CRISPR/cas9 mRNA Therapy gene mutation SRD5A3 in Hepato-cellular carcinoma

Sengthong Mounphoumi[1], Caoke[2],Phonesouda Souphamith[3], Viengsavanh Khammounheuang[4], Douangphrachan Sathathone[5], Phetsamay Phommaseng[6]

[1]Ministry of Health, Mitaphab Hospital, National Cancer Center, Vientiane Capital, Lao P. D. R,
[2]Central South of University, China, Third Xiangya Hospital (Xiangya Campus), Oncology,
[3]Department of Computer Science, Faculty of Natural Sciences, National University of Laos Vientiane Capital, Lao P. D. R

Abstract

A primary hepatocellular carcinoma (HCC, liver cancer for short) is a common malignant tumor that seriously threatens human life and health. It is the sixth most common malignant tumor in humans and ranks third in the global cause of cancer-related deaths. However, the relationship between the expression of SRD5A3 and platinum-based chemotherapy resistance of liver cancer remains to be further explored. Whether the inhibition of centrosome-associated protein SRD5A3 kinase gene by small interfering RNA can inhibit the occurrence and development of hepatocellular carcinoma, the effect of SRD5A3 on the biological behavior of liver cancer cells has not been reported at home and abroad. If the changes in SRD5A3 can be used to guide the diagnosis and treatment of hepatocellular carcinoma and evaluate the prognosis of patients, the related research of SRD5A3 will play a major role in the diagnosis and treatment of hepatocellular carcinoma. These researches use CRISPR/Cas9 (Clustered Regularly Inter Spaced Short Palindromic Repeat-CRISPR associated Cas protein) technology to construct a stable liver cancer cell line with Cas9 protein, analyze the expression difference of SRD5A3 gene in liver cancer and corresponding adjacent tissues, and explore the relationship between its expression and the clinical prognosis of liver cancer, and further study the expression of SRD5A3 gene through cell experiments effects on the proliferation, cycle and invasion of liver cancer cells.

Keywords: CRISPR/Cas9, Liver cancer, proliferation, cycle, invasion.

I. INTRODUCTION

The primary hepatocellular carcinoma (HCC, liver cancer for short) is a common malignant tumor that seriously threatens human life and health. It is the sixth most common malignant tumor in humans and ranks third in the global cause of cancer-related deaths. In gastric cancer and esophageal cancer [1,2,3,7,8].In the United States, there are than 800,000 newly diagnosed cases each year, and more than 250,000 liver cancer patients die. Liver cancer is an increasingly serious global public health problem [4,5,6,8]. Although great progress has been made in the diagnosis and treatment of liver cancer in recent years, the prognosis of liver cancer patients is still poor [7,8]. One of the important reasons for the high mortality and poor prognosis of liver cancer is that the molecular mechanism of the occurrence and development of liver cancer is still poorly understood [9]. However, with the development of modern cell molecular biology, it has been proved that the formation of liver cancer is mostly accompanied by a series of abnormalities in molecular and signaling pathways [10]. Therefore, exploring the mechanism of the occurrence and development of liver cancer at the molecular level and using characteristic molecules as anti-tumor targets can open up new ways for clinical prevention and treatment of liver cancer.

Steroid 5a reductase 3 (steroid, 5aredutase3, SRD5A3) is a membrane protein, which is mainly located in the cell microsomal and nucleus. It is a member of the steroid 5a reductase family. It is a microsomal enzyme

and is provided by NADPH. Reduced hydrogen participates in the reduction reaction of steroid compounds. Studies have shown that mutations of 5a reductase at the gene level led to abnormal male external genitalia development, which promotes prostate cancer by regulating androgen metabolism, and is closely related to male spermatogenesis. Because of its influence on steroid hormones Metabolism, which affects the initiation of childbirth in women, and plays a role in the occurrence of polycystic ovary. In addition, SRD5A3 can reduce Polypentenol toPolyterpencalcohol and participate in N. The synthesis of glycosylatedoligosaccharide precursors and changes in SRD5A3 gene levels can cause congenital glycosylation disorders in patients, manifested as metabolic disorders of sugar, fat and protein, and various organ functions are impaired to varying degrees [11]. SRD5A3 is also involved in the occurrence of various malignant biological behaviors of human tumors, and is a key regulator of the occurrence and development of tumors. Immunohistochemicallymethods were used to detect the expression difference of SRD5A3 in 18 cases of being tissues and 26 cases of tumor of tumor tissues. SRD5A3 was found in malignant tumor tissues. The protein level in SRD5A3 is higher than that in benign control tissues. SRD5A3 can be used as biomarker of malignant tumors. [12] Yamana, K et al. clarified that the expression level of SRD5A3 is up-regulated in peripheral tissues and breast cancer cells [17]. SRD5A3 is in metastatic Prostate cancer tissue has a higher expression level. Using small interfering is RNA transient transfection technology to knock down the expression level of SRD5A3 in hormone-resistant Prostatecncer HRPC cells significantly reduce the level of DHT secreted by cancer cells and weaken the prostate. The proliferation of cancer cells makes it a promising target for the treatment of prostate cancer. Platinum-based chemotherapy resistance is a complex process caused by changes in the DNA, mRNA and protein levels of malignant tumor cells. In the course of treatment of ovarian cancer, with the increase in the number of times or doses of platinum drugs, patients gradually appear to be less sensitive to platinum drug as acquired drug resistance. There is a certain correlation between the acquired resistance of chemotherapeutic drugs and the up-regulation of SRD5A3 expressing during the treatment of prostate cancer with chemotherapeutic drugs [13].

The CRISPR/Cas system (Clustered Regularly Inter Spaced Short Palindromic Repeat-CRISPR associated Cas protein) is the antiviral defense system of bacteria. In 2012, scientists Doudna and Charpentier pioneered the discovery that it can cut double stranded DNA in vitro, thus opening a new pattern of gene editing technology. It can be used to effectively cut DNA at sites of interest and target different target sites. It is necessary to design a short guide RNA (gRNA) complementary to the target site sequence. CRISPR/Cas9 technology was quickly applied to mammalian cells [14-17], thus simplifying the process of gene editing. The CRISPR/Cas system is the innate immune system [18-22] in archaea and bacteria. In short, the response of CRISPR/Cas includes three stages: (1) Adaptation stage. The Cas1-Cas2 protein complex (including other subunits in some system) cuts the foreign target DNA into a fragment (called a protospacer) and insert the fragment into the 5’ end of the CRISPS array between repeating sequences, creating new intervals. (2) Expression and processing stage. A CRISPS array and many spacers are transcribed and grown together. The transcript is called the precursor of CRISPRRNA (pre-CRISPRRNA, pre-crRNA), which is then covered by a unique Cas protein complex (in same case, it will also there are additional proteins and RNA molecules involved) processed into mature small CRISPRRNAs (crRNAs). (3) Interference stage. A Cas protein complex (usually a modified complex) that uses crRNAs as a guide to cut the target DNA or RNA. Similar to other defense mechanisms, the CRISPR/Cas system continues to evolve in competition with variable genetic elements, resulting in extremely diverse Cas protein sequences and CRISPR/Cas locus structures [23-28]. The CRISPR/Cas system has obvious functional and evolutionary modular characteristics. The adaptation module is responsible for obtaining the interval and has limited variation in different CRISPR/Cas system. However, the effector module of the CRISPR/Cas system mediates the maturation of crRNAs and recognition and cleavage of the target. It has diversity in genetic composition and locus, which is why the CRISPR/Cas system is divided into two categories.

Usually, the effector complex of the first type system consists of Cas protein subunits in a non-uniform stoichiometry. The typical feature of the second type of system is an effector module, which is a single multi-domain protein. Such a relatively simple effect complex feature makes the second type of CRISPR/Cas system a new generation of gene editing tools [29]. Eukaryotic cells use a precise network of gene and gene regulatory elements to perform a variety of biological functions, such as cell growth and death, organelle

formation and organization, metabolites and microenvironment sensing. Precise manipulation of the genome is necessary to understand complex and dynamic cellular processes. Recent advances in genetic engineering have opened up a revolution in biological research and transformation. Broadly speaking, genetic engineering can include gene editing, epigenetic editing, transcription regulation, and chromosome structure manipulation. The realization of these goals requires a "molecular toolbox" that is easy to construct and easy to deliver into cells to perform the above functions. The CRISPR/Cas9 system is widely used due to its ease of operation and high efficiency. The CRISPR/Cas9 gene editing technology is a "Precision scalpel" for modifying or changing the genome, which can avoid semi-random genome insertion caused by traditional viral vector-mediated gene transfer therapy (for example, nearby proto-oncogenes are activated ectopically [30], [31], Genotoxicity caused by knockout of tumor suppressor genes or interference of normal splicing). In addition, genes introduced or corrected by CRISPR/Cas9 technology are regulated by endogenous promoters, so that gene expression is more in line with the physiological environment101. It has been reported that the use of CRISPR/Cas9 technology to change the splicing method of the mutant dystrophin gene, or directly repair then mutation of the dystrophin gene, so as to repair the Duchenne muscular dystrophy mouse model. In addition, diseases caused by dominant-negative mutations cannot be treated by gene introduction, but gene editing technology should be used for gene repair. CRISPR/Cas9 technology can also be used for the editing of germ cell lines, that is gene editing of germ cells, gametes, fertilized eggs or embryos, and the resulting new individuals can pass the edited to the offspring.

However, the relationship between the expression of SRD5A3 and platinum-based chemotherapy resistance of liver cancer remains to be further explored. Whether the inhibition of centrosome-associated protein SRD5A3 kinase gene by small interfering RAN can inhibit the occurrence and development of hepatocellular carcinoma, the effect of SRD5A3 on the biological behavior cancer cells has not been reported at home and abroad. If the changes in SRD5A3 can be used to guide the diagnosis and treatment of hepatocellular carcinoma and evaluate the prognosis of patients, the related research of SRD5A3 will play a major role in the diagnosis and treatment of hepatocellular carcinoma. In this project these researches use CRISPR/Cas9 technology to construct a stable liver cancer cell line with Cas9 protein, analyze the expression difference of SRD5A3 gene in liver cancer and corresponding adjacent tissues, and explore the relationship between its expression and the clinical prognosis of liver cancer, and further study the expression of SRD5A3 gene through cell experiments effects on the proliferation, cycle and invasion of liver cancer cells.

II. MATERIALS AND METHODS

The CRISPR/Cas system, Clusters Regularly Interspaced Short Palindromic Repeats/CRISPR associated, clusters of regularly spaced short palindromic repeats, is an immune defense system found in bacteria and arches that can be used to resist foreign DNA invasion.Compared with zinc finger nuclease technology(ZFNs) and transcriptional activator-like effector nucleases (TALENS), the CRISPR/Cas9 system has the advantages of simplicity, efficiency, and precision cutting. It is widely used in scientific experiments. The system passes crRNA the dimer formed with tracrRNA guides the Cas9 protein to recognize the target gene to achieve the purpose of precise cutting. This topic usesCRISPR/Cas9, the most popular gene editing technology with the highest precision and cutting efficiency, to knock out the SRD5A3 gene of liver tumor cells, so that it cannot form a heterodimer with SRD5A3 and play a role, so as to study the SRD5A3 gene. How does it affect the invasion and migration of liver tumor cells?It provides new ideas for the diagnosis and targeted therapy of liver tumors.

Immunohistochemistry experiment

Embedding tissue and sectioning: Place the liver cancer and adjacent tissues to be embedded in liquid paraffin, arrange them neatly and then freeze them, and the paraffin will become solid. Note that the tissue should be fully immersed in paraffin. Take out the embedded tissue and slice it on a paraffin microtome. Sections should be deparaffinized and hydrated: Paraffin sections should be treated to water: xylene I, xylene II for 20 minutes each, then treated with absolute ethanol I and absolute ethanol II for 10 minutes each, and then used Different concentrations of alcohol are processed in an orderly manner, the alcohol concentrations are respectively: 95%, 90%, 80%, 70%, and each is processed in order for 5 minutes. After the above

treatment is over, the final cleaning is done with distilled water. Antigen retrieval steps: According to the operation instructions of the retrieval solution, transfer an appropriate amount of EDTA antigen retrieval solution to the retrieval box. The sections of the tissue after the above treatment should be placed in the antigen retrieval solution and heated in a microwave oven for antigen retrieval. First, apply medium heat until the repair solution boils, then cut off the power and stop the fire for 10 minutes, and continue to heat the repair solution with a medium-low heat until the repair solution boils. During this process, do not over-evaporate the repair solution to dry slices. After the slices are naturally cooled, wash the slices with PBS solution 3 times, 5min/time. Note that they should be placed on a horizontal shaker and shaken to wash them evenly. Peroxidase blocking process: After the above treatment, the slices should be incubated in H2O2 (concentration 3%) solution for 20 minutes, and should be placed at room temperature and protected from light. After that, under shaking on a rocking table, the slices were washed 3 times with PBS solution, 5 min/time. Add the primary antibody: Dilute the primary antibody in a certain proportion, and take an appropriate amount of diluted primary antibody reagent and drop it on the sliced tissue. At this time, to prevent the antibody from flowing out of the tissue, put a histochemical pencil on the periphery of the sliced tissue, so that the antibody can evenly cover the sliced tissue. After that, the sections can be placed in the antibody incubator and incubated overnight 4℃. Incubation of the secondary antibody: The sections incubated overnight with the primary antibody were washed 3 times in PBS solution, 5 min/time. After drying the section with water, add the secondary antibody to cover the section tissue in the same way as primary antibody, and incubate the secondary antibody at room temperature for about 50 minutes. DAB reagent for color development: After the incubation time of the secondary antibody is reached, the sections are placed in PBS buffer and washed 3 times, 5min/time. After drying the section with water, add DAB reagent in the same way as the primary antibody for color reaction. At this time, pay attention to the real-time observation under the microscope, the section color development, but the positive reaction of the section (brown-yellow) is obvious, so it should be stopped in time Color rendering. And record the time of this color development. Nuclear staining: Hematoxylin is used for nuclear staining. At this time, the nuclear counter staining should also be observed under the microscope in real time. Generally, the counter staining is about 3 minutes. Rinse the dye solution with tap water, and rinse it with hydrochloric acid and alcohol differentiation treatment. The ammonia water is used to turn blue to rinse off the above dye solution. Re-dehydration: Treat different concentrations of alcohol in an orderly manner. The alcohol concentrations are respectively: 70%, 80%, 90%, 95%, each in order for 5 minutes; then use anhydrous ethanol I and anhydrous ethanol II for each Treat it for 5 minutes, and finally treat it with xylene I and xylene II for 5 minutes, and then dehydrate the slices until they are transparent. Mounting treatment: Take out the slices and let them dry naturally, add a drop of neutral gum, cover the slides, and mount the slides. Take care to prevent air bubbles from being generated when covering the film.

Judgment method of immunohisto chemistry results

Immunohistochemical reaction score (IRS) = staining intensity x percentage of positive cells staining intensity is divided into 4 levels: 0 (no positive cells), l (weak positive), 2 (medium positive), 3 (strong positive). The percentage of positive cells is divided into 5 levels: 0 (none), 1 (<10%), 2 (10%-50%), 3 (50%-80%), 4 (>80%). IRS is divided into four levels: negative 0 points; weakly positive ≤ 3 points; moderately positive 3-6 points; strong positive ≥ 6 points. Among them, the score<6 is the SRD5A3 low expression group (Low), and the score ≥6 is the SRD5A3 high expression group (High); the immunohisto chemistry results are read and scored by two or more professional pathologists.

Western blot analysis (Western blot)

Extract protein from liver cancer and adjacent tissue samples: First weigh an appropriate amount (10-100mg) of liver cancer and adjacent tissues, rinse with PBS buffer solution 3 times, use clean scissors to cut small tissue pieces, and put them into a clean glass homogenizer. Add 0.5-1mL of lysis buffer to the homogenizer, homogenize the tissue to crush. Pipette 0.5mL tissue lysate to 1.5mL centrifuge tube, take 1mL of protein extraction reagent, mix well, and let stand. Centrifuge at 4℃ at 10,000 rpm for 10 minutes. The liquid in the centrifuge tube isdivided into upper and lower layers, but the membrane is located between the two layers.

Pipette the upper and lower layers of liquid into a new centrifuge tube, taking care to avoid the middle membrane. Then add 0.2mL 3% SDS solution to the centrifuge tube, boil until the protein is completely dissolved in the SDS solution. In order to avoid repeated freezing and thawing, the protein solubilization solution can be grouped into EP tubes and stored at -20℃. Determination of protein concentration by BCA method: Read the instructions of the BCA protein quantification kit carefully, and according to its operating instructions, prepare the appropriate amount of BCA assay solution with reagents A and B in the specified ratio (49:1). To dissolve protein standards on ice, you can take 10μL of protein standards and dilute to 100μL, or make the final concentration of protein standard working solution 0.5mg/mL. Note that the protein sample solution to be tested should be the same as the standard dilution solution. Add protein standard working solution according to 0,1,2,4,8,12,16 into 96-well plate, and add ddH2O to make up to 20uL; add 200μLBCA working solution to each well in each tube, and let stand at room temperature for 3-5 minutes Use a microplate reader to measure the absorbance value of 562μm, and calculate the protein concentration of the sample according to the standard curve drawn in the standard well.

Table 1: BCA protein Quantification: Standard Curve Setup

Project	1	2	3	4	5	6
BCA working fluid	200μL	200μL	200μL	200μL	200μL	200μL
Standard protein	0μL	1μL	2μL	4μL	8μL	12μL
ddH_2O	20μL	19μL	18μL	16μL	12μL	8μL

SDS-PAGE gel electrophoresis

With glue: Clean the glass plate. Hold the glass plate to be cleaned tightly with both hands, gently scrub the front and back sides of the glass plate with detergent or soapy water, then rinse the glass plate repeatedly with clean water, and finally rinse the glass plate with distilled water, and place it in a ventilated place to dry naturally. Install the glass plate carefully. Gently put two clean and dry 1mm glass plates into the glass plate clamp. Pay attention to the alignment of the two sides of the glass plate. The glass plate can be clamped evenly on one or both sides at the same time, but attention should be paid to prevent the glass plate from leaking. Equipped with separating glue. According to the molecular size of the target gene, reasonable preparation of separation gel and concentrated gel, in order to better separate the target gene from the internal control, choose 10% separation gel, specific configuration materials and reagents see the main reagent SDSPAGE gel configuration content. This operation and the following glue dispensing process should be carried out in a fume hood to avoid toxicity. Pouring separation glue. Use a 1mL pipette to absorb the above-mentioned proportioned separation glue, and slowly inject it along the gap between the two glass plates. Pay attention to the uniform application force and speed to avoid bubbles in the separation glue. Wait until the level of the separation glue rises to When a certain height is reached, the injection of separation glue should be stopped. Sealing and separating glue. After stopping the injection of the separating glue, immediately continue to use the pipette to slowly inject an appropriate amount of absolute ethanol to seal the glue. It should be noted that when the sealing glue is added with absolute ethanol, the action should be gentle to avoid deformation of the separating glue level. After sealing, pay attention to observe whether there is any leakage, and try to avoid moving. With concentrated glue. Observe whether the dividing line between the separating glue and absolute ethanol is formed. Generally, the dividing line between the two can be seen for about 30 minutes at room temperature, which indicates that the separating glue has solidified. Gently pour the upper layer of absolute ethanol, while the remaining liquid can be sucked up between the two plates with filter paper. At the same time, configure the 5% concentrated gel according to the SDS-PAGE gel configuration of the above main reagents. Filling the concentrated glue, the specific operation is the same as filling the separating glue. Immediately after filling the glue, insert the comb with both hands smoothly to ensure that the lower end of the comb enters the concentrated glue horizontally. After the concentrated glue has solidified, the glass plate can be taken out and set aside.

Electrophoresis and transfer membrane: Add samples. Prepare the protein sample to be tested and the protein Marker. The protein sample can be boiled in a water bath for 5 minutes and centrifuged at 12000g for 10 minutes to fully denature it. Then use a pipette of appropriate size to add the protein sample and the protein Marker to the gel according to the purpose of the experiment. Pay attention to the gentle and even movements to prevent the pipette tip from damaging the concentrated gel. Electrophoresis. Pour an appropriate amount of 1*electrophoresis solution into the electrophoresis tank, and connect the electrophoresis tank and the electrophoresis instrument. Turn on the power supply and adjust the voltage of the electrophoresis apparatus. When the sample and the marker are in the concentrated gel, the electrophoresis can be performed at a constant voltage of 80V for about 30 minutes. When the sample and the marker are in the separation gel, or after the marker is separated, the constant voltage should be adjusted to 120V and run until the target protein is completely separated. Turn off the power after the end. Unloading and cutting glue. After the electrophoresis is finished and the power is turned off, take out the electrophoresis holder, carefully and gently remove the glass plate, and gently pry the glass plate from a corner between the two glass plates with a rubber cutting plate. Cut off the gel bands of the internal control and the target gene and mark them for easy distinction. Cut the film. Compare the size of the gel band, cut the PVDF membrane of the corresponding size with clean scissors, and soak the cut PVDF membrane in a 10% methanol solution for several minutes until the membrane is completely wetted and turned. Preparation before transfer membrane. Place the items needed for the transfer film, such as transfer film clips, filter paper, sponge gaskets, etc., into the bend tray with 1*transfer film solution, and stack them in the order of sandwiches. Transfer the above cut gel to the filter paper of the transfer clip, and cover the PVDF membrane on it, and then close the transfer membrane in the order of the transfer clip, sponge, filter paper, glue, PVDF membrane, filter paper, sponge, and white surface folder. Note that the operation should be gentle to prevent the gel and PVDF membrane from rupturing or breaking. At the same time, closely observe whether there are bubbles on the layer between the membrane and the glue. If there are bubbles, roll them out in time. Transfer filmConnect the film transfer equipment, pour an appropriate amount of 1*film transfer liquid into the film transfer tank, and place an appropriate size ice bag in the film transfer tank to ensure that the film transfer is performed at a low temperature. The placement of the film transfer clamp should be careful not to reverse the current direction, so the black surface of the film transfer clamp can correspond to the black surface of the film transfer tank. Adjust the constant current according to the transfer experience and exploration conditions, and set the transfer time according to the size of the target protein molecule. Therefore, set the constant current and time of film transfer to 260mA and 100min respectively.

Western Blot method to detect protein expression: Transfer the protein to the PVDF membrane; First immerse the SDS-PAGE gel in the transfer buffer. Put the PVDF membrane in methanol for polarization for 1 min, then place it in ddH2O for depolarization for 1 min, and then put it in the transfer solution for 10 min. Assemble the electro-transfer membrane clamp: open the electro-transfer membrane clamp, pad the special Scotch-Brite pad and filter paper soaked with the transfer solution, place the gel flat on the filter paper on the cathode side, and place the balanced PVDF membrane flat on the gel. Insert the assembled electro-transfer film clamp into the transfer tank, put the transfer tank into the electro-transfer instrument according to the correct polarity direction, and add the transfer liquid to connect to the power supply. Under the condition of cooling in a mixture of ice and water, 300mA electric transfer for 100 minutes, and the specific current and time can be adjusted according to the molecular weight. After the film transfer is finished, turn off the power, cool at room temperature, and take out the transfer film. Second Detection of protein. Configure a blocking solution containing PBST (containing 5% skimmed milk powder), seal the transfer membrane, and seal at room temperature for 2 hours. Dilute the blocking solution, incubate the primary antibody, and incubate overnight in a refrigerator at 4℃. The primary antibody HES5 was diluted at a ratio of 1:500, and the dilution ratio of GAPDH was 1:3000. On the second day, after taking out the transfer film, rewarm it at room temperature for 30 minutes, and wash the film 3 times with PBST for 15 minutes each time. Incubate the transfer membrane with the HRP-conjugated secondary antibody at a dilution ratio of 1:10000, and incubate for 2 hours at room temperature in the dark. Wash the membrane with PBST washing solution, once every 15min, 3 times in total. Odyssey imager performs scanning and imaging, machine development, and scanning or photographing. Compare the gray level of the target protein with the gray level of the internal reference protein, and the obtained ratio is the relative expression of the target protein.

Immunohisto chemical detection

Baked slices the required liver cancer tumor tissue is fixed by 4% formalin, dehydrated, transparent and waxed with LEICA automatic dehydrator, embedded into wax blocks, sliced, and cut into slices with a thickness of 4~5um, the section should be flat, try to avoid tissue scratches caused by the knife gap, so as not to affect the morphology of the tissue cells, and non-specific staining occurs. The slides have been treated in advance to prevent stripping: soak in detergent for 8~24 hours, rinse overnight in running water, rinse twice in distilled water, then soak in 95% alcohol overnight, then wash twice in distilled water, and finally the glass slides are arranged neatly and dried, and are treated with POLY-L anti-dropping sheets. Bake the paraffin slices in a 60°C constant temperature electric drying oven for 6 to 8 hours.

Dewaxing and hydration before dewaxing again, place it in a 60°C constant temperature electric drying oven for 60~90 minutes, and start dewaxing after the paraffin is melted. Place the xylene in an electric heating constant temperature drying oven at 60°C in advance for 15 minutes, then place the paraffin sections in No. 1 xylene for 15 minutes. While dewaxing, preheat the No. 2 xylene for 15 minutes. The paraffin sections in toluene were quickly taken out, placed in preheated No. 2 xylene, 15 minutes, after taking out, the gradient alcohol hydration was performed, and the dewaxing was required to be complete. The sequence is: 10 minutes in absolute ethanol *2 times→95% ethanol for 5 minutes *2 times→80% ethanol for 5 minutes→70% ethanol for 5 minutes → rinse with tap water→ddH2O for 5 minutes→0.01M histochemistry with PBS 3 minutes *3 times. The paraffin sections were then placed in citrate buffer. Antigen retrieval: Boil water in a pressure cooker in advance, completely immerse the paraffin slices in citrate buffer, put it in the pressure cooker, heat for 3 minutes, take out the specimen, and place it in an ice-water mixture to cool to room temperature naturally. Wash thoroughly with PBS for histochemistry. Closed. Prepare the skimmed milk sealing solution, take out the PVDF membrane after the mold transfer, and transfer it to the petri dish of the milk sealing solution. Note that the PVDF membrane should be completely immersed in the milk sealing solution. When there are more membranes, try not to overlap them. Shake slowly on a horizontal shaker at room temperature and close for 2 hours. Incubate the primary antibody. After the blocking time is up, remove the PVDF membrane in the blocking solution, and use 1*TBST to wash the membrane for 5 minutes. At the same time, make a primary antibody incubation bag, and dilute the primary antibody according to the recommended dilution concentration of the primary antibody or the adjusted dilution concentration. Put the washed PVDF membrane into the primary antibody incubation bag for incubation. Care should be taken to immerse all the PVDF membrane in the primary antibody diluent while expelling the air from the membrane and incubate overnight in a refrigerator at 4°C. Wash the membrane. Take out the PVDF membrane from the primary antibody diluent and place it in a petri dish containing 1*TBST solution while recovering the primary antibody diluent. Wash the membrane 3 times on a shaker, each time about 10 minutes. Incubate the secondary antibody. Dilute the secondary antibody with a milk powder solution, and then completely immerse the washed PVDF membrane in the diluted secondary antibody solution. Take care not to incubate the secondary antibody by mistake. The incubation time is too long or too short, and incubate at room temperature on a slow horizontal shaker. About 2h, the incubation time can also be adjusted according to the experimental results. After the incubation, wash the membrane with 1*TBST solution 3 times, about 10 minutes each time. Note that the 1*TBST solution should be replaced each time.

Chemiluminescence detection, development and fixing

Prepare before entering the darkroom. Prepare the chemiluminescent solution, developer solution, fixing solution, pipette, etc., and at the same time take out the PVDF membrane after washing with the secondary antibody, and transfer it to the press holder with clean plastic wrap. Pay attention to the PVDF membrane surface with protein surface Upward, the luminescence reaction is carried out. Add chemiluminescence reagents dropwise. According to the instructions of the luminescence kit, add reagents A and B to the EP tube at a ratio of 1:1, pipette and mix them evenly; open the tablet holder with the PVDF membrane, and mix the chemiluminescence reagents with a pipette The liquid drops uniformly on the membrane, so that the target protein on the PVDF membrane can be fully combined with the luminescent reagent. Turn off the infrared light and observe whether there is protein green fluorescence on the membrane. According to the intensity of the fluorescence, the compression time can be roughly estimated. This operation and the following operations

should be carried out in a dark room. Cover X film. After dropping the luminescent reagent, cover the upper layer of fresh-keeping film, and cut out the appropriate size X film according to the size of the PVDF film, and mark it. Cover the film carefully on top of the PVDF film, pay attention to the simple and fast operation, after placing it, close the press clamp. Perform compression, and the compression time is estimated based on the intensity of fluorescence. Generally, it can be compressed for about 1-5 minutes and then exposed. Exposure. After pressing the film, take out the X film and put it in the developing solution for development. Observe whether there are black protein bands on the film during development, and observe the thickness and density of the protein bands. Take out the X film for fixing in time. After the operation is over, the X film becomes transparent and taken out, washed with water, dried, and analyzed the experimental results.

Judgment criteria for immunohisto chemistry results

Observation of pathological slices All slices were observed and judged by two deputy directors and above in the pathology department. All results were independently observed and judged under the condition that the pathologist did not understand the patient's clinical and prognosis. For cases with inconsistent judgments, observe together to achieve a consistent result. a. Judgment criteria of pathological type: According to 2015WHO lung cancer histopathological classification, it is mainly divided into: squamous cell carcinoma, adenocarcinoma and adenosquamous carcinoma. Adenocarcinoma: The cancer tissue has the characteristics of adenoid differentiation such as mature tubular, alveolar, columnar cell lined papillary structure, or there is mucus secreted by cancer cells. Squamous cell carcinoma: The cancer tissue has squamous epithelial differentiation characteristics such as keratinization and the existence of intercellular bridges. Keratinization can be the formation of keratinized beads in the cancer nest. b. Judgment criteria for the degree of differentiation: highly differentiated, moderately differentiated and poorly differentiated. Adenocarcinoma: well-differentiated: cancer tissue has the characteristics of adenoid differentiation; moderately differentiated: the degree of differentiation of cancer tissue is between well-differentiated and poorly differentiated; poorly differentiated: well-differentiated characteristics are not obvious, more realistic areas, visible cells Internal mucus. c. Immunohistochemical results judgment criteria: Observe the results under a microscope, observe 5 high-power fields of each tissue, and count 500-1000 cells for statistics. HES5 is positive for yellow particles in the nucleoplasm. The evaluation of the staining positive rate of HES5 uses the staining intensity of the positive cells and the positive rate of the cells to be comprehensively judged (immunohistochemical semi-quantitative scoring method). Positive cell rate score: 1 point: <50%; 2 points: 50-75%; 3 points: >75%. Staining intensity score of positive cells: 0 points: no staining or weak staining; 1 point: moderate staining; 2 points: strong staining. Immune response score (IS) = positive cell rate score *staining intensity score. IS>3 is the high expression group, IS≤3 is the low expression group. Ki67 judges the expression level by the positive rate, where the positive rate>50% is high expression, and ≤50% is low expression.

Statistical analysis

Data analysis was carried out using SPSS17.0 statistical software, and the statistical results were all expressed in the form of mean±standard deviation. Paired t test was used to compare the expression data of SRD5A3 in liver cancer and adjacent tissues. The correlation between SRD5A3 expression and clinical data was tested by $\chi 2$ (chi-square) test. When $P<0.05$, the difference between the two groups can be considered to be statistically significant.

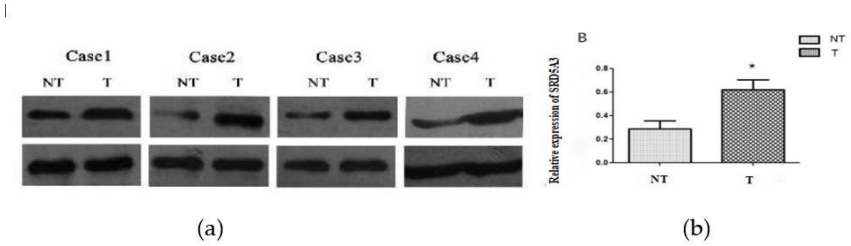
III. RESULTS

Western Blot detection results in the detected liver cancer tissues and corresponding adjacent liver tissues, the expression level of SRD5A3 protein in liver cancer tissues was significantly higher than that in corresponding adjacent tissues, and the expression of SRD5A3 protein in adjacent tissues was higher than that in cancer tissues. Immunohistochemical test results: In 89 cases of liver cancer tissue, the high expression rate of SRD5A3 was 60.7% (54/89), the high expression rate in adjacent tissues was 39.3% (35/89), and the high expression rate of SRD5A3 in liver cancer tissue was significantly higher. It has a high expression rate in adjacent tissues and is statistically significant ($P<0.05$). Analyze the relationship between

the expression of SRD5A3 and various clinical parameters, and found that the expression of SRD5A3 is positively correlated with tumor size and metastasis, and negatively correlated with the degree of differentiation. The results of qRT-PCR and Western Blot indicated that the expression of SRD5A3mRNA and protein in each liver cancer cell line was higher than that of normal liver cells; and the expression of SRD5A3 in the liver cancer cell line M HCCLM3 was higher than that of other liver cancer cell lines. RNA interferes with the expression of SRD5A3 in MHCCLM3 liver cancer cell lines, qRT-PCR and Western Blot detect the interference effect of SRD5A3 at the mRNA and protein expression levels, and screen out the best RNA interference series. CCK8 detection, flow cytometry technology, Trans well method detection found that reducing the expression of SRD5A3 gene, the proliferation, cycle and invasion of liver cancer cells were significantly inhibited.

The expression of SRD5A3 in liver cancer

Western Blot was used to detect the expression of SRD5A3 at the protein level in 40 cases of liver cancer tissues and corresponding adjacent liver tissues (Figure 1a). The results showed that the expression of SRD5A3 protein in 29 cases (72.5%) of cancer tissues was significantly higher than that in the corresponding adjacent tissues, and the expression of SRD5A3 protein in 11 cases (27.5%) of adjacent tissues was higher than that in cancer tissues. Analyze the gray value of SRD5A3 protein and the internal reference β-actin, and after standardizing the results of the internal control, the statistical results of liver cancer tissues and adjacent tissues are obtained, and the paired t-test statistical analysis is used to find that the expression of SRD5A3 in liver cancer tissues is higher than the corresponding adjacent tissues. Tissue expression was significantly increased ($P<0.05$) show in Figure 1b.



(a) (b)

Figure 1 Western Blot detects the expression of SRD5A3 in liver cancer tissues and corresponding adjacent

The expression of SRD5A3 in liver cancer tissues and corresponding adjacent liver tissues was detected by immunohistochemistry. Immunohistochemistry detected 89 cases of liver cancer and adjacent tissues. The SRD5A3 protein positive reaction material was brown-yellow particles. The SRD5A3 protein positive signal was mainly located in the cytoplasm, most of which were diffusely distributed in the whole plasma, and a few were distributed in the nucleus or around the nucleus. See the materials and methods section for the scoring criteria. According to the staining intensity and the number of positive cells, 60.67% (54/89 cases) were highly expressed in liver cancer tissues and 39.3% (35/89 cases) in adjacent tissues, and the high expression rate in liver cancer tissues was significantly higher than that in adjacent tissues The rate is statistically significant ($P<0.05$) show in Figure 2

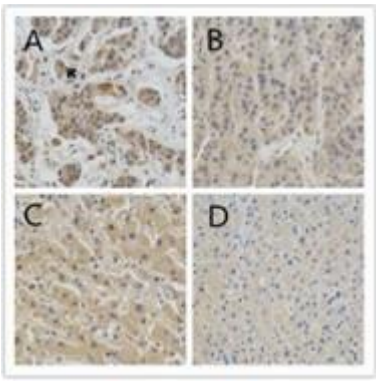


Figure 2 Immunohistochemical method to detect the expression of SRD5A3 in liver cancer and adjacent tissues (╳400). Among them, A is high expression in liver cancer tissue, B is low expression in liver cancer tissue, C is high expression in adjacent tissues, D is low expression in adjacent tissues, and the arrow is SRD5A3 positive staining

The relationship between SRD5A3 gene expression and clinicopathological parameters of liver cancer

Collect 89 89 hepatocellular carcinoma tissue specimens detected by the above immunohistochemistry, and divide them into low expression group (35 cases) and high expression group (54 cases) according to the scoring standard in the method section. The clinical data was collected, combined with relevant clinical data for statistical analysis, and chi-square test was used. The results showed that the expression of SRD5A3 had nothing to do with the patient's gender, age, HBsAg infection, serum AFP, and liver cirrhosis, but tumor size, tumor differentiation and metastasis Correlation (P<0.05) (Table 2).

Table 2 The relationship between the expression of SRD5A3 and the clinical pathological parameters of patients.

Clinic pathological parameters	N	SRD5A3 expression		Title 3
		Low expression	High expression	P value
Number of cases	89	35	54	
Gender				0.508
male	55	20	35	
Female(age)	34	14	20	0.709
<50	30	12	18	
≥50	59	20	39	
HBsAg				0.219
feminine	36	12	24	
positive	53	25	28	
Serum AFP (U/L)				0.654
<400	31	13	18	
≥400	57	31	26	
Liver cirrhosis				0.310
without	40	28	12	
Have	49	25	24	
<5	38	20	18	
>5	51	26	25	
Differentiation				0.040*
Poorly differentiation	21	3	18	
Moderate differentiation	39	19	20	
Well differentiated	29	5	24	
Transfer				0.007*

without	54	26	28
Have	35	6	29

Expression of SRD5A3 gene in liver cancer cell lines

QRT and western Blot methods were used to detect the expression of SRD5A3 in various liver cancer cell lines, so as to determine the differential expression of SRD5A3 in various liver cancer cell lines at the mRNA level and protein level. The results are shown in the figure3. It can be seen from the experimental results that the mRNA and protein expression of SRD5A3 in various liver cancer cell lines are different. The expression of SRD5A3 in each liver cancer cell line is higher than that of normal liver cells, and it is more aggressive in liver cancer cells. Line (LM3) has the highest expression level, as shown in Figure 3.

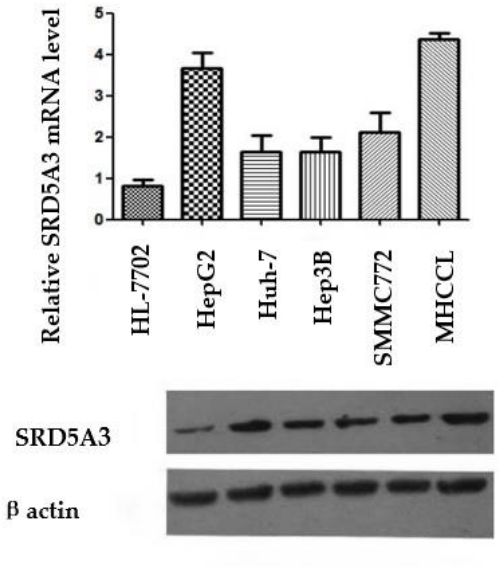
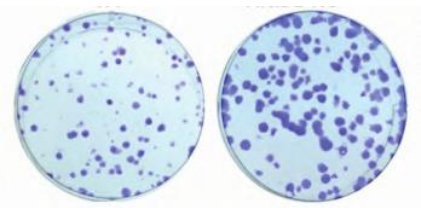


Figure 3 qRT-PCR and Western Blot to detect the expression of SRD5A3 in various liver cancer cell lines

Clone formation results

By culturing the cells, when the cells have grown to 90%, trypsin zing the cells and counting the cells, inoculating them into a 6-well plate, the number of cells inoculated in each well is 200, doing three parallel experiments, using the three results to count (Table 3) cells with strong cloning ability will form more clones in the figure bellow data analysis was performed with SPSS software, using T test, and represented by a histogram shown in the figure below, $P<0.05$ difference it is statistically significant. The results showed that the ability of SK-Hep1 to form clones was stronger than that of knocked out cells ($p<0.05$). this show that knocking out SRD5A3 can affect the ability of SK-Hep1 to form clones.



Ko (a) SRD5A3 (b)

Figure 4 Clone formation results.

Table 3 Statistics of cell colony formation (three parallel experiments)

	Group	Number of counts	Cell count	T value	Title 3
Clone Formation experiment	SRD5A3	3	36.45±4.56	3.17	0.038
	KO	3	20.08±4.09		

Scratch test to detect cell migration

SRD5A3 SRD5A3 was used as the control group, and the SRD5A3 knock-out group was used as the experimental group to perform a scratch experiment, and the scratch area at 0h and 24h was recorded respectively. As shown in the figure below, over time, the migration area of SRD5A3 was significantly higher than the migration area of SRD5A3 knockout cells. After calculation, it was found that the migration rate of SRD5A3 knockout cells was lower than that of SRD5A3 cells at 24h after scratching ($P<0.01$, the result was statistically significant). The results of the scratch experiment indicated that after the SRD5A3 gene was knocked out, the migration ability of the cells was reduced, and it was concluded that the SRD5A3 gene has the effect of promoting the migration of Sk-Hep1 cells.

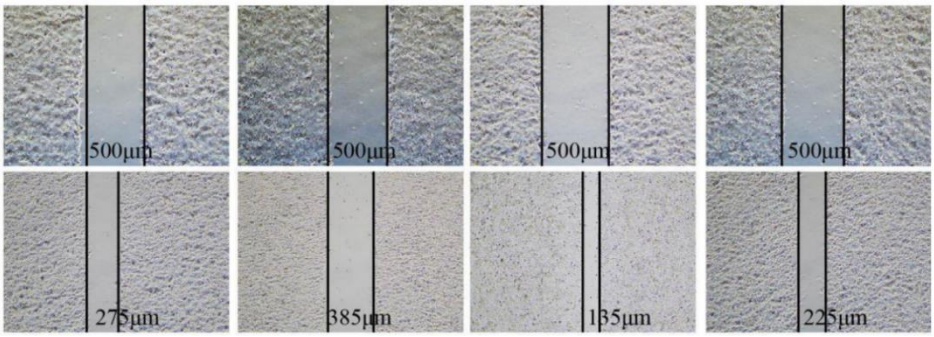


Figure 5 Scratch test to detect cell migration.

The effect of SRD5A3 knockout on the migration and invasion ability of SK-Hep1 cells

Observe the results of the Transwell migration experiment under an inverted microscope with 200-time magnification, and count the number of penetrating cells. The number of cells penetrating the membrane reflects the cell's migration ability. After statistics, it was found that after SRD5A3 gene knockout, the number of SK-Hep1 knockout cells penetrating the membrane was significantly less than that of SRD5A3 gene non-knockout cells, and the result was statistically significant ($P<0.01$). it shows that after the SRD5A3 gene is knocked out, the migration ability of SK-Hep1 cells is inhibited.

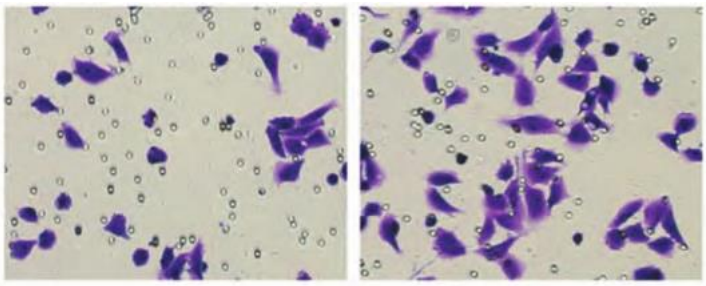


Figure 6 The effect of SRD5A3 knockout on the migration and invasion ability of SK-Hep1 cells.

Table 4 Trans well migration experiment cell penetration number

Group	Cell count	P value
SRD5A3	268.00±5.01	0.000
KO	189.00±5.56	

IV. DISCUSSION

Although the treatment methods and levels of liver cancer have been significantly improved in recent years, surgery is still the main method of treatment of liver cancer, but the high recurrence rate and metastasis rate of liver cancer, the prognosis of liver cancer is still unsatisfactory. Factors closely related to the prognosis of liver cancer mainly include liver cirrhosis, tumor size and local tissue infiltration [32]. A large number of studies hope to find abnormally expressed proteins or factors in liver cancer tissues, which can be used to judge the malignant degree of liver cancer, so as to help judge the prognosis of liver cancer patients [33-35]. For example, alpha-fetoprotein (AFP) is used as a clinical application. Diagnosis of liver cancer and the most effective prognostic marker. The viewpoints of precision medicine and individualized medicine all hope to propose more effective tumor treatment plans for patients. Through basic research, scholars hope to find more effective targeting molecules to intervene and treat the metastasis and recurrence of liver cancer in a timely manner [36]. In recent years, VEGF (vascular endothelial growth factor) has been a research hotspot, and its expression is also closely related to the prognosis of patients, and thus serves as an indicator for judging high-risk factors. High recurrence rate and high metastasis rate have always been the focus and difficulty of the research on the occurrence and development mechanism of liver cancer. By studying the mechanism of its metastasis and recurrence, it may provide some new references and ideas for tumor research. In this study, it was found that the expression level of SRD5A3 was correlated with the cloning ability of the plate. The number of SK-Hep1 cell clones that knocked out the SRD5A3 gene was lower than that of the control group. Through the results of the scratch experiment and the Transwell migration experiment, we can see that the SRD5A3 gene after knockout, the migration ability and penetration ability of liver cancer cells were significantly reduced, and the difference was statistically significant ($P<0.05$). the experimental results further proved from the genetic level that the invasion and migration ability of nasopharyngeal cancer cells will be reduced after the SRD5A3 gene is knocked out.

V. CONCLUSIONS

SRD5A3 gene is highly expressed in liver cancer tissues, and its expression is related to the invasion and malignancy of liver cancer. It plays an important role in the occurrence and development of liver cancer. SRD5A3 gene is highly expressed in liver cancer cell lines. The expression of SRD5A3 can promote the proliferation, cycle and invasion of liver cancer cells. This project detects the expression of SRD5A3 in liver cancer tissues and analyzes the clinical data of patients with liver cancer. It is found that the expression of SRD5A3 is closely related to the occurrence and development of liver cancer. At the same time, through cell biology function experiments, it is confirmed that the expression of SRD5A3 can promote the proliferation, cycle and metastasis of liver cancer cells; it further reveals that the expression of SRD5A3 gene plays an important role in the progression of liver cancer.

At present, we have a preliminary understanding of the characteristics of SRD5A3 and the mechanism involved in the formation of various diseases and tumors. With the advancement of science and technology and people's in-depth exploration of SRD5A3's involvement in tumor occurrence and development, as well as experimental verification in a variety of tumors, we found that SRD5A3 is very likely to become a new target and a new tumor market for tumor molecular targeted therapy. However, there is no relevant research on the relationship between SRD5A3 and lung cancer. We need to explore more deeply, and further analyze and experiment. Verification, strive to achieve early diagnosis and precise treatment of tumors as soon as possible, to more accurately assess survival time for patients, and to benefit patients to a greater extent

VI. ACKNOWLEDGMENT

I sincerely thank you to the anonymous referees, for their guidance and support. Special thanks to the participants and to my colleagues, family, and friends for their encouragement. Your contributions made this work possible.

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