The Proliferation and Migration-Enhancing Effects of Vitronectin in SMMC 7721 Cells: A Pilot Study

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Abstract: To understand the effects of Vitronectin on cell proliferation and migration in the cell line of hepatocellular carcinoma, SMMC 7721, the effects of Vitronectin on cell proliferation rate or on the prevention of the cells from the apoptotic stimuli were appraised with WST-1 assay; and the morphology of β-Tubulin was observed with con-focal microscope. The effect on migration was detected with transwell chamber. The results show that Vitronectin helps the cells adhere to Petri dish as well as the sustaining of the morphology of β-Tubulin. Vitronectin could enhance the proliferation rate of SMMC 7721 with the concentration-effect mode, and could protect the cells from the stimuli of apoptosis inducer. As to cell migration, the results show that Vitronectin enhance cell migration across the membrane of transwell chamber. According the results, the conclusion could be made that Vitronectin might play important roles in the following biological effects, such as sustaining the morphology of the tumor cells, enhancing the proliferation as well as the migration.

Keywords: Vitronectin, Hepatocellular Carcinoma, Proliferation, Tumor Cell Migration.

Hepatocellular carcinoma (HCC) is one of the major diseases causing serious health problems in the world. According to the statistics, almost 0.626 million new cases suffering from liver cancer with 0.598 million deaths each year worldwide, ranking sixth for the cancer incidence and third for cancer mortality in the world [1, 2]. At present, no effective preventive and treatment measures for advanced liver cancer, postoperative recurrence and metastasis of the early liver cancer. Therefore, early diagnosis and treatment of liver cancer are of great significant. Early diagnosis of cancer depends on effective biological markers, currently, the most common biological marker associated with HCC is serum alpha-fetoprotein (AFP) level [3]. However, previous studies show that AFP has various limitations such as a low sensitivity and a high false negative rate, resulting in high unreliability in identifying liver cancer and other liver diseases [4]. Thus, to find better biomarkers became a research hotspot.

Vitronectin (VTN) also called serum spreading factor or complements S-protein, is a cell adhesion and spreading factor found in serum and tissues [5]. One of the most important roles of VTN is to participate in the formation of extracellular matrix. In normal liver cells, VTN expresses only in portal system and central vein subendothelial matrix. But when HCC occurs, VTN becomes the main component of the extracellular matrix [6], and has a high level of expression especially in the cavernous-like vascular [7]. The dramatic difference in VTN expression suggests that it may have important significance in cancer [8]. However, there are few reports about effects of VTN on liver cancer cell lines. Therefore, we simulate the actual action mode of VTN in physiological processes [9], trying to observe the growth and the metastasis ability of SMMC 7721 cells when cultured in VTN-coating Petri dishes in order to understand whether VTN has biological significances such as promoting tumor cell growth, metastasis as well as protecting tumor cells from apoptotic stimuli.

MATERIALS AND METHODS

Reagents

VTN was purchased from Invitrogen (Grand Island, NY). arginine-glycine-asparginine peptide(RGD) was supplied by Sigma-Aldrich (St Louis, MO). DIM was purchased from LKT (St. Paul, MN) and was prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich, MO). Penicillin, streptomycin, RPMI 1640, Insulin-Transferrin-Selenium (ITS) and EDTA were purchased from Gibco (Grand Island, NY). Cell proliferation reagent (WST-1) was supplied by Roche (Germany). Antibodies against β-Tubulin were purchased from Cell Signaling Technology (Beverly, MA).

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Cell Lines

The human hepatocellular carcinoma cell lines SMMC 7721 was obtained from the Type Culture Collection of the Chinese Academy of Science (Shanghai, China). The passage numbers of the cells were from 6~30. The cells were cultured in RPMI 1640 medium with 3% ITS, a standard additive for cells grown in serum-free conditions, and supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in the present of 5% CO_2.

VTN Coating

For cell growth experiments, 500 μl of VTN solutions diluted in PBS were aliquoted into the dishes (diameter 3 cm, NUNC, Rochester, NY) or 100 μl into the 96-well dishes, left at room temperature for 18 h and then at 4°C for another 18 h. Then the dishes were incubated with 0.1% BSA in PBS for 30 min at 37°C to block non-coated areas of plastic. After that, the dishes were washed several times with PBS and air-dried for using [9, 10].

Cell Attachment and Cytoskeletal Morphology

Dishes were coated with VTN and 10,000 cells were inoculated into each dish and incubated at 37 ˚C for 24 h. After wash with PBS for 5 min, the cells were fixed for 10 min with methanol-acetic acid (3:1) fixative and blocked with BSA for 20 min. After addition of 2 ml 0.5% Triton X-100 at room temperature for 20 min, 50 μl rabbit anti-human β-Tubulin monoclonal antibody was added and incubated at 4 ˚C overnight. After wash with PBS, the cells were incubated with fluorescent-labeled secondary antibody at room temperature for 20 min followed by DAPI staining and wash with PBS. The cells were observed with a laser con-focal microscope.

Cell Growth

About 10,000 cells were incubated in a 96-well culture dish coated with VTN solution at 37 ºC for 24 h. WST-1, a cell proliferation reagent, was added into every well and cultured for 2 h. Colorimetric analysis was performed in 450 nm (measure wavelength) and 690 nm (reference wavelength) after full mixing.

Abate Apoptotic Stimuli

About 10,000 cells were incubated in a 96-well culture dish coated with VTN solution at 37 ºC for 24 h followed by treating with various concentration of DIM (5, 10, 15, 20, 30, 40 and 50 μM) for 24 h. WST-1 was added into every well and cultured for 2 h. Colorimetric analysis was performed after full mixing.

Cell Migration

Migration assay were performed essentially as described by Leavesley *et al*. Briefly, $1X10^5$ cells cultured in serum-free medium for 4 h were seeded into the upper chamber of an 8.0-μm-pore Costar Transwell (24-well plate format). VTN was added into the lower chamber. After 12 h of incubation at 37 ˚C in 5% CO_2, Cells that migrated to the lower chamber, were fixed, then stained with Trypan Blue. The number of cells migrated was estimated by cell counting [11, 12].

Statistical Analysis

Data was expressed as mean ± standard deviation, and SPSS 11.0 software was used for statistical analysis. The data of cell attachment and migration rate were analyzed using a one-way ANOVA and post hoc analysis was done using S-N-K t test. The a priori significance level was set at $p < 0.05$.

RESULTS

Effects of VTN-Coating on Cell Adherence and the Morphology of β-Tubulin

Our results showed that adherent cells in VTN-coated group grew well. The spreading cell presented spindle or polygonal shape. Round-shrink cells were rare in the dishes coated with VTN. But the round-shrink cells increased when the dilution ratio of VTN increases. However, RGD-coating had no effect on cell adherence. The cells were round-shaped shrinkage and the same as the control group (1% BSA coated group) (Figure **1**).

VTN solutions allowed the cells adhere to the Petri dish in 2 h and fully extended into the normal shape of spindle or polygonal. Compared with the control group, the difference in cell adhesion rate was statistically significant ($F = 37.526$, $p < 0.01$). However, RGD-coating was not helpful for cell adherence. The cell adhesion rates in RGD solutions of various dilution were close to those of their control group (1% BSA coated group) ($F = 8.221$, $p = 0.762$). S-N-K test showed that when compared with the corresponding RGD-coated groups, the increase in cell adherent rates of VTN-coated cells was statistically significant (Figure **2**).

As compared with un-coated cells, VTN-coating was good for adherence and the cells had more typical

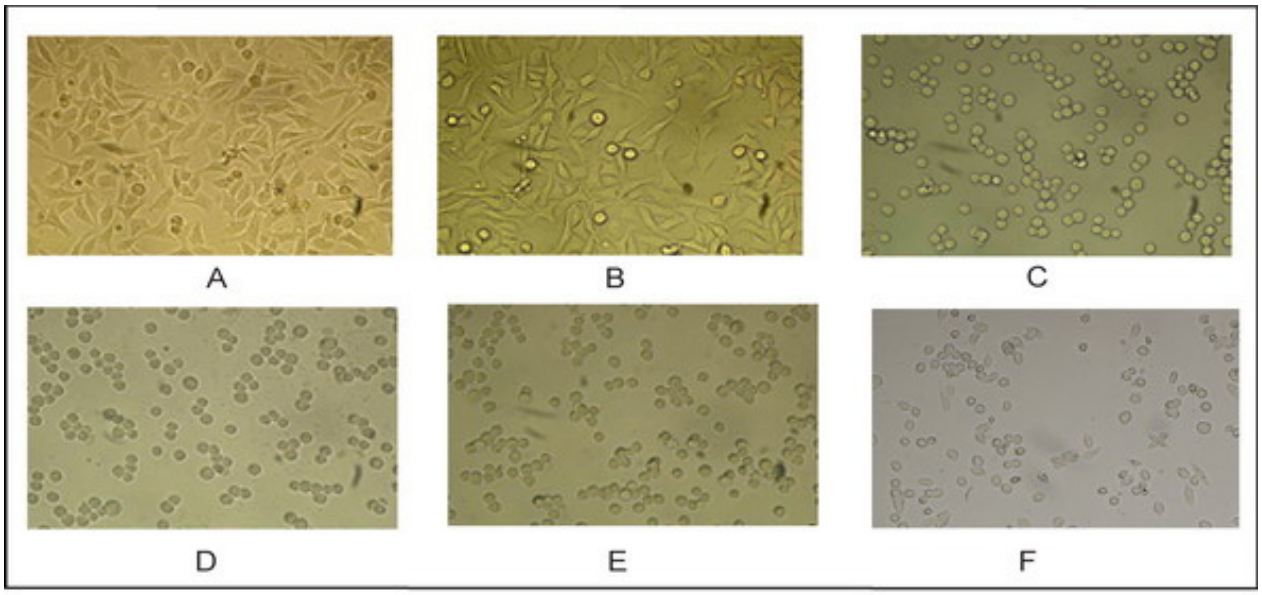

Figure 1: The effects of VTN-coating on the adherence of SMMC 7721 to Petri dish. SMMC 7721 cells were cultured in 96-well dish coated with VTN or RGD solution with the dilution of 1:200 and1:1600 for 2 h, the morphology of cells was observed with microscope. **A**. Cells coated with VTN (the dilution is 1:200); **B**. Cells coated with VTN (the dilution is 1:1600); **C**. Cells non-coated; **D**. Cells coated with RGD (the dilution is 1:200); **E**. Cells coated with RGD (the dilution is 1:1600); **F**. Cells coated with 1%BSA.

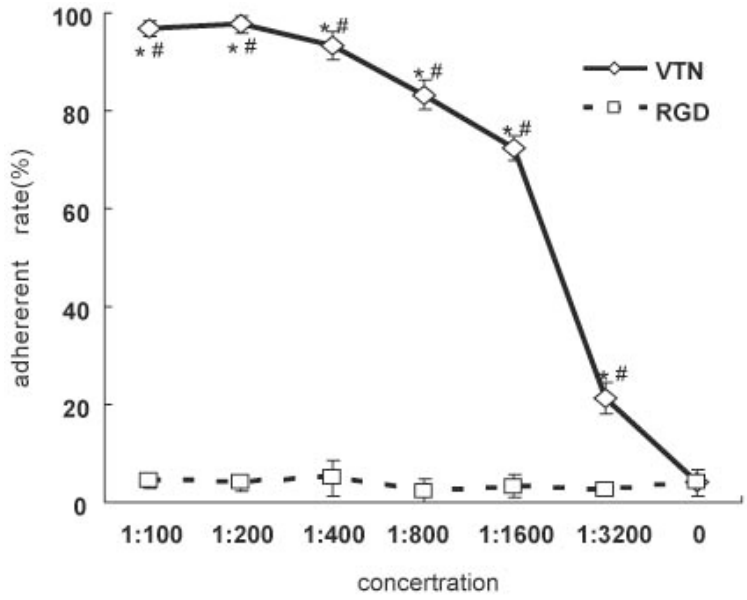

Figure 2: The effects of VTN-coating on the adherent rate of SMMC 7721 to Petri dish. SMMC 7721 cells were cultured in 96-well dish coated with VTN or RGD solution with the dilution of 1:200 and1:1600 for 2 h, adherent cells were washed, digested and counted. Calculated the cell adhesion rate and mapping with the coating concentrations as X-axis and cell adhesion rate as Y-axis.

spindle or polygonal structure. In contrast, the spindle or polygonal shape of the non-coated cultured cells was not typical and poor stretching. Spindle cells were rare and most cells presented short spindle or cone (Figure **3**). Accordingly, β-Tubulin, which plays an important role in cell morphology, movement of cells and organelles as well as material transportation, was changed in some extent. β-Tubulin has a network and filamentous structure arranging from perinuclear to the surrounding. As indicated in Figure **2G-I**, VTN-coating favored the maintenance of normal β-Tubulin structure. Under the general culture conditions without VTN coating, the normal filamentous structure of microtubules is not obvious and presents form of

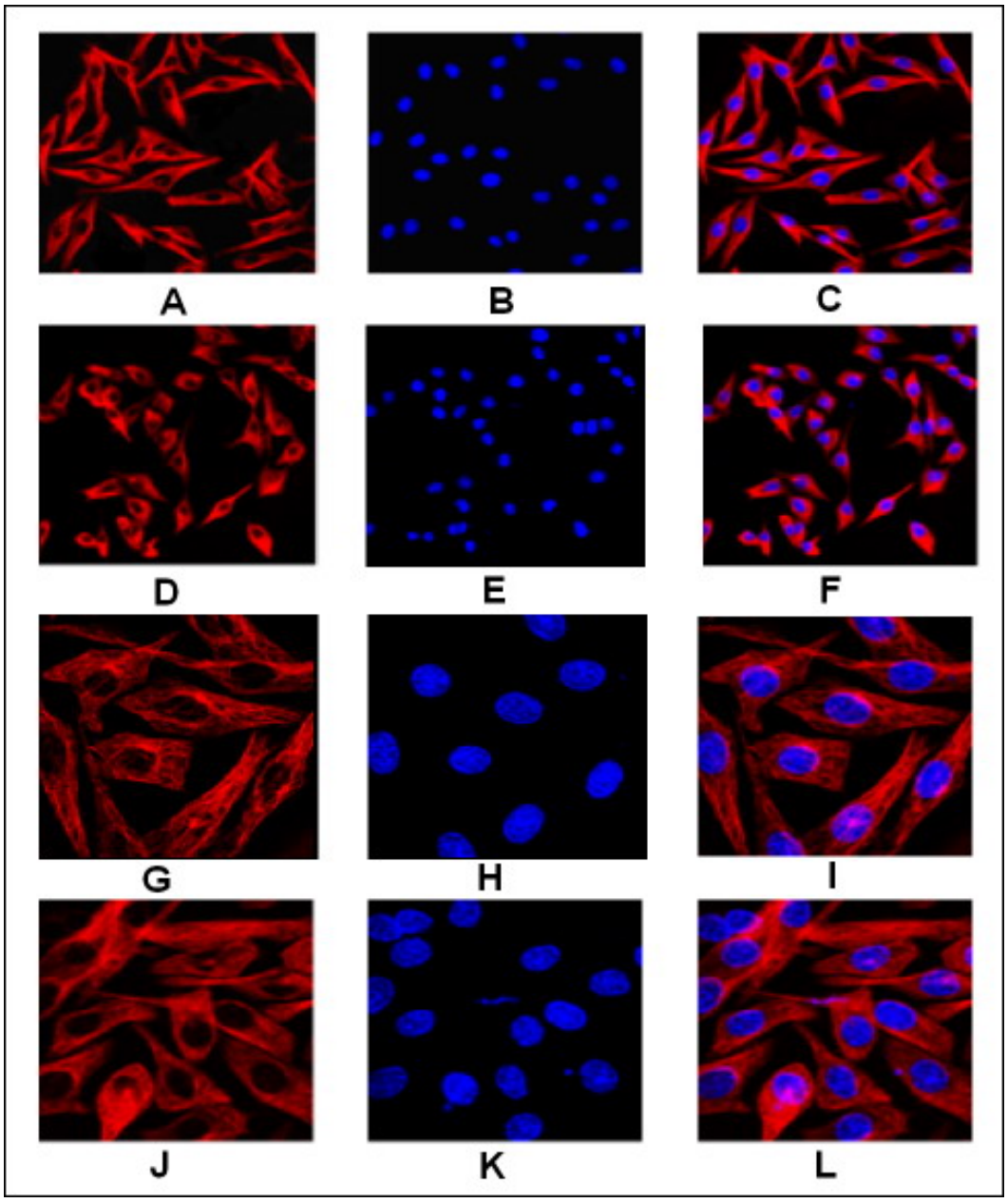

Figure 3: The effects of VTN on the morphology of SMMC 7721 and β-Tubulin. Petri dishes were coated with VTN or RGD solution with a 1:200 dilution and 10,000 cells were inoculated and incubated at 37 ˚C for 24 h. Then the cells were incubated with β-Tubulin monoclonal antibody and with fluorescent-labeled secondary antibody followed by DAPI staining. The cells were observed with a laser con-focal microscope. Red fluorescence represents β-Tubulin and blue fluorescence represents nuclear. **C** is merging image of **A** and **B**; **F** is merging image of **D** and **E**; I is merging image of **G** and **H**; **L** is merging image of **J** and **K**.

pellets or frosted glass with a less regular stretching radial structure (Figure **3**).

Effects of VTN on Cell Growth

Figure **4A** indicated that VTN-coating plays a certain role in promoting cell growth ($F = 61.224$, $p < 0.01$), and that cell proliferation was inversely proportional to the VTN dilution ratio, i.e. the smaller the dilution ratio, the levels of cell proliferation were higher. The cell proliferation level under the lowest dilution (1:100) was 1.7-fold higher than that of the control group (1% BSA). In addition, RGD-coating has no promoting effect on cell growth ($F = 18.370$, $P=0.813$).

Protective Effects of VTN on Liver Cancer Cell Growth Induced by Different Concentrations of DIM

To observe the protective effects of VTN on the cells which were pretreated with apoptotic stimuli, the fixed concentration of VTN-coating (1:200) were used.

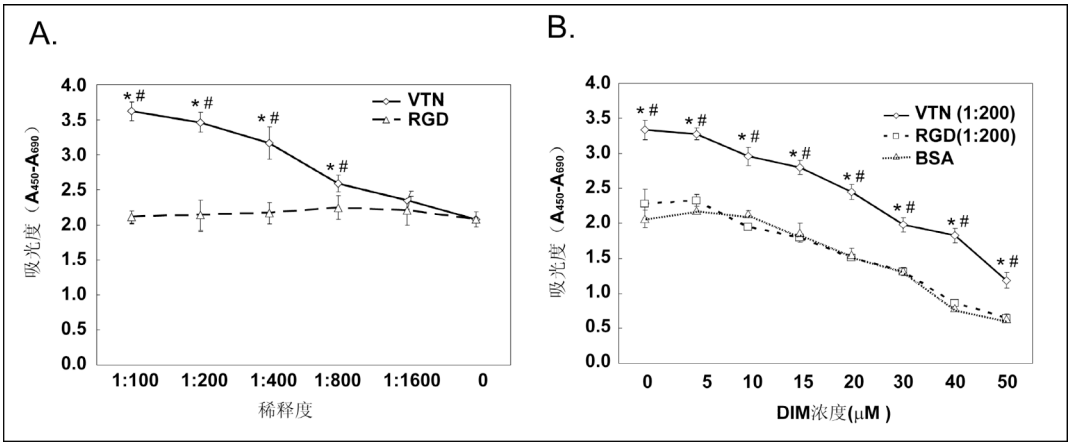

Figure 4: The growth-promotion effects and apoptosis-inhibitory effects of VTN. A. SMMC 7721 cells were cultured in 96-well dish coated with VTN or RGD solution with the dilution of 1:100, 1:200, 1:400, 1:800, and 1:1600, then the cells were incubated with WST-1 for 2 h. Colorimetric analysis was performed in 450 nm and 690 nm wavelengths after full mixing; **B**. SMMC 7721 cells were cultured in 96-well dish coated with VTN or RGD solution with the dilution of 1:200, followed by treating with DIM at different concentrations for 24 hrs. Then the cells were incubated with WST-1 for 2 hrs. Colorimetric analysis was performed in 450 nm and 690 nm wavelengths after full mixing.

The results showed that VTN coating could reduce the inhibitory effect of apoptosis-inducing agent DIM on SMMC 7721 cell proliferation ($F=106.192$, $P<0.001$). In addition, RGD-coating had no significant protective effect on DIM inhibition of SMMC 7721 cell protection ($F=12.167$, $P=0.979$) (Figure **4B**).

Effect of VTN-Coating on the Migration of SMMC 7721 Cells

SMMC 7721 cells were inoculated in the upper chambers of Transwell chamber. Serum-free medium with different concentrations of VTN solution were added in the lower chambers and incubated for 12 h. The number of cells transferring from upper chambers to lower chambers through the membrane was counted. As indicated in Figure **5**, different concentrations of VTN, RGD, or BSA had a different promoting effect on SMMC 7721 cell migration from the upper chamber into lower chamber ($F = 197.647$, $p < 0.001$). One-way ANOVA analysis showed that compared with the BSA group, various concentration of VTN was capable of promoting SMMC 7721 cell migration, whereas RGD has no similar effect.

DISCUSSION

It is well known that malignant transformed cells cannot properly undergo apoptosis [13, 14] is an

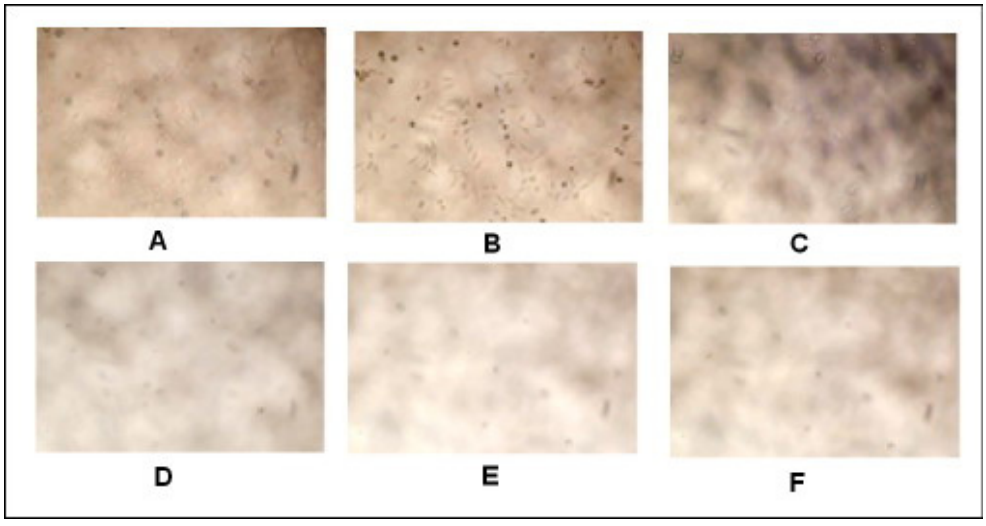

Figure 5: The effect of VTN-coating on the migration of SMMC 7721 cells. Fifty thousand cells cultured in serum-free medium for 4 h were seeded into the upper chamber of a 8.0–μm-pore Costar Transwell. VTN, RGD or BSA was added into the lower chamber. Cells that migrated to the lower chamber after 12 hr of incubation were fixed and stained with Trypan Blue. (**A**) VTN(1:200), (**B**) VTN(1:800), (**C**) VTN(1:1600), (**D**) RGD(1:200), (**E**) RGD(1:800), (**F**) 1%BSA.

important reason of tumor development, however, uncontrolled cell proliferation and cell differentiation abnormalities play significant roles in the process of neoplasia. In glioma cells, abnormal proliferation could be induced when coated with VTN and the highest protective effect could be find when the coating concentration is 500 ng/cm^2 [10]. At present, the effect of VTN and whether it produces similar inhibition of apoptosis-inducing effects of agents on human hepatocellular cells have not been reported.

Tumor cell migration is a prerequisite of tumor invasion and metastasis. As an extracellular matrix molecule, VTN is involved in extracellular matrix composition, and has a potential impact on tumor cell metastasis. Pervious studies show that VTN promotes tumor cell metastasis through its receptor molecule Alpha V integrin and other signaling molecules. The most important active sites in the molecular structure of the VTN are located in its N-terminal three peptide structure Arg-Gly-Asp, namely RGD sequence [15]. Interaction of VTN and its main cell surface receptor Alpha V integrin depends on this structure site [16]. A variety of extracellular matrix components including fibronectin, laminin, VTN and collagen contain RGD sequence, which is very widely distributed in human body [17]. In this study, in order to determine that promotion of cell adhesion, cell growth, cell transfer and inhibition of tumor cell apoptosis are attributed to VTN, RGD-coating was used as a control in the studies. The results demonstrate that RGD-coating has no impact on any effect mentioned above, suggesting that the phenomena observed in this study are induced by VTN itself.

The main active components of cruciferous plants, indole-3-carbinol (I3C) and its derivatives such as 3,3'-Diindolylmethane (DIM), can inhibit cell proliferation and induce apoptosis of various tumor cells. *In vitro* studies indicate that I3C and DIM affect activation of carcinogens and initiation and progress of tumor *via* a variety of ways, inducing apoptosis in various tumor cells such as breast cancer, prostate cancer, endometrial cancer, colorectal cancer and leukemia cells etc. [18-21]. Previous studies by our group show that I3C and DIM can inhibit proliferation of poorly differentiated nasopharyngeal carcinoma cell line CNE1, human hepatoma cell lines HepG2 and SMMC 7721 cell as well as induce apoptosis of these types of cells [22] in a concentration-dependent manner and time-dependent manner. In the present study, we employed DIM as an apoptosis-inducing agent to observe the impact of VTN-coating on its role. Our results show that DIM inhibits proliferation of SMMC 7721 cell and induces apoptosis in a concentration-dependent manner. VTN-coating inhibits apoptosis, suggesting that the extracellular VTN can promote tumor cell growth and inhibit effect of apoptotic stimuli on tumor cells.

VTN is a normal component of serum. To avoid the impact of VTN in fetal bovine serum on the experimental results and to ensure nutritional factors required for cell growth, we employed Insulin-Transferrin-Selenium (ITS), a basic nutritional support material [23], to domesticate cells by gradually reducing the serum concentration with gradual addition of ITS. In addition, to prevent cell matrix proteins including VTN from hydrolysis by trypsin, we used a non-enzymatic digestion chelating agent-EDTA in this study to digest the cells and used low-speed centrifugation to remove EDTA in the cell suspension, preventing cell damage induced by intracellular calcium and magnesium ions in the EDTA chelation cells.

Furthermore, the current studies show that VTN can promote cell adhesion and contribute to the maintenance of Tubulin structures. Since Tubulin plays extremely important roles in the constitution of the cell stents, maintaining cell shape, displacement of organelles and participating in material transport, therefore, we speculate some biological roles of VTN such as promoting cell growth and metastasis of tumor cells may be achieved by regulating Tubulin structure.

In conclusion, VTN might function importantly in the growth, anti-apoptosis and migration of hepatocellular carcinoma cell line, SMMC 7721. The mechanism underlying the above effects may attribute to the sustaining the morphology of cytoskeleton as well as the attachment-helping roles.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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