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doxorubicin 과 flavopiridol 의
상승적 항암효과 규명

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Synergistic anti-tumor efficacy of doxorubicin and flavopiridol in hepatocellular carcinoma

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Seoul National University
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Min-Sun Kwak

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지도 교수 윤 정 환

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Synergistic anti-tumor efficacy of doxorubicin and flavopiridol in hepatocellular carcinoma

by
Min-Sun Kwak

A thesis submitted to the Department of Internal Medicine in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Medicine at Seoul National University College of Medicine

January, 2014

Approved by Thesis Committee:

Professor _____Chairman
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논문 제목: Synergistic anti-tumor efficacy of doxorubicin and flavopiridol in hepatocellular carcinoma

학위구분: **석사 □ · 박사 ▣**
학 과: 의학과 내과학
학 번: 2011-30533
연 락 처:
저 작 자: 곽 민 선 (인)

제 출 일: 2014 년 1 월 10 일

서울대학교총장 귀하

ABSTRACT

Synergistic anti-tumor efficacy of doxorubicin and flavopiridol in hepatocellular carcinoma

Min-Sun Kwak

Department of Internal Medicine

Seoul National University College of Medicine

Introduction: Hypoxic hepatocellular carcinoma (HCC) cells have been reported to be less sensitive than normoxic HCC cells to doxorubicin due to N-myc downstream-regulated gene-1 (NDRG1) expression. Additionally, a previous *in vitro* study showed that flavopiridol, a cyclin-dependent kinase inhibitor, suppresses NDRG1 expression and sensitizes hypoxic HCC cells to doxorubicin cytotoxicity by increasing apoptosis. Based on this, we investigated whether the combination treatment of doxorubicin and flavopiridol has a synergistic anti-tumor efficacy against an *in vivo* model of HCC.

Methods: An *in vivo* HCC mouse model was established by

implanting C3H/He mouse with MH134 HCC cells. The mice were divided into 4 groups, and each group was administered DMSO (control group), doxorubicin, flavopiridol, or the doxorubicin + flavopiridol combination. We evaluated necrotic area and hexokinase II expression to evaluate whether hypoxic condition was achieved in HCC. The anti-tumor efficacy was evaluated by measuring tumor volumes, and the anti-tumor mechanism was investigated by quantifying apoptotic cells, microvessel densities, and NDRG1 expression through immunohistochemical staining of tumor tissue. In the *in vitro* portion of the study, HCC cell viability was assessed using the MTS assay. The apoptotic signaling pathway and expression of NDRG1 were evaluated with immunoblotting.

Results: In all mouse HCC tumor tissue, hypoxic condition was achieved. Tumor growth was significantly suppressed in the doxorubicin+flavopiridol combination group compared to the control, doxorubicin-only, or flavopiridol-only group. On immunohistochemical staining of tumor tissue, the percentage of apoptotic cells was significantly high in the doxorubicin+flavopiridol combination group; however, microvessel densities were not statistically different across

groups. Additionally, flavopiridol suppressed the expression of NDRG1 in mouse tumor tissue. In the *in vitro* study, the doxorubicin+flavopiridol combination treatment significantly suppressed cell viability by increasing apoptosis through the caspase 7-dependent pathway. Flavopiridol also suppressed NDRG1 expression in MH134 cells *in vitro*.

Conclusions: These results show that combination treatment of doxorubicin and flavopiridol has a synergistic anti-tumor effect in an HCC model, both *in vivo* and *in vitro*. This can be interpreted that flavopiridol killed doxorubicin-resistant cells under hypoxic condition, which continued to exist after doxorubicin treatment. Synergistic effect of doxorubicin and flavopiridol combination therapy was attributed to increased apoptosis by suppressing NDRG1 expression rather than decreased angiogenesis.

Keywords: Hepatocellular carcinoma, doxorubicin, flavopiridol, apoptosis, hypoxia

Student number: 2011-30533

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LIST OF ABBREVIATIONS

HCC: hepatocellular carcinoma

TACE: transarterial chemoembolization

NDRG1: N-myc downstream-regulated gene-1

NONMEM: nonlinear mixed effect modeling

H&E: hematoxylin and eosin

MVD: microvessel density

HK II: hexokinase II

MTS: 3,4-(5-dimethylthiazol-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt

JNK: c-Jun N-terminal kinase

eIF2α: eukaryotic initiation factor 2α

SD: standard deviations

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignant disorder worldwide and causes nearly 1 million deaths a year (1). HCC is generally considered to be a hypervascular tumor; thus, transarterial chemoembolization (TACE) is employed as a standard treatment when radical therapies for HCC are not feasible. However, TACE is occasionally ineffective for advanced infiltrative HCCs or locally recurrent nodules after TACE. It has been thought to be associated with profound hypoxic insults in these tumor types. These tumors sometimes grow more rapidly and portend a poorer prognosis than mass-forming hypervascular tumors (2, 3). Unfortunately, systemic chemotherapy suggested as an alternative treatment option for these tumors is also ineffective; no definite evidence that cytotoxic drugs improve survival compared to supportive care has been presented (4). Doxorubicin, an anthracycline agent that has been commonly tried for advanced HCC, showed only a response rate of about 15-20% (6, 7). Previous *in vitro* study showed that hypoxic HCC cells are more resistant to doxorubicin (5), which could be a plausible explanation the reason why infiltrative-type HCC

are expected to be highly resistant to doxorubicin. Therefore, there is a need to develop a more efficient strategy to improve treatment outcomes, especially for infiltrative HCC or HCC in hypoxic insult.

A previous study showed that N-myc downstream-regulated gene-1 (NDRG1), which is a hypoxia-inducible carcinogenesis-related protein, was responsible for doxorubicin resistance in HCC cells, particularly under hypoxic conditions (5). This study showed that hypoxia enhanced NDRG1 expression, and doxorubicin sensitivity increased with suppression of NDRG1 by siRNA or a drug (flavopiridol) (5).

Flavopiridol, a semisynthetic flavonoid, is an inhibitor of several cyclin-dependent kinases and displays unique anticancer properties with various mechanisms of action such as cyclin-dependent kinase inhibition, apoptosis induction, DNA interaction, and others (8). In human colon cancer cells, flavopiridol was shown to suppress NDRG1 expression and to increase the sensitivity of colon cancer cells to CPT-11, a chemotherapeutic agent. Similarly, flavopiridol can be effective in HCC. In fact, flavopiridol enhanced doxorubicin sensitivity by suppressing NDRG1 expression in hypoxic HCC cells (5, 9).

However, there were few studies showing these effects in animal models.

The aim of this study was to confirm the synergistic effect of doxorubicin and flavopiridol in an *in vivo* HCC mouse model in hypoxic microenvironment. We also repeated the *in vitro* study for a mouse MH134 HCC cell line.

Materials and methods

1. Cell culture and reagents

MH134 cells, a mouse HCC cell line, were used for the *in vivo* and *in vitro* experiments. MH134 cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin (100,000 U/L), and streptomycin (100 mg/L). Cells were serum starved overnight to avoid serum-induced signaling prior to all experiments performed in this study. Cells were incubated under standard condition (20% O_2 and 5% CO_2 at 37℃) or hypoxic culture condition (1% O_2 and 5% CO_2 and 94% N_2, at 37℃). Doxorubicin and flavopiridol were purchased from Sigma-Aldrich (St. Luis, MO, USA).

2. Animals

Animal experiments were performed using 5-week old male C3H/He mice (Charles River Laboratories, Wilmington, MA, USA). Mice were housed under specific pathogen-free conditions, and treated in accordance with the guidelines issued by the Institutional Animal Care and Use Committee of Seoul National University Hospital (12-0176).

3. Mouse HCC model

We used a previously established subcutaneous HCC mouse model (10). Briefly, 2.5 x 10^5 viable MH134 cells suspended in 0.1 mL of RPMI-1640 were injected subcutaneously to produce a bleb in right flanks of C3H/He mice. When tumor volumes reached 0.5~1.0 cm^3, doxorubicin (4mg/kg) and/or flavopiridol (5mg/kg) was administered intraperitoneally daily for 6 consecutive days as follows; In flavopiridol group, flavopiridol (5 mg/kg/d) was administered every other day for 3 days. In doxorubicin group, doxorubicin (4mg/kg) was administered every other day for 3 days. In combination group, flavorpidiol (5 mg/kg/d, on days 1, 3, and 5) and doxorubicin (4 mg/kg/d, on days 2, 4 and 6) were administered alternatively. Control group was administered 1.0% DMSO/sodium chloride solution without drugs. The adequate dose of medication was based on previous *in vivo* study (11). Previous study showed an intra-tumoral hypoxic environment in this mouse model (12). Mice were checked daily for tumor volume, body weight, behavior, water/food consumption. Seven days after administering doxorubicin and/or flavopiridol, mice were killed by

exsanguination via cardiac puncture under general anesthesia induced by isoflurane inhalation. Tumor masses and liver tissues were harvested, fixed in 10% formaldehyde, and cryopreserved.

4. Tumor growth kinetics

An exponential model was selected to describe tumor growth kinetics and the equation used after doxorubicin and/or flavopiridol administration was as follows (12, 13): $V = V0 \times \exp(k \times T)$ where V0 and V are the tumor volumes at baseline and T days later, respectively, and k is the growth rate constant related to tumor doubling time. Data were analyzed using a nonlinear mixed effect modeling (NONMEM) software program (version V, level 1.1, Double Precision), using the first order conditional estimation method and the PRED routine (14). Inter-individual variabilities for V0 and k were modeled using exponential random effect models. For example, the tumor volume at baseline was modeled as $V0i = V0 \times \exp(\eta i)$, where $V0i$ is the tumor volume at baseline for individual i, V0 is the typical value for tumor volume at baseline for the population, and ηi is a normally distributed random variable with mean zero

and variance ω^2_{V0}:. In addition, the residual variability was modeled using a combined additive and proportional error model. The combined error model is described by $Y_{ij} = \hat{Y}_{ij} \times (1 + \varepsilon_{ija}) + \varepsilon_{ijp}$ where Y_{ij} is the jth observed tumor volume in individual i, $\hat{Y}_{ij}$ is the jth predicted tumor volume in individual i, and ε_{ija} and ε_{ijp} are normally distributed random variables for individual i and measurement j, with mean zero and variances σ^2_a and σ^2_p; respectively.

5. Quantitative evaluation of the necrotic area

Hematoxylin and eosin (H&E) stain was applied to assess the morphological features of the necrotic tumor regions. ScanScope CS (Aperio Technologies, Inc., Vista, CA, USA) was used to scan the entire tissue sections at ×40 magnification. The histological images of the tissue sections were saved as digital images (in Joint Photographic Experts Group format) in a computer for the measurement of the percentage of necrosis. Aperio ImageScope v11.0.2.725 (Aperio Technologies, Inc., Vista, CA, USA) was used to draw boundaries around the entire tumor region and the region-defining interface between necrotic and viable tissues by hand and to measure the necrotic

area of each tumor tissue section. The pink amorphous areas with glassy homogeneous appearance on the H&E staining were considered to represent necrotic tissues, and the purple pixel area were considered to represent viable tissues (15). The percentage of necrotic tissue from the H&E-stained sections was calculated by dividing the necrotic area with the total tumor area (15).

6. Apoptosis

Apoptosis in tumor tissue was investigated by TUNEL staining using ApopTag *In Situ* Apoptosis Detection Kits (Millipore, Temecula, MA, USA) after fixing fresh tissue in 4% paraformaldehyde. Positive TUNEL cells were counted in six different high-power fields at x400 and averaged. Cell numbers are expressed as percentages of total cells and these are referred to as apoptotic indexes.

7. Microvessel density (MVD)

Immunohistochemical staining for anti-CD 31 (Vector Laboratories, Burlingame, CA, USA) on paraffin-embedded sections was performed using Vectastain Elite ABC Kits

(Vector Laboratories). CD 31-positive microvessels within the most vascular areas of tumor tissues were counted in six different high-power fields at x400 and averaged. Intratumoral mean MVD was expressed as numbers of microvessel/mm^2.

8. Immunohistochemical staining of NDRG1 or hexokinase II (HK II)

Immunohistochemical staining for mouse anti-NDRG1 (Cambridge, MA, USA) or goat anti-HK II (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in paraffin-embedded sections was done with the Vectastain Elite ABC Kit (Vector Liboratories). Anti-NDRG1 positivity was quantified with Aperio ImageScope v11.0.2.725 (Aperio Technologies, Inc., Vista, CA, USA) in six different randomly selected high-power fields at x400 and averaged. Aperio ImageScope calculated the extent of the strong positive, positive, weak positive, and negative. Anti-NDRG1 or anti-HK II positivity was defined as the extent of positive (strong positive, positive, weak positive) cells divided by the total.

9. Cell viability test

Cell proliferation was measured using the CellTiter 96 Aqueous One Solution cell proliferation assay (Promega, Madison, WI, USA), based on the cellular conversion of the colorimetric reagent, 3,4-(5-dimethylthiazol-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) into soluble formazan by dehydrogenase enzymes found only in metabolically active, proliferating cells. Following each treatment, 20 μL of dye solution was added into each well in a 96-well plate and incubated for 1 hour. Subsequently, optical densities were recorded at 490 nm using an ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA).

10. Immunoblot analysis

Cells were lysed for 20 min on ice with lysis buffer (50mM Tris-HCL[pH 7.4]; 1% Nonidet P-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM phenyl-methylsulfonyl fluoride; 1 μM/mL aprotinin, leupeptin, and pepstatin; 1 mM Na3VO4; and 1 mM NaF) and centrifuged at 14,000g for 10 min at 4℃. Samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred

to nitrocellulose membranes, and blotted with appropriate primary antibodies. The blots were incubated with peroxidase-conjugated secondary antibodies (Biosource International, Camarillo, CA, USA). Bound antibodies were visualized using a chemiluminescent substrate (ECL; Amersham, Arlington Heights, IL, USA) and exposed to Kodak X-OMAT film. The primary antibodies were as follows: rabbit anti-caspase 9 and mouse anti-phospho-c-Jun N-terminal kinase (JNK) obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); rabbit anti-phospho-eukaryotic initiation factor 2α (eIF2α), rabbit anti-caspase 7, rabbit anti-phospho-p38 MAP kinase and rabbit-ati-phospho-42/44 MAPK obtained from Cell Signaling Technology Inc. (Denver, MA, USA); rabbit anti-caspase-8 obtained from R&D Systems (Minneapolis, MN USA); and anti-NDRG1 obtained from Abcam (Cambridge, MA, USA). The arbitrary units were calculated by densitometric scanning of the intensity of caspase 7 relative to actin intensity, taking that of control as 1.

11. Statistical analysis

All data represent at least three independent experiments and are expressed as the mean ± standard deviation (SD). Analysis of variance with post hoc analysis was done to compare the quantitation of the necrotic area, immunohistochemical staining (TUNEL assay, microvessel density, NDRG1 and HK II), and cell viability test (MTS assay), among the groups. To reduce the type I error, the LSD method was used in the post hoc analyses. All statistical analyses were done with SPSS, version 19.0 software (SPSS, Inc., Chicago, IL, USA) and Stata version 12.1 (StataCorp, College Station, Tex, USA). *P* values < 0.05 were considered statistically significant.

Results

1. *In vivo* study

1.1 The synergistic anti-tumor efficacy of doxorubicin and flavopiridol in the *in vivo* hypoxic HCC mouse model.

We evaluated the synergistic anti-tumor effect of doxorubicin and flavopiridol against the *in vivo* mice HCC model. Indeed, the mean tumor volume was significantly reduced in the doxorubicin+flavopiridol combination group compared with the control, doxorubicin-only, or flavopiridol-only treated mice (Figure 1). The synergistic effect was examined with NONMEM assuming an exponential tumor growth kinetic curve. The growth rate constants were significantly lower for the doxorubicin+flavopiridol-treated mice than for the untreated controls ($P < 0.001$), doxorubicin-only ($P = 0.001$), or flavopiridol-only ($P = 0.007$) treated mice, showing that doxorubicin and flavopiridol acted synergistically to inhibit tumor growth (Figure 1, Table 1). Body weight change ($P = 0.707$), behavior, and water/food consumption were not different across the 4 groups.

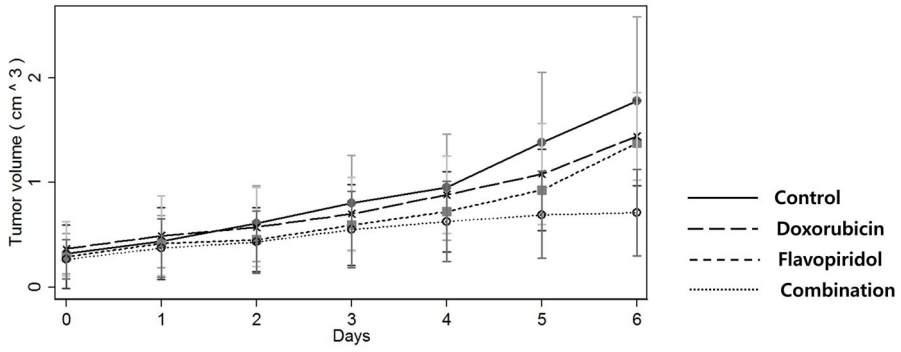


Figure 1. The synergistic anti-tumor effect of doxorubicin and flavopiridol in the *in vivo* model of HCC. An *in vivo* model of HCC was established in C3H mice by implanting MH134 cells subcutaneously. Doxorubicin and/or flavopiridol were administered to the mice for 6 days after tumor volumes had reached 0.5~1.0 cm^3. The tumor volumes were calculated as, 0.5 x L (cm) x W^2 (cm^2), where L and W represent the maximum length and width, respectively. Seven to eight mice were randomly allocated to each group. Points, mean tumor volumes; bars, SD

Table 1. Development of the tumor growth kinetics model by forward addition to the base model and backward elimination from the full model (group 1= control, group 2= flavopiridol, group 3= doxorubicin, group 4= combination).

Hypothesis	−2 * log−likelihood	df	Diff (−2* log−likelihood)	Chi−square (α=0.05)	*P*−value (Adjusted *P*−value)*	Conclusion
Base Model						
K value of each group was identical. (k1=k2=k3=k4)	−88.0	2				
Full Model						
Was k different according to treatment group?	−112.3	5	−24.3	7.81 (df=3)	<0.0001	YES
Backward elimination from the Full Model						
Was k different between group 1 & group 2?	−112.1	4	0.2	3.84 (df=1)	0.6547 (0.9983)	NO
Was k different between group 1 & group 3?	−107.1	4	5.2	3.84 (df=1)	0.0226 (0.1281)	NO
Was k different between group 1 & group 4?	−92.2	4	20.1	3.84 (df=1)	<0.0001 (<0.0001)	**YES**

Was k different between group 2 & group 3?	−108.2	4	4.1	3.84 (df=1)	0.0429 (0.2312)	NO
Was k different between group 2 & group 4?	−93.0	4	19.3	3.84 (df=1)	<0.0001 (0.0001)	**YES**
Was k different between group 3 & group 4?	−101.7	4	10.6	3.84 (df=1)	0.0011 (0.0068)	**YES**

*Adjusted P−value by sidak method

1.2. The synergistic anti-tumor effect of doxorubicin and flavopiridol is mediated by increased apoptosis, but not decreased angiogenesis.

Because hypoxic insult in HCC cells increases doxorubicin resistance, we first evaluated whether an intra-tumoral hypoxic condition was achieved in the mice model. Thus, we evaluated 1) whether a necrotic area implying a hypoxic condition was generated in the HCC tissue, and 2) whether HK II expression, which is increased in hypoxic condition, was expressed in the HCC tumor tissue. Central necrosis was observed in all mice HCC tissues, implying an intra-tumoral hypoxic microenvironment was present. The proportion of necrotic area per total tumor area was different across the 4 groups, with control, doxorubicin-only, flavopiridol-only, and doxorubicin+ flavopiridol combination groups each having 17.5% vs. 24.0% vs. 18.0% vs. 38.5% of proportional necrosis, respectively. The proportion of central necrosis was higher for the mice treated with the doxorubicin+flavopiridol combination compared with control, doxorubicin, or flavopiridol groups (P = 0.004, P = 0.037 and P = 0.005, respectively, Figure 2). HK-II expression was also demonstrated in all 4 groups, implying the

existence of hypoxic microenvironment in tumor tissue.

To identify the mechanism behind the synergistic effect from doxorubicin and flavopiridol, we quantitatively compared the level of apoptosis and angiogenesis, which are well-known effectors of tumor progression, in the 4 groups. We evaluated the apoptotic levels in the 4 groups with TUNEL staining of the tumor tissues. The apoptotic index were 1.5%, 3.3%, 3.2%, and 5.1% in control, doxorubicin, flavopiridol, and combination groups, respectively. As expected, the percentages of TUNEL positive cells were significantly increased in the doxorubicin+flavopiridol combination group compared with the other 3 groups ($P < 0.001$, Figure 4). However, the anti-angiogenic effect evaluated by microvessel density showed no significant differences across the 4 groups ($P = 0.879$, Figure 5).

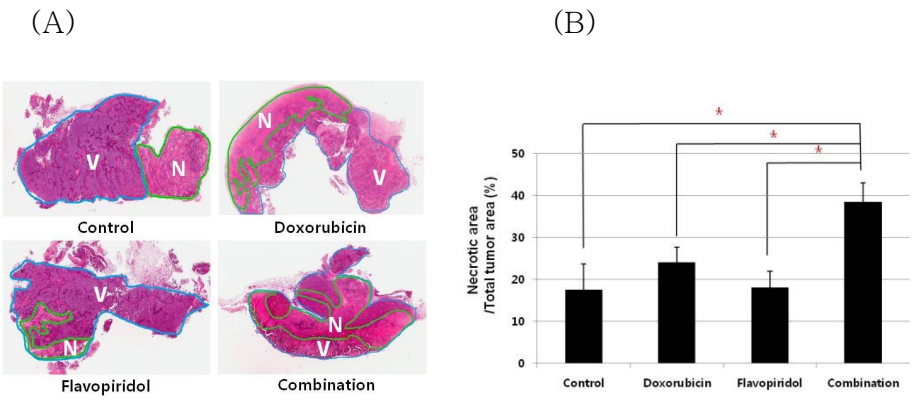


Figure 2. Central necrosis of mouse HCC tissue. (A) The H&E staining image shows that central necrosis (area designated N) developed within the tumor tissue in the control, flavopiridol-only, doxorubicin-only, and doxorubicin+flavopiridol groups (original magnification, ×40). (B) Central necrosis was estimated by dividing the necrotic area with the total tumor area. Central necrosis area was significantly higher in mice treated with the doxorubicin+flavopiridol combination compared to the control, doxorubicin-only and flavopiridol-only treated mice ($P < 0.05$). Columns, the means of 7 or 8 independent cases; bars, SD. *, $P < 0.05$. N represents necrotic tissue. V represents viable tissue.

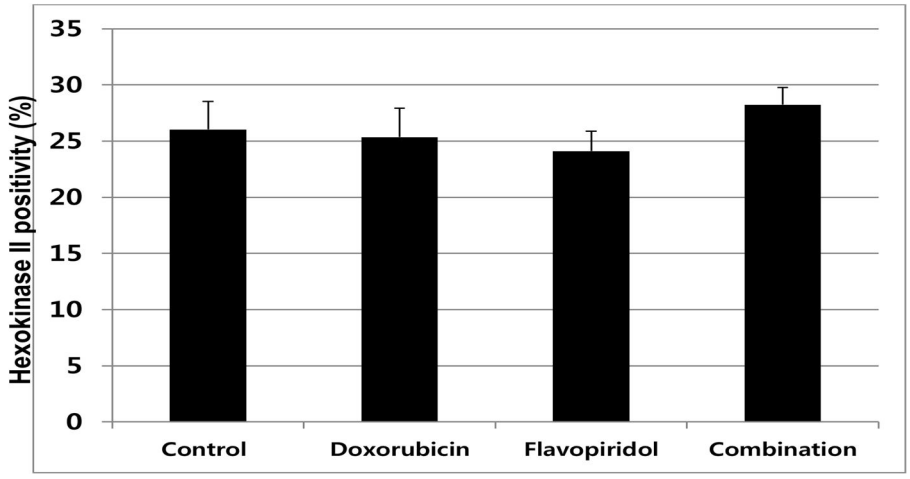


Figure 3. HK II expression in mouse HCC tissue. Immunohistochemical staining for mouse anti-HK II was performed on paraffin-embedded mouse HCC tissues. Anti-HK II expression was evaluated in 6 different randomly selected high-power fields at x400 and averaged. Anti-HK II positivity was quantified using Aperio ImageScope v11.0.2.725 (Aperio Technologies, Inc., Vista, CA, USA). Anti-HK II expression was similarly elevated across the four groups (P=0.481). Columns, mean values of each group; bars, SD.

(A) (B)

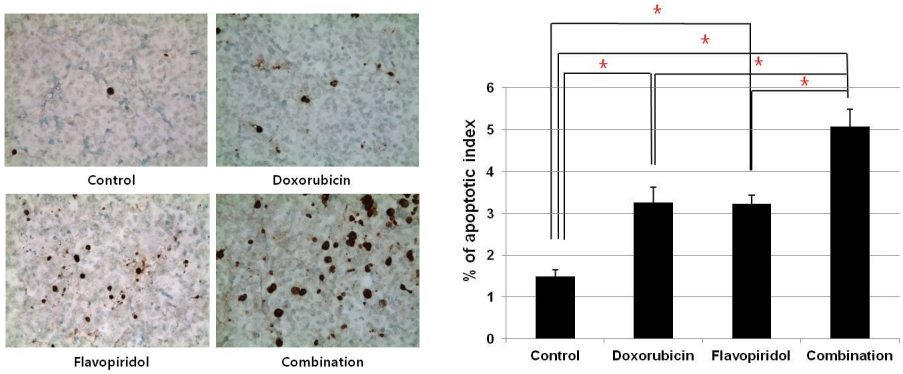


Figure 4. Flavopiridol enhances doxorubicin-induced intra-tumoral apoptotic cell death. (A) Apoptosis in the tumor tissues were assessed by TUNEL staining after fixing fresh tissues in 4% paraformaldehyde. Positive stained cells were counted in 6 different randomly selected high-power fields (×400) and averaged. (B) Cell numbers are expressed as percentages of the total cells, and are referred to as apoptotic indexes. The apoptosis index (TUNEL-positive cell proportions) was significantly increased in mice treated with the doxorubicin+flavopiridol combination compared to the other 3 groups.

Columns, mean values of each group; bars, SD. *, $P < 0.001$.

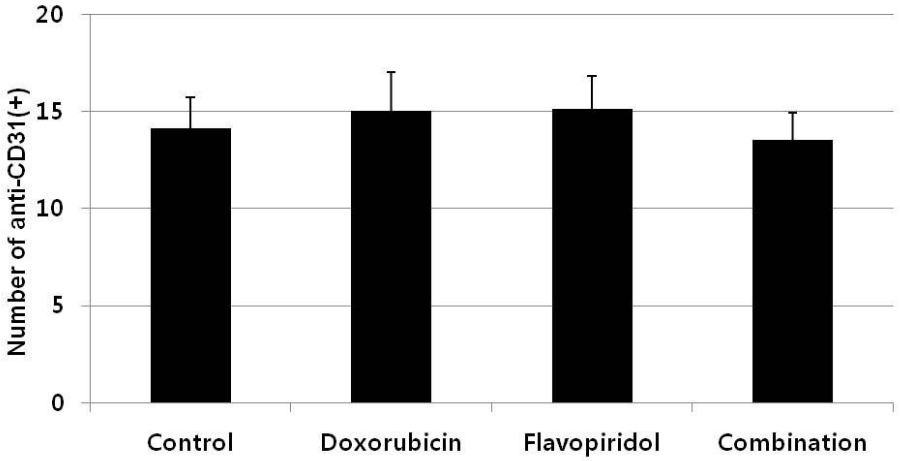


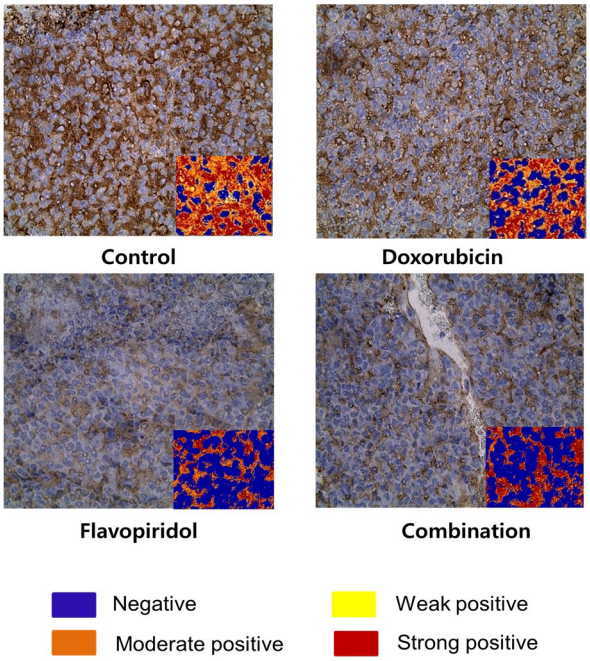
Figure 5. No effect of doxorubicin or flavopiridol on intra-tumoral angiogenesis. Intra-tumoral micorovessel densities (MVDs) were compared by immunohistochemical staining. The mean MVDs were determined by counting CD31 + vessels in 6 different randomly selected high-power fields (×400) and averaged. There was no significant difference in four groups (P = 0.879).

Columns, mean values of each group; bars, SD.

1.3. The increased apoptosis by doxorubicin and flavopiridol is through the suppression of NDRG1 expression.

Because a previous *in vitro* study showed that adding flavopiridol to doxorubicin induces HCC cell apoptosis by suppressing NDRG1 expression, we investigated whether NDRG1 expression in HCC tissues *in vivo* was also significantly decreased when flavopiridol was given as a treatment. As expected, flavopiridol significantly suppressed NDRG1 expression ($P < 0.05$, Figure 6).

(A)


Control
Doxorubicin
Flavopiridol
Combination
Negative
Weak positive
Moderate positive
Strong positive

(B)

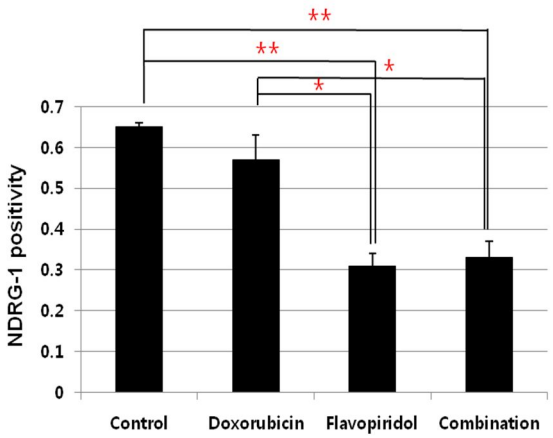

**
**
*
*
NDRG-1 positivity
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
Doxorubicin
Flavopiridol
Combination

Figure 6. Flavopiridol suppresses the expression of NDRG1 in mouse HCC tissue. (A) Immunohistochemical staining for mouse anti-NDRG1 was performed on paraffin-embedded mouse HCC tissues. Anti-NDRG1 expression was evaluated in randomly selected 6 different high-power fields at x400 and averaged. Anti-NDRG1 positivity was quantified using the Aperio ImageScope v11.0.2.725 (Aperio Technologies, Inc., Vista, CA, USA). The Aperio ImageScope calculated the extent of strong positive, positive, weak positive, and negative. Anti-NDRG1 positivity was defined as the extent of positive cells (strong positive, positive, weak positive) divided by the total. (B) Anti-NDRG1 expression was significantly lower in the groups treated with flavopiridol ($P < 0.05$).

Columns, mean values of each group; bars, SD. *, $P < 0.05$ and **, $P < 0.001$

2. *In vitro* study

Because investigators have previously published *in vitro* results by which NDRG1 suppression by flavopiridol was shown to increase doxorubicin sensitivity in hypoxic HCC cells (5), we repeated the main components of the *in vitro* study.

2.1. The doxorubicin and flavopiridol suppressed HCC cell survival in the *in vitro study.*

In the MTS assay, cell viability was significantly decreased for doxorubicin+flavopiridol compared to the control, doxorubicin-only, and flavopiridol-only treated group ($P < 0.001$, $P = 0.039$, and $P < 0.001$, respectively, Figure 7).

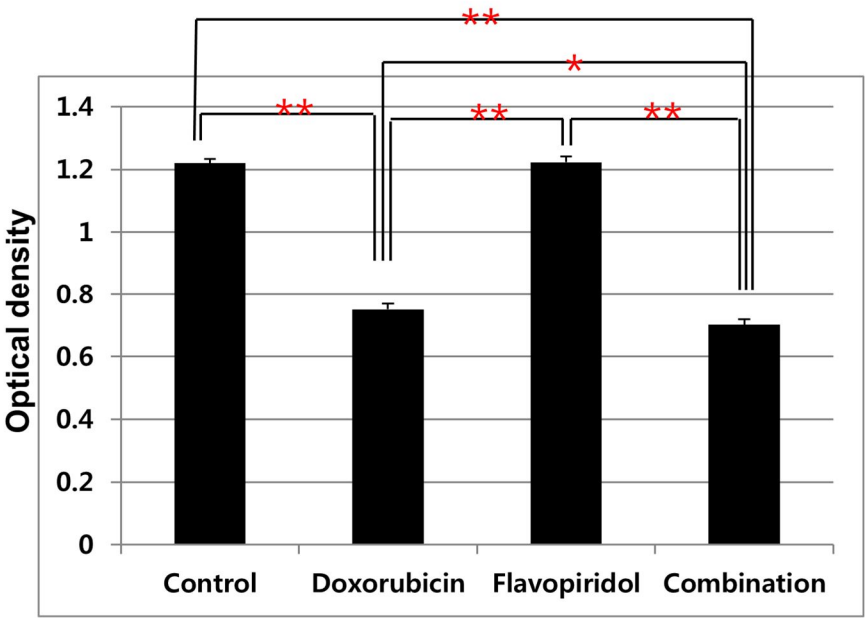


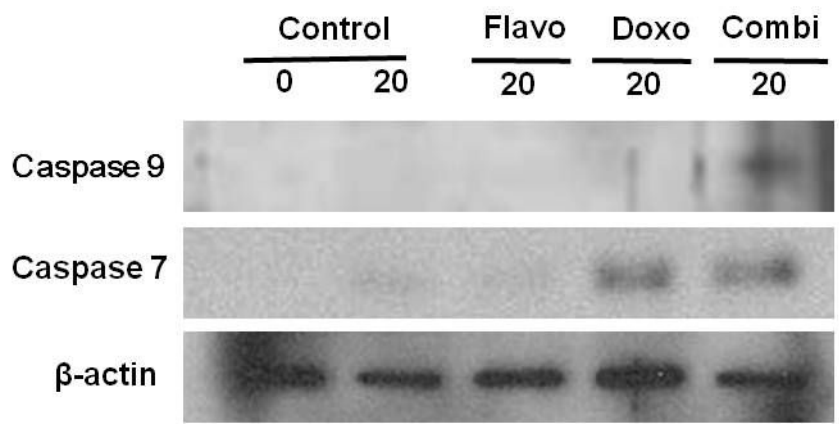
Figure 7. Flavopiridol enhanced doxorubicin-induced cell death in the MTS assay. MH134 cells were incubated in the absence or presence of flavopiridol (300 nM) for 4 hours, then treated for 24 hours with doxorubicin (2 μM) under hypoxic culture conditions. Cell viability was measured by MTS assay, which was decreased when flavopiridol was added to doxorubicin-treated cells ($P = 0.039$) in 1 way ANOVA analysis

Columns, mean values of each group; bars, SD. *, $P = 0.039$ and **, $P < 0.001$

2.2. Doxorubicin and flavopiridol combination treatment activated the caspase 7 apoptotic pathway

The enhanced apoptosis from the doxorubicin+flavopiridol combination treatment is attributable to a more profound activation of caspase 7 in the combination group (Figure 8). Densitometry analysis also showed enhanced activation of caspase 7 in the combination group.

(A)



(B)

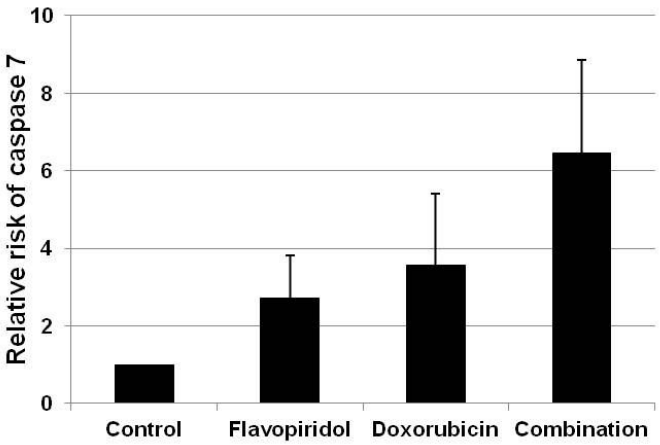


Figure 8. Doxorubicin and flavopiridol enhances apoptosis of HCC cells through the caspase 7-mediated apoptotic pathway. (A) Mouse MH134 cells were cultured in the absence or presence of flavopiridol (300 nM) for 4 hours, then treated for

20 hours with doxorubicin (2 μM) under hypoxic culture conditions. Cell lysates were immunoblotted with anti-caspase 9, 7, and 8, anti-phospho-eIF2α, anti-phospho-p38, anti-phospho-p42/44, anti-phospho-JNK and anti-actin antibody. The enhanced apoptosis could be attributed to a more profound activation of caspase 7 in the doxorubicin+flavopiridol combination group. (B) Densitometry analysis was performed and data are expressed as the mean ± SD of the relative ratios of caspase 7 to actin.

2.3. Increased doxorubicin sensitivity in mouse HCC cells from flovopiridol-induced NDRG1 suppression.

Flavopiridol effectively suppressed NDRG1 expression in mouse HCC cells (Figure 9).

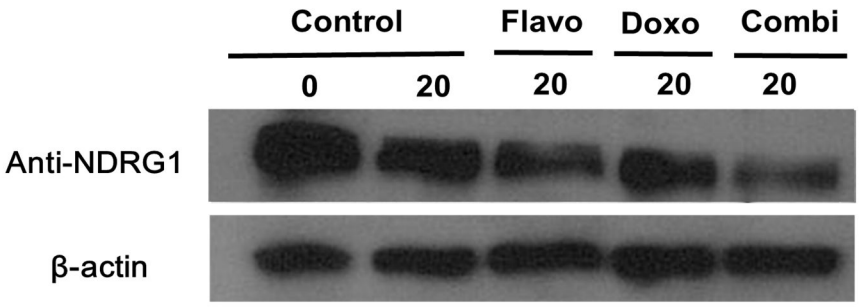


Figure 9. Flavopiridol suppresses NDRG1 expression. Mouse MH134 cells were cultured in the absence or presence of flavopiridol (300nM) for 4 hours and then treated for 20 hours with doxorubicin (2μM) under hypoxic culture conditions. The cell lysates were immunoblotted with the anti-NDRG1 and anti-actin antibodies. The data show the suppressive effect of flavopiridol on NDRG1 expression.

Discussion

The main finding of this study is the synergistic anti-tumor efficacy of doxorubicin and flavopiridol against HCC in hypoxic microenvironment, especially in the *in vivo* model. This synergistic effect was attributed to enhanced apoptosis from suppressed NDRG1 expression.

Doxorubicin is an anthracycline drug used in cancer chemotherapy which inhibits DNA/RNA synthesis by intercalating between base pairs of DNA strands. It has been reported that the effect of doxorubicin was insufficient as a systemic chemotherapeutic agent in palliative treatment for advanced HCC (4, 16). In this *in vivo* study, tumor volume was decreased in doxorubicin-only treatment group compared to the control group, however this difference was not statistically significant after adjustment for confounding variables (Table 1). The previous *in vitro* study showed that hypoxic HCC cells are more resistant to doxorubicin compared to normoxic HCC cells (5). Therefore, it can be hypothesized that doxorubicin-sensitive cells were killed by doxorubicin-treatment reducing HCC tumor volume partially; however, doxorubicin-resistant cells in hypoxic condition remain viable, making the effect of

doxorubicin therapy insufficient. The addition of flavopiridol to doxorubicin enhanced cytotoxicity against those doxorubicin-resistant HCC cells, and consequently decreased tumor volume additionally.

Flavopiridol, a semisynthetic flavonoid, is an inhibitor of several cyclin-dependent kinases and exhibits unique anticancer properties with various mechanisms of action such as CDK inhibition, apoptosis, DNA interaction, and others (8). In addition, flavopiridol is known to potentiate the effects of chemotherapy in several types of cancer cell lines (17). For example, flavopiridol sensitized colon cancer xenograft cells to CPT-11 and sensitized sarcoma cells or breast cancer cells to doxorubicin-induced cell killing (18-20). In HCC cells, flavopiridol is known to enhance doxorubicin cytotoxicity (5), and another study also showed the flavopiridol sensitivity in HCC cells which was treated with and eventually became refractory to doxorubicin (21). However, there has been no *in vivo* study for the effect of flavopiridol on HCC. As such, this is the first *in vivo* study showing the synergistic anti-tumor effect of doxorubicin and flavopiridol in HCC.

The action mechanism of each drug is cancer-type or cell-

type specific. In this study, increased apoptosis in the combination group lead to synergistic anti-tumor effect. Previous studies also showed flavopiridol as a potent apoptotic agent using diverse pathways: inducing both caspase-dependent and-independent mitochondrial cell death pathways to cause cell death in cycling as well as non-cycling tumor cells; down-regulation of important cell survival proteins, such as survivin, through the inhibition of phosphorylation at Thr34; inhibiting the activation of p-Akt which in turn inhibits the activation of NF-κB; increasing the sensitivity of S phase cells to drug treatment by modulating the E2F-1 transcription factor activity in tumor cells, etc. (22) This study showed decreased cell viability and increased expression of caspase 7 in the combination group, indicating increased apoptosis through the caspase dependent pathway. This is consistent with a previous *in vitro* result using Huh-7 HCC cell lines, which showed caspase-dependent apoptosis for the doxorubicin+flavopiridol combination treatment (5). However, flavopiridol did not have any effect on angiogenesis in the MH134 HCC cell line in this study, although several previous studies had reported an anti-angiogenesis effect of flavopiridol in other cancer types through

the induction of apoptosis in endothelial cells or inhibition of the hypoxic induction of vascular endothelial growth factor (22).

In this study, flavopiridol suppressed NDRG1 expression in both *in vivo* HCC tissue and the MH134 HCC cell line. NDRG1 has diverse roles including cell differentiation, early life development, inflammation and stress-responsive (23). With regard to carcinogenesis, NDRG1 has a role as a tumor suppressor or oncogenic function, which is tissue specific or cell-type specific. In HCCs, previous studies have shown NDRG1 to be associated with aggressive HCC features. High expression of NDRG1 was associated with vascular invasion, metastasis, and shorter overall survival in HCC (24). Furthermore, silencing of NDRG1 reduced proliferation and invasion *in vitro*, and inhibited tumor growth *in vivo* (23, 25, 26). Previously, the authors showed the association between increased NDRG1 expression and doxorubicin resistance and the association between suppressed NDRG1 expression with increased doxorubicin sensitivity; both associations indicating that NDRG1 is one of the molecules causing doxorubicin-resistance. NDRG1 is induced under hypoxic conditions, which is the usual microenvironment inside solid tumors and is

associated with tumor resistance to chemotherapy (5). Intra-tumoral hypoxic environment in this *in vivo* model (12) induced NDRG1 expression, and flavopiridol significantly suppressed NDRG1 expression in the *in vivo* HCC tissue as expected from the results of previous *in vitro* study. This result supports the hypothesis that suppressing NDRG1 by flavopiridol is a mechanism to overcome HCC resistance of doxorubicin in hypoxic condition.

Although downstream pathway of NDRG1 was not evaluated in this study, previous *in vitro* study showed glutredoxin2 as a downstream molecule of NDRG1 dependent resistance of HCC cell cytotoxicity (5). Glutaredoxin2 is a known thiol/disulfide oxidoreductase component of the glutathione system and plays a crucial role in the regulation of mitochondrial redox status and regulation of cell death at the mitochondrial checkpoint (27, 28). Previous study showed that the selective suppression of glutaredoxin2 expression increased sensitivity to doxorubicin cytotoxocity (27), and that glutaredoxin2 expression was suppressed when NDRG1 siRNA or flavopiridol was administered to HCC cells (5). These results suggest that flavopiridol suppresses NDRG1 and the downstream

glutaredoxin2 molecule, which then increases susceptibility of HCC cells to doxorubicin.

Taken together, the findings suggest that the suppression of NDRG1 expression by flavopiridol may be therapeutically useful in the treatment of HCCs by increasing doxorubicin sensitivity. In addition, flavopiridol has been shown to be relatively safe because it is already used in many clinical trials, especially in hematologic malignancies (29). With further studies on the efficacy and safety of the doxorubicin and flavopiridol combination therapy, flavopiridol with doxorubicin treatment could be considered in clinical applications for infiltrative type HCC.

In conclusion, this study shows that the combination treatment of doxorubicin and flavopiridol has a synergistic anti-tumor effect against HCC both *in vivo* and *in vitro* models. This effect is attributed to increased apoptosis through caspase 7 by suppressing NDRG1 expression in the combination group rather than decreasing angiogenesis.

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국문 초록

서론: 저산소성 환경에 있는 간세포암, 즉 반복적인 화학 색전술 후에 남아 있는 저혈관성 간세포암이나 침윤형 간세포암은 일반적인 과혈관성 간세포암에 비하여 예후가 나쁘고 항암제에 잘 반응하지 않는다. 기존 연구에서 간암세포는 저산소성 환경에서 doxorubicin 항암제에 저항성을 보임이 밝혀진 바 있으며 이는 저산소성 환경에서 발현되는 NDRG1단백의 발현 증가와 연관되어 있음이 보여진 바 있다. 한편 flavopiridol은 cyclin dependent kinase inhibitor의 일종으로 여러 종류 암의 발암 및 항암 기전에 작용한다. 기존에 간암 세포주를 이용한 실험에서 flavopiridol을 doxorubicin에 병합 투여시 NDRG1의 발현이 억제되는 것을 확인하였으며, 이를 통하여 간암 세포주의 세포 자멸사가 증가되는 것이 증명된 바 있다. 따라서 이러한 생체 외 실험에 근거하여, 본 연구에서는 생쥐의 저산소성 간암 생체 모델에서 doxorubicin과 flavopiridol 병합요법이 상승적 항암 효과를 가지는지 알아보고자 하였고, 그 기전을 규명해보고자 하였다.

방법: 본 연구에서 간세포암 생체 모델을 수립하기 위하여 C3H 생쥐의 피하에 MH134 세포를 접종하였다. 생체 모델이 수립된 후에 생쥐를 대조군, doxorubicin 단독 투여군, flavopiridol 단독 투여군,

그리고, doxorubicin 과 flavopiridol 병합 투여군의 네 군으로 나누어 종양의 부피를 측정하였고 항종양 효과의 기전을 평가하기 위하여 실험 종료 후 적출된 종양 조직으로부터 세포 자멸사 및 종양 내부 혈관 밀도 (microvessel density)를 정량적으로 분석하였다. 아울러 종양 내 저산소 환경이 조성되었는지를 확인하기 위하여 종양 내부 괴사 정도와 저산소 신호 중 하나인 HK II 에 대한 면역화학염색을 시행하였으며, NDRG1 에 대한 면역화학염색을 시행하여 flavopiridol 이 NDRG1 발현에 미치는 영향을 밝히고자 하였다. 또한 약제가 MH134 세포의 생존 (cell viability)에 미치는 영향은 MTS assay 를 통하여 측정하였고, 자멸사 신호체계 및 NDRG1 발현은 면역점적법을 이용하여 확인하였다.

결과: 모든 생쥐 간세포암 조직에서 저산소성 환경이 조성되는 것을 확인할 수 있었으며, doxorubicin과 flavopiridol을 병합 투여한 군에서는 대조군이나 약제 단독 투여군에 비하여 통계적으로 유의하게 종양의 증식이 억제되는 것을 확인하였다. 병합 투여군의 적출된 종양 조직에서 TUNEL-양성 세포가 다른 군에 비하여 통계적으로 유의하게 증가된 것을 관찰할 수 있었으나 MVD는 각 군에서 유의한 차이가 없었다. 또한, 종양 조직 면역 화학염색에서 flavopiridol은 NDRG1의 발현을 억제하였다. 생체 모델 결과와 유사하게, MH134 세포주를 이용한 생체 외 실험 (*in vitro*)에서도

doxorubicin, flavopiridol 병합군에서 유의하게 세포의 생존이 감소하는 것을 관찰하였으며, 이는 caspase 7 신호 증가에 의한 세포 자멸사 증가에 의한 것임을 확인할 수 있었다. 또한 면역점적법을 이용하여 flavopiridol이 NDRG1 발현을 억제하는 것을 확인할 수 있었다.

결론: 결론적으로, 간세포암에서 doxorubicin과 flavopiridol을 병합하여 사용하였을 때 생체 모델과 생체 외 실험 모두에서 상승적 항암 효과를 가진다. 이는 저산소성 환경에서 살아남은 doxorubicin에 저항성을 보이는 세포들에 flavopiridol을 처리하였을 때 세포 자멸사가 증가함으로써 나타나는 현상으로 생각된다. 또한, 이 과정에서 flavopiridol이 저산소 환경에서 발현된 NDRG1을 억제함으로써 작용하는 것임을 확인할 수 있었다. 따라서 doxorubicin과 flavopiridol의 병합요법은 향후 간세포암의 치료에 유용하게 사용 될 수 있을 것으로 생각되며, 특히 반복적인 경동맥 색전술 후에 남아 있는 저혈관성 간세포암이나 침윤성 간세포암의 치료에 기여할 수 있을 것으로 기대된다.

주요어: 간세포암, 독소루비신 (doxorubicin), 플라보피리돌 (flavopiridol), 자멸사, 저산소 상태
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