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Study on the Mechanism of Positive Regulation of YAP by CD166 in Promoting Occurrence and Development of Liver Cancer

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Abstract: This study aims to investigate the role and molecular mechanism of the cell surface molecule CD166 in the occurrence and development of liver cancer. As a malignant tumor with high incidence and mortality, early diagnosis and treatment of liver cancer are of great significance. CD166, known as ALCAM, belongs to the immunoglobulin superfamily, which is intertwined with the ability of tumor cells to proliferate, migrate, and invade. In this study, the expression difference of CD166 in HCC tissues and adjacent normal tissues was analyzed by immunohistochemistry and Western Blot technology. It was found that the expression of CD166 in HCC tissues was significantly upregulated. In further experiments, CD166 in HCC cells was knocked down by RNA interference technology. It was also observed that cell proliferation was inhibited and apoptosis increased, which indicates that CD166 plays a vital role in regulating the growth and survival of HCC cells. The results provide a scientific basis for CD166 as a potential molecular target for early diagnosis and treatment of liver cancer, which is beneficial to promoting liver cancer treatment.

Keywords: CD166; Liver Cancer; Cell Proliferation; Apoptosis; Molecular Target.

1. Introduction

1.1. Research Background

Liver cancer, a malignant tumor that occurs in the liver, has a high morbidity and mortality in China. It is developed by most patients based on hepatitis B cirrhosis, and the number of hepatitis C patients is also on the rise [1-3]. The disease often lacks obvious symptoms in the early stage and is difficult to detect in time. In the late stage, patients often show clinical symptoms such as liver pain, fatigue, weight loss, jaundice and ascites. In view of the extremely high mortality rate and extremely low early diagnosis rate of liver cancer patients, it is particularly urgent to reveal its molecular pathogenesis and explore early molecular diagnosis targets [4]. This study focuses on the role of the cell surface molecule CD166 (Cluster of differentiation 166), also known as Activated leukocyte cell adhesion molecule (ALCAM), in the occurrence and development of liver cancer. CD166 belongs to the immunoglobulin gene superfamily, whose gene is located in the long arm of human chromosome 3, with a total length of about 210kb and 16 exons [5]. The molecular weight of the encoded transmembrane glycoprotein is about 100-105KD, which binds to T cell differentiation antigen CD6 and participates in cell adhesion and migration [6-8]. Studies have shown that CD166 is abnormally expressed in many malignant tumors, especially in liver cancer. Its high expression is also intertwined with tumor progression, migration, proliferation and metastasis [9, 10]. Therefore, CD166 is intertwined with tumor proliferation, metastasis, invasion, migration and prognosis, with its up-regulation related to the dysregulation of related signaling pathways.

1.2. Research Purpose and Significance

The main goal of this study is to explore the molecular mechanism of CD166 in the development of liver cancer. As a cell surface molecule, CD166 plays a crucial role in tumor biology, especially in liver cancer. The change of its expression is closely related to the proliferation, migration and invasion ability of tumors. By deeply analyzing the expression pattern of CD166 in liver cancer and its influence on tumor cell behavior, its molecular mechanism in the development of liver cancer can be revealed. Assessing the potential of CD166 as a molecular indicator for the diagnosis of liver cancer is equally important. Early diagnosis of liver cancer is essential to improve patient survival, and high expression of CD166 is associated with poor prognosis of liver cancer, making it a promising biomarker candidate. Studying the expression of CD166 in the early stage of liver cancer can provide a new molecular basis for its early diagnosis, and then may improve the formulation of the treatment plan and prognosis evaluation. This study aims to analyze the expression of CD166 in HCC tissues by experimental methods such as immunohistochemistry and Western Blot, so as to explore its role in the development of liver cancer and its feasibility as a diagnostic molecular index. These studies can provide a new perspective for the molecular diagnosis and treatment of liver cancer, and help to promote liver cancer treatment.

2. Experimental Materials and Experimental Methods

2.1. Research Ideas

The core of this study is to explore the expression and biological function of CD166 in HCC cells and tissues. CD166, a cell surface molecule that plays a key role in tumor development, especially in liver cancer, is closely related to changes in the proliferation, migration and invasion ability of tumor cells. Using immunohistochemistry and Western Blot techniques, this study will compare and analyze the expression of CD166 in HCC tissues and adjacent normal tissues, so as to reveal its potential role in the development of liver cancer and further deepen its understanding of its function in tumor biology.

Furthermore, this study will use RNA interference technology to specifically knock down CD166 in HCC cells to observe the changes in cell proliferation and apoptosis. Through MTT assay and flow cytometry, the effect of CD166 knockdown on cell proliferation rate and apoptosis rate will be quantitatively analyzed, and the role of CD166 in regulating the growth and survival of HCC cells will be evaluated, so as to provide experimental support for CD166 as a potential target for liver cancer treatment.

This study will also delve into the mechanism of CD166 in the development of liver cancer. By analyzing the changes in cell signaling pathways after CD166 knockdown, this study aims to reveal the possible pathways of action of CD166 in liver cancer, improve the understanding of the molecular mechanism of CD166, and lay a theoretical foundation for the development of novel therapeutic strategies. Through these comprehensive experimental methods, this study hopes to comprehensively analyze the expression and function of CD166 in liver cancer, provide a new perspective for its diagnosis and treatment, and thus promote the research progress in this field.

2.2. Experimental Materials

The selection of experimental materials is important for the study of liver cancer. The materials involved in this study include HCC cell line, HCC tissue, adjacent normal tissue and cell culture medium, all of which are the basis of experimental design. HCC cell lines provide an in vitro model to simulate the biological characteristics of liver cancer, while HCC tissues and adjacent normal tissues provide actual samples for comparative research. Cell culture medium is an essential medium for maintaining cell growth and conducting experimental manipulations. Through these carefully selected materials, this study will be able to deeply analyze the expression pattern of CD166 in liver cancer and its impact on tumor cell behavior, and then provide a solid experimental foundation for the molecular mechanism study and the discovery of therapeutic targets of liver cancer.

2.3. Experimental Methods

The molecular biology and cell biology techniques adopted in this study aim to accurately explore the expression pattern and biological function of CD166 in liver cancer. The designed experimental steps are all focused on ensuring the accuracy and reliability of the results, which lays a solid scientific foundation for the study of molecular mechanisms of liver cancer and the identification of potential therapeutic targets. These rigorous experimental procedures can comprehensively analyze the role of CD166 in the development of liver cancer and evaluate its feasibility as a therapeutic intervention target.

2.3.1. Cell Culture

All cells were cultured using DMEM+10% FBS+1% PS complete medium, and passaging began when the cell density reached 80% to 90%. During the passage process, the culture medium was first discarded, washed once with PBS, and then 0.05% trypsin was added for digestion until the cells became round and loose. The trypsin was discarded, a complete culture medium was added to stop digestion, and the cells were evenly blown and inoculated into Petri dishes of different specifications, and the cell adherence growth was observed the next day.

2.3.2. Western Blot

2.3.2.1 Cell Lysis and Protein Extraction

The cell total protein extraction steps are as follows. The first step is to discard the culture medium for cells to be treated, wash it with PBS once, and add the culture medium after trypsin digestion. Then, it's time to blow the cells evenly into a 1.5ml EP tube with a pipette gun, centrifuge at 350rcf for 3 minutes, discard the culture medium, resuspend and wash the cells with PBS once. After that, the cells were centrifuged at 350rcf for 3 minutes without PBS before adding an appropriate amount of pre-cooled Western blot/IP lysate (containing protease inhibitor) to lyse on ice for 30 minutes, or placed in a refrigerator at -80°C for later storage. The lysed cells were centrifuged on a 4°C at 12,000 rcf for 10 minutes, and the protein-containing supernatant was taken into a new 600 ul EP tube and placed on ice to prepare protein quantification.

2.3.2.2 Protein Quantification

Protein standard BSA is as follows. The first step is to melt 2 mg/ml BSA at room temperature and dilute 4-fold with Western blot/IP lysate to a final concentration of 0.5 mg/ml. The preparation of chromogenic solution BCA mixture MIX requires 50 parts of solution A and 1 part of solution B. The preparation of the protein standard curve requires a 96-well plate, with each reagent added in the volume shown in the table below:

Table 1. The preparation of the protein standard curve requires a 96-well plate, with each reagent added in the volume

Double Distilled Water (ul)	20	19	18	16	12	8	4	0
Protein Standard Solution (ul)	0	1	2	4	8	12	16	20
BCA Working Solution (ul)	200	200	200	200	200	200	200	200

It's needed to mix well, incubate at 37°C for 30 minutes, read the absorbance value at 562nm in the microplate reader, and draw the protein standard curve to detect the protein content of the sample. Then, take the above extracted protein and dilute it 10 times respectively. Take 20ul of the diluted sample, add 200ul of BCA mixture (A + B), and make 2 double wells for each sample. After that, mix well, incubate at 37°C for 30 minutes, measure the absorbance value with a microplate reader at 562nm, and calculate the protein content of the sample according to the protein standard curve.

2.3.2.3 Western Blot Detection

(1) Sample processing before loading: The protein sample was mixed with 6xSDS loading buffer at a volume of 5:1, boiled in a 95°C water bath for 10 minutes, and centrifuged

for 1 minute. The sample load volume of each group is 10-50ug.

(2) Preparation of 10% lower layer separation gel and 5% upper layer concentrated gel:

As for 10% AP, lgAP powder is dissolved in 10ml of double-distilled water, stored in a refrigerator at -20℃ for a long time, or stored at 4℃ (within two weeks). The formulation of 10% separation gum and 5% concentration gum is:

Table 2. The formulation of 10% separation gum and 5% concentration gum

Reagent Name	10% Separation Gel	5% Concentrated Gum
30% Acr/Bis	3.3 ml	0.85 ml
$1.5MTis-HCl(PH=8.8)$	2.5 ml	0ml
$0.5MTis-HCl(PH=6.8)$	0ml	1.25 ml
10% SDS	100ul	50ul
10% AP	50ul	25ul
TEMED	5ul	5ul
ddH2O	4.1 ml	2.85 ml

Finally, add TEMED before mixing well and filling glue immediately. After the separated glue solidifies, the concentrated glue can be poured and inserted into a 10-hole or 15-hole comb.

(3) Preparation of Electrophoresis Solution:

Preparation of the 1x Electrophoresis solution

Table 3. Preparation of the 1x Electrophoresis solution

Tris	3.03g
Glycine	14.4g
SDS	1g
ddH2O	1000ml

Pull off the comb on the concentrated gel, rinse the gel hole with electrophoresis buffer, and load the sample according to the above sample quantification results, with 80V as the constant pressure of the concentrated gel. When the sample runs to the separation gel, the voltage is adjusted to 120V.

(4) Film Transfer

Preparation of 1x membrane transfer solution

Table 4. Preparation of 1x membrane transfer solution

Tris	3.03g
Glycine	14.4g
methanol	200ml
ddH2O	至 1000ml

After electrophoresis, carefully pry open the thin plate and thick plate, leave the glue on the thin plate, cut off the upper layer of concentrated glue, wash the glue in clean water for 30 seconds to remove SDS in the electrophoresis solution, and prepare nitrocellulose membrane (NC) and filter paper with appropriate size to the glue. Sandwiches (negative to positive) should be placed in the following order: sponge→3 layers of filter paper→glue→NC membrane →3 layers of filter paper→sponge. Try to eliminate bubbles in each layer to ensure the success of film transfer. Constant current 200mA is kept for 60 to 90 minutes.

(5) Blocking: Prepare 5% skimmed milk with PBST and block it at room temperature for at least 1 hour or 4℃ for 4 hours.

(6) Primary antibody: Dilute the primary antibody to a suitable concentration with a blocking solution, evenly cover the surface of the NC membrane with the primary antibody and incubate in a wet box at 4°C overnight.

(7) Secondary antibody: Take out the NC membrane the next day, recover the primary antibody, and wash it 3 times with PBST for 15 minutes each time. The secondary antibody with horseradish peroxidase was incubated at a dilution ratio of 1: 2000. Incubate at room temperature for 60 minutes with PBST wash 3 times for 15 minutes.

(8) Color Exposure

Ready-to-use ECL color development solution is required. It's needed to mix solution A and solution B in equal volumes, add the mixed solution to the NC membrane, and react for 30 seconds to 5 minutes. Then put the NC film into the transparent plastic film inside the cassette, remove the bubbles, keep the NC film flat, and close the cassette. Enter the dark room to prepare for exposure, and prepare the

developer and fixer in advance. Put the Kodak film in the cassette, wait for a few seconds to minutes to take out the film, put it in the developer and wash it for 30 seconds, then wash it with tap water. Finally, put it in the fixer and wash it for 30 seconds with tap water. The results were analyzed after the film was air-dried.

2.3.3. Cellular Immunofluorescence (IF)

(1) Soak and disinfect the 14mm round coverslip with 75% alcohol, wash it three times with PBS, seed the cells into a 24-well plate containing the coverslip, and let the cells grow on the coverslip.

(2) After the cells adhere to the wall, discard the culture medium, wash once with PBS, fix with 4% PFA for 15 minutes, and wash 3 times with PBS for 5 minutes each time.

(3) Blocking solution (1.5 ml FBS, 500ul goat serum, 50ul Triton X-100, mixed to 50ml with PBS) was blocked at room temperature for 1 hour.

(4) The primary antibody was diluted with a blocking solution and incubated at 4°C overnight. The next day, PBS was washed 3 times for 5 minutes each time.

(5) The secondary fluorescent antibody was diluted at 1:500, incubated at room temperature and protected from light for 1 hour, and washed 3 times with PBS for 5 minutes each time.

(6) Place the coverslip in the 24-well plate on the slide with DAPI in advance for nucleus staining and sealing. Nail polish then fixes the edge of the coverslip.

(7) Observe the cell staining under a laser confocal microscope or fluorescence microscope.

2.3.4. Immunohistochemistry (IHC)

(1) Tissue section: xylene 10 minutes→100% ethanol 5 minutes→95% ethanol 5 minutes→70% ethanol 5 minutes→clear water 5 minutes→PBS soaking minutes.

(2) The slices are placed on the sheet rack, and the sheet rack is placed in 1x improved sodium citrate antigen repair solution, and finally put in a rice cooker and boil for 2 hours.

(3) Take out the tablet rack, naturally cool it to room temperature, wash it with clean water 3 times for 5 minutes each time, and then wash it once with PBS for 5 minutes.

(4) The slices were placed in 3% hydrogen peroxide solution and soaked warmly for 18 minutes. Preparation of 3% methanol hydrogen peroxide solution requires 45ml methanol, 5ml 30% H2O2, and 3 times washing of PBS for 5 minutes each time.

(5) Suck off excess PBS with toilet paper and draw circles around the tissue with a PAP pen.

(6) Blocking solution (5% BSA, 1% goat serum, 0.1% Tween 20) was added to the tissue and blocked for 1 hour.

(7) Dilute the primary antibody with the blocking solution (1:100-500), aspirate the blocking solution on the tissue, add the diluted primary antibody to the tissue, incubate at 4°C overnight, and wash 3 times with PBS the next day for 5 minutes each time.

(8) Dilute the biotin-labeled secondary antibody with blocking solution at a dilution ratio of 1:1000, add it dropwise to the sections, incubate at room temperature for 1 hour, and wash 3 times with PBS for 5 minutes each time.

(9) Prepare ABC reagent, add a drop of reagent A to 2.5 ml PBS, and mix well. Then, add another drop of reagent B, mix well, and let stand at room temperature for 30 minutes.

(10) Add ABC reagent to tissue sections, incubate at room temperature for 30 minutes, and wash 3 times with PBS for 5 minutes each time.

(11) Preparation of a DAB reagent: Add 1 drop of DAB Buffer to 2, 5ml distilled water, mix well, add 2 drops of DAB and mix well. Finally, add a drop of H2O2, mix well and set aside.

(12) Add DAB reagent to tissue sections, observe the DAB reaction under a microscope, and immerse the sections in clear water after the appropriate reaction to stop the reaction. The sections were stained in hematoxylin for 4.5 minutes and washed with water several times. Differentiate with 1% hydrochloric acid alcohol for a minute, and wash with clean water several times.

(13) The slices were sequentially soaked in 70%, 95%, and 100% ethanol for 5 minutes each, and in xylene for 15 minutes. Finally, neutral gum sealing and microscopic examination.

2.3.5. Construction of Lentiviral Interference Vector

(1) Obtain the Target Fragment

CD166 shRNA2 was cloned into the PLKO.1 vector as shown in Figure 1. The siRNA interference sequences of the corresponding genes were obtained from http://jura. wi. mit.edu/bioc/siRNAext/. The principles for designing primers are shown in Figure 2. Appropriate siRNA sequences are selected to synthesize oligonucleotide sequences by Shanghai Biotech. The primer sequences are as follows:

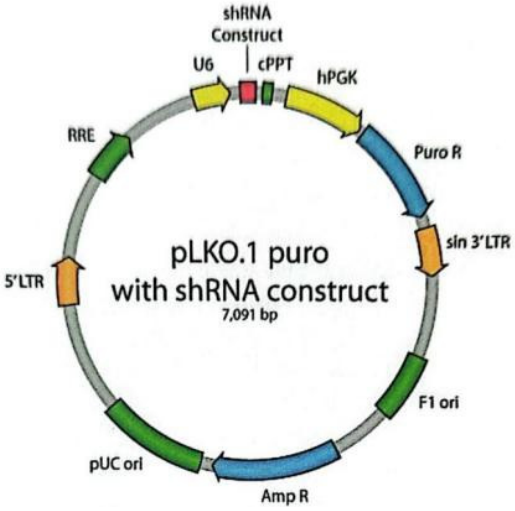

Figure 1. The Map of PLKO.1

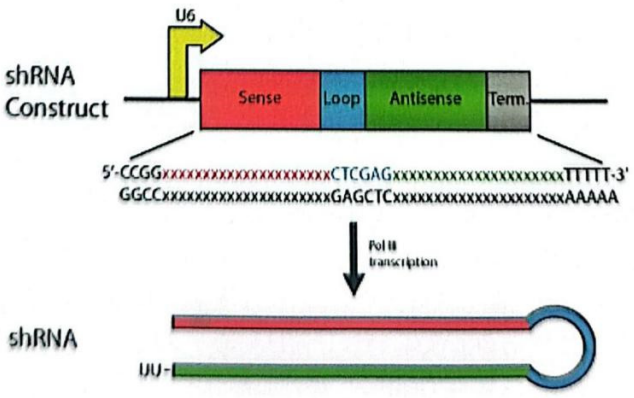

Figure 2. The Principle of shRNA Primer Design

CD166 shRNA Primer Sequence

Table 5. CD166 shRNA Primer Sequence

Primer Name	Sequence (5 '→ 3')
CD166-shRNA2-F	CCGGTCAAGCAACCATCTAAACCTGCTCGAGCAGGT
CD166-shRNA2-R	TTAGATGGTTGCTTGATTTTTG
	AATTCAAAAATCAAGCAACCATCTAAACCTGCTCGA
	GCAGGTTTAGATGGTTGCTTGA

(2) Annealing of Upstream and Downstream Primer Oligonucleotide Strands

The reaction system is as follows:

Table 6. The reaction system

Forward Oligo (20uM)	5ul
Reverse Oligo (20uM)	5ul
5XAnnealing buffer for DNA oligos	10ul
ddH20	30ul
Total	50ul

Reaction conditions; 95°C 4 minutes→70°C 10 minutes→slowly down to room temperature.

(3) PLKO, 1 double enzyme digestion reaction system

Table 7. PLKO, 1 double enzyme digestion reaction system

PLKO, 1	5ug
AgeI-HF	3ul
EcoRI-HF	3ul
10x Cutsmart Buffer	To 5ul

ddH_2O to 50ul

Reaction conditions: It's needed to react at 37°C for 2 hours with 1% agarose gel electrophoresis, 7 kb and 1.9 kb bands appeared, and 7 kb fragments were recovered.

(4) Ligation of PLKO.1 Digestion Product and Oligonucleotide Annealing Product

Table 8. Ligation of PLKO.1 Digestion Product and Oligonucleotide Annealing Product

PLKO.1	1ug
Annealing product	1ul
Ligase	1ul
10x Ligase buffer	1ul
ddH_2O	To 10ul

Reaction conditions: Ligation overnight at 16 °C.

(5) Transformation requires single clone: The procedure is the same as that of 2.2. 2.6. 2 above.

(6) Identification of positive clones with the plasmid double digestion reaction as follows:

Table 9. Identification of positive clones with the plasmid double digestion reaction

Vector	lug
EcoRI-HF	0.5 ul
NcoI	0.5 ul
10x Cutsmart Buffer	1ul
ddH_2O	To 10ul

Reaction conditions: It's needed to react at 37°C for 1 hour with 1% agarose gel electrophoresis identified. The appearance of 5kb and 2kb bands indicated that the interference vector was constructed.

(7) Lentiviral Packaging and Infection of Target Cells

a. The day before transfection, 1.5×106 HEK-293T cells were plated on a 6-well plate.

b. On the second day of transfection, the confluence of 293T cells should reach 80%-90%.

Preparing a transfection mixture:

Table 10. Preparing a transfection mixture

Liquid A		Liquid B	
Lenti Virus Vector	2ug	Lipofectamine 2000	10ul
PCMV-dR8.74	1.325 ug	DMEM	375ul
pMD2-VSVG	0,6625ug		
DMEM	375ul		

Solution A and solution B were incubated at room temperature for 5 minutes, and then mixed together and incubated at room temperature for 20 minutes.

The medium to be transfected with 293T was discarded, and the above transfection medium was added. After 4-6 hours, it was changed to complete medium (containing serum and double antibody) for culture.

c. 24 hours after the solution change, the medium containing virus particles was aspirated with a disposable needle tube, and fresh complete medium was added to continue the culture. The above-mentioned culture solution containing virus particles is filtered with a 0.45 uM filter, and the filtered virus solution can immediately infect the target cells, or it can be frozen in a refrigerator at-80 ℃ for later use.

d. Infection of target cells: The cells to be infected are laid in a 6-well plate the day before infection, and the next day when the cell density reaches 50%-60% can be infected (the cells should not be too dense or too thin, otherwise it will affect the infection efficiency). The virus solution and the culture medium were mixed at a ratio of 1:1, 1xpolybrene was added (the storage solution was 0.4 mg/ml, 100x) and incubated at room temperature for 5 minutes after mixing. The mixed solution was added to the target cells.

e. 24 hours after infection, start screening with medium containing puromycin and collecting cellular RNA or protein after 2 days of screening.

2.3.6. MTT Cell Proliferation Experiment

(1) Cells from different treatments were counted, with 1×10^4 cells added to each well (48-well plate).

(2) Cells were incubated in 5% CO_2 at 37°C for 3 to 5 days, or until there was a significant difference in cell growth between the treatment group and the control group.

(3) Preparing MTT reagent: The MTT powder was prepared with complete medium to a final concentration of 5 mg/ml. After use, the medium in the cells was aspirated, washed once with PBS, and then 200μl of the prepared MTT reagent was added to each well.

(4) Incubate at 37°C with 5% CO2 for 4 hours, or black precipitate visible to the naked eye can appear.

(5) Aspirate the medium containing MTT reagent, add

100μl DMSO, shake and lyse the cells on a shaker for 10 minutes, fully dissolve the black crystals, read the absorbance value of each well at the wavelength of 595nm in the microplate reader, and draw the cell proliferation curve.

3. Experimental Results

Studies have found that the expression of CD166 is up-regulated in liver tissues and cells with anti-apoptotic effects. In this study, the experimental results showed the expression of CD166 in liver tissues and cells and its effect on cell behavior. Immunohistochemical analysis revealed a significant up-regulation of CD166 expression in liver tissues compared with adjacent normal liver tissues (Figure 3). This finding highlights the potential role of CD166 in the development of liver cancer.

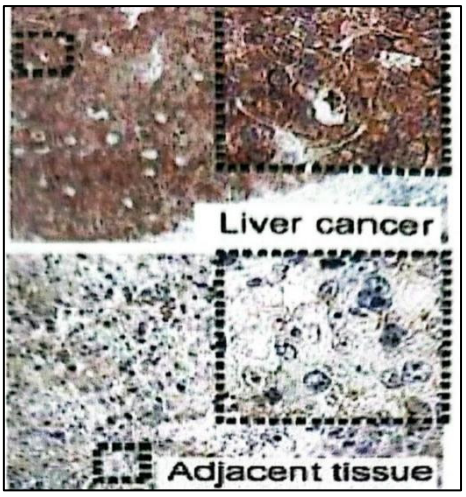

Figure 3. The Expression of CD166 in HCC Tissues and Adjacent Normal Liver Tissues

The Western Blot technology further verified the high expression of CD166 protein in HCC cell lines. The CD166 protein levels in 7402, 7721 and HepG2 were significantly higher than those in normal hepatocytes (Figure 4), which may be closely related to the biological characteristics of HCC cells.

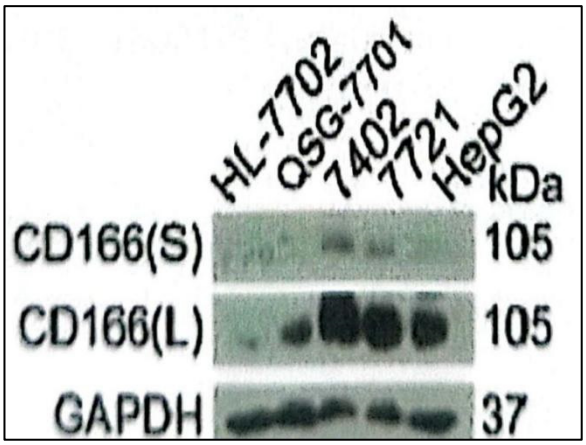

Figure 4. The Expression of CD166 in HCC Cell Lines and Normal Hepatic Cell Lines

In further experiments, after knocking down CD166 by RNA interference technology, it was observed that the proliferation ability of HCC cells was inhibited, and the number of apoptotic cells increased. Specifically, after knockdown of CD166 in the 7402-cell line, the number of cells was significantly reduced, the morphology changed, and the cells became unhealthy (Figure 5).

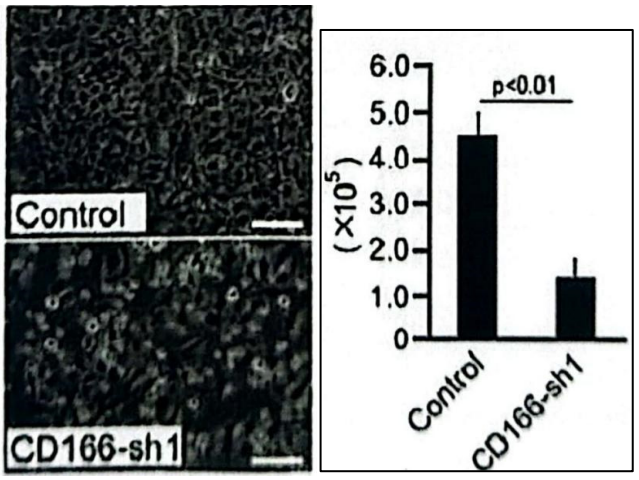

Figure 5. 7402 Cells with CD166 Knocked Down Became Unhealthy with Decreased Cell Numbers

The expression level of the anti-apoptotic protein Bcl2 was downregulated (Figure 6), whereas upregulation of Cleaved Caspase 3 indicated increased apoptosis (Figure 7). These results indicate that CD166 is key to regulating the growth and survival of hepatocellular carcinoma cells, which provides experimental evidence for CD166 as a potential molecular target for hepatocellular carcinoma therapy. Through these experiments, not only the expression pattern of CD166 in hepatocellular carcinoma was verified, but also its functional role in the development of hepatocellular carcinoma was preliminarily explored, laying a foundation for an in-depth understanding of the molecular mechanism of hepatocellular carcinoma and the development of new therapeutic methods.

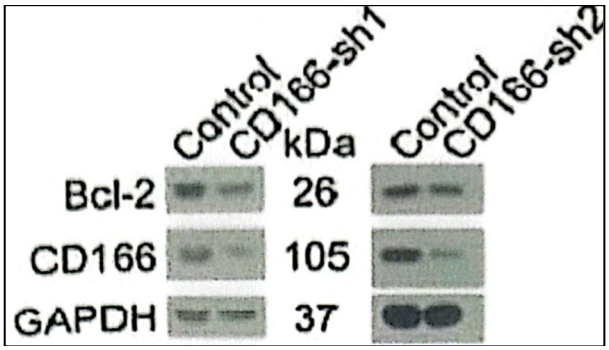

Figure 6. The Expression of Bcl2 in 7402 Cells with CD166 Knocked Down Compared with Control Cell

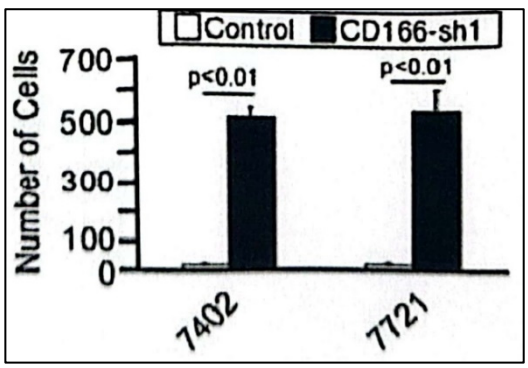

Figure 7. CD166 Knockdown Induced Apoptosis as Measured by 7402, 7721

4. Conclusion

This study profoundly explored the role of cell surface molecule CD166 in liver cancer and its regulatory mechanism. It was found that the expression of CD166 in HCC tissues was much higher than that in adjacent normal tissues, and its high expression was closely related to the proliferation and anti-apoptosis ability of HCC cells. By knocking down CD166 by RNA interference technology, it was observed that the proliferation of HCC cells was inhibited and the apoptosis increased, which revealed the positive regulatory effect of CD166 on the growth and survival of HCC cells. These findings provide strong evidence for CD166 as a potential molecular target for early diagnosis and treatment of liver cancer with important implications for developing new therapeutic strategies for liver cancer.

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