Up-Regulation of VCAN Promotes the Proliferation, Invasion and Migration of HCC and Serves as a Biomarker

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Abstract

Hepatocellular carcinoma(HCC) is the world's most common cause of cancer death. Therefore, more molecular mechanisms need to be clarified to meet the urgent need to develop new detection and treatment strategies. We selected three liver cancer transcriptome database GSE124535, GSE136247, GSE144269, and analyze the overexpressed genes contained in them. The overlapping genes were found by Venn map, and two interacting networks module, were found by Mcode. Module1 is mainly related to mitosis and cell cycle, and module2 is mainly related to EMT, angiogenesis, glycolysis and so on. We found that the seed gene in module2 is VCAN. The purpose of this study is to study the expression characteristics of VCAN gene in HCC, and to explore its role in the occurrence and development of HCC and its possible mechanism. Data from TCGAportal shows that compared with normal tissues, the expression of VCAN is up-regulated in HCC tissues. The patients with high expression of VCAN had shorter distant recurrence-free survival and overall survival. The effects of VCAN expression on cell proliferation, invasion and migration were evaluated in vitro by using gene knockout and overexpression strategies. Multiple possible VCAN interactions have also been identified. These result reveal that the level of VCAN is higher in the subtypes of HCC with higher malignant degree and is connected to the poor prognosis. In addition, the treatment of VCAN with DNA methyltransferase inhibitors and transcription factor inhibitors may improve prognosis of patients with liver cancer.

Introduction

HCC(HCC) is the world's most common cause of cancer death, the fifth most common cancer in the United States, and the only cancer with an increasing annual incidence among the top five fatal cancers(1). The prognosis of HCC is extremely poor, with a 5-year average survival rate of less than 10% (2). In addition, only 5% to 15% of patients with HCC are eligible for surgical resection at the early stage, while most patients with HCC are diagnosed with advanced cancer. Advanced treatment includes transarterial chemoembolization (TACE) and oral sorafenib chemotherapy. However, less than 1/3 of patients benefited from treatment, and drug resistance was evident within six months after the start of treatment(3). Therefore, exploring biomarkers with high specificity and sensitivity or looking for new molecular targets can not only help clinicians to predict the prognosis of patients, but also clarify the potential mechanism of HCC, which has long-term significance.

Versican (VCAN) is a chondroitin sulfate proteoglycan, a major component of extracellular matrix (ECM), which provides hydration and loose matrix in disease progression and critical events(4,5). VCAN refers to a complex molecule that covers the glycosaminoglycan side chain and modular core protein domain, and has a series of synthetic procedures and processes to regulate these elements(6). VCAN can affect the process of cell adhesion, proliferation, migration and angiogenesis, which seriously affects the morphogenesis and maintenance of tissue(7).In addition, VCAN involves many pathological steps, including axonal outcome, central nervous mechanism injury, hair follicle circulation, tendon remodeling and atherosclerotic vascular disease(8,9). However, the detailed function and molecular mechanism of

VCAN in HCC are still unclear. Therefore, in this study, we studied the expression, molecular mechanism and clinical correlation of VCAN in HCC.

Result

VCAN may be related to EMT, angiogenesis and glycolysis.

We selected three liver cancer transcriptome database GSE124535, GSE136247, GSE144269, and used GEO2R to analyze the overexpressed genes contained in them. The overlapping genes were found by Venn map (Figure 1A), and two interacting networks module, were found by Mcode (Figure 1B). Module1 is mainly related to mitosis and cell cycle (Figure 1C-D), and module2 is mainly related to EMT, angiogenesis, glycolysis and so on (Figure 1E-F). We found that the seed gene in module2 is VCAN.

VCAN is over-expressed in HCC

The three database GSE124535, GSE136247, GSE144269 all showed that the expression of VCAN in HCC tissue is higher than that in normal tissue (Figure 2A). Next, we used UALCAN to conduct a more comprehensive analysis of VCAN mRNA expression in HCC. Subgroup analysis based on race, nodal metastasis status and histological subtypes showed significantly higher VCAN mRNA levels in LIHC patients than in healthy people (Figure 2B-D). Analysis of Human Protein Atlas data indicated that VCAN staining is stronger in LIHC tissue than in normal liver tissue (Figure 2E). According to the human protein atlas database, VCAN is located in the vesicles in U-2 OS, U-251 MG and A-431 cells (Figure 2F).

Research results of VCAN in single cell

We studied the expression of VCAN at the single cell level. Stellate is a subtype of fibroblasts. Studies have shown that VCAN is expressed in fibroblasts and has a similar expression pattern to classical fibroblast markers such as COLA1/2, DCN (Figure 3A). Further data indicate that there is a strong correlation between VCAN and FB markers in LIHC (Figure 3B). In addition to fibroblasts, VCAN was also highly expressed in myeloid (Figure 3C). Some correlation between VCAN and myeloid markers in LIHC is exhibited in Figure 3D.

VCAN expression is strongly associated with clinical outcome

The prognostic potential of VCAN in LIHC was further examined using Kaplan-Meier Plotter. Results indicated that patients with higher VCAN expression had higher recurrence rates as well as shorter RFS and OS (Figure 4A-D).

VCAN plays a promoting role in HCC cells in vitro

The results of scratch assay showed that in HCC cell line, the scratch closure rate of inhibiting VCAN was significantly lower than that of the control group (Figure 5A). Compared with the control group in the confluence monolayer Transwell experiment of cultured HCC cell line, si-VCAN inhibited the relative

migration and invasion rate of VCAN (Figure 5B and C). Plate cloning and CCK-8 assay showed that VCAN gene knockout significantly inhibited the proliferation of YY-8103 and LM3 cells compared with the control group (Figure 5D). Overexpression of VCAN has the opposite effect (Figure 6A-D). These results suggest that inhibition of VCAN can delay the proliferation, invasion and migration of HCC in vitro.

Mechanistic prediction of TF, hub genes, miRNA, and circRNA for VCAN in HCC

The transcription factors (TF) that may affect the transcription of VCAN gene

In order to identify the members of the molecular network that may regulate the expression of VCAN, we detected the transcription factor (TF) that may affect the transcription of VCAN gene. First, the 20 most regulated TF in human cancers were identified using Cistrome DB Toolkit (Figure 7A). We reviewed the relevant literature and found that transcription factors such as SOX2, SMAD3, CTNNB1, TP53 have been reported to play an important role in liver cancer. Previous studies have shown that the high expression of SOX2 is associated with metastasis and low survival rate of HCC. Hepatoma cells overexpressing SOX2 are characterized by active epithelial-mesenchymal transition, showing stronger ability of transpire invasion, soft Agar colonization and spheroid formation(10). Hepatoma cells release exosomes containing SMAD family member 3 (SMAD3) protein and mRNA, and transfer them to isolated hepatoma cells to promote their adhesion. These exosomes can induce the enhancement of SMAD3 signal transduction and adhesion ability of the recipient hepatoma cells. In addition, The research also found that there are abundant SMAD3 exosomes in the peripheral blood of patients with hepatocellular carcinoma, and its level is related to the stage of the disease and the expression of Smad3 in the primary tumor(11). In addition to these, in HCC, Wnt/CTNNB1 mutation is a characteristic of immune rejection (cold tumor) and may be a biomarker for predicting drug resistance of immunosuppressants at immune checkpoints(12).

VCAN mRNA expression is positively correlated with DNMT's

Previous studies have shown that DNA methylation plays an important role in HCC(13). In addition, the methylation of CpG island in the promoter region of the gene prevents some TF from binding to DNA, thus inhibiting gene transcription. Therefore, we used MEXPRESS to examine the DNA methylation modification of VCAN gene in HCC (Figure 7C). Interestingly, in the GEPIA 2 database, there was also a positive correlation between the expression of VCAN and DNA methyltransferase (DNMT) in HCC (Figure 7B). These results suggest that transcription factors and DNA methylation may play an important role in the process of HCC by regulating the expression of VCAN.

miRNA, circRNA, and RBP interact with VCAN in HCC

By mining the three databases of LinkedOmics, Starbase and Targetcan, it was found that four common miRNA were down-regulated in HCC: hsa-miR-144-3p, hsa-miR-455-5p, hsa-miR-944 and hsa-miR-186-5p (Figure 8A). Previous studies have shown that the expression of hsa-miR-144-3p in hepatocellular carcinoma is significantly higher than that in adjacent tissues, and the ratio of HSA144-3p/hsA-miR-21-5p

increases significantly during the occurrence of hepatocellular carcinoma, which is even better than alpha-fetoprotein in ROC curve analysis, suggesting that HSA144-3p may be an excellent predictive marker of liver cancer(14). In addition, HSA-miR-455-5p has also been proved to be involved in the occurrence and development of liver cancer(15). Further analysis of Starbase showed that the expression of the four miRNAs was negatively correlated with the expression of VCAN in HCC (Figure 8B). Since circRNA can further regulate gene expression through sponge miRNAs, 10 circRNAs of sponge hsa-miR-455-5p and hsa-miR-144-3p are also identified in HCC (Figure 8C).

RNA binding proteins (RBP) are important post-transcriptional regulators, and different RBPs can interact with many RNA binding domains. The development of cancer is often accompanied by abnormal interactions between RBPs and RNA (16). We used starBase to mine 20 RBP most likely to interact with VCANmRNA in hepatoma cell line HepG2.

VCAN expression was correlated with immune factors

Existing studies have confirmed that the immune system is closely related to the occurrence and development of tumors. Therefore, we studied the relationship between the expression of VCAN and immune factors. Figure 9A-C show that there is a strong correlation between the expression of immunoinhibitors, immunostimulator and lymphocyte and the expression of VCAN.

Materials And Methods

VCAN expression level analysis

TCGAportal (www.tcgaportal.org) was used to study the expression of VCAN in different tumor tissues and corresponding paracancerous tissues. Human protein map (<https://www.proteinatlas.org/>) database contains pathological and genetic information from many reports from a variety of tissues and cells. We used it to detect the expression of VCAN in different tissues and the localization of VCAN mRNA in cells. Next, we used UALCAN (<http://ualcan.path.uab.edu/>) to compare the expression of VCAN in patients with HCC of different races, ages, and histological subtypes. Finally, the significance of the observed difference was evaluated by Wilcoxon rank sum test.

Relapse and survival analysis

KaplanMeier Plotter (<http://kmplot.com/analysis/index.php?p=background>) is a free online database, built by using gene expression data and survival data from a variety of cancers patients including HCC(17-19). We used this online database to explore the relationship between the expression of VCAN and OS and RFS of patients with HCC. Kaplan-Meier survival plots were used to compare OS and RFS in HCC patients with high VCAN expression and those with low VCAN expression, and 95% confidence interval hazard ratios and log-rank P values were calculated.

TF identification

The Cistrome DB Toolkit database (<http://dbtoolkit.cistrome.org>) is a resource of human and mouse cis-regulatory information, including about 47,000 human and mouse samples with about 24,000 newly collected data sets compared with two years ago. User can use this database to search for TFs related to the regulation of target genes in order to identify binding factors, histone modifications, and chromatin accessibility in a genomic interval of interest up to 2 Mb in length. Once users provide the overlap with the particular genomic interval sets, the similar ATAC-seq, DNase-seq, and ChIP-seq samples can be determined(20,21). We used the Cistrome Database Toolkit to search for TFs that were most likely to increase VCAN expression.

DNA methylation modification analysis

MEXPRESS (<https://mexpress.be/>), a user-friendly database tool for the the visualization and interpretation of TCGA data, can be used to study TCGA expression, DNA methylation status and clinical data and the relationships between them(22). In this research, we use this database tool to study the methylation status of VCAN mRNA and the relationships between VCAN mRNA expression and different clinical characteristics in HCC patients.

Gene correlations analysis

GEPIA2 (<http://gepia2.cancer-pku.cn/#index>), an openaccess dataset, can be used to study RNA sequencing expression data from 9,736 tumors and 8,587 normal samples driven from the TCGA and GTEx projects. The dataset provides tumor/normal differential expression analysis, profiling according to cancer types or pathological stages, patient survival analysis, similar gene detection, correlation analysis, and dimensionality reduction analysis. In this study, we used GEPIA2 to synthetically analyzed the correlations between all important genes.

Identification of miRNAs and circRNAs that target VCAN

TargetScanHuman (http://www.targetscan.org/vert_71/) has an ability of searching for the presence of conserved 8mer, 7mer, and 6mer sites that match the seed region of each miRNA to predict biological targets of miRNAs (Lewis et al., 2005). starBase v3.0 (<http://starbase.sysu.edu.cn/index.php>), an open-source platform for the identification of the interactions between miRNA to lncRNA, RBP to lncRNA, miRNA to mRNA, RNA to RNA, ncRNA to RNA, and RBP to mRNA from CLIP-seq, degradome-seq, and RNA-RNA interactome data. These two databases was used to confirm the potentialable miRNAs that bind to VCAN mRNA. In addition, starBase v3.0 was used to perform circRNA prediction, miRNA survival analysis, and analysis of correlations between miRNAs and VCAN mRNA.

Protein-protein interaction and functional enrichment analysis

Metascape (<http://metascape.org/gp/index.html#/main/step1>), a web portal, combines 40 independent knowledgebases's functional enrichment, interactome analysis, gene annotation, and membership search. It promotes comparative analysis of multiple independent and orthogonal experiments across datasets(23). STRING (<https://string-db.org/cgi/input.pl>) is a database that you can use to search for protein-protein interactions you are interested in, including direct (physical) and indirect (functional) connections; The conclusions obtained are comprehensively calculated and predicted, knowledge transfer between organisms and interactions summarized in other (main) databases(24). We use STRING to create an interaction network between VCAN and other important proteins.

Immune-related analysis

DISIDB (<http://cis.hku.hk/TISIDB/index.php>) is a web portal. Multiple heterogeneous data types were integrated to analyze the interaction between tumor and immune system in this web portal(25). We use it to analyze the spearman correlations between VCAN expression and immunostimulator, immunoinhibitors, and lymphocyte across human cancers

Cells culture and transfection

We used RPMI 1640 medium, contains 10% fetal bovine serum, to cultivate YY-8103 and LM3 cells lines in 5% CO₂ incubator with penicillin(100 IU/mL) and streptomycin(100 mg/mL). The small interfering RNA target to VCAN (si-VCAN) and untargeted control siRNA were produced by HongXin company in Nanjing China. We used the solution manufacturing by Applied biological materials company(Canada) and the Opti-MEM (Gibco, USA) to transfer. We mined the data use the cells after transfection for 48 hours. The target sequence of si-VCAN we obtained is as follows: si-RNA1: 5'-GGAAAUGGAAGAUGGCUATT-3'; si-RNA2: 5'-GGAAAGAUUGAAAGAGATT-3'; si-RNA3: 5'-GGAUAGGCCUCAUGACAATT-3'.

Cell proliferation experiments

In CCK8 experiment, we firstly transfected the YY-8103 and LM3 cells line and incubated at 37°C. Then put the CCK8 solution (Biosharp, China) into each hole and incubate for two hours. The absorbance was detected on 450nm at 0, 24, 48, 72 hours. We did all the experiments three times.

Transwell migration and invasion assays

In accordance with the manufacturer instructions, we vaccinated the YY-8103 and LM3 cells line at the upper chamber, and the culture was performed on 200 microliter serum-free 1640 medium. The matrigel mix (BD Biosciences, San Jose, CA, USA) covers transwell chamber (Corning, NY, USA) so that the invasion test can be realized and the matrigel mix is not needed for migration experiment. The HCC cell chemical inducers made by RPMI 1640 medium and 10% FBS was lured to the bottom of the chamber. Incubation for 24 hours, we fixed the colour of upper chamber. Then crystal violet (Kaigen, Nanjing, China) was used to dyeing for 15 minutes. We photographed and counted the cells in five fields in order to implement visualization.

Wound healing assay

After culture on a six-well plate, we transfected YY-8103 and LM3. The artificial linear wound in monolayer fused cell was removed by the standard 20 μ l pipetting device. The free floating cells and debris in well bottom was removed slowly. Inject it into the medium and put the well in an incubator to incubate at 37 degrees celsius. The width of scratch clearance was recorded by inverted microscope and taking pictures at 0, 24, 48 hours. The difference between the width of original wound and the width of process of quantitative cell migration was done three times.

Statistical Analysis

The SPSS 25.0 (IBM, SPSS, and Chicago, IL, USA) and GraphPad Prism 8 were used to analyse the data. We think the data had statistical significance when the p value less than 0.05. Independent t-test was used to compare continuous information between the two groups. Besides, we used chi-square test to dive into categorized data. Corresponding significance level was showed in those figures.

Discussion

Previous studies have shown that VCAN is an EMT-related gene, which plays a role in promoting, leukemia, breast cancer, non-small cell lung cancer and other cancers. However, the research of VCAN in HCC is still relatively rare, so we used a variety of databases to explore the expression of VCAN in HCC. As expected, VCAN is highly expressed in HCC.

We use some databases to observe the co-localization of VCAN and tubulin in cells. Some previous studies have shown that tubulin plays an important role in cell cycle and cell proliferation. Many anti-tumor drugs kill cancer cells by changing the microstructure of cancer cells(26, 27). Therefore, in the development and progression of HCC, VCAN is likely to interact with the microtubule structure to promote cancer. The above reminds us that the focus of future research can be on the co-localization and interaction of VCAN and tumor tubulin.

In vitro experiments showed that VCAN gene knockout inhibited the proliferation, invasion and migration of HCC cells, while overexpression of VCAN had the opposite effect. The theory of cancer stem cells (CSC) provides a new perspective on the mechanism of tumorigenesis and metastasis(28). Some recent studies have pointed out that DNA methylation is a potential epigenetic mechanism to maintain CSC. In addition, studies have shown that DNA methyltransferase (DNMT) plays a vital role in CSC, and knocking out DNMT can reduce and inhibit the occurrence of tumors by limiting and reducing the CSC pool(29–31). In summary, targeting epigenetic modifiers, especially DNA methylation, is a potential way for humans to overcome cancer. Research on colorectal cancer shows that 5-Aza-2'-deoxycytidine (5-AzaDC) is a DNMT inhibitor, which can significantly reduce the number and activity of colorectal CSCs, and can inhibit the progression of colorectal cancer(32). Therefore, we envision whether 5-AzaDC is also an anti-cancer treatment approach for patients with HCC.

The role of immune cells in tumors has received more and more attention. A large number of studies have shown the important role of immune regulation in HCC, and immune-related anti-tumor drugs are also appearing repeatedly. Studies have shown that CSF1 receptor (CSF1R)-mediated signal transduction plays an irreplaceable role in the differentiation and survival of the mononuclear phagocyte system, especially macrophages (33). CSF1R belongs to the type III protein tyrosine kinase receptor family, and binding to CSF1 or the more recently identified ligand IL-34 can induce receptor homodimerization and subsequently activate receptor signal transduction(34). Some studies have confirmed that CSF1R + macrophages are associated with poor survival of various tumor types(35, 36), so therapies targeting CSF1R-related signal transduction pathways such as CSF1R inhibitors have been proven effective against cancer(37). In our study, we confirmed the correlation between CSF1R($\text{Rho} = 0.396$, $p = 9.32\text{e-}16$) and VCAN in HCC through DISDB, which implies their positive correlation. Our research shows that low expression of VCAN can significantly improve the survival time of HCC patients, which implied that VCAN may be the downstream or upstream target of CSF1R in HCC, and is partly involved in its cancer-promoting effect. In addition, we have also unearthed many immunoinhibitors like CSF1R related to VCAN, such as CD96, PDCD1, CD271, etc., which are positively correlated with the expression of VCAN. In addition to immunoinhibitors, we also found that some immunostimulator such as CD28, CD86, CD27, lymphocytes such as macrophages, Act-DC are all positively correlated with the expression of VCAN. It is suggested that VCAN plays an important role in immune regulation in HCC. Therefore, the combination of inhibitors against these immunological checkpoints and VCAN inhibitors may potentially enhance the anti-cancer effect in patients with HCC.

As mentioned in this article, the expression of VCAN is increased in many cancers, including HCC, which is significantly related to the poor prognosis of patients with HCC. At the same time, we found a variety of miRNA and circRNA, that can regulate VCAN and promote the progression of HCC. Our study provides a new anti-hepatoma idea-to find some DNA methyltransferase inhibitors and TF inhibitors that can effectively down-regulate the expression of VCAN. To sum up, VCAN has great potential to become a prognostic marker and therapeutic target for HCC.

Declarations

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Disclosure

The authors report no conflicts of interest in this work.

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Figures

Figure 1

VCAN may be related to EMT, angiogenesis and glycolysis. (A) We selected three liver cancer transcriptome database GSE124535, GSE136247, GSE144269, and used GEO2R to analyze the overexpressed genes contained in them. The overlapping genes were found by Venn map. (B) Two interacting networks module were found by Mcode. (C-D) Module1 is mainly related to mitosis and cell cycle. (E-F) Module2 is mainly related to EMT, angiogenesis, glycolysis and so on.

Figure 2

VCAN expression overview. (A) The expression level of VCAN mRNA in Hepatocellular carcinoma is obviously more than that in normal tissues. (B-D) Discrepancy in VCAN mRNA expression is hinged on race, nodal metastasis status and histological subtypes. (E) The expression level of VCAN in normal tissue and HCC tissue. (F) VCAN is in the same position as microtubule proteins in the cytoplasm of U-2 OS, A-431 and U-251 MG cells.

Figure 3

Research results of VCAN in single cell. (A) VCAN is expressed in fibroblasts and has a similar expression pattern to classical fibroblast markers such as COLA1/2, DCN. (B) There is a strong correlation between VCAN and FB markers in LIHC. (C) VCAN is highly expressed in myeloid. (D) Some correlation between VCAN and myeloid markers in LIHC.

Figure 4

Clinical role of VCAN in HCC. (A-D) The patient have poor OS and poor RFS with high level of VCAN.

Figure 5

Artificial reduction of VCAN expression can effectively inhibit the proliferation of HCC cells. (A) Scratch assay was used to reduce the expression of VCAN gene. (B) When VCAN expression was inhibited, the migration of HCC cells was also inhibited. (C) By knocking down the expression of VCAN, the invasion of HCC was effectively prevented. (D) CCK8 assay confirmed that the inhibition of VCAN by Si-VCAN could slow down the proliferation of HCC cells. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Figure 6

Artificial up regulation of VCAN expression can effectively accelerate the proliferation of HCC cells. (A) Scratch assay was used to increase the expression of VCAN gene.. (B) When VCAN expression was up regulated, the migration of HCC cells was also promoted. (C) By up regulating the expression of VCAN, the invasion of HCC was effectively promoted. (D) CCK8 assay confirmed that the over expression of VCAN could accelerate the proliferation of HCC cells.* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Figure 9

Three types of cancer-related immune factor are related to VCAN. (A) Correlation between VCAN expression and immunoinhibitors in HCC. (B) Correlation between VCAN expression and immunostimulator in BC. (C) Correlation between VCAN expression and lymphocyte in BC.