The role of caspase-10 in cancer pyroptosis

Wonjin Woo

Department of Medical Science

The Graduate School, Yonsei University

The role of caspase-10 in cancer pyroptosis

Wonjin Woo

Department of Medical Science

The Graduate School, Yonsei University

The role of caspase-10 in cancer pyroptosis

Directed by Professor Lark Kyun Kim

The Master's Thesis
submitted to the Department of Medical Science
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Master of Medical Science

Wonjin Woo

December 2021

This certifies that the Master's Thesis of
Wonjin Woo is approved.

Thesis Supervisor : Lark Kyun Kim

Thesis Committee Member#1 : Hyun Woong Lee

Thesis Committee Member#2 : Jungmin Choi

The Graduate School
Yonsei University

December 2021

ACKNOWLEDGEMENTS

본 논문이 완성되기까지 항상 세심한 지도와 열정과 정성으로 이끌어 주신 김락균 교수님께 진심으로 감사드리며, 본 연구를 위하여 각별한 조언을 아끼지 않으시고 항상 긍정적으로 생각할 수 있도록 도움을 주신 도소희 박사님께 깊은 감사를 드립니다. 또한 졸업 논문 심사를 맡아주신 이현웅 교수님과 최정민 교수님께도 감사를 전하고 싶습니다. 교수님의 도움이 있었기에 졸업을 무사히 마무리 할 수 있게 되었습니다.

그리고 매일 같은 실험실 생활 속에서 삶의 활력소가 되어준 민정, 영운, 지연, 효진, 정원이에게 고마움을 전합니다. 많은 분들의 도움이 있었기에 어려움을 극복하고 석사 졸업이라는 마무리를 지을 수 있었던 것 같습니다. 석사 과정 동안 많은 것을 배웠고 느꼈습니다. 실험실 식구들 항상 좋은 일만 가득하기를 진심으로 바랍니다.

마지막으로 항상 저를 믿어주시고 부산에서 응원을 보내 주셨던 엄마, 아빠, 동생 항상 고맙고 앞으로 더 발전하는 모습 보여줄 수 있도록 노력 하겠습니다.

우원진 드림

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

The role of caspase-10 in cancer pyroptosis

Wonjin Woo

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Lark Kyun Kim)

Liver cancer is the leading cause of cancer mortality worldwide. Despite the improved treatment of HCC, the 5-year survival rate is relatively lower than other cancers, therefore further research to identify a new target is needed.

Caspase-10 is a human-only protein that is highly homologous to caspase-8. Both caspase-10 and caspase-8 are known to activate apoptosis. Given that caspase-10 is highly homologous to caspase-8, it has been reported that there is overlap between the substrate of caspases. However, recent studies reported that caspase-10 might have a distinct role in cancer cell death that differs from that of caspase-8.

To elucidate the mechanism of caspase-10 in cancer, we generated a caspase-10 knock-out cell line and performed RNA-seq analysis. In this study, we identified that caspase-10 induces pyroptosis. We show that caspase-10 mediates pyroptosis by cleaving GSDME under the treatment of sorafenib.

Recently, switching from apoptosis to other types of programmed cell death such as pyroptosis has emerged as a new strategy for cancer treatment.

Our findings suggest that targeting caspase-10 can be a potential therapy for patients with liver cancer.

Key words : Caspase-10, pyroptosis, HCC

The role of caspase-10 in cancer pyroptosis

Wonjin Woo

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Lark Kyun Kim)

I. INTRODUCTION

Liver cancer or hepatocellular carcinoma (HCC) is one of the most common high-mortality cancers in the world [1]. Globally, HCC is the fifth most common cancer and the fourth most common cancer in South Korea. The mortality rate for HCC was 21.1 per 100,000 of the population in 2016, which is the second highest cancer mortality rate after lung cancer in South Korea [2,3]. The most common risk factors for HCC include chronic hepatitis B virus (HBV) and chronic hepatitis C virus (HCV) infection and alcohol abuse [4]. Despite the improved treatment of HCC, the five-year survival rate in Korea is relatively low compared with other cancers [5]. Thus, further research to identify a new target in liver cancer is needed.

Previously, apoptosis was regarded as the only form of programmed cell death (PCD) that is different from necrosis. Apoptosis is a physiological programmed cell death (PCD) that is responsible for the removal of damaged cells [6]. Apoptosis is characterized by shrinkage of the cell, phosphatidylserine externalization, chromatin condensation, cellular budding, fragmentation into apoptotic bodies, rapid phagocytosis and DNA fragmentation into units of approximately 200 base pairs [7,8]. Apoptosis can be triggered by a variety of stimuli such as cytokines, hormones, drugs, and viruses [9].

Over the past few decades, researchers have identified several types of programmed cell death (PCD) in contrast to apoptosis including pyroptosis, necroptosis, ferroptosis and autophagy [10]. These non-apoptotic programmed cell deaths (PCDs) are pro-inflammatory cell deaths because cells secrete intracellular contents including inflammatory cytokines and damage-associated molecular patterns (DAMPs). These non-apoptotic cell deaths are induced by different types of stimuli [11]. Over the past few years, various approaches to induction cancer cell death through apoptosis have been used as a common cancer therapies However, one of the hallmarks of cancer is evading apoptosis [12], thereby leading to the failure of cancer therapy [13]. Due to limitations of apoptosis in cancer therapy, inducing other types of programmed cell death (PCDs) such as pyroptosis in cancer cells is emerging as a new therapeutic strategy.

Caspases are cysteine-dependent endo-proteases that cleave their substrates on following aspartic acid residues. The majority of caspases participate in the induction of apoptosis [14]. Caspases are classified into two types: initiator caspases, and effector caspase. Caspase-8, -9, -10 are initiator caspases that activate effector caspases like caspase-3, -7. Activated effector caspases cleave death substrate and induce apoptosis [15]. Caspase-10 is a human-only protein which shows a high homology to caspase-8. As caspase-8 is a well-known initiator of the extrinsic apoptotic pathway. Caspase-10, which is homologous to caspase-8, is regarded as an initiator caspase function that is similar to caspase-8. However, the role of caspase-10 is still controversial and not yet fully understood [16]. Recent advances have revealed that caspase-10 can act as a possible tumor suppressor in cancer [17]. But the specific mechanism of caspase-10 in cancer remains unknown and should be further explored.

Pyroptosis is a form of inflammatory programmed cell death (PCD) that is

mainly caused by microbial infection [18]. Pyroptosis is morphologically characterized by pore formation in the plasma membrane leading to cell swelling and membrane disruption, triggering the release of damage-associated intracellular molecules, and inflammation [19]. Gasdermins (GSDMs) are the key mediator molecule proteins of pyroptosis which assemble membrane pore. The gasdermin family consists of GSDMA, GSDMB, GSDMC, GSDME, GSDME, and PJVK. It is reported that each gasdermin is differently expressed in a variety of cell types and tissues [20]. Gasdermin can be cleaved by caspase and granzymes. Recent studies revealed that different family of gasdermins can be cleaved by different caspases or granzymes [21]. Once the inactive form of gasdermins has been proteolytically cleaved by caspases or granzymes, its N-terminal fragment is possible to assemble in plasma membrane and permeabilize and disrupt membrane. In recent years, approaches to targeteting pyroptosis other than apoptosis have emerged to overcome apoptosis resistance in cancer [22]. Recent findings reported that pyroptosis facilitates the cytotoxic lymphocytes to kill tumor cells and reprograms the tumor microenvironment to an immunostimulatory state [23]. In this study, by examining the role of caspase-10 in liver cancer pyroptosis, we expect that caspase-10 can be a potential new target for cancer treatment. Our findings suggest the possibility of pyroptosis as a potential cancer treatment strategy rather than apoptosis. Therefore, it is crucial to understand the mechanism of pyroptosis

Liver cancer or hepatocellular carcinoma (HCC) is one of the most common high-mortality cancers in the world [1]. Globally, HCC is the fifth most common cancer and the fourth most common cancer in South Korea. The mortality rate for HCC was 21.1 per 100,000 of the population in 2016, which is the second highest cancer mortality rate after lung cancer in South Korea [2,3]. The most common risk factors for HCC include chronic hepatitis B virus (HBV) and chronic hepatitis C virus (HCV) infection and alcohol abuse [4]. Despite the improved treatment of HCC, the five-year survival rate in Korea is relatively low compared with other

cancers [5]. Thus, further research to identify a new target in liver cancer is needed.

Previously, apoptosis was regarded as the only form of programmed cell death (PCD) that is different from necrosis. Apoptosis is a physiological programmed cell death (PCD) that is responsible for the removal of damaged cells [6]. Apoptosis is characterized by shrinkage of the cell, phosphatidylserine externalization, chromatin condensation, cellular budding, fragmentation into apoptotic bodies, rapid phagocytosis and DNA fragmentation into units of approximately 200 base pairs [7,8]. Apoptosis can be triggered by a variety of stimuli such as cytokines, hormones, drugs, and viruses [9].

Over the past few decades, researchers have identified several types of programmed cell death (PCD) in contrast to apoptosis including pyroptosis, necroptosis, ferroptosis and autophagy [10]. These non-apoptotic programmed cell deaths (PCDs) are pro-inflammatory cell deaths because cells secrete intracellular contents including inflammatory cytokines and damage-associated molecular patterns (DAMPs). These non-apoptotic cell deaths are induced by different types of stimuli [11]. Over the past few years, various approaches to induction cancer cell death through apoptosis have been used as a common cancer therapies However, one of the hallmarks of cancer is evading apoptosis [12], thereby leading to the failure of cancer therapy [13]. Due to limitations of apoptosis in cancer therapy, inducing other types of programmed cell death (PCDs) such as pyroptosis in cancer cells is emerging as a new therapeutic strategy.

Caspases are cysteine-dependent endo-proteases that cleave their substrates on following aspartic acid residues. The majority of caspases participate in the induction of apoptosis [14]. Caspases are classified into two types: initiator caspases, and effector caspase. Caspase-8, -9, -10 are initiator caspases that activate effector caspases like caspase-3, -7. Activated effector caspases cleave death substrate and

induce apoptosis [15]. Caspase-10 is a human-only protein which shows a high homology to caspase-8. As caspase-8 is a well-known initiator of the extrinsic apoptotic pathway. Caspase-10, which is homologous to caspase-8, is regarded as an initiator caspase function that is similar to caspase-8. However, the role of caspase-10 is still controversial and not yet fully understood [16]. Recent advances have revealed that caspase-10 can act as a possible tumor suppressor in cancer [17]. But the specific mechanism of caspase-10 in cancer remains unknown and should be further explored.

Pyroptosis is a form of inflammatory programmed cell death (PCD) that is mainly caused by microbial infection [18]. Pyroptosis is morphologically characterized by pore formation in the plasma membrane leading to cell swelling and membrane disruption, triggering the release of damage-associated intracellular molecules, and inflammation [19]. Gasdermins (GSDMs) are the key mediator molecule proteins of pyroptosis which assemble membrane pore. The gasdermin family consists of GSDMA, GSDMB, GSDMC, GSDME, GSDME, and PJVK. It is reported that each gasdermin is differently expressed in a variety of cell types and tissues [20]. Gasdermin can be cleaved by caspase and granzymes. Recent studies revealed that different family of gasdermins can be cleaved by different caspases or granzymes [21]. Once the inactive form of gasdermins has been proteolytically cleaved by caspases or granzymes, its N-terminal fragment is possible to assemble in plasma membrane and permeabilize and disrupt membrane. In recent years, approaches to targeteting pyroptosis other than apoptosis have emerged to overcome apoptosis resistance in cancer [22]. Recent findings reported that pyroptosis facilitates the cytotoxic lymphocytes to kill tumor cells and reprograms the tumor microenvironment to an immunostimulatory state [23]. In this study, by examining the role of caspase-10 in liver cancer pyroptosis, we expect that caspase-10 can be a potential new target for cancer treatment. Our findings suggest the possibility of pyroptosis as a

potential cancer treatment strategy rather than apoptosis. Therefore, it is crucial to understand the mechanism of pyroptosis.

II. MATERIALS AND METHODS

1. Cell line and cell culture

HepG2 cells were maintained in DMEM (Hyclone, Logan, Utah, USA) supplemented with 10% FBS (Gibco) and 1% penicillin–streptomycin. Puromycin (2μg/ml) (InvivoGen #ant-pr) was added to the medium to maintain caspase knock-out HepG2 cell lines. The cells were incubated at 37°C in 5% CO2.

2. Generation of caspase-10 and casasp-8 knock-out HepG2 cell line using the CRISPR/Cas9 system

The gRNA sequences of caspase-10 and caspase-8 were designed using the CHOPCHOP tool. gRNAs were cloned into the Lenti-CRISPR vr2 (Addgene #52961). Constructs were transduced into HepG2 cells, using the Neon™ Transfection System (Life Technology; 1400 V, 10 ms, and 3 pulses). For caspase-10 and caspase-8 knock-out cell selection, puromycin (2μg/ml) was added to the medium. After puromycin selection, caspase-10 and caspase-8 knock-out sequences were confirmed by Sanger Sequencing and western blot. HepG2 cells were maintained in DMEM (Hyclone, Logan, Utah, USA) supplemented with 10% FBS (Gibco) and 1% penicillin–streptomycin. Puromycin (2μg/ml) (InvivoGen #ant-pr) was added to the medium to maintain caspase knock-out HepG2 cell lines. The cells were incubated at 37°C in 5% CO2.

3. Antibodies and reagents

Anti-caspase-10 (#ab177475), anti-GSDME (#ab215191) and anti-6X His

(#ab9108) are obtained from Abcam; anti-GAPDH (sc-25778) and anti-myc (#sc-40) are obtained from Santa Cruz Biotechnology; anti-caspase-8 (#M058-3) is obtained from MBL. All antibodies for the western blot analyses were used at 1:1000 dilution.

Recombinant human TNF-α protein (#210-TA-100) is obtained from R&D Systems. 5-Fluorouracil (#F6627); Cisplatin (#C2210000) and Sorafenib (SML2653) are all obtained from Sigma-Aldrich.

4. Flow cytometry

To measure the cell death response in HepG2, cells were treated with TNF-α, 5-Fluorouracil, cisplatin, and sorafenib at the desired concentrations. After exposure to the drugs for 48 hours, the cells were collected using trypsin-EDTA (Gibco #25200056) and centrifuged and washed twice with 4℃ PBS. Then, HepG2 cells were stained for 15 min with Annexin V-FITC and PI (Annexin V: FITC Apoptosis Detection Kit #BD 556547) at room temperature in the dark.

For ROS detection, cells were treated with 100ng/ml TNF-α at 37°C and 5% CO2. Then, cells were stained for 3 hr with 5 μM with MitoSOX™ Red (Invitrogen #M36008) at 37°C for 20 min 37 °C in the dark. After staining, the cells were analyzed by flow cytometry (BD Biosciences FACS Canto™ II). Data files were analyzed using FlowJo V10.

5. Cell proliferation assay

For IncuCyte® cell proliferation assay, HepG2 cells were plated in a 96-well plate for three days. Cells were imaged by IncuCyte® Live-Cell proliferation assays and phase area confluence (%) was quantified by IncuCyte® 3 Live-Cell analysis System (Sartorious).

For MTT assay, reagents (DoGenBio #EZ-Cytox) are added in medium with

HepG2 cells and incubated for 30 min at 37 °C.

For migration assay, 1 X 105 LX-2 HSCs were plated in an ImageLock 96-well plate (Sartorious 4379). The cell-free area is created in a confluent monolayer by the wound maker (Essen BioScience #4563) and cell migration was imaged using the IncuCyte® S3 Live-Cell analysis System (Sartorious).

For trypan blue staining assay, cells were stained by trypan blue (Sigma #T8154) and measured by Vi-CELL™XR 2.04 cell counter (Beckman Coulter).

6. RNA-sequencing analysis

The RNA-sequencing data was generated with the paired-end sequencing method. The read of the RNA-sequencing data was aligned by HISAT2 (version 2.2.1) according to the provided index file from HISAT2 (https://genome-idx.s3.amazonaws.com/hisat/grch38_tran.tar.gz). The SAM files were processed by SAMtools (version 1.10) for read sorting. Read quantification of sam files was conducted by HTSeq (version 0.11.1) to generate the read count data. The read count data was normalized and the differentially expressed genes (DEGs) between each sample were analyzed by DESeq2. Only genes with at least 1.5-fold expression difference and adjusted p-value of less than 0.05 were selected. The DEGs were divided with K-means clustering and visualized by Morpheus (https://software.broadinstitute.org/morpheus/). To investigate the functional roles of each DEG cluster, we conducted pathway analysis by using:Profiler (https://biit.cs.ut.ee/gprofiler/gost) and gene set enrichment analysis by using GSEA (https://www.gsea-msigdb.org/gsea/index.jsp).

7. Western blot analysis

HepG2 cells were lysed in RIPA buffer (CST #9806S), and 50μg of the total protein from each lysate was then loaded to SDS-PAGE. Primary antibodies targeting Caspase-10, -8, GSDME, GAPDH, 6XHis, and Myc (all 1:1000 dilution) were incubated at 4°C overnight. The membrane was then washed three times with TBST buffer for 10 min per wash and incubated with a secondary antibody at room temperature for 1 h. LightCycler 480 (Roche) was used for detection.

8. Overexpression of Caspase and GSDME

Transfection was performed using Lipofectamine 2000 (Invitrogen #11668019) following the manufacturer's protocols. 2.5μg of empty vector was co-transfected as a control. The medium was changed after 6 h and cells were collected after 48 h of transfection.

III. RESULTS

1. Generation of caspase knock-out cell line

First, to investigate the role of caspase-10 in liver cancer, we generated caspase-10 and caspase-8 knock-out HepG2 cell line using CRISPR/Cas9 technology (Figure 1). We confirmed caspase knock-out HepG2 by performing genome sequencing and western blot (Figure 2).

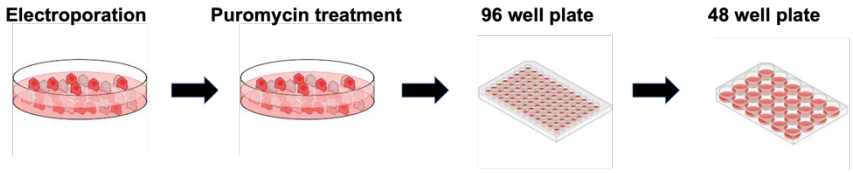

Figure 1. Schematic flow of generation knock-out cell line.

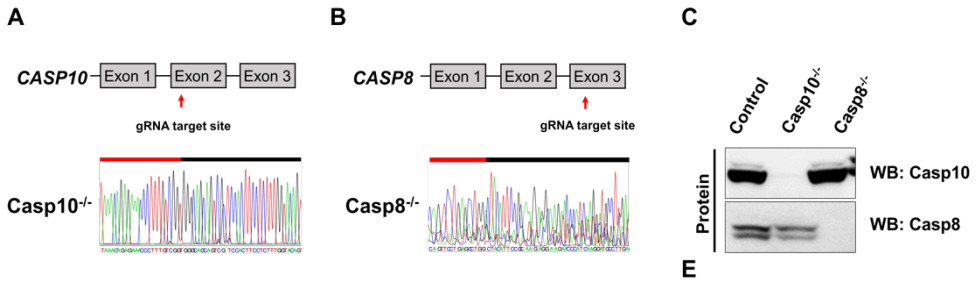

Figure 2. Generation of caspase-10 knock-out cell line. (A, B) Caspase knock-out cell line confirmed by Sanger sequencing. (C) Expression of caspase-10, -8 in HepG2 control cell and caspase knock-out cell

Table 1. gRNA target sequences

Targeted gRNA	Sequence (5'-3')
Caspase-10	CACCGAGAGAAACCCTTTGTCGGGT
Caspase-8	AAACACCCGACAAAGGGTTTCTCTC

Table 2. Generation of caspase knock-out cell line (%)

gRNA		Colony	Total Colony
Caspase-8	Caspase-8 KO	11 (47.8%)	23
Caspase-10	Caspase-8 KO	22 (52.4%)	42
Caspase-8 / Caspase-10	Caspase-8 KO	7 (25%)	28
	Caspase-8 KO	7 (25%)	
	Caspase-8/10 DKO	0	

2. Caspase-10 regulates TNF-α-induced programmed cell death

As a preliminary study to investigate cell death response in liver cancer, HepG2 cells were treated with various stimuli [24]. Since the TNF receptor family is also known as CD95, it is well known to induce cell death including apoptosis [25]. As sorafenib is the first-line treatment for advanced hepatocellular carcinoma [26,27], 5-fluorouracil (5-FU) and cisplatin are common chemotherapy drugs, we carried out annexin-V/propidium iodide (PI) staining under each stimulus. Annexin-V/propidium iodide (PI) staining shows that all stimuli induced HepG2 cell death. Especially, we observed that TNF-α stimulation markedly increased annexin V-positive populations (Figure 3). Our observation is consistent with the fact that TNF-α is known to induce programmed cell death including apoptosis. Given that TNF-α stimulation markedly induces programmed cell, we next investigated the roles of caspase-10 in the TNF-α-induced programmed cell death.

We found that annexin-V positive populations are decreased in caspase-10 knock-out HepG2 (Figure 4). This result indicates that caspase-10 is crucial for inducing apoptosis, and it may also be involved in other types of programmed cell death.

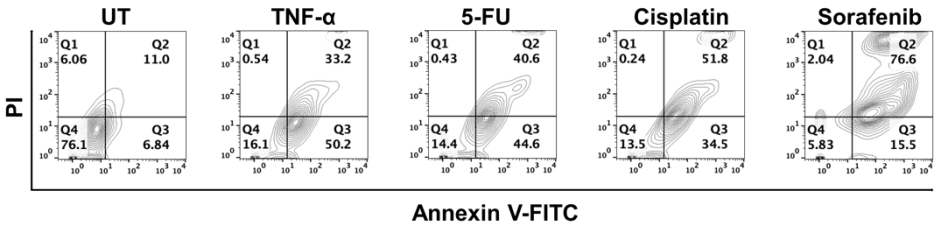

Figure 3. Cell death response of HepG2 cells treatment with various stimuli. HepG2 cells were treated with 100ng/ml TNF- α, 10ng/ml 5-FU, 50 μM cisplatin, 60uM sorafenib for 24h. Cell death are measured by Annexin-V/PI staining followed by flow cytometry.

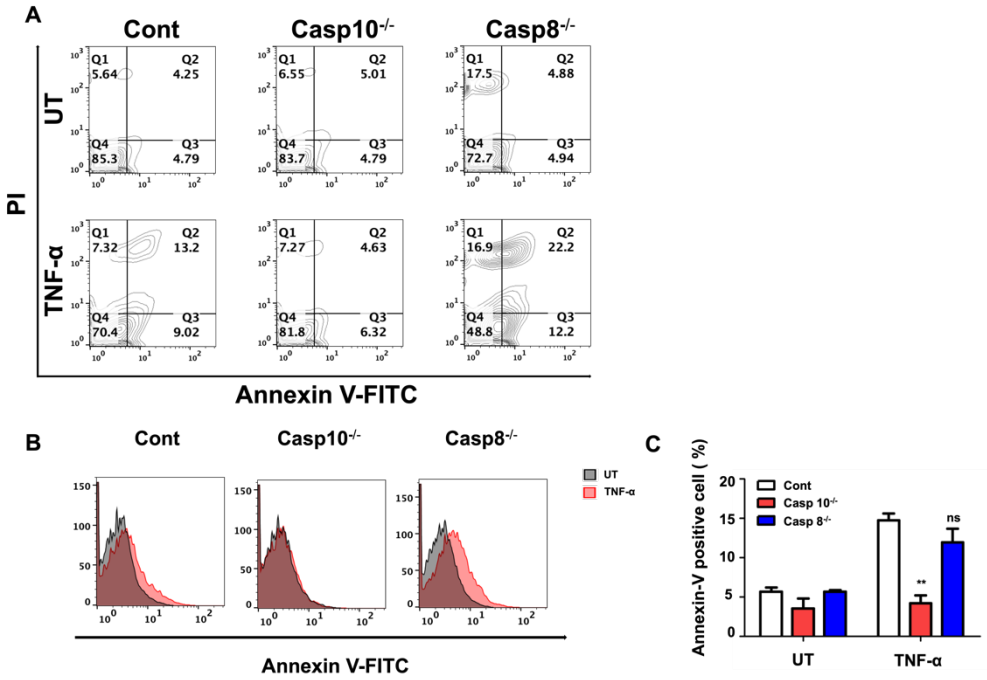

Figure 4. Caspase-10 regulates programmed cell death under TNF-α stimulation. (a) HepG2 cells were treated with 100 ng/ml TNF-α for 24 h. Cell death is measured by Annexin-V/PI staining followed by flow cytometry. Represented in the contour plot. (b) Represented in the histogram. (c) Quantitative analysis of programmed cell death is measured by the percentage of Annexin-V. For comparisons between HepG2 control cell caspase-10 knock-out cell, a Student's t-test was employed (For a p-value of <0.05 was considered statistically significant. *$p < 0.05$, **$p < 0.01$).

3. Proliferation rates are increased in caspase-10 knock-out HepG2 in steady state

Next, in order to investigate the roles of caspase-10 in cancer cell death, we performed RNA-seq in caspase knock-out HepG2 cells. We first analyzed caspase-10 knock-out HepG2 in steady state. Our RNA-seq analysis shows that cell proliferation related genes were up-regulated in caspase-10 knock-out HepG2 in steady state (Figure 5). We confirmed that caspase-10 regulates cancer proliferation by proliferation assays including IncuCyte® proliferation assay, MTT, migration assay and trypan blue staining assay (Figure 6). It appears that the proliferation rates were much higher in caspase-10 knock out HepG2. In contrast, proliferation rates were much lower in caspase-8 knock-out HepG2. These results suggest that caspase-10 acts as a tumor suppressor that differs from caspase-8. Also, caspase-10 could be a potential target to regulate tumor growth.

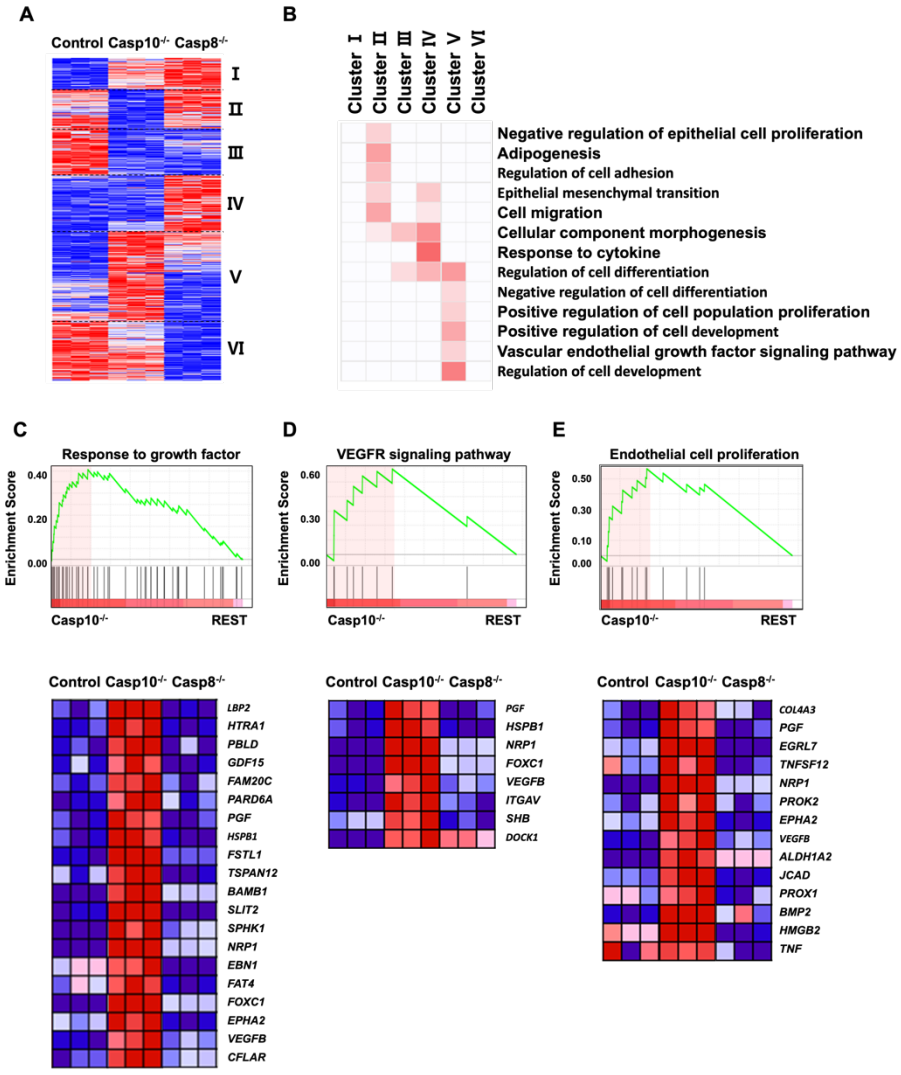

Figure 5. Cell proliferation-related genes are up-regulated in caspase-10 knock-out HepG2. (A) The cluster heat map of expression profiles of mRNA at steady state. (B) Pathway analysis at steady state. (C-E) Gene Set Enrichment Analysis (GSEA) on cluster V.

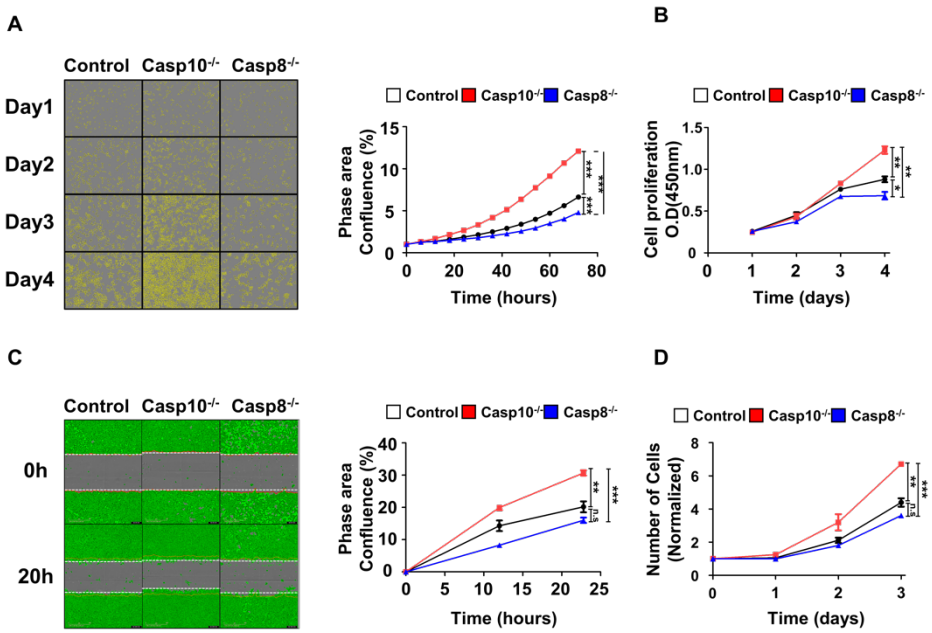

Figure 6. Increased cell proliferation in caspase-10 knock-out HepG2. (A) An Incucyte cell proliferation assay measuring confluence over time. (B) Cell proliferation of HepG2 cell was determined by MTT assay. (C) The effects of caspase-10 knock-out on cell migration were evaluated with the wound healing assay using the IncuCyte® analyzer in HepG2. (D) Cell proliferation of HepG2 cells was determined by Trypan blue exclusion assay.

4. ROS level is increased in caspase-10 knock-out HepG2 under TNF-α stimulation

We next analyzed increased gene expression in caspase knock-out HepG2 under TNF-α stimulation (Figure 7). We discovered the components of the mitochondrial electron transport chain (ETC) including cytochrome B, cytochrome oxidase subunits, mitochondrial membrane ATP synthase, and NADH-ubiquinone oxidoreductase chain 4L were up-regulated in caspase-10 knock-out HepG2 (Cluster2) (Figure 8). Some electrons from the mitochondrial electron transport chain (ETC) are directly transferred to O2 leading to generation of reactive oxygen species (ROS) [28]. We hypothesized that the level of ROS is increased in caspase-10 knock-out HepG2. We therefore performed ROS detection assay. Indeed, we confirmed that the level of ROS is increased in caspase-10 knock-out HepG2 under TNF-α stimulation. As signaling molecules, ROS play a pivotal role in the number of cellular processes. Under normal physiological conditions, the ROS level is homeostatically controlled by scavenging systems [29]. It is well known that elevated levels of ROS can cause mitochondrial dysfunction, damage to DNA, lipids, and proteins, and ultimately lead to cell death[30]. It has been reported that there are two faces of ROS in cancer. A high level of ROS can cause cell damage and cell death. However, a moderate level of ROS can cause tumor promotion by activating cancer cell proliferation, oncogene activation and activation of cell survival-signaling cascade [31]. We expected that elevated level of ROS in caspase-10 knock-out HepG2 would correlate with ROS contribution as a tumor promotion.

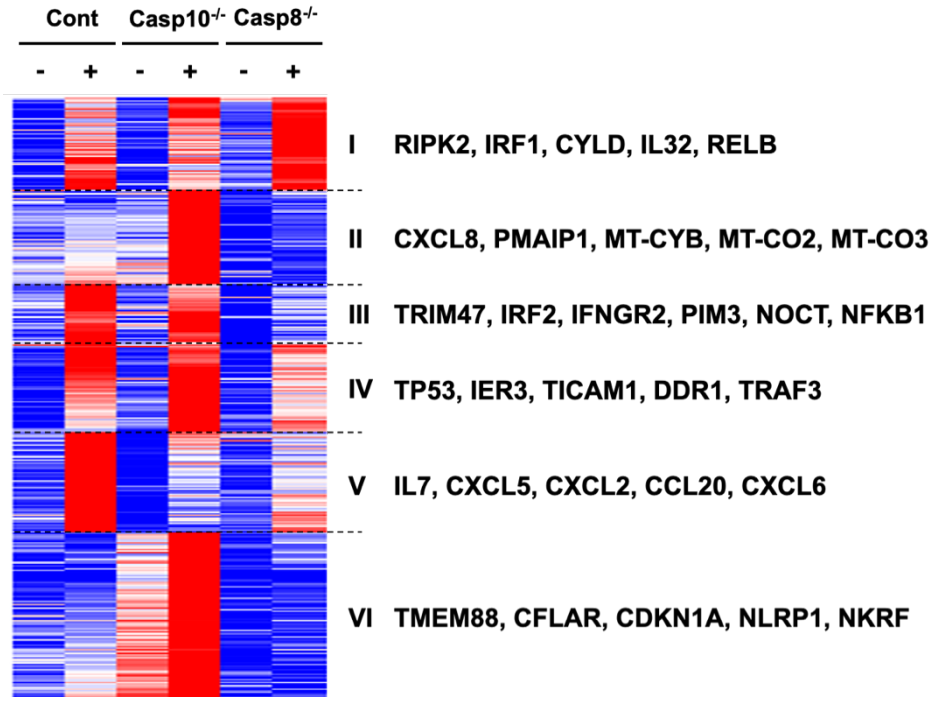

Figure 7. RNA-seq analysis on HepG2 cells under TNF-α stimulation. The cluster heat map of the expression profiles of the mRNA level under TNF-α stimulation. Representative genes in the cluster heat map are shown.

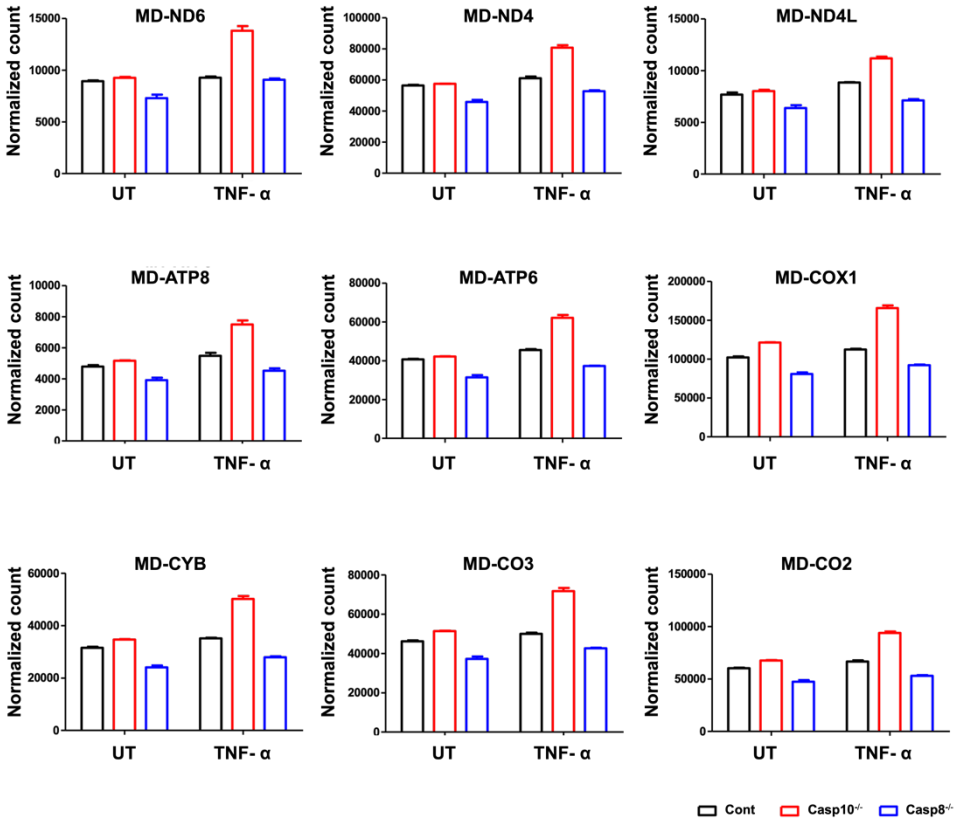

Figure 8. Up-regulation of ETC genes in caspase-10 knock-out HepG2. Representative genes in Cluster 2 are shown with a bar graph. The read count data was normalized and the differentially expressed genes (DEGs) between each sample were analyzed by DESeq2.

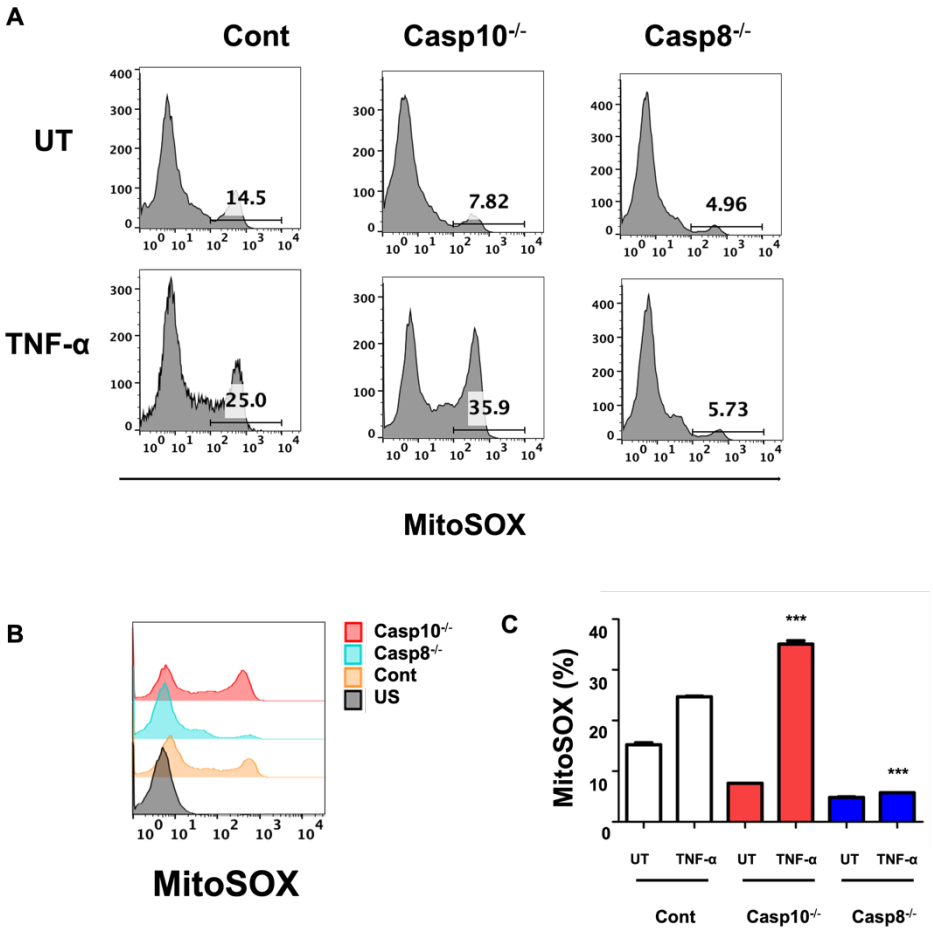

Figure 9. Caspase-10 regulates generation of ROS. Cells were treated with 100 ng/ml TNF-α for 3 h before being stained with MitoSOX. (A, B) Quantitative ROS were analyzed by flow cytometry, represented in the histogram. (C) Elevated ROS in caspase-10 knock-out HepG2 represented in a bar graph. For comparisons between HepG2 control cell caspase-10 knock-out cell, a Student's t-test was employed (A p-value of <0.05 was considered statistically significant. *p < 0.05, **p < 0.01.).

5. Chemokines are decreased in caspase-10 knock-out HepG2 under TNF-α stimulation

We next analyzed decreased gene expression in caspase-10 knock-out HepG2 under TNF-α stimulation. We found that the expression levels of chemokines including IL-7, IL-5, IL-2, IL-6 were decreased in caspase-10 knock-out HepG2 (Figure 10). Since chemokines are known for activating the immune system by regulating the migration of immune cells into tissues [32], we expected that the immune system was suppressed in caspasep10 knock-out HepG2, indicates that caspase-10 regulates activation of the immune system.

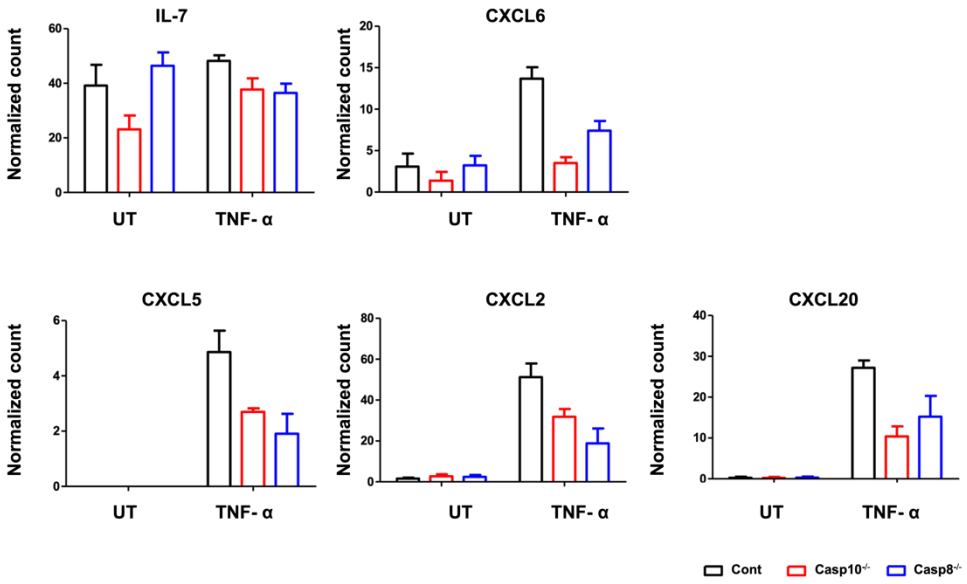

Figure 10. Down-regulation of chemokines in caspase-10 knock-out HepG2. Representative genes in Cluster 5 are shown with a bar graph. The read count data was normalized and the differentially expressed genes (DEGs) between each sample were analyzed by DESeq2.

6. Caspase-10 cleaves GSDME to induce pyroptosis

Caspases were recently identified as the mediators of pyroptosis by cleaving gasdermin family members. We next hypothesized that caspase-10 might be involved in the pyroptosis process. To test our hypothesis, caspase-10 and caspase-8 are overexpressed in 293T cells with GSDME. We found out that caspase-10 cleaves GSDME. Interestingly, we observed caspase-8 also cleaves GSDME but with lower efficiency. This suggests that caspase-10 might have a distinct role in pyroptosis (Figure 11).

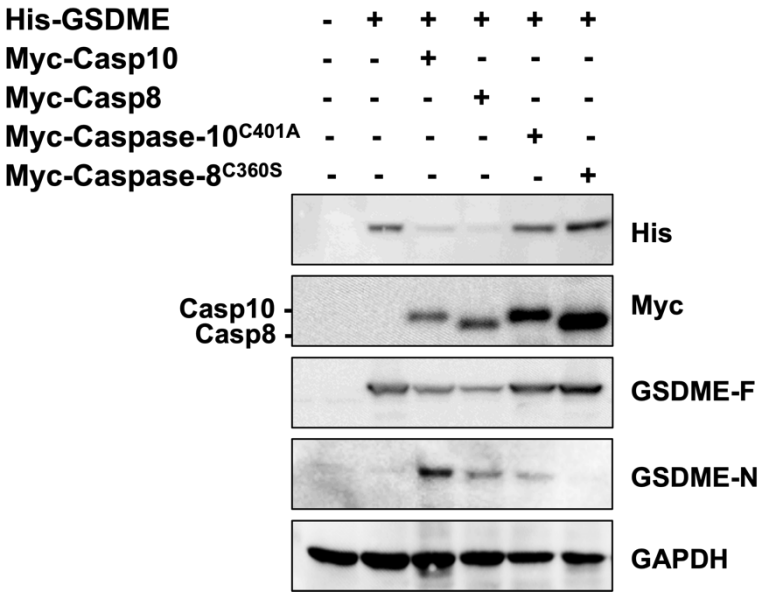

Figure 11. Caspase-10 cleaves GSDME to induce pyroptosis. Western blot was performed with caspase-10 and caspase-8 overexpression 293T cells.

7. Sorafenib-induced caspase-10-dependent pyroptosis

Next, we investigated whether GSDME is cleaved by endogenous caspase-10 in HepG2. We observed that activated caspase-10 cleaved GSDME treatment with sorafenib but not cisplatin, indicating that HepG2 underwent a different cell death mechanism between sorafenib and cisplatin. In addition, we confirmed that caspase-8 shows lower efficiency than caspase-10, corresponding to Figure 11.

Given that sorafenib is the first-line drug for HCC and received FDA approval in 2007 [33,34], caspase-10 mediates pyroptosis with sorafenib treatment that gives potential therapeutic benefits to patients with liver cancer.

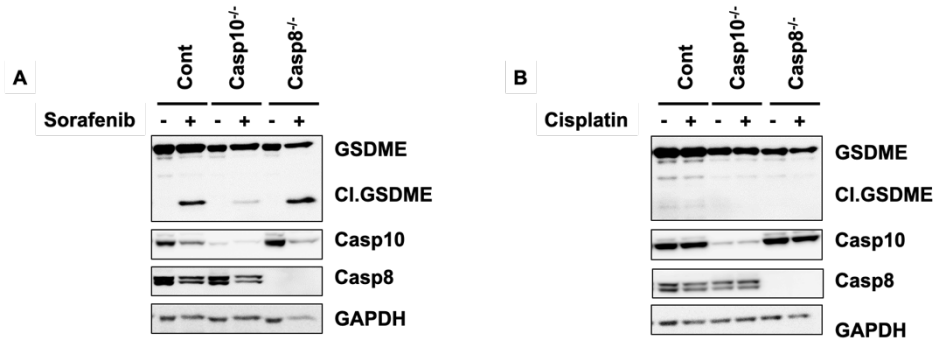

Figure 12. Caspase-10 cleaves GSDME treatment with sorafenib. Western blot was performed in caspase-10 and caspase-8 knock-out HepG2 cells. (A) Cells were treated with 60uM sorafenib for 24 h. (B) 50 μM cisplatin for 24 h.

IV. DISCUSSION

Apoptosis was generally considered to be the only form of programmed cell death [35,36]. It is well studied that apoptosis can be triggered by an intracellular signal such as DNA damage or by extrinsic signals such as tumor necrosis factor-α (TNF-α) [37]. Apoptosis has long been considered as a promising target for anti-cancer therapy. However, resistance to apoptosis and immune evasion in cancer are considered to be hallmarks of cancer [38]. Moreover, recent studies have revealed that cells that are adjacent to apoptosis occurs may undergo sustained compensatory proliferation until the wound healing therefore, leads to failure of cancer therapy [39]. Due to imitations in apoptosis, approaches to inducing other types of programmed cell death are emerging as new cancer therapy [40].

Studies have reported that caspase not only mediates apoptosis but also regulates other types of programmed cell death (PCD) such as necroptosis and pyroptosis [41]. Recent studies have reported that caspase-8 acts as a molecular switch between apoptosis and necroptosis [42]. Caspase-10, which shows high homology to caspase-8, functions as an initiator caspase in death receptor-mediated apoptosis similar to caspase-8 [43]. However, the role of caspase-10 is not well elucidated. It has been considered that caspase-10 may have a different role in cancer cell death [44]. We hypothesized that caspase-10 might be involved in necroptosis. Necroptosis is a programmed form of necrosis that results in membrane permeabilization, ultimately releases intracellular component including DAMPS [45].

Necroptosis is mediated by receptor-interacting proteins (RIP)1, RIP3, and mixed-lineage kinase domain-like (MLKL). The assembly of RIP1, RIP3 with other proteins formation oligomeric complex termed necrosome. Activated MLKLs translocate to plasma membrane and increase membrane permeability lead to membrane disruption [18]. It has been reported that RIP3 expression is silenced in the majority of cancers as one of the cancer evasion mechanisms.

Recent studies have reported that the transcription start site (TSS) of RIP3 is methylated in HepG2, but it can be recovered by treatment with hypomethylating agents such as 5-AD[46]. We show Above Data that caspase-10 mediated programmed cell death under various stimuli, and we concluded that necroptosis did not occur by caspase-10 in HepG2 since RIP3, which is a key molecule of the necroptosis, is silenced in HepG2.

Given that GSDMC[47], GSDMD cleaved by caspase-8 [48], we hypothesized that caspase-10 might be involved in inducing pyroptosis. To investigate our hypothesis, we performed overexpression of caspase-10 in 293T cells. Indeed, we observed cleavage of GSDME within caspase-10 overexpression HepG2 cells. Notably, we observed that caspase-8 also cleavage GSDME to induce pyroptosis. However, cleavage efficiency was higher with overexpression of caspase-10. This suggests that caspase-10 can be a potential target to induce pyroptosis better than caspase-8.

We next show that sorafenib induces caspase-10-dependent pyroptosis in HepG2 cells but not cisplatin. Considering that our preliminary observation under various cell death inducers including TNF- α, 5-FU, cisplatin, sorafenib were markedly induce programmed cell death, we hypothesized that both sorafenib and cisplatin may induce pyroptosis. But only sorafenib induces caspase-10-dependent pyroptosis.

Further studies will be needed to examine the role of caspase-10 in cancer pyroptosis; other stimuli such as TNF-α and chemotherapy drugs need to be investigated. In addition, in this study we show caspase-10-dependent induction of pyroptosis only *in-vitro.* For further support for our findings, xenograft as an *in-vivo* experimental model will need to be done as well as clinical research.

V. CONCLUSION

In summary, we showed that caspase-10 mediates cancer cell death.

First, we showed that caspase-10 regulates generation of ROS under TNF- α stimulation.
Second, our study suggested that caspase-10 regulates chemokines to activate immune system under TNF- α stimulation.
Finally, we showed that caspase-10 induces pyroptosis by cleaving GSDME under sorafenib treatment in HepG2.

Therefore, our findings suggested that caspase-10 may be a new target for inducing pyroptosis in liver cancer.

REFERENCES

1. Forner A, Reig M, Bruix J. Hepatocellular carcinoma. Lancet 2018;391:1301-14.
2. Korean Liver Cancer Association (KLCA); National Cancer Center (NCC) G, Korea. 2018 Korean Liver Cancer Association-National Cancer Center Korea Practice Guidelines for the Management of Hepatocellular Carcinoma. Korean J Radiol 2019;20:1042-113.
3. Lin L, Yan L, Liu Y, Qu C, Ni J, Li H. The Burden and Trends of Primary Liver Cancer Caused by Specific Etiologies from 1990 to 2017 at the Global, Regional, National, Age, and Sex Level Results from the Global Burden of Disease Study 2017. Liver Cancer 2020;9:563-82.
4. Yamashita T, Kaneko S. [Liver Cancer]. Rinsho Byori 2016;64:787-96.
5. Yoon SK, Chun HG. Status of hepatocellular carcinoma in South Korea. Chin Clin Oncol 2013;2:39.
6. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972;26:239-57.
7. Teraki Y, Shiohara T. Apoptosis and the skin. Eur J Dermatol 1999;9:413-25; quiz 26.
8. Van Cruchten S, Van den Broeck W. Morphological and Biochemical Aspects of Apoptosis, Oncosis and Necrosis. Anatomia, Histologia, Embryologia 2002;31:214-23.
9. Pistritto G, Trisciuoglio D, Ceci C, Garufi A, D'Orazi G. Apoptosis as anticancer mechanism: function and dysfunction of its modulators and targeted therapeutic strategies. Aging (Albany NY) 2016;8:603-19.
10. Wiernicki B, Dubois H, Tyurina YY, Hassannia B, Bayir H, Kagan VE, et al. Excessive phospholipid peroxidation distinguishes ferroptosis from other cell death modes including pyroptosis. Cell Death & Disease 2020;11:922.
11. Tang D, Kang R, Berghe TV, Vandenabeele P, Kroemer G. The molecular machinery of regulated cell death. Cell Res 2019;29:347-64.
12. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646-74.
13. Pommier Y, Sordet O, Antony S, Hayward RL, Kohn KW. Apoptosis defects and chemotherapy resistance: molecular interaction maps and networks. Oncogene 2004;23:2934-49.

14. Van Opdenbosch N, Lamkanfi M. Caspases in Cell Death, Inflammation, and Disease. Immunity 2019;50:1352-64.
15. Kumar S. Caspase function in programmed cell death. Cell Death Differ 2007;14:32-43.
16. Horn S, Hughes MA, Schilling R, Sticht C, Tenev T, Ploesser M, et al. Caspase-10 Negatively Regulates Caspase-8-Mediated Cell Death, Switching the Response to CD95L in Favor of NF-κB Activation and Cell Survival. Cell Rep 2017;19:785-97.
17. Kumari R, Deshmukh RS, Das S. Caspase-10 inhibits ATP-citrate lyase-mediated metabolic and epigenetic reprogramming to suppress tumorigenesis. Nat Commun 2019;10:4255.
18. Ding J, Wang K, Liu W, She Y, Sun Q, Shi J, et al. Pore-forming activity and structural autoinhibition of the gasdermin family. Nature 2016;535:111-6.
19. Abe J, Morrell C. Pyroptosis as a Regulated Form of Necrosis: PI+/Annexin V-/High Caspase 1/Low Caspase 9 Activity in Cells = Pyroptosis? Circ Res 2016;118:1457-60.
20. Feng S, Fox D, Man SM. Mechanisms of Gasdermin Family Members in Inflammasome Signaling and Cell Death. J Mol Biol 2018;430:3068-80.
21. Bergsbaken T, Fink SL, Cookson BT. Pyroptosis: host cell death and inflammation. Nat Rev Microbiol 2009;7:99-109.
22. Wu D, Wei C, Li Y, Yang X, Zhou S. Pyroptosis, a New Breakthrough in Cancer Treatment. Frontiers in Oncology 2021;11.
23. Tsuchiya K. Switching from Apoptosis to Pyroptosis: Gasdermin-Elicited Inflammation and Antitumor Immunity. Int J Mol Sci 2021;22.
24. Ikeda M, Morizane C, Ueno M, Okusaka T, Ishii H, Furuse J. Chemotherapy for hepatocellular carcinoma: current status and future perspectives. Jpn J Clin Oncol 2018;48:103-14.
25. Dai ZJ, Tang W, Lu WF, Gao J, Kang HF, Ma XB, et al. Antiproliferative and apoptotic effects of β-elemene on human hepatoma HepG2 cells. Cancer Cell Int 2013;13:27.
26. Uchibori K, Kasamatsu A, Sunaga M, Yokota S, Sakurada T, Kobayashi E, et al. Establishment and characterization of two 5-fluorouracil-resistant hepatocellular carcinoma cell lines. Int J Oncol 2012;40:1005-10.
27. Raoul JL, Adhoute X, Penaranda G, Perrier H, Castellani P, Oules V, et al. Sorafenib: Experience and Better Manage-ment of Side Effects Improve Overall Survival in Hepatocellular Carcinoma Patients: A Real-Life Retrospective Analysis. Liver Cancer 2019;8:457-67.

28. Zhao RZ, Jiang S, Zhang L, Yu ZB. Mitochondrial electron transport chain, ROS generation and uncoupling (Review). Int J Mol Med 2019;44:3-15.
29. Yu Z, Yang Y, Wang Y, Yin J, Qiu J. Reactive oxygen species-scavenging system is involved in l-amino acid oxidase accumulation in Pseudoalteromonas sp. B3. 3 Biotech 2017;7:326.
30. Singh A, Kukreti R, Saso L, Kukreti S. Oxidative Stress: A Key Modulator in Neurodegenerative Diseases. Molecules 2019;24.
31. Reczek CR, Chandel NS. The Two Faces of Reactive Oxygen Species in Cancer. Annual Review of Cancer Biology 2017;1:79-98.
32. Sokol CL, Luster AD. The chemokine system in innate immunity. Cold Spring Harb Perspect Biol 2015;7.
33. Lang L. FDA approves sorafenib for patients with inoperable liver cancer. Gastroenterology 2008;134:379.
34. Kane RC, Farrell AT, Madabushi R, Booth B, Chattopadhyay S, Sridhara R, et al. Sorafenib for the treatment of unresectable hepatocellular carcinoma. Oncologist 2009;14:95-100.
35. Busciglio J, Yankner BA. Apoptosis and increased generation of reactive oxygen species in Down's syndrome neurons in vitro. Nature 1995;378:776-9.
36. Han J, Zhong C-Q, Zhang D-W. Programmed necrosis: backup to and competitor with apoptosis in the immune system. Nature Immunology 2011;12:1143-9.
37. Bodmer J-L, Holler N, Reynard S, Vinciguerra P, Schneider P, Juo P, et al. TRAIL receptor-2 signals apoptosis through FADD and caspase-8. Nature Cell Biology 2000;2:241-3.
38. Gong Y, Fan Z, Luo G, Yang C, Huang Q, Fan K, et al. The role of necroptosis in cancer biology and therapy. Mol Cancer 2019;18:100.
39. Huang Q, Li F, Liu X, Li W, Shi W, Liu F-F, et al. Caspase 3-mediated stimulation of tumor cell repopulation during cancer radiotherapy. Nature Medicine 2011;17:860-6.
40. Jiang M, Qi L, Li L, Li Y. The caspase-3/GSDME signal pathway as a switch between apoptosis and pyroptosis in cancer. Cell Death Discovery 2020;6:112.
41. Tummers B, Mari L, Guy CS, Heckmann BL, Rodriguez DA, Ruhl S, et al. Caspase-8-Dependent Inflammatory Responses Are Controlled by Its Adaptor, FADD, and Necroptosis. Immunity 2020;52:994-1006 e8.
42. Fritsch M, Günther SD, Schwarzer R, Albert M-C, Schorn F, Werthenbach JP, et al. Caspase-8 is the molecular switch for

apoptosis, necroptosis and pyroptosis. Nature 2019;575:683-7.
43. Wang J, Chun HJ, Wong W, Spencer DM, Lenardo MJ. Caspase-10 is an initiator caspase in death receptor signaling. Proceedings of the National Academy of Sciences 2001;98:13884-8.
44. Mohr A, Deedigan L, Jencz S, Mehrabadi Y, Houlden L, Albarenque SM, et al. Caspase-10: a molecular switch from cell-autonomous apoptosis to communal cell death in response to chemotherapeutic drug treatment. Cell Death Differ 2018;25:340-52.
45. Liu Y, Liu T, Lei T, Zhang D, Du S, Girani L, et al. RIP1/RIP3-regulated necroptosis as a target for multifaceted disease therapy (Review). Int J Mol Med 2019;44:771-86.
46. Koo GB, Morgan MJ, Lee DG, Kim WJ, Yoon JH, Koo JS, et al. Methylation-dependent loss of RIP3 expression in cancer represses programmed necrosis in response to chemotherapeutics. Cell Res 2015;25:707-25.
47. Hou J, Zhao R, Xia W, Chang CW, You Y, Hsu JM, et al. PD-L1-mediated gasdermin C expression switches apoptosis to pyroptosis in cancer cells and facilitates tumour necrosis. Nat Cell Biol 2020;22:1264-75.
48. Shiromizu K, Thorgeirsson SS, Mattison DR. Effect of cyclophosphamide on oocyte and follicle number in Sprague-Dawley rats, C57BL/6N and DBA/2N mice. Pediatr Pharmacol (New York) 1984;4:213-21.

ABSTRACT(IN KOREAN)

암세포 파이롭토시스에 대한 캐스페이스-10의 역할 규명

<지도교수 김락균 >

연세대학교 대학원 의과학과

우원진

간암은 전 세계적으로 높은 사망률을 보이는 암 종이다. 간암에 대한 많은 치료법이 개발되었지만 암 5년 상대 생존율은 타 암 종에 비해서 낮은 수준을 보이고 있다. 따라서 간암 치료법에 대한 더 많은 연구가 이루어져야 한다.

Caspase-10은 인간에게만 있는 프로테아제로서, 유전자 서열에 있어 caspase-8과 높은 상동성을 가지고 있다. Caspase-10과 caspase-8은 apoptosis를 유도하는 것으로 이미 알려져 있다. 때문에 이 효소에 대한 기질 또한 비슷할 것으로 생각되고 따라서 비슷한 역할을 할 것으로 생각되었다. 하지만 최근 연구들에서 caspase-8과 구별되는 caspase-10만의 역할이 있다고 보고되고 있으며 우리는 이 연구를 통하여 암세포에서 caspase-10의 역할을 규명하고자 하였다.

이를 위해 먼저 간암 세포 주인 HepG2세포를 이용하여 caspase knock-out 세포 주를 제작하였다. 그 후 제작한 caspase knock-out 세포 주에 대한 RNA-seq을 수행하였다.

이 연구를 통해 caspase-10이 GSDME를 활성화 시킴으로써 파이롭토시스를 일으키는 것을 확인 할 수 있었다. 또한 이러한 세포 사멸은 sorafenib 자극을 주었을 때 유도될 수 있음을 확인할 수 있었다.

이전까지 암 치료 요법으로서 암세포의 apoptosis를 유도하는 방법이 일반적으로 많이 이용되어왔으나, 암세포의 apoptosis 저항성과 같은 한계점이 존재한다. 근 보고되는 연구에서 암 치료 요법으로서 pyroptosis와 같은 다른 유형의 세포사멸을 유도하는 것이 새로운 치료방법으로 떠오르고있다.

이 연구를 통하여 Caspase-10를 조절하는 것이 새로운 간암 치료 방법이 될 수 있음을 시사하였다.

핵심되는 말 : 캐스페이즈-10, 파이롭토시스, 간암