석 사 학 위 논 문

Clinical Value of EZH2 in Hepatocellular Carcinoma and its Potential for Target Therapy

계 명 대 학 교 대 학 원

의 학 과

배 안 나

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이 논문을 석사학위 논문으로 제출함

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배 안 나

배안나의 석사학위 논문을 인준함

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Table of Contents

List of Tables

List of Figures

1. Introduction

Hepatocellular carcinoma (HCC) is one of the leading life-threatening malignancies and the second most common cause of cancer-related deaths globally (1). The global incidence of HCC has been increasing, with an estimated 600,000-800,000 new cases occurring annually (2). The development of surgical techniques has improved the prognosis of patients with HCC (3). But, the prognosis of HCC remains dismal despite the great advances in HCC treatment. Thus, the need for reliable biomarkers for diagnosing HCC and novel strategies for the effective treatment of patients with HCC is pressing.

HCC-associated genes, along with a subset of their neighbors, are generally developed into a gene interaction network (4). Research has shown that various molecules play key roles in the development and progression of HCC: For example, there are genes such as signal transducer and activator of transcription 3 (STAT3) and Centrosomal protein of 55 (CEP55) (1,5). In HCC, STAT3 and CEP55 are known to be involved in cell migration and invasion (2).

Enhancer of zeste homolog 2 (EZH2) is the catalytic subunit of polycomb repressive complex 2, and it has been functionally associated with the regulation of the cell cycle (6). EZH2 is regulated by its involvement in the S and G2-M phases (7). As EZH2 regulates cell cycle progression, its dysregulation accelerates cell proliferation and prolongs cell survival, which may lead to carcinogenesis and cancer development (8). Recently, several studies have shown that EZH2 is aberrantly upregulated in various malignant tumors, such as prostate and breast cancer, and is associated with advanced stages and poor prognosis (6). EZH2 overexpression was first identified in prostate

cancer and is associated with poor clinical outcomes (8). Further studies have shown EZH2 overexpression in several other cancer types, including breast cancer, esophageal cancer, gastric cancer, anaplastic thyroid carcinoma, nasopharyngeal carcinoma, and endometrial carcinoma (8). Especially, EZH2 is overexpressed in HCC, and this correlates with poor prognosis (6). However, its clinical significance and molecular mechanisms have not been elucidated to date. In this regard, understanding the role of EZH2, as part of one of the molecular signaling pathways involved in the pathogenesis of hepatocellular carcinoma, in carcinogenesis will facilitate the discovery of prognostic biomarkers and therapeutic targets.

In this study, I aimed to analyze the clinical and prognostic values of EZH2 expression in hepatocellular carcinomas using the Total Cancer Genome Atlas (TCGA) data. I also analyzed the association between EZH2 and STAT3. Furthermore, based on these data, cell invasion and migration in HCC cell lines transfected with EZH2 siRNA were investigated to clarify the precise underlying mechanism of HCC. The results of this study potentially reveal new targets and strategies that can be applied to HCC diagnosis and treatment.

2. Materials and Methods

2.1. The Cancer Genome Atlas (TCGA) Data Analysis:

I investigated the publically available TCGA datasets, and the relevant data were downloaded from the TCGA Data Porta (9). The microarray and RNA-Seq experiments and clinical data were downloaded directly from the TCGA website in March 2021.

2.2. Cell culture and siRNA transfection:

HepG2 and Huh1 cells were cultured in Dulbecco's modified Eagle medium supplemented with 1 % penicillin/streptomycin solution and 10 % fetal bovine serum (FBS) (Gibco BRL., Grand Island, NY, USA) in a humidified 5 % CO2 incubator at 37 °C.

Negative-control (NC) siRNA (AllStars Negative control siRNA, Cat.no. 1027280) and EZH2-specific siRNAs were obtained from Qiagen. The targeting sequence of EZH2-siRNA was as follows: 5-AACCATGTTTACAACTATCAA-3 (Qiagen, Cat.no. SI02665166). The HCC cell lines reached 70 % confluence in 6-well plates and were transfected with negative-control siRNA or EZH2-specific siRNA using Lipofectamine 2000 (Invitrogen, Camarillo, CA, USA) according to the manufacturer's instructions. Lipofectamine (Invitrogen) and 200 nM siRNAs were mixed in Opti-MEM (Thermo Scientific, Rockford, IL, USA). After 6 h of transfection, the medium was replaced with a standard culture medium. After 48 h of EZH2-siRNA transfection, the RNA was extracted. The total cellular RNA was extracted using a

QIAzol lysis reagent (Qiagen, CA, USA) according to the manufacturer's protocol. For RNA extraction, 500 µl of the QIAzol lysis reagent was added to a 6-well plate after media suction. Harvesting was performed after 5 min at room temperature, and 1/5 (100 µl) of QIAzol with chloroform (Sigma-Aldrich, St Louis, MO, USA) was added and immediately vortexed for 15 seconds. After heating to room temperature for 3 min, the mixture was centrifuged at 12000 g at 4 °C for 15 min. Carefully pipetting only the transparent supernatant of the tube, 10 ml of the supernatant was added, and the mixture was immediately vortexed gently. The tube was centrifuged at full speed for 20 min, and the white pellet of the tube was checked. The solution was discarded in the tube and 1 ml of 70 % ethanol was added, followed by centrifuging at full speed. Ethanol was removed from the tube and evaporated as much as possible, followed by elution with 20 µl RNA free water, light tapping, vortexing several times, and measurements using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, MA, USA). The NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, MA, USA) was used to determine the quantity and quality of the isolated total cellular RNA.

2.3. Quantitative Real-Time PCR analysis (RT-qPCR):

Reverse-transcription reactions were conducted using the ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). The expression levels of EZH2, STAT3, and GAPDH were measured by RT-qPCR. RT-qPCR analysis was performed using a CFX Connect RT-PCR System (Bio-Rad, Hercules, CA, USA). The primers used for RT-qPCR were synthesized by Bionics (Seoul, Korea). The primer sequences used

for RT-qPCR are listed in Table 1. Each PCR contained 2 μL of template cDNA (10 ng/μL), 1 μL of the forward primer, 1 μL of reverse primer, 10 uL 2×SYBR Green Master Mix (Toyobo, Osaka, Japan), and DNase/RNase-free distilled water to a total volume of 20 μL. The PCR amplification cycles were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 60 s and 72 °C for 30 s. GAPDH expression was used as an endogenous control to normalize mRNA expression using the $2^{-\Delta\Delta Ct}$ method. Each experiment was performed in triplicate.

2.4. Cell viability assay:

HepG2 and Huh1 cells were seeded into 24-well plates at a density of 1×10^5 cells/well. After 24 h, 48 h, and 72 h after siRNA transfection at 37 °C, the cells were subsequently incubated with 100 μL of 5 mg/ml MTT for 4 h. Following the MTT incubation, the purple formazan crystals were dissolved by the addition of 700 μL dimethyl sulfoxide (DMSO, Sigma-Aldrich, St Louis, MO, USA) solution. Cell viability was subsequently analyzed at a wavelength of 570 nm using an Asys UVM 340 microplate reader (Biochrom, Cambridge, UK). Each experiment was performed in triplicate.

2.5. Wound healing assay:

HepG2 and Huh1(1×10^5) were seeded in 12-well plates at 70-80 % confluence for the wound healing assay. A linear wound was created using a 1 mL micropipette tip and washed three times to remove unattached cells. After washing, the cells were transfected with 200 nM negative control siRNA or EZH2 siRNAs using Lipofectamine 2000

(Invitrogen, Camarillo, CA, USA) according to the manufacturer's instructions. After 6 h of transfection, the medium was replaced with a standard culture medium. The cells were incubated at 37 °C for 24 h, 48 h, and 72 h. Light microscope images of three locations of the marked wounds were obtained, and the migrated cells were counted.

2.6. Statistical Analysis:

The chi-squared test, Fisher's exact test, and Mann-Whitney U-test were used to analyze the association between variables. Univariate survival analysis was performed using the log-rank test with Kaplan-Meier curves. Overall survival was defined as the time between diagnosis and mortality. Disease-free survival was defined as the time between diagnosis and disease recurrence or the development of distant metastasis. Statistical significance was set at $p < 0.05$.

Table 1. Primer sequences used for RT-qPCR in this study

Name	Primer (5 to 3')
EZH2	Forward: GACCTCTGTCTTACTTGTGGAGC Reverse: CGTCAGATGGTGCCAGCAATAG
STAT3	Forward: GCTTTTGTCAGCGATGGAGT Reverse: ATTTGTTGACGGGTCTGAAGTT
GAPDH	Forward: GAAAGGTGAAGGTCGGAGTC Reverse: GTTGAGGTCAATGAAGGGGTC

3. Results

3.1. Clinical characteristics of EZH2 expression in hepatocellular carcinoma:

Based on the TCGA data, EZH2 expression was associated with the prognoses for various cancers, and this was most significant for HCC. The average expression level in each cancer varied from 123.29 to 1041.46. Stratified by survival, HCC ranked 36th, with statistical significant of prognofic value of EZH2 in cancer types at $p < 0.001$ (Table 2).

The clinical data for HCC were obtained from TCGA (9), and they included age, gender, grade, clinical stage, and TNM stage of 360 HCC patients. The association between age, sex, T stage, N stage, M stage, AFP and Child-pugh class is presented in Table 3. The results showed that EZH2 expression was positively correlated with age ($p = 0.027$), N stage ($p = 0.045$), and alpha-fetoprotein (AFP) > 20 ng/ml ($p < 0.01$) (Table 3). The EZH2 expression levels were higher in patients younger than 65 years than in those older. Regarding AFP, which is used as a marker for HCC, the level of EZH2 expression was higher in patients with concentrations of > 20 ng/ml than of < 20 ng/ml. In addition, the level of expression of EZH2 at N1 was higher than that at N0. The other clinical features showed no significant differences.

3.2. Prognostic Value of EZH2 mRNA Expressions in HCC:

Survival analysis of HCC was performed to determine the prognostic value of the EZH2 mRNA expression. When the association between EZH2 expression and overall survival of HCC patients was investigated, the Kaplan Meier analysis found that high expression of EZH2 was correlated with significantly poorer prognoses of patients with high EZH2 expression (χ^2 = 16.10, p < 0.001) (Figure 1A). In addition, the recurrence rate was also significantly higher in patients with high EZH2 expression than in those with low expression (χ^2 = 12.70, p < 0.001) (Figure 1B). High EZH2 expression was associated with a poor prognosis.

3.3. Correlation with downstream genes related to EZH2:

Quantitative correlation analysis was performed using clinical parameters. The gene-gene correlation analysis based on the TCGA data analysis showed that EZH2 expression was negatively correlated with STAT3 expression (R = -0.163, p = 0.002) (Figure 2A). I also examined the association between EZH2 and the CEP55 gene, a subgene of EZH2. The levels of expression of EZH2 and CEP55 were positively correlated (R = 0.601, p < 0.001) (Figure 2B).

3.4. Effect of EZH2 Silencing on STAT3 mRNA Expression:

To investigate the association between EZH2 and STAT3, mRNA expression of STAT3 was analyzed in EZH2-silencing HepG2 and Huh1 cells. EZH2 was knocked down to determine its association with STAT3 expression. The HepG2 and Huh1 cells were transfected with Negative Control (NC) siRNA and EZH2-siRNA. To evaluate the potential role of EZH2 in regulating STAT3, I first investigated the effect of its expression on the efficacy of EZH2-siRNA by qPCR. As shown in Figure 3, EZH2 mRNA was significantly lower in HepG2 and Huh1 cells transfected with EZH2-siRNA than in cells transfected with NC-siRNA. Also, EZH2 silencing induces a decrease in mRNA levels of STAT3 in both cells. These result suggest that the function of EZH2 is related to the STAT3 pathway in HCC progression.

3.5. EZH2 knockdown reduces HCC Cell viability and Cell recovery :

The viability of the HepG2 and Huh1 cells was assessed using an MTT assay. EZH2 knockdown significantly decreased HepG2 and Huh1 cell viability after 24 h, 48 h, and 72 h (Figure 4). These findings suggest that EZH2 regulates HCC cell viability. In addition, wound healing analysis was used to determine the extent of resilience after

treatment with EZH2-siRNA knockdown (Figure 5). The resilience in HepG2 was significantly decreased (Figure 5A), but the resilience in Huh1 did not show any significant results (Figure 5B).

Table 2. Statistical significances of prognostic value of EZH2 in various cancers types

Cancer type	*P*
Bladder urothelial carcinoma	0.726
Breast invasive carcinoma	0.402
Cervical squamous cell carcinoma	0.293
Colon adenocarcinoma	0.333
Esophageal carcinoma	0.837
Glioblastoma multiforme	0.994
Head and neck squamous cell carcinoma	0.183
Kidney renal clear cell carcinoma	<0.001
Kidney renal papillary cell carcinoma	0.00794
Acute myeloid leukemia	0.378
Brain lower grade glioma	0.00212
Liver hepatocellular carcinoma	**<0.001**
Lung adenocarcinoma	0.476
Lung squamous cell carcinoma	0.0946
Ovarian serous cystadenocarcinoma	0.526
Pancreatic adenocarcinoma	0.145
Rectum adenocarcinoma	0.959
Sarcoma	0.415
Skin cutaneous melanoma	0.182
Stomach adenocarcinoma	0.0874
Uterine corpus endometrial carcinoma	0.144

Table 3. Clinical characteristics of EZH2 expression in hepatocellular carcinoma

	EZH2 expression		
	High (%, N)	Low (%, N)	*P* value
Age			**0.027**
<65	56.0 (98)	44.0 (77)	
≥65	44.3 (82)	55.7 (103)	
Sex			0.176
Male	47.5 (116)	52.5 (128)	
Female	55.2 (64)	44.8 (52)	
T stage			0.386
T1	51.4 (90)	48.6 (85)	
T2	43.0 (40)	57.0 (53)	
T3	56.0 (42)	44.0 (33)	
T4	50 (7)	50 (7)	
N stage			**0.045**
N0	49.4 (117)	50.6 (120)	
N1	100 (4)	0 (0)	
M stage			0.614
M0	51.4 (129)	48.6 (122)	
M1	40.0 (2)	60.0 (3)	
AFP			**<0.001**
<20ng/ml	34.9 (51)	65.1 (95)	
≥20ng/ml	65.9 (85)	34.1 (44)	
Child-Pugh class			0.543
A	44.9 (97)	55.1 (119)	
B	45.0 (9)	55.0 (11)	
C	100 (1)	0 (0)	

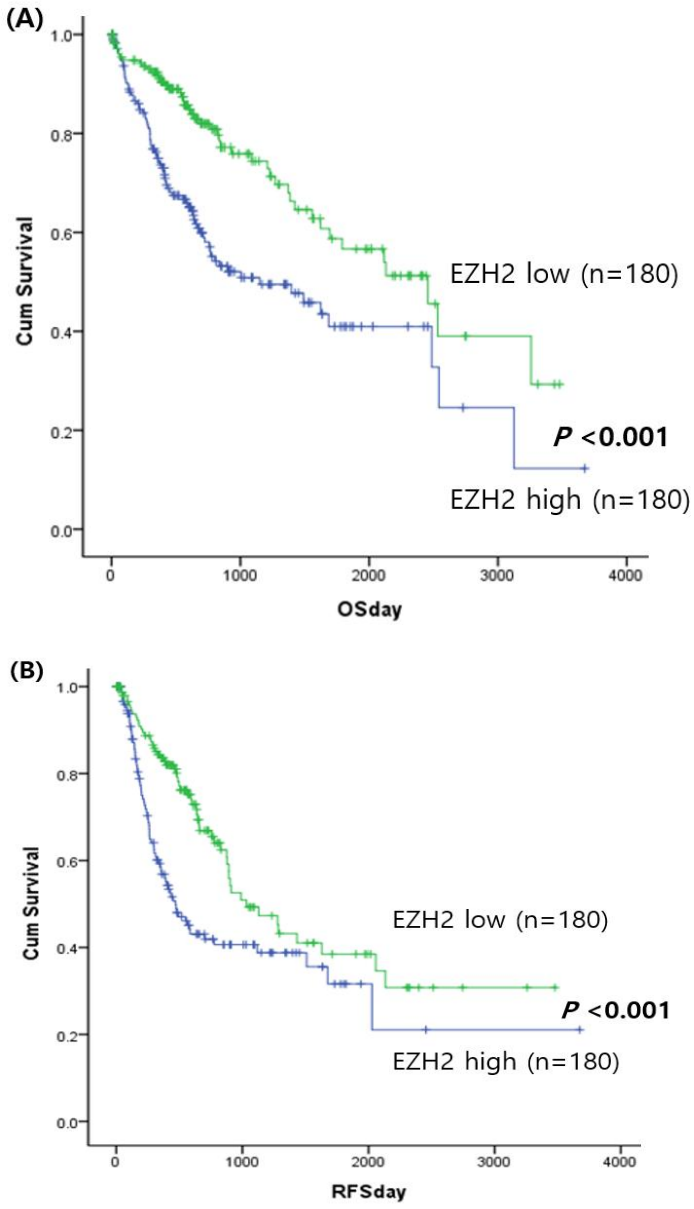

Figure 1. Survival analysis in HCC. (A) Overall survival of EZH2 expression (B) Disease free survival of EZH2 expression.

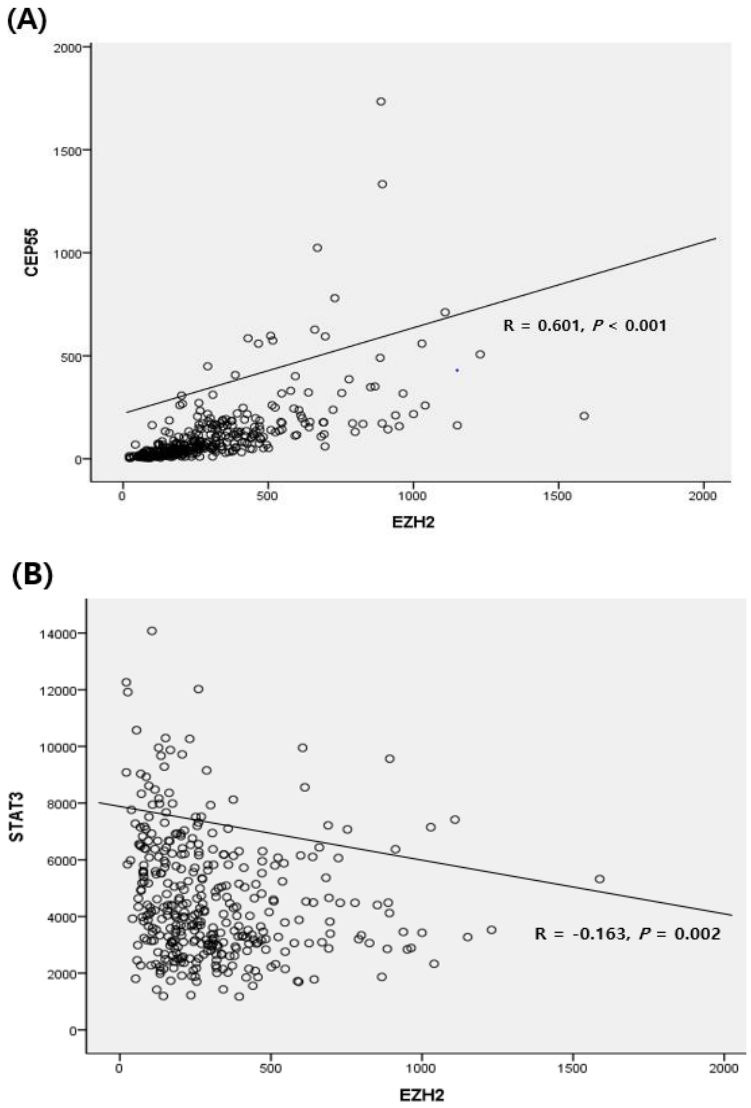

Figure 2. Correlation analysis. (A) Between EZH2 expression and CEP55. (B) Between EZH2 and STAT3.

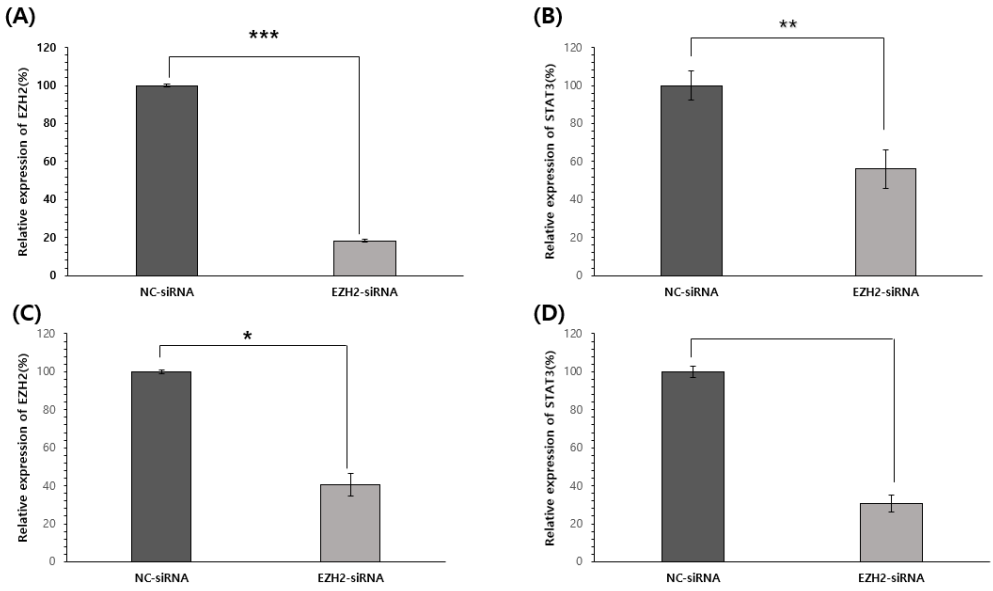

Figure 3. siRNA knockdown of EZH2 in HCC cells and the association between EZH2 and STAT3 mRNA expression. (A) Detection of EZH2 mRNA expression in HepG2 cells at 48 h after transfection. (B) After EZH2 knockdown, STAT3 mRNA expression in HepG2. (C) Detection of EZH2 mRNA expression in Huh1 cells at 48 h after transfection. (D) After EZH2 knockdown, STAT3 mRNA expression in Huh1.

* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ by Student's t-test.

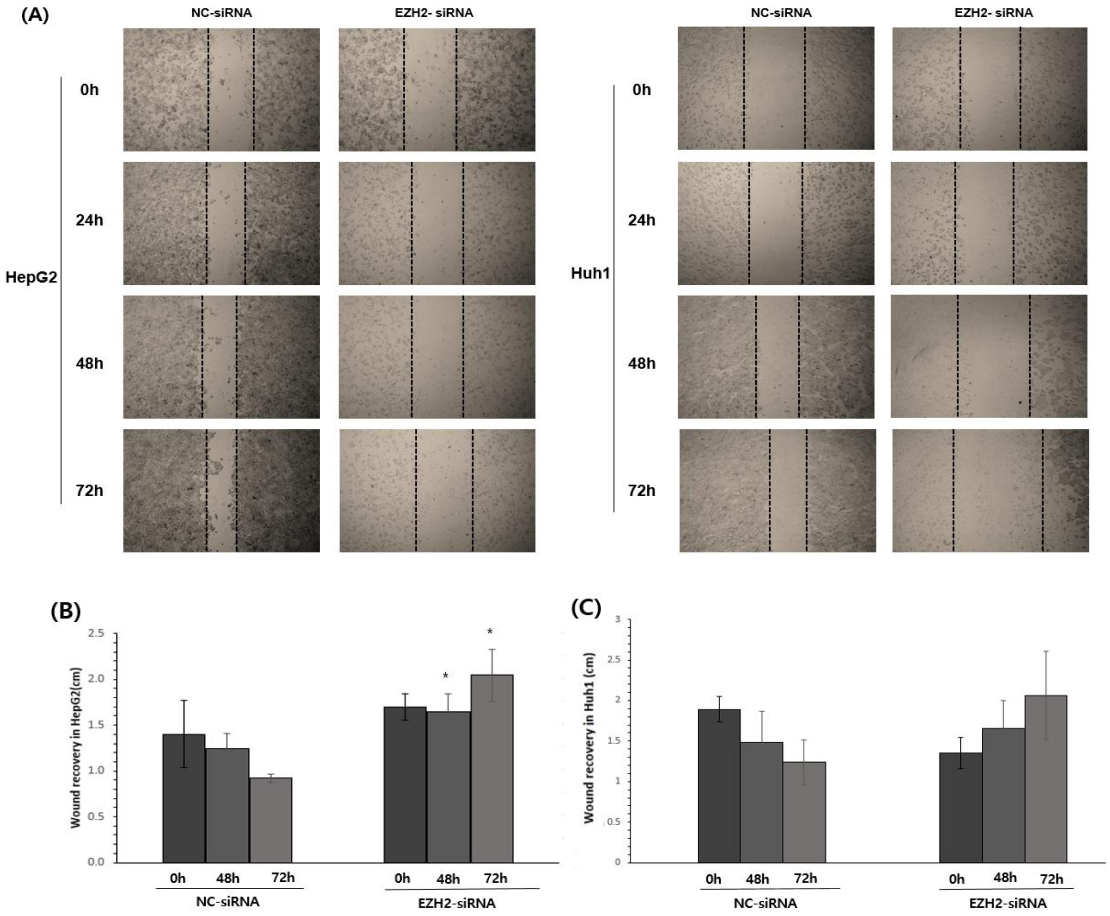

Figure 4. Cells migration in cell scratch wound healing assay. EZH2 inhibited cell migration in HepG2. (A) Wound healing assay of HepG2 and Huh1 upon (200 nM) for 24h, 48h, and 72h. The HCCs treated with NC-siRNA were used as control. (B) Quantification of wound area in control and EZH2 treated HepG2 (C) Quantification of wound area in control and EZH2 treated Huh1.

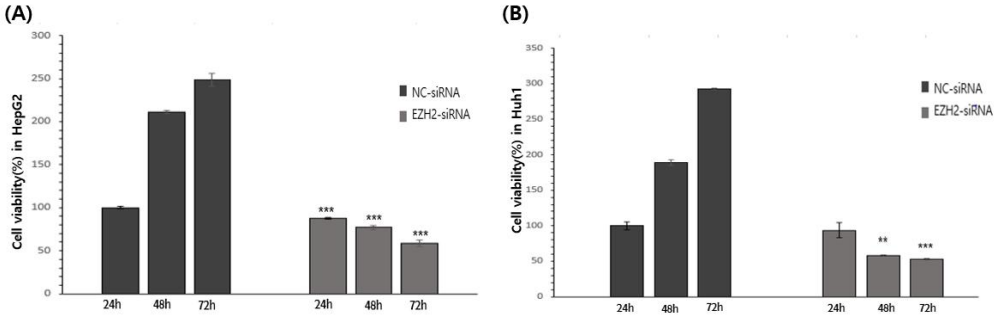

Figure 5. siRNA knockdown of EZH2 and growth of HCC cells. (A) Detection of cell viability after transfection in HepG2 (B) Detection of cell viability after transfection in Huh1

** $p < 0.01$, and *** $p < 0.001$ by Student's t-test.

4. Discussion

In this study, the clinical characteristics of HCC were confirmed using TCGA and the function of EZH2 was investigated using HCC cell line. As a novel and potential target for cancer therapy, EZH2 has become extensively researched (8). An increasing number of functions and roles of EZH2 in multiple types of cancer has been revealed. Several new drugs targeting EZH2 are being developed and evaluated in clinical trials. Therefore, targeting the carcinogenic activity of EZH2 in HCC may improve diagnosis and prognosis.

The analysis of clinical features showed that it was related to the degree of metastasis of lymph nodes, and it was also significantly related to an increase in the concentration of AFP. AFP is a representative biomarker in HCC, suggesting that elevated serum AFP concentrations (> 20 ng/mL) correlate with an increase in the risk of HCC development (10). Given this result, the expression of EZH2 seems to affect AFP. Although the exact mechanism is not known yet, the expression of EZH2 is thought to affect lymph node metastasis and deteriorate the prognosis of HCC. According to previous research, EZH2 upregulation was associated with HCC progression and multiple HCC metastatic features, including venous invasion, direct liver invasion, and the absence of tumor encapsulation (11). In addition, the results of previous studies on the correlation between EZH2 and lymph node metastasis showed that the correlation was stronger for EZH2-expressing tumor cells in lymph nodes than for matched primary tumor cells (12). Therefore, high expression levels of EZH2 were associated with the pathological grade of tumors and lymph node metastasis.

EZH2 has downstream genes involved in signaling, and EZH2 interacts with these genes to cause carcinogenesis. Among related genes, it has been previously reported that overexpression of CEP55 may worsen the prognosis in HCC (13,14). When looking at the association between CEP55 and EZH2 gene, there is a positive correlation. The relationship between the two genes has been reported in lung, but there is no study in HCC (15). Moreover, STAT3 is known to cause carcinogenesis by binding to EZH2 and being activated. EZH2 binds to and methylates STAT3, leading to the enhancement of its activity by increasing its tyrosine phosphorylation. STAT3 and EZH2 are potential molecular biomarkers for tumor progression and serve as poor predictors of outcomes. Previous research has suggested that STAT3 and EZH2 are closely associated with cell proliferation, invasion, and metastasis. The activation of EZH2 and STAT3 is significantly correlated with TNM stage and patient survival, suggesting that a combination of STAT3 and EZH2 expressions may determine the clinical TNM stage and predict disease outcomes (16).

I wanted to know why EZH2 and STAT3, which were negatively correlated in big data, were different in vitro. The HCC cell lines, HepG2 and Huh1 cells, were transfected with siRNA. Subsequently, their migration ability was determined using a wound-healing assay and their invasion ability was determined using the transwell assay. The results showed that the knockdown of EZH2 markedly decreased the migration and invasion abilities of the HepG2. However, when observed visually in Huh1, the cell mobility and recovery power seemed to decrease, but quantification did not show any significant results. More research is needed in this regard.

STAT3, which is closely related to cancer cell invasion and metastasis, was downregulated by EZH2 knockdown (8). My study

suggests that the downregulation of EZH2 and STAT3 can be utilized as new therapeutic target candidates for HCC. The present study demonstrates that EZH2 regulates cell viability, mobility, and resilience in HCC cell lines, which suggests that EZH2 can be used as a potential biomarker for HCC diagnosis and prognostication. The downregulation of EZH2 promotes apoptosis through suppression. Similarly, resilience was reduced in HCC. These in vitro results indicate that EZH2 may play an important role in the development and progression of HCC.

In conclusion, the overexpression of EZH2 was an independent biomarker for poor outcomes of HCC. Based on the results, EZH2 may be used as a therapeutic target in patients with HCC. However, more in vivo studies are required to identify the downstream target genes in HCC to improve our understanding of the biological role of EZH2 in HCC.

5. Summary

EZH2 is overexpressed in HCC, and this correlates with poor prognosis. However, its clinical significance and molecular mechanisms have not been elucidated to date. The aim of this study understanding the role of EZH2, as part of one of the molecular signaling pathways involved in the pathogenesis of hepatocellular carcinoma, in carcinogenesis was facilitate the discovery of prognostic biomarkers and therapeutic targets. I aimed to analyze the clinical and prognostic values of EZH2 expression in hepatocellular carcinomas using the TCGA data. I also analyzed the association between EZH2 and STAT3. Furthermore, based on these data, cell invasion and migration in HCC cell lines transfected with EZH2 siRNA were investigated to clarify the precise underlying mechanism of HCC. The results showed that EZH2 expression was positively correlated with age, N stage and alpha-fetoprotein. To investigate the association between EZH2 and STAT3, mRNA expression of STAT3 was analyzed in EZH2-silencing HepG2 and Huh1 cells. EZH2 mRNA was significantly lower in HepG2 and Huh1 cells transfected with EZH2-siRNA than in cells transfected with NC-siRNA. Also, EZH2 silencing induces a decrease in mRNA levels of STAT3 in both cells. The overexpression of EZH2 was an independent biomarker for poor outcomes of HCC. Based on the results, EZH2 may be used as a therapeutic target in patients with HCC.

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Clinical value of EZH2 in hepatocellular carcinoma and its potential for target therapy

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(Abstract)

Enhancer of zeste homolog 2 (EZH2) is the catalitic subunit of polycomb repressive complex 2 and has been associated with the regulation of the cell cycle mediated gene silencing. EZH2 is overexpressed in hepatocellular carcinoma (HCC) and is correlated with poor prognosis. However, its clinical significance and molecular mechanism have not been studied in HCC. In this study, clinical and prognostic values of EZH2 was studied using Total Cancer Genom Atlas (TCGA) data and then, theses data were confirmed in Huh1 and HepG2 cell lines. According to TCGA, EZH2 had a prognostic value in various cancers, especially in HCC. And EZH2 in HCC was correlated with N stage and AFP level. However, a negative association between EZH2 and age was found. The overall survival result of HCC was

significantly poorer in patients with high EZH2 expression. In addition, the recurrence rate was also significantly higher in patients with high expression of EZH2 than those with low expression. EZH2 expression was negatively correlated with STAT3 expression among EZH2-associated genes. To confirm effect of EZH2 in HCC cell lines, Huh1 and HepG2 cells were treated with EZH2 specific siRNA. EZH2 expression level was down-regulated to 50% or less compared to the control group treated negative siRNA. MTT assays showed that EZH2-siRNA did affect on the viability of HCC cell line significantly. Futhermore, STAT3 mRNA was significantly decreased in Huh1 and HepG2 cells. These findings demonstrate that EZH2 is a potential prognostic biomarker and therapeutic target.

간세포 암종에서 EZH2의 임상적 가치 및 표적 치료 가능성

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(초록)

Zeste homolog 2 (EZH2)의 증강 인자는 polycomb 억제 복합체 2의 catalitic subunit이며 세포주기 매개 유전자 침묵의 조절과 관련이 있다. EZH2는 간세포 암종 (HCC)에서 과발현되며 불량한 예후와 관련이 있다. 그러나 그 임상적 중요성과 분자 메커니즘은 HCC에서 연구되지 않았다. 본 연구에서는 Total Cancer Genome Atlas (TCGA) 데이터를 사용하여 EZH2의 임상 및 예후 값을 연구한 후 Huh1 및 HepG2 세포주에서 이러한 데이터를 확인했다. TCGA에 따르면 EZH2는 다양한 암, 특히 간세포 암종에서 예후 적 가치가 있었다. 그리고 HCC의 EZH2는 N 단계 및 AFP 수준과 관련이 있다. 그러나 EZH2와 나이 사이에 부정적인 연관성이 발견되었다. HCC의 전반적인 생존 결과는 EZH2 발현이 높은 환자에서 유의하게 더 나빴다. 또한 EZH2의 발현이 높은 환자에서 발현이 낮은 환자보다 재발률이 유의하게 높았다. EZH2 발현은 EZH2 관련 유전자 중 STAT3 발현과 음의 상관 관계를 보였다. HCC 세포주에서 EZH2의 효과를 확인하

기 위해 Huh1 및 HepG2 세포에 EZH2 특이적 siRNA를 처리했다. EZH2 발현 수준은 음성 siRNA를 처리한 대조군에 비해 50 % 이하로 하향 조절되었다. MTT 분석은 EZH2-siRNA가 HCC 세포주의 생존력에 크게 영향을 미치는 것으로 나타났다. 그러나 STAT3 mRNA는 Huh1 및 HepG2 세포에서 유의하게 감소했다. 이러한 발견은 간암에서 EZH2가 잠재적인 예후 바이오 마커 및 치료 표적임을 입증한다.