0.047$ ; VPA:  $0.56\pm 0.03$ ,  $P=0.017$ ) and PD-L2 (control:  $1.04\pm 0.10$ ; vorinostat:

0.71±0.04,  $P=0.005$ ; VPA: 0.43±0.01,  $P<0.001$ ). The experiments were repeated three times. **b.** Immunoblot analyses of PD-L1 and PD-L2 were also performed in SNU-761 cells treated with VPA (5 mM) or vorinostat (2  $\mu$ M). Protein expression levels were also decreased following VPA or vorinostat treatment.

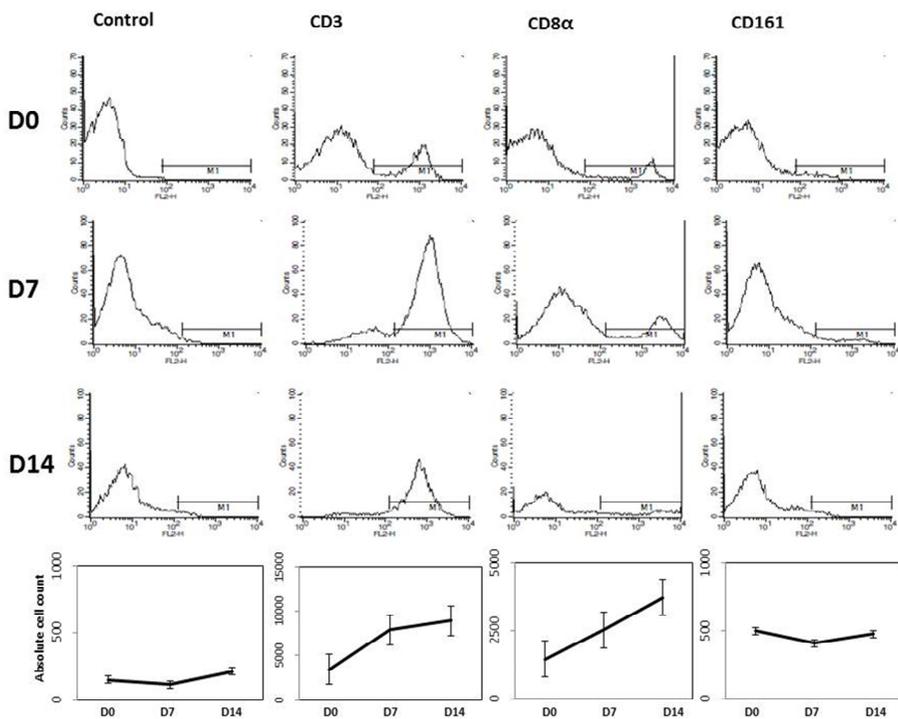


**Figure 4. In vitro cytotoxicity assay.** SNU-761 cells were incubated with VPA (5 mM) or vorinostat (2  $\mu$ M) for 40 hours, and then,  $2 \times 10^5$  target cells/ml were prepared. Human CIK cells (effector cells) were obtained from mononuclear cells cultured with interleukin 2 and immobilized monoclonal antibody to CD3, and then cells were mixed to  $3 \times 10^6$  cells/ml with washing medium. For in vitro cytotoxicity assay, each well consisted of 50  $\mu$ l of target cells and 100  $\mu$ l of effector cells. After 4 hours incubation, treated cells were centrifuged and optical densities of supernatant were measured. VPA (5 mM) or vorinostat (2  $\mu$ M) significantly increased CIK cell cytotoxicity on SNU-761 cells (control:  $22.2 \pm 7.6$ ; VPA:  $60.40 \pm 10.16$ ,  $P < 0.001$ ; vorinostat:  $56.54 \pm 9.73$ ,  $P < 0.001$ ).

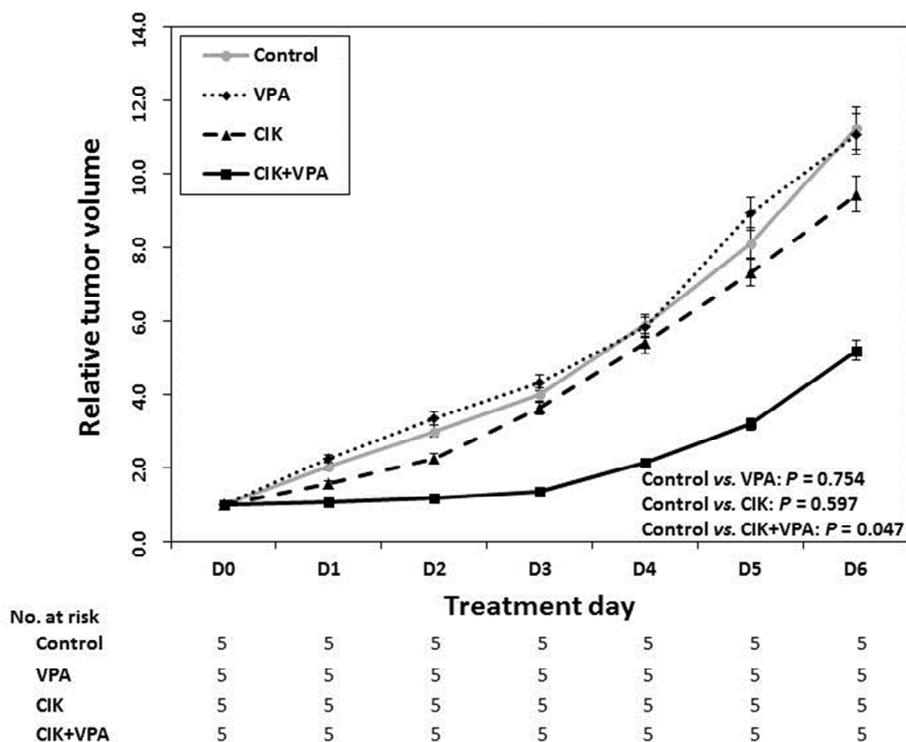


| No. at risk  | Treatment day |   |   |   |   |   |   |
|--------------|---------------|---|---|---|---|---|---|
| Control      | 5             | 5 | 5 | 5 | 5 | 5 | 5 |
| VPA 10 mg/kg | 5             | 5 | 5 | 5 | 5 | 5 | 5 |
| VPA 30 mg/kg | 5             | 5 | 5 | 5 | 5 | 5 | 5 |

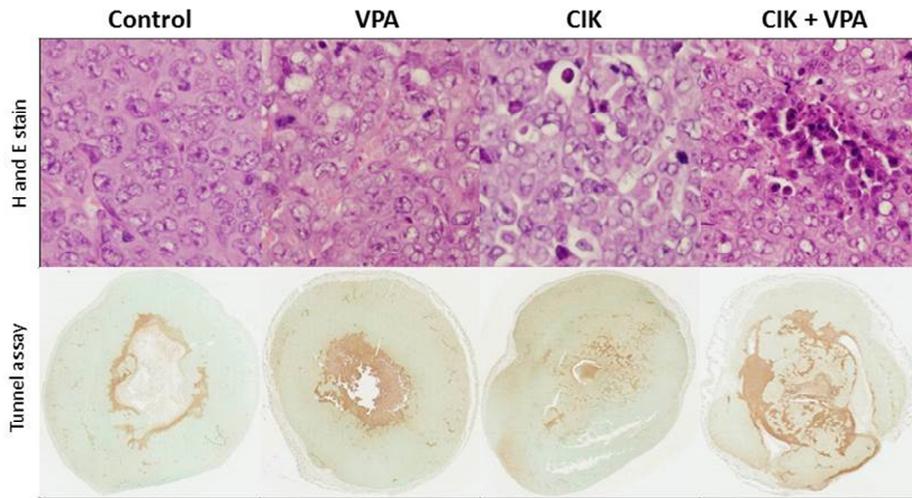
**Figure 5. Identification of maximum VPA dose without cytotoxicity *in vivo*.** After every 7 day of VPA application, control group and 10 mg/kg/day group were not different in the tumor volume change ( $P=0.100$ ). The tumor volume was significantly reduced in 30mg/kg/day VPA application group compared to control group ( $P=0.008$ ).



**Figure 6. Generation of mouse CIK cells.** Four spleens of 5-week-old C3H mice were acquired and incubated with interferon  $\gamma$  100 U/ml for 24 hours. After then, cell dishes were moved to CD3 monoclonal antibody coated dishes followed by interleukin 2 (500 U/ml). CD3, CD8 $\alpha$ , and CD161 were checked to identify the CIK cell differentiation. CIK cells were proliferated well compared to isotype IgG control.



**Figure 7. Synergistic effect of CIK with VPA *in vivo*.** Relative tumor volume of VPA single or CIK single treatment group was not statistically different from that of control group ( $P= 0.754$  and  $P= 0.597$  respectively). The combination therapy of CIK cells + VPA produced a significant synergistic effect ( $P= 0.047$ ). The relative tumor volume of combination therapy group increased to 5.20 while that in the control group increased to 11.25.



**Figure 8. *CIK cells infiltration and apoptosis in tumor tissue.*** More CIK cell infiltration and apoptosis were observed in CIK cells + VPA combination treatment group than CIK or VPA single treatment mice tissue. Apoptosis of each group was evaluated in TUNEL assay. Percent of positivity is  $24.31 \pm 6.28\%$  in control,  $28.31 \pm 2.30\%$  in VPA treated mice,  $28.77 \pm 4.50\%$  in CIK cell treated mice, and  $38.30 \pm 2.79\%$  in combination treatment mice.

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## 요약 (국문 초록)

**배경:** Cytokine-induced killer (CIK) 세포는 실험실에서 만들 수 있으며, NK 세포와 T 림프세포의 특성을 모두 지녀서 항암효과를 가지고 있는 면역세포이다. 그러나 CIK 세포 단독으로는 충분한 항암효과를 보여주지 못하는 것으로 알려져 있다. Valproate는 CIK 세포가 인지하는 간세포암 세포의 natural killer group 2D (NKG2D) 리간드를 증폭시킬 수 있다. 본 연구에서는 CIK 세포와 valproate의 병합을 통해 나타나는 항암 시너지효과를 쥐 모델에서 밝혀내고자 하였다.

**방법과 결과:** 이 연구에서는 쥐와 인간의 간세포암 세포주인 MH134와 SNUH761 등을 이용하여 실험을 시행하였다. NKG2D 리간드의 증폭은 FACS를 통해서 확인하였고, 인간 CIK 세포를 이용하여, 세포독성시험을 확인하였다. 이후 쥐에서 피하-간세포암 모델을 만들어 CIK 세포와 valproate의 항암효과를 평가하였다. 세포 실험에서 간세포암 세포주에 valproate를 처리하였을 때, NKG2D 리간드의 유의미한 증폭을 확인할 수가 있었고, 세포독성실험에서 CIK 세포 단독처리군 보다 CIK세포와 valproate 병합 처리군에서

세포독성의 유의미한 증가를 확인할 수 있었다. 쥐의 피하-간세포암 모델에서는 대조군, valproate 단독 처리군, CIK 세포 단독 처리군, CIK 세포와 valproate 병합 처리군에서 각각 종양의 성장을 7일간 비교하였고, 대조군에 대비하였을 때 CIK 세포와 valproate 병합치료 군에서만 유의한 종양억제가 확인되었다. 이후 시행한 종양조직 병리검사에서도 TUNEL염색시에 병합 처리군에서 높은 세포자멸사를 확인할 수 있었다.

**결론:** Valproate는 간세포암 세포주에서 NKG2D 리간드를 증폭시키는 것을 확인할 수 있었다. 또한 CIK 세포와 valproate를 병합하여 치료를 하면, 단독 치료와 비교하여 항암 효과가 증대되는 것을 밝혀내었다.

**주요어:** 간세포암, 면역치료, Cytokine-induced killer 세포, Valproate

**학번:** 2015-21968