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Effects of Long Non-coding Ribonucleic Acid Plasmacytoma Variant Translocation 1 Regulating Octamer Binding Transcription Factor 4 Expression on Hepatocellular Carcinoma Cells

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KEYWORDS Hepatocellular Carcinoma. Invasion. Long Non-coding Ribonucleic Acid. Migration. Radiosensitivity

ABSTRACT The researchers aimed to evaluate the effects of long non-coding ribonucleic acid plasmacytoma variant translocation 1 (lncRNA PVT1) regulating octamer binding transcription factor 4 (OCT4) expression on hepatocellular carcinoma (HCC) cells. It was discovered that in contrast to the blank group, si-PVT1 group had lower PVT1 and OCT4 expressions, number of invading cells and wound healing rate, and higher radiosensitivity ($P<0.05$). OCT4 served as a gene targeted by lncRNA PVT1. The PVT1 group exhibited declined luciferase activity compared with the NC group after transfection with wild-type OCT4 ($P<0.05$). In comparison with those of the sh-NC group, the number of invading cells and wound healing rate dropped in the sh-OCT4 group ($P<0.05$). At the same radiation dose, a lower survival fraction than that in the sh-NC group was observed in the sh-OCT4 group ($P<0.05$). Silencing lncRNA PVT1 significantly suppresses HCC cell invasion as well as migration and elevates their radiosensitivity, probably by down-regulating OCT4 expression.

INTRODUCTION

Given the most frequently-occurring malignancy in clinical practice, hepatocellular carcinoma (HCC) has a mortality rate ranking third among all cancers (Kim et al. 2020; Sivakumar et al. 2021). Due to insidious symptoms, over 80 percent of patients suffer from HCC that has already evolved into the middle-advanced stage upon diagnosis, greatly affecting the prognosis (Guo et al. 2021b). Currently, HCC is primarily treated with radiotherapy, but the surviving cancer cells become resistant, leading to tumor recurrence and unsatisfactory outcome (Weiler et al. 2020). Long non-coding ribonucleic acids (lncRNAs) exert regulatory effects on the radiosensitivity of tumor cells, but the underlying mechanism remains unclear (Liang et al. 2020a). With specific spatiotemporal expressions, the nucleus and cytoplasm possess extensive expressions of lncRNAs (Ahadi 2021). Due to the lack of obvious open reading frames, however, lncRNAs control protein-coding genes from the aspect of expression by virtue of RNAs (Liang et al. 2020b). Plasmacytoma variant translocation 1 (PVT1) serves as a newly discovered lncRNA which is associated with the incidence and advancement of diversified tumors (Lv et al. 2019). It has been revealed that lncRNA PVT1 has overexpression in malignancies such as cancers of the lung, stomach and breast, which can enhance the proliferation while inhibiting cancer cell apoptosis *via* participating in DNA rearrangement and interacting with oncogene Myc (Qiu et al. 2020). On the other hand, octamer binding transcription factor 4 (OCT4), as a POU transcription factor family member, can bind the OCT domain of target gene, thereby regulating the expressions of downstream target genes (Čančer et al. 2019). OCT4 can maintain embryonic stem cells regarding the pluripotency besides self-renewal (Ma et al. 2021). Moreover, OCT4 expression is potent in various tumor tissues (Lu et al. 2020), but it is still largely unknown in HCC cells, so is the mechanism.

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Objectives

As for the purposes of this study, therefore, the expressions of lncRNA PVT1 and OCT4 in HCC cells, as well as whether lncRNA PVT1 can regulate the biological activity and radiosensitivity of HCC cells by modulating OCT4, were explored.

METHODOLOGY

Cell Lines

Human hepatocyte LO2 and human HCC SMMC-7721, Huh7, PLC and HepG2 cell lines (batch No. 20190923) were offered by Shanghai Cell Bank, Chinese Academy of Sciences.

Reagents and Apparatus

The following reagents and apparatus were used: lncRNA PVT1 small interfering RNA (si-PVT1) together with negative control (si-NC) (Shanghai Chuangyi Biotechnology Co., Ltd., China), short hairpin RNA targeting OCT4 (sh-OCT4) plus sh-NC (Shanghai GenePharma Co., Ltd., China), OCT4 antibody (Sino Biological, China), RPMI-1640 medium, DMEM, trypsin and fetal bovine serum (Wuhan Procell Life Science & Technology Co., Ltd., China), inverted fluorescence microscope (Shanghai Xinmao Instrument Co., Ltd., China), low-temperature high-speed centrifuge (Shandong Xinbeixi Scientific Instrument Co., Ltd., China), and X-ray irradiator (Rad Source, USA).

Cell Culture

The cells were all cultured in medium mixed with 10 percent fetal bovine serum, streptomycin at 100 μg/mL and penicillin at 100 μg/mL using an incubator (5% CO_2, 37°C, 24 h), which was changed once every 2 d. With the wall adhesion and the covering of the bottom of the bottle observed, the cells were subjected to digestion, and those in the logarithmic growth phase entered into experiments implemented afterwards.

Cell Grouping and Transfection

Following culture, implantation into a 6-well plate and transfection using Lipofectamine™ 2000 kit were conducted for HepG2 cells which were assigned as blank group (cultured normally with no transfected plasmid), si-PVT1 group (transfected with si-PVT1), si-NC group (plus si-NC transfection), sh-OCT4 group (together with transfection of sh-OCT4 plasmid) and sh-NC group (on the basis of sh-NC plasmid transfected). All the cells were cultured for 24 h.

Detection of Expressions of lncRNA PVT1 and OCT4 in Cells by qRT-PCR

The HCC cells were digested and lysed by TRIzol lysis buffer to extract the total RNA, for which reverse transcription was then performed to acquire cDNA by means of reverse transcription kit and the following primers: lncRNA PVT1 F: 5'-TGAGAACTGTCCTTACGTGACC-3', R: 5'-AGAGCACCAAGACTGGCTCT-3'. OCT4 F: 5'-ACATCAAAGCTCTGCAGAAAGAACT-3', R: 5'-CTGAATACCTTCCCAAATAGAACCC-3'. GAPDH F: 5'-TGTTCGTCATGGGTGTGAAC-3', R: 5'-ATGGCATGGACTGTGGTCAT-3'. The primers were centrifuged, added deionized water, mixed well and prepared into 100μmol/L stock solution. The aforementioned primers were prepared into working solution (final concentration: 10 μmol/L) through dilution in EP tubes. The conditions of PCR were determined as follows: 15 min of pre-denaturation (95°C), then 20 s of denaturation (95°C)and 34 s of annealing (60°C), totally 40 cycles. Finally, $2^{-\Delta\Delta CT}$ was adopted for analysis of lncRNA PVT1 and OCT4 expression levels.

Detection of Cell Invasion Capability by Transwell Assay

The cells in the blank, si-PVT1 and si-NC groups were inoculated into 24-well plates after the density was adjusted to 6×10^4/mL. Then the medium (500 μL) blended with 10 percent fetal bovine serum was suplemented, and the cells were digested and diluted into 5×10^5. Next, another plate (24-well) was added with Transwell chamber, inoculated with the cell suspension treated with dilution, and placed on a culture plate with 10 percent fetal bovine serum, followed by 24 h of culture in the incubator. Finally, the cells were wiped off from the chamber, prior to the observation of plate under a microscope to count the invading cells.

Detection of Cell Migration Capability *via* Wound Healing Assay

Cells in blank, si-NC and si-PVT1 groups were inoculated into 24-well plates after density adjustment (at 5×10^4/mL), followed by culture in a 37°C incubator under 5 percent CO_2. Upon reaching 80 percent confluence, the supernatant was discarded, a sterile pipette tip (20 μL) was employed to scratch the cells, and the detached ones were eliminated with PBS. Subsequent to 24 h of culture by virtue of serum-free medium, the cells were photographed at 0 h and 24 h in the same position by means of an inverted microscope. Finally, the wound width (W_{0h}, W_{24h}) was measured, and the wound healing rate (%) was calculated: $(W_{0h}-W_{24h})/W_{0h}\times100\%$. The assay was repeated 3 times.

Detection of Cell Radiosensitivity by Colony Formation Assay

The cells in blank, si-NC and si-PVT1 groups were inoculated into 6-well plates, which were irradiated using 6 mV X-ray (SAD: 100 cm, dose rate: 200 cGy/min, irradiation field: 20 cm×20 cm, and source-skin distance: 100 cm). After irradiation, the cells were further cultured until colonies formed. After fixing of the cells with formaldehyde and crystal violet staining (totally 30 min), and the inverted microscope was utilized for observation, followed by enumeration of the colonies containing over 50 cells. Moreover, the survival fraction was calculated to plot survival curves, so as to compute the sensitization enhancement ratio (SER): $DO_{control\ group}/DO_{experimental\ group}$. DO refers to the mean lethal dose, namely the radiation dose required to kill 63 percent of cells in the survival curve, which can assess the sensitivity of cells to radiation damage.

Dual-luciferase Reporter Assay

It was predicted by Target Scan 7.1 software that OCT4 was the target gene of lncRNA PVT1, and there were binding sites with lncRNA PVT1 in OCT4 3'-UTR. Hence, wild-type sequence (wt-OCT4 3'-UTR) together with mutant sequence (mut-EZH2 3'-UTR) was designed and synthesized, and the gene fragments were cloned into pmirGLO luciferase reporter vector to construct OCT4 3'-UTR dual-luciferase reporter vector (pmirGLO-wt-survivin) and corresponding mutant vector (pmirGLO-mut-survivin).

Wt-OCT4 3'-UTR, si-PVT1, mut-EZH2 3'-UTR and si-NC were used for joint transfection of cells with Lipofectamine™ 2000. About 48 h later, the determination of luciferase activity was accomplished. The grouping for co-transfection is listed in Table 1.

Table 1: Grouping for co-transfection

Group	*Co-transfected gene*
NC	pmir GLO-wt-*OCT4* 3'-UTR
PVT1	pmir GLO-wt-*OCT4* 3'-UTR
NC	pmir GLO-mut-*OCT4* 3'-UTR
PVT1	pmir GLO-mut-*OCT4* 3'-UTR

Statistical Analysis

The statistical analysis was executed through SPSS 22.0 software. Mean ± standard deviation ($\bar{x} \pm s$) was adopted as the expression format of measurement data, which were subjected to one-way analysis of variance for multi-group comparison, and the comparison between two groups was achieved by the Tukey's test. $P<0.05$ signified a statistically significant difference.

RESULTS

Expressions of PVT1 and OCT4 in Normal Hepatocytes as well as HCC Cells After Transfection

Compared with LO2 cells (1.00±0.06), the expressions of PVT1 significantly rose in SMMC-7721 (1.32±0.08), Huh7 (1.83±0.10), PLC (2.41±0.12) and HepG2 cells (3.76±0.16) ($P<0.05$) (Fig. 1A). Compared with LO2 cells (0.98±0.05), the expressions of OCT4 also significantly increased in SMMC-7721 (1.41±0.08), Huh7 (1.71±0.09), PLC (2.02±0.11) and HepG2 cells (2.81±0.13) ($P<0.05$) (Fig.1B). Since the expressions were lowest in HepG2 cells, they were selected for subsequent experiments.

No prominent differences were observed regarding PVT1 and OCT4 expressions between si-NC and blank groups ($P>0.05$). Compared with the blank group (3.97±0.15, 2.85±0.11), the expressions of PVT1 and OCT4 in the si-PVT1 group significantly reduced (1.23±0.12, 1.06±0.09) ($P<0.05$),

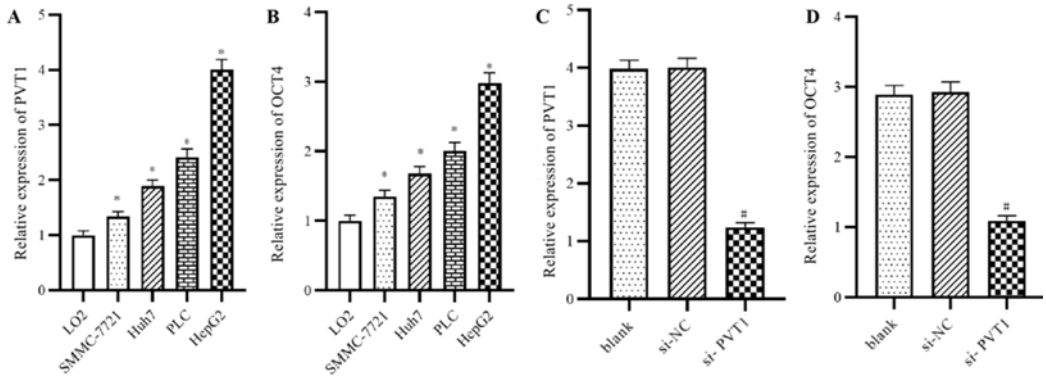

Fig. 1. Expressions of PVT1 and OCT4 in normal hepatocytes and HCC cells. A: Expression of PVT1 in normal hepatocytes and HCC cells. B: Expression of OCT4 in normal hepatocytes and HCC cells. *P<0.05, compared with LO2 cells

suggesting that the transfection was successful (Fig. 1C and D).

Effect of Inhibited lncRNA PVT1 Expression on Invasion Capability of HCC Cells

The cell invasion capability in the blank group was not remarkably different from that in the si-NC group (P>0.05). Besides, the si-PVT1 group (117.26± 7.15) exhibited a significantly smaller number of invading cells compared to the blank group (416.32±10.24) (P<0.05) (Fig. 2).

Influence of Inhibited lncRNA PVT1 Expression on Migration Capability of HCC Cells

As for the blank and si-NC groups, the wound healing rate at 24 h had no apparent variation (P>0.05), whereas it dropped significantly in the si-PVT1 group (20.14±2.35) in contrast with that in the blank group (57.32±5.24) (P<0.05) (Fig. 3).

Role of Inhibited lncRNA PVT1 Expression in Influencing Radiosensitivity of HCC Cells

At the same radiation dose, no evident difference in the survival fraction of HCC cells was detected in the blank group from the si-NC group (P>0.05), but a notably lower survival fraction was obtained from the si-PVT1 group compared with that from the blank group (P<0.05), with an SER of 2.085 (Fig. 4). Therefore, inhibiting lncRNA PVT1 expression can enhance HCC cell radiosensitivity.

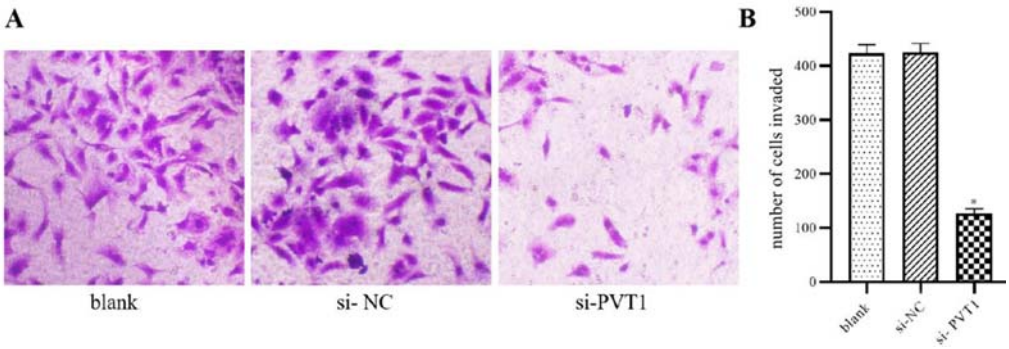

Fig. 2. Cell invasion capability detected by Transwell assay. A: Transwell assay results. B: Number of invading cells. *P<0.05, compared with blank group

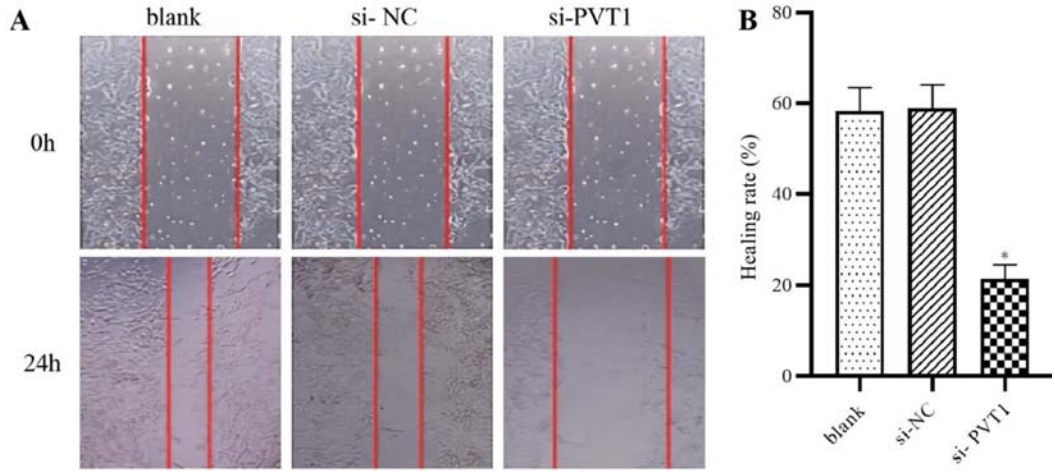

Fig. 3. Cell migration capability detected by wound healing assay. A: Wound healing assay results. B: Wound healing rate of HCC cells. *P<0.05, compared with blank group

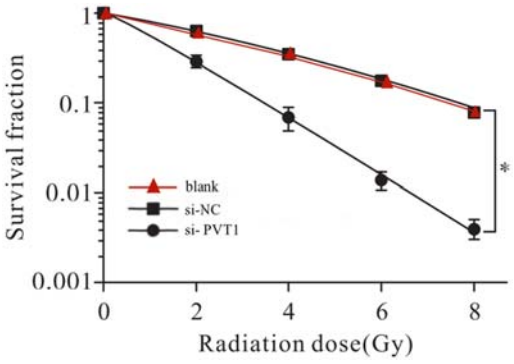

Fig. 4. Effect of inhibited lncRNA PVT1 expression on radiosensitivity of HCC cells. *P<0.05, compared with blank group

Prediction and Identification of Targeting Relationship Between lncRNA PVT1 and OCT4

There were binding sites with lncRNA PVT1 in OCT4 3'-UTR (Fig. 5A). In comparison to that of the NC group (1.00±0.09), the luciferase activity of the PVT1 group (0.32±0.03) declined markedly after transfection with wild-type OCT4 (OCT4-WT) (P<0.05), while no distinct variation occurred the two groups after transfection with mutant OCT4 (OCT4-MUT) (P>0.05) (Fig. 5B).

Function of Inhibited OCT4 Expression in Affecting Migration, Invasion as well as Radiosensitivity of HCC Cells

The invading cell count together with wound healing rate significantly decreased in the sh-OCT4 group (123.25±3.46, 19.86±1.87) compared with those in the sh-NC group (415.62±10.47, 58.67±2.43) (P<0.05) (Fig. 6A and B). In the case of an identical radiation dose to that in the sh-NC group, the sh-OCT4 group manifested an overtly lower survival fraction of HCC cells (P<0.05), with an SER of 1.972 (Fig. 6C).

DISCUSSION

HCC is characterized by high morbidity and mortality rates, and more than 80 percent of HCC patients are from the Asia-Pacific region (Mariadoss et al. 2021; Ding et al. 2022). At present, HCC is primarily treated with radiotherapy in clinical practice, among which stereotactic radiotherapy and radioembolization are most commonly used (Kim et al. 2020; Yang et al. 2021). However, the inevitable radiation damage during radiotherapy poses a huge challenge to the treatment of HCC. Acute radiation-induced liver injury can generally be relieved in a short time, but the disease may aggravate into fatal liver

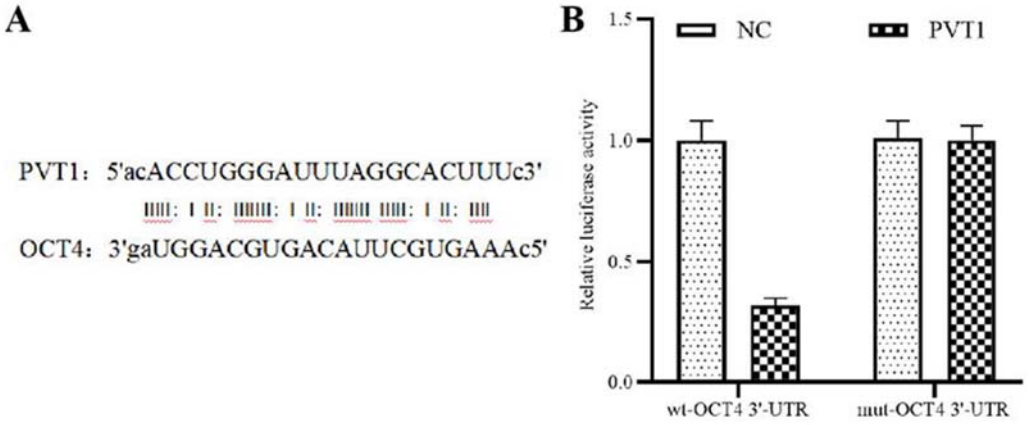

Fig. 5. Targeting relationship between lncRNA PVT1 and OCT4. A: Binding sites between lncRNA PVT1 and OCT4 predicted online. B: Targeting relationship between lncRNA PVT1 and OCT4 validated by luciferase assay

failure (Hasuzawa et al. 2021). The resistance to radiological agents is also a major obstacle in the treatment of HCC (Preston and Shaida 2021). Therefore, it is of great significance to enhance the radiosensitivity of HCC cells.

The onset and progression of HCC involve changes at the genome level, among which lncRNAs can regulate genetic expression at the transcriptional, epigenetic and post-transcriptional levels, thereby getting implicated in tumor proliferation, invasion, metastasis as well as other pathological processes (Yan et al. 2020). LncRNAs belong to non-coding RNAs catalyzed using RNA polymerase II (Tan et al. 2021). As the most studied molecule among lncRNAs, PVT1 contains more than three thousand bases, and can form more than twenty variants through post-transcriptional alternative splicing (Guo et

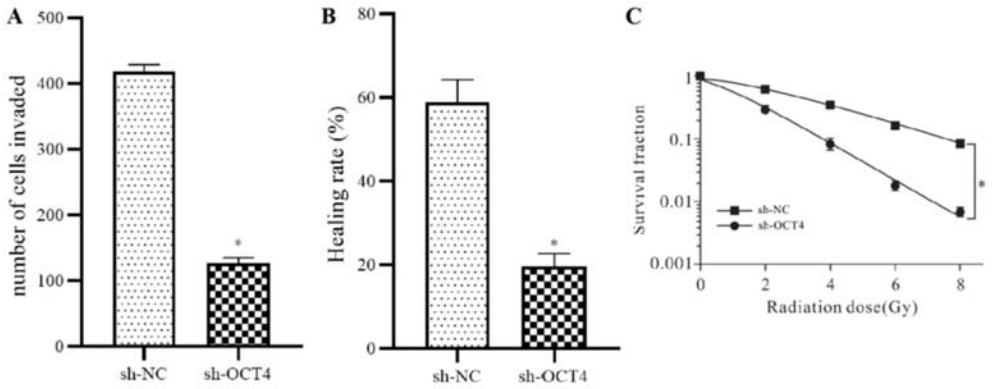

Fig. 6. Effect of inhibited OCT4 expression on invasion, migration and radiosensitivity of HCC cells. A: Number of invading cells. B: Wound healing rate. C: Cell survival fraction curves. *P<0.05, compared with sh-NC group

al. 2021a). It has been confirmed that suppressing the expression of PVT1 can result in embryonic death, indicating that PVT1 mediates the growth and development and facilitates cell differentiation (Xiong et al. 2021). LncRNA PVT1 can, through interacting with Myc, mediate the occurrence of tumors, and its increased expression in gastric cancer tissues has been confirmed, which is highly intimate with metastasis of lymph nodes, tumor TNM stage and degree of invasion (Dahai et al. 2020). LncRNA PVT1 is at a high level in terms of expression concerning ovarian cancer cells, and the inhibited expression can suppress the invasion plus proliferation of ovarian cancer cells while prominently strengthening their apoptosis (Chen et al. 2021). According to the findings of Li et al. (2015), HCC tissues were detected with highly expressed lncRNA PVT1, where a higher expression indicated higher risk related to postoperative recurrence. Likewise, in this study, silencing PVT1 inhibited HepG2 cell invasion besides migration. Additionally, the survival fraction of HCC cells in the si-PVT1 group significantly reduced, with an SER of 2.085, suggesting that inhibiting the HCC cell radiosensitivity was intensified by means of lncRNA PVT1 expression repression. Cancers are clinically treated mainly by radiotherapy, and DNA repair ability is one of the main factors affecting radiosensitivity (Hauth et al. 2022). PVT1 can regulate the sensitivities of various tumors to chemotherapy and radiotherapy (Wang et al. 2018). It was corroborated by Yang et al. (2019) that suppressing PVT1 expression augmented the radiosensitivity of hepatocytes by inhibiting HCC cell multiplication while promoting their apoptosis, possibly being related to inhibited DNA repair.

OCT4 encoded by POU5F1 consists of 5 exons, which is discovered with extensive expression in germ cells and embryonic stem cells. OCT4 can regulate embryonic stem cells from the aspects of renewing and differentiating themselves, in addition to the expressions of its downstream target genes at the gene level and the activities of other proteins (Takayam et al. 2021). As revealed by previous reports, OCT4 in bladder cancer T24 cells had a significantly increased expression (El-Anwar et al. 2016). Moreover, Chen et al. (2020) found that OCT4 expression in HepG2 cells significantly rose. Consistently, the researchers herein found that OCT4 had significantly increased expression in HCC cells. Besides, silencing PVT1 in HCC cells inhibited OCT4 expression. Therefore, the researchers postulated that silenced PVT1 was capable of restricting both invasion and migration of HCC cells, probably by inhibiting OCT4 expression. To confirm the above postulation, it was predicted by bioinformatics that lncRNA PVT1 bound the OCT4 3'-UTR sequence. After the dual-luciferase plasmid inserted with the OCT4 3'-UTR sequence and si-PVT1 was used for transfection into HCC cells together, significantly inhibited luciferase activity of OCT4 3'-UTR was acquired, verifying that PVT1 bound the OCT4 3'-UTR sequence and lncRNA PVT1 targeted the expression of OCT4. After transfection of HepG2 cells using sh-OCT4, HepG2 cells were also inhibited from the perspectives of invasion along with migration, and their radiosensitivity was enhanced. The above findings further validated that silencing PVT1 inhibited HCC cell invasion as well as migration and elevated their radiosensitivity through negatively modulating OCT4 expression. Zhou et al. (2019) confirmed that lncRNA PVT1 regulated both self-repair and activity of hepatic cancer stem cells by modulating the Wnt signaling pathway. Accordingly, lncRNA PVT1 can target and regulate diversified signaling pathways, so as to exert impacts on the biological activities of tumor cells.

CONCLUSION

In conclusion, lncRNA PVT1 in HCC cells is at a high level of expression. Silencing lncRNA PVT1 can significantly suppress the invasion and migration of HCC cells while strengthening their radiosensitivity, probably by down-regulating the expression of OCT4.

RECOMMENDATIONS

Further *in vivo* and clinical studies are in need to validate the findings herein and to provide valuable evidence for HCC treatment.

ABBREVIATIONS

HCC: hepatocellular carcinoma; lncRNA: long noncoding RNA; NC: negative control;

OCT4: octamer binding transcription factor 4; PVT1: plasmacytoma variant translocation 1; RT-qPCR: real-time quantitative polymerase chain reaction; SER: sensitization enhancement ratio.

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