Int J Clin Exp Pathol 2015;8(7):7786-7797
www.ijcep.com /ISSN:1936-2625/IJCEP0010271

Original Article

Down-regulation of cancer/testis antigen OY-TES-1 attenuates malignant behaviors of hepatocellular carcinoma cells *in vitro*

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Received May 16, 2015; Accepted June 26, 2015; Epub July 1, 2015; Published July 15, 2015

Abstract: Cancer/testis (CT) antigens are normally expressed in testis and overexpressed in various tumor types. However, their biological function is largely unknown. OY-TES-1, one of cancer/testis (CT) antigens, is reported overexpression in hepatocellular carcinoma (HCC). And we assumed that OY-TES-1 contribute to oncogenesis and progression of HCC. In this study, we knocked down OY-TES-1 by small interference RNA (siRNA) in HCC cell lines (HepG2 and BEL-7404) to verify this assumption and evaluate its potential as therapeutic targets for HCC. We showed that down regulation of OY-TES-1 decreased cell growth, induced the G_0/G_1 arrest and apoptosis, and prevented migration and invasion in the two HCC cell lines. Further analysis revealed that down regulation of OY-TES-1 increased expression of apoptosis-regulated protein caspase-3, and decreased expression of cell cycle-regulated protein cyclin E, migration/invasion-regulated proteins MMP2 and MMP9. These findings may shed light on the gene therapy about the OY-TES-1 expression in HCC cells.

Keywords: Cancer/testis antigen, OY-TES-1, hepatocellular carcinoma, siRNA

Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer mortality in China, and the third worldwide [1, 2]. Even combination therapy including surgical procedures and chemotherapy is ineffectual in HCC, as demonstrated by low response rates and no significant survival advantage. Currently, rapid growth, high invasion and metastasis are still the main causes of HCC mortality. However, the mechanism underlying the tumorigenesis and highly aggressive metastasis of HCC remain unclear.

Cancer/testis (CT) antigens, a heterogeneous group of more than 70 families with at least 200 antigens, have been identified so far (http://ctadatabase.com). CT antigens hold a unique expression pattern being expressed in human tumors of different histological origin, but not in normal tissues except for germ cells of testis [3-5]. This tumor-restricted pattern of expression, together with their strong *in vivo* immunogenicity, served CT antigens as ideal targets for tumor-specific immunotherapy. Nowadays several clinical studies of CT antigens based vaccine therapy have been developed [6, 7]. Surprisingly, although the CT antigens have been identified already 20 years ago, almost nothing is known about their biological function in CT antigen-positive tumor cells [8].

OY-TES-1, also known as acrosin binding protein (ACRBP), is one of CT antigens [9-11]. The gene of OY-TES-1 located on chromosome 12p12-13 and encoded a protein with the C-terminal homologous to the sperm protein 32 (sp32) [12]. Therefore, OY-TES-1 was initially identified as the human homologue of proacrosin binding protein precursor. Sp32 was found in the sperm head and considered as a binding protein to

proacrosin for packaging and condensation of the acrosin zymogen in the acrosomal matrix, involving in spermatogenesis and sperm capacitation [10, 12]. It is also reported that OY-TES-1, expresses in mouse spermatogenic cells and epididymal sperm, present two functional forms produced by pre-mRNA alternative splicing and may play different role in spermiogenesis and fertilization [13]. In tumors, OY-TES-1 mRNA was detected in various tumors including live cancers with 40% positive rate, but only trace amount of OY-TES-1 mRNA was detected in some of normal tissues except for testis [10, 11]. It was a report that high ACRBP expression is significantly correlated with reduced survival time and faster relapse among ovarian cancer patients [14]. Recently, we also found that OY-TES-1 protein expression was correlated with tumor invasion stage as well as histological grade in colon cancer [15]. Moreover, we have demonstrated that OY-TES-1 was expressed in marrow-derived mesenchymal stem cells, and interestingly, its down-regulation by small interfering RNA (siRNA) resulted in a series of changes, such as inhibiting cell growth, inducing apoptosis, arresting cell cycle and attenuating migration [16]. Therefore, implication from these results is that OY-TES-1 might somehow support tumor cells malignancy or aggressiveness.

In order to assess the functional properties of OY-TES-1, we investigated the effects of OY-TES-1 down-regulation by siRNA in HCC cell lines and evaluated its potential as therapeutic targets for HCC.

Materials and methods

Cell lines

Human HCC cell lines Bel-7404, HepG2, QGY-7703, QGY-7701, BEL-7402 and SMMC-7721 were purchased from the Type Culture Collection of Chinese Academy of Science (Shanghai, China) and cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco).

siRNA transient transfection

siRNA transient transfection and siRNA sequence targeting OY-TES-1 were performed as described previously [16]. Briefly, Bel-7404 and HepG2 cells were cultured in the 24-well plate. When the cell confluence reached around 50%, siRNA transfection was performed with the ratio of siRNA to transfection reagent (1 μg: 5 μL). Both scrambled siRNA and FAM-labeled scrambled siRNA (for transfection efficiency test) were also used in parallel. The cells were harvested at 24 h, 48 h and 72 h post-transfection, respectively. The group that cells were transfected into OY-TES-1-specific siRNA abbreviated as OY siRNA. The controls were set up as following: first group cells (MOCK1) were only cultured in ordinary culture medium, and second group cells (MOCK2) were treated with transfection reagent only, while third group cells (Ctrl siRNA) were transfected into a scrambled siRNA.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using Trizol (Invitrogen, USA) according to the manufacture's recommendation. Two μg of total RNA was reverse transcribed using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Canada). Obtained cDNA was tested for integrity by amplification of p53 transcripts. The expression level of OY-TES-1 was normalized to p53 [17]. The sequences of OY-TES-1-specific primer and PCR condition were referenced [15].

Immunocytochemistry (ICC)

Immunostaining with polyclonal anti-human OY-TES-1 antibody produced by our laboratory was performed as previously described [12]. Cells were cultured in the cover slide and fixed with 4% paraformaldehyde for 15 min. Then cells were permeabilized with 0.1% Triton X-100 in PBS for 30 min. After raising, 3% H_2O_2 was applied on the cells for inactivation of endogenous peroxidase. Then cells were incubated with 1:200 dilution of anti-OY-TES-1 antibody overnight at 4°C. Astreptavidin-biotin horseradish peroxidase (HRP) based 3, 3'-diaminobenzidine (DAB) kit (Maixin, China) was used for detection of immunoreactivity. All slides were lightly counterstained with hematoxylin. Negative controls were performed by using preimmune serum of rabbit.

Western blot

Western blotting was performed as described before [18]. The polyclonal primary antibodies were as followed: OY-TES-1 antibody as above mentioned, anti-human MMP2 and MMP9 anti-

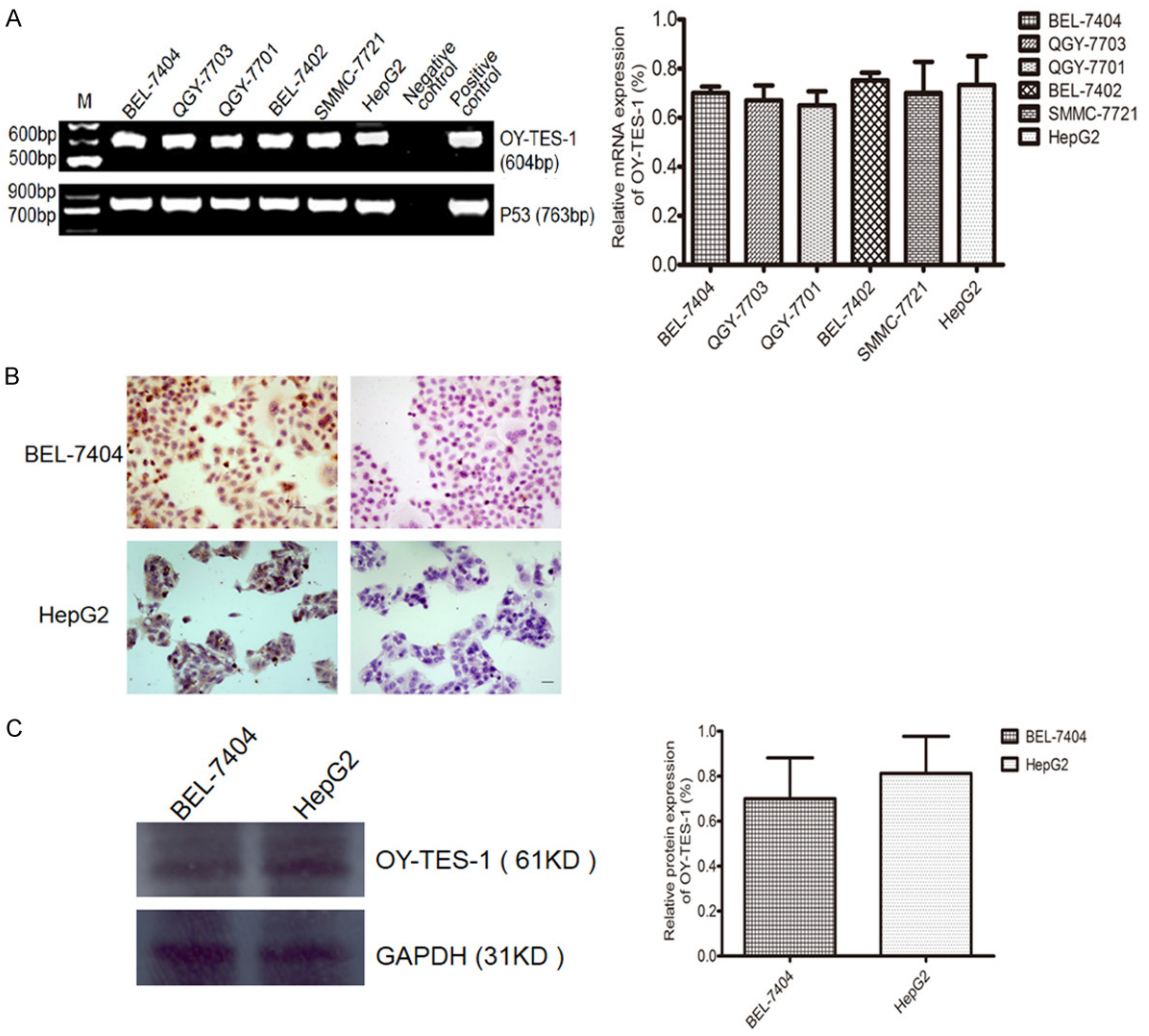

Figure 1. The mRNA expression of OY-TES-1 in six different cell lines has no significant difference, the protein expression of OY-TES-1 in BEL-7404 and HepG2 has no significant difference. A. RT-PCR analysis of OY-TES-1 mRNA expression in six different cell lines of HCC. B. Immunocytochemical staining of OY-TES-1 protein. OY-TES-1 protein (left). Preimmune serum of rabbit as negative control (right). C. Western blot analysis of OY-TES-1 expression. The OY-TES-1 expression level was presented as the ratio to p53 in mRNA and GAPDH in protein, respectively. Marker (M); PCR without template (Negative control); PCR with testis cDNA (positive control). Bar: 10 μm. The columns show the mean for three separate experiments; bars, sd.

bodies (1:1000 dilution, Cell Signaling Technology, USA), anti-human cyclin E antibody, (1:800 dilution, Proteintech Group, USA), and anti-human cyclin D1 antibody (1:1000 dilution), anti-human caspase-3 antibody (1:500 dilution) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:1000 dilution) purchased from Beyotime, China. The goat anti-Rabbit IgG conjugated with HRP (1:1000 dilution, Changdao, China) and donkey anti-Rabbit IgG conjugated with HRP (1:5000 dilution, Jackson Immuno Research, USA) were used as secondary antibodies. The levels of target proteins were normalized to GAPDH.

Cell viability assay

The Cell viability assay was performed as reported previously [16].

Cell cycle and apoptosis assays

The Cell viability assay were performed as reported previously [16].

Invasion assay

Invasion assay was carried out using 24-well transwell chambers (Corning, USA), which were

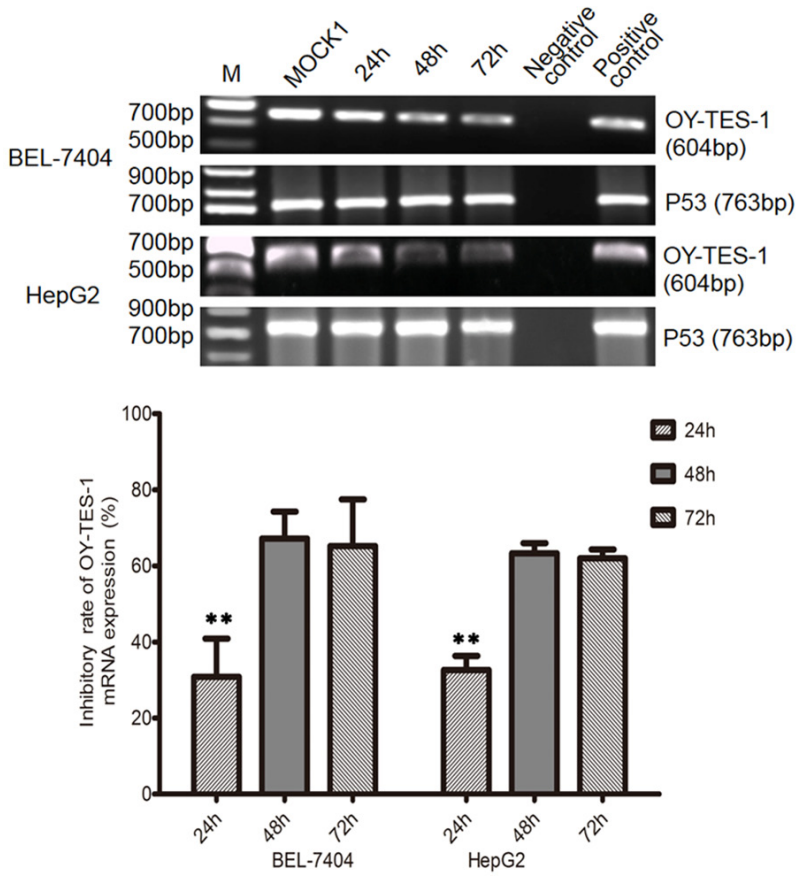

Figure 2. The inhibitory rate of OY-TES-1 expression at 48 h and 72 h was higher than that at 24 h after transfection. The expression level was presented as the ratio of OY-TES-1 to p53 in mRNA and GAPDH in protein, respectively. Marker (M); PCR without template (Negative control); PCR with testis cDNA (positive control); cells without transfection (MOCK 1); cells transfected with OY siRNA targeting OY-TES-1 after 24 hours (24 h); cells transfected with OY siRNA targeting OY-TES-1 after 48 hours (48 h); cells transfected with OY siRNA targeting OY-TES-1 after 72 hours (72 h). The columns show the mean for three separate experiments; bars, sd. $^{**}P < 0.01$.

precoated with Matrigel (BD, USA). Cells (1×10^5/well) were seeded in the upper chamber, while the lower chamber was filled with 600 μl medium containing 10% FBS. After 24 h of incubation, cells on the upper surface of filter were wiped off by gently scrubbing with a cotton swab. Cells invaded to the lower surface of the filter were stained with crystal violet and then counted in 5 random different fields under microscope.

Migration assay

Ability of cell migration was tested by transwell and wound healing assay, respectively. The procedure of transwell assay was as above described instead of using uncoated filter. For wound healing assay, cells were plated into a 24-well plate at 2×10^5 cells/well. After reaching confluence, cells were carefully wounded by a sterile pipette, and the detached cells were removed. The plate was continuously cultured in DMEM without FBS. Serial photographs were taken by an inverted microscope in the same field at 0 h, 12 h and 24 h, respectively. The wound width (μm) was measured using Image pro-plus 6.0 software. The wound closure rate = wound width (at 0 h)-wound width (at 12 h or 24 h)/wound width (0 h) ×100%.

Adhesion assay

96-well plates were precoated with Matrigel (BD, USA). Cells were seeded at a density of 4×10^3/well and then incubated for 60 min, 90 min and 120 min, respectively. Three duplicate wells were set up for each group. After washing non-adherent cells, remaining cells on the plate were counted under a microscope in five randomly-chosen fields.

Statistical analysis

All statistical analyses were performed using the SPSS16.0 software (SPSS, USA). The data were obtained from three independent experiments and presented as the means ± standard deviation of the mean (mean ± SD). The statistical significance of differences was determined by one-way analysis of the variance (ANOVA) and a value of $P < 0.05$ was considered statistically significant.

Results

OY-TES-1 was expressed in liver cancer cell lines and suppressed by OY siRNA

As a prerequisite for functional analysis of OY-TES-1 in HCC, we firstly investigated the mRNA expression of OY-TES-1 in six different

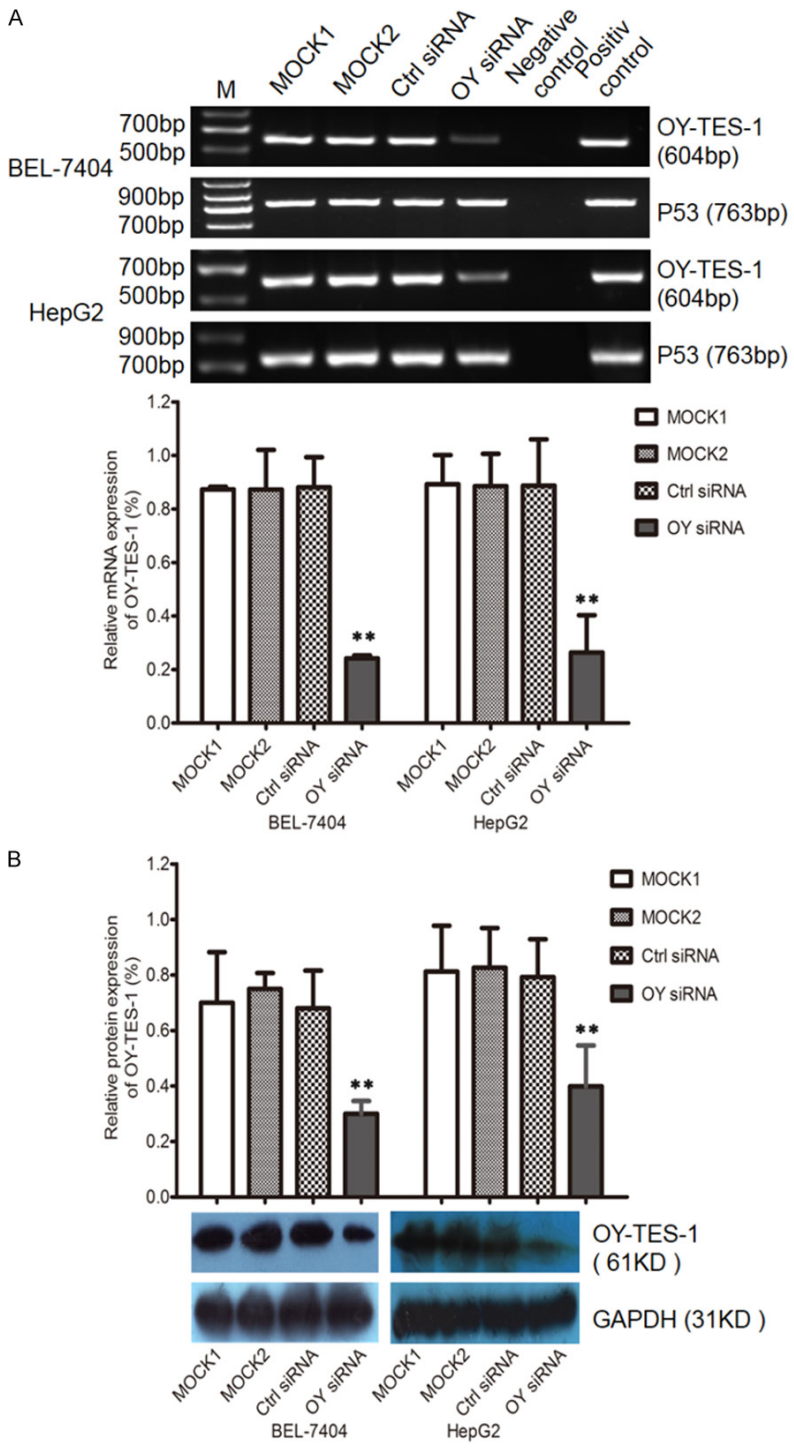

Figure 3. OY-TES-1 expression level was significantly reduced by transfection with OY siRNA targeting OY-TES-1. A. RT-PCR analysis of OY-TES-1 mRNA expression in cells treated after 48 h. B. Western blot analysis of OY-TES-1 expression in cells treated after 48 h. The expression level was presented as the ratio of OY-TES-1 to p53 and GAPDH respectively in mRNA and protein level. Marker (M); PCR without template (Negative control); PCR with testis cDNA (positive control); cells without transfection (MOCK 1); cells treated with transfection reagent only (MOCK2), cells transfected into scrambled siRNA (ctrl siRNA), cells transfected into OY-TES-1-specific siRNA (OY siRNA). The columns show the mean for three separate experiments; bars, sd. $^{**}P < 0.01$.

cell lines of liver cancer. Since OY-TES-1 was present with no significant difference in all liver cancer cell lines available to us (**Figure 1A**), we could not perform the functional analysis of OY-TES-1 gene by transfecting it into OY-TES-1 negative liver cancer cell lines. Therefore, we decided to select two cell lines (BEL-7404 and HepG-2) to perform knock-down experiments using interfering RNA (**Figure 1C**). We detected that OY-TES-1 protein was located at the cytoplasm of the both two kinds of HCC cell lines by ICC (**Figure 1B**).

To begin of the exploration, the efficiency of siRNA for down-regulation of OY-TES-1 was tested. The results showed that OY siRNA can more effectively suppressed OY-TES-1 mRNA expression at 48 h and 72 h than at 24 h after transfection ($P < 0.01$) (**Figure 2**). Therefore, 48 h cells after transfection were harvested for later experiments. After optimization of siRNA transfection condition, the OY-TES-1 expression in both mRNA and protein level was further tested by RT-PCR and Western blot. The result demonstrated that OY-TES-1 mRNA (**Figure 3A**) and protein (**Figure 3B**) was down-regulated in both Bel-7404 and HepG2 compared to the controls ($P < 0.01$).

OY-TES-1 knockdown inhibited cell proliferation and cell cycle

CCK8 assay was performed to investigate the cell growth. Although cell

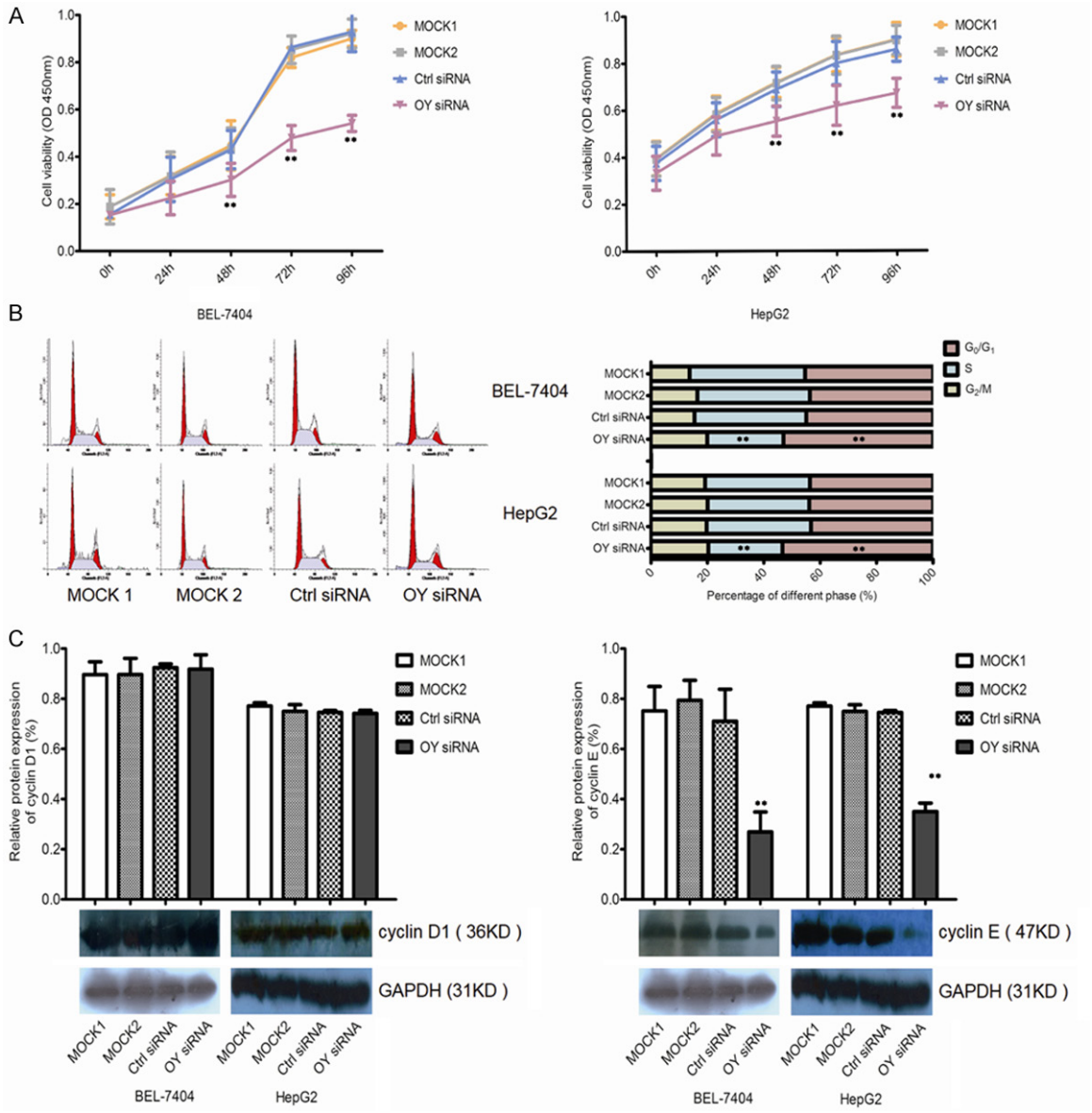

Figure 4. OY-TES-1 knockdown inhibited the proliferation and cell cycle by down-regulating cylin E. A. CCK8 assay of cell viability. The viability of OY siRNA group cells was significantly reduced compared to control since 48 h. B. Flow cytometric analysis of cell cycle and the percentage of different phase. C. Western blot analysis of cyclin D1 and cyclin E levels. The columns show the mean for three separate experiments; bars, sd. $^{**}P < 0.01$.

growth was not affected after OY siRNA treatment for 24 hours, the cell viability was significantly reduced at the time points of 48 h, 72 h and 96 h compared to the controls (**Figure 4A**).

To determine whether the growth-inhibitory effects of OY-TES-1 could result from changes in the cell cycle, flow cytometry was used to analyze the cell cycle. The result showed that suppression of OY-TES-1 caused a significant decrease in S phase in both cell lines, along with a concomitant accumulation of cells in G_0/G_1 phase as compared to the controls ($P < 0.01$). No significant difference was observed in the proportion of cells proportion in the G_2/M phase (**Figure 4B**); thereby, the cell cycle was blocked in the G_0/G_1 phase.

As the results described above, we then detected the expression of cyclin E and cyclin D1, respectively. As shown in **Figure 4C**, the expression of cycin E was significantly reduced in par-

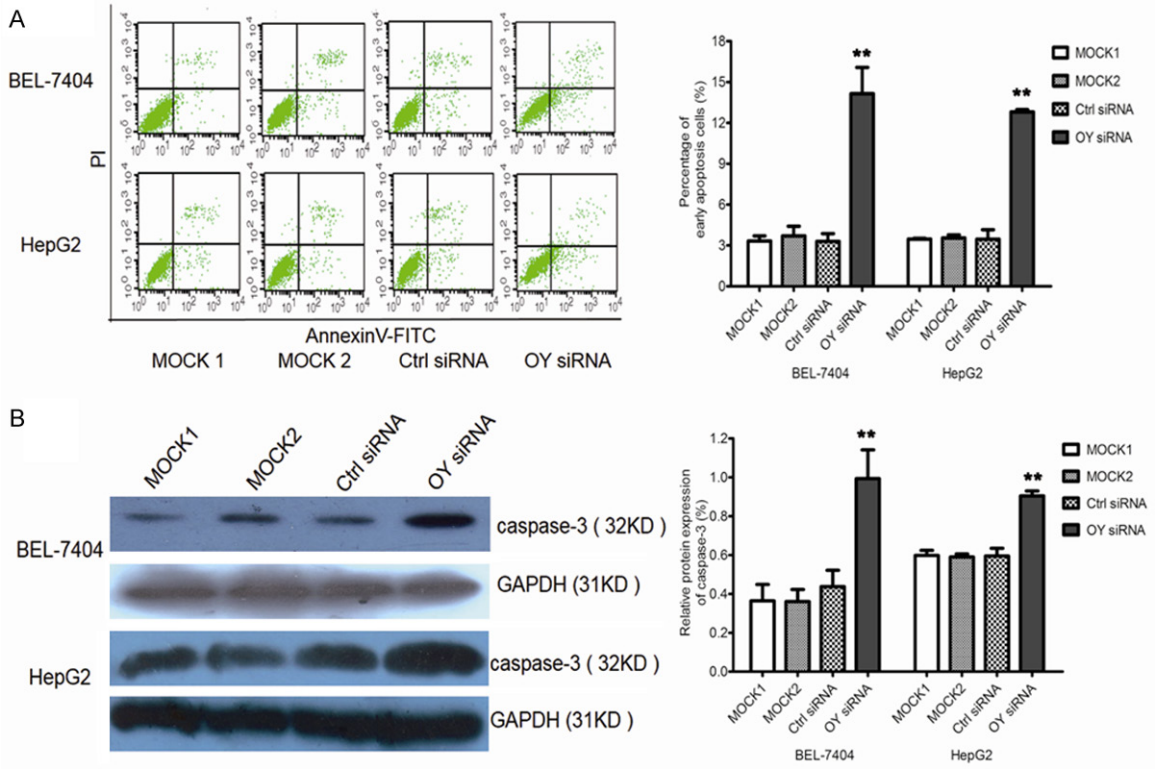

Figure 5. OY-TES-1 knockdown enhanced the cell apoptosis by up-regulating caspase-3. A. Flow cytometric analysis of cell apoptosis and the percentage of early apoptosis cells. B. Western blot analysis of caspase-3 levels. The columns show the mean for three separate experiments; bars, sd. $**P < 0.01$.

allel to the down-regulation of OY-TES-1, whereas cyclin D1 did not show any significance change in both of cells treated with OY siRNA.

OY-TES-1 knockdown enhanced apoptosis

As OY-TES-1 knockdown resulted in cell growth delay and cell cycle arrest, we further used flow cytometric analysis to detect apoptosis with AnnexinV-FITC/PI double staining. The result revealed that AnnexinV-FITC positive cells increased remarkably after OY-TES-1 knockdown (**Figure 5A**). Then, caspase-3, a protein related to apoptosis, was also examined. Up-regulation of caspase-3 expression was demonstrated in cells transfected with OY siRNA but not in controls ($P < 0.01$) (**Figure 5B**).

OY-TES-1 knockdown inhibited cell migration and invasion

In the wound healing assay and Transwell assay performed, suppression of OY-TES-1 resulted that wound healing capacity of cells was decreased and fewer cells migrated into the lower chamber in the Transwell assay (**Figure 6A**). These findings indicated that down-regulation of OY-TES-1 inhibited migration capability of tumor cells.

Then, cell invasion was examined by Matrigel invasion assay and the results showed that the down-regulation of OY-TES-1 significantly suppressed cell invasion (**Figure 6B**). As MMPs play critical roles in the extracellular matrix degradation and are considered closely relate to tumor cells invasion, we detected the expression of MMP2 and MMP9 by Western blot. The results demonstrated that down-regulation of OY-TES-1 was accompany with decrease of MMP2 as well as MMP9 (**Figure 6C**).

OY-TES-1 knockdown did not affect cell adhesion

We finally determined whether OY-TES-1 might play a role in cell adhesion. There was no difference observed in any groups after 60 min, 90 min and 120 min of incubation ($P > 0.05$) (data not shown). This result indicated that treatment of cells with OY siRNA did not affect cell adhesion.

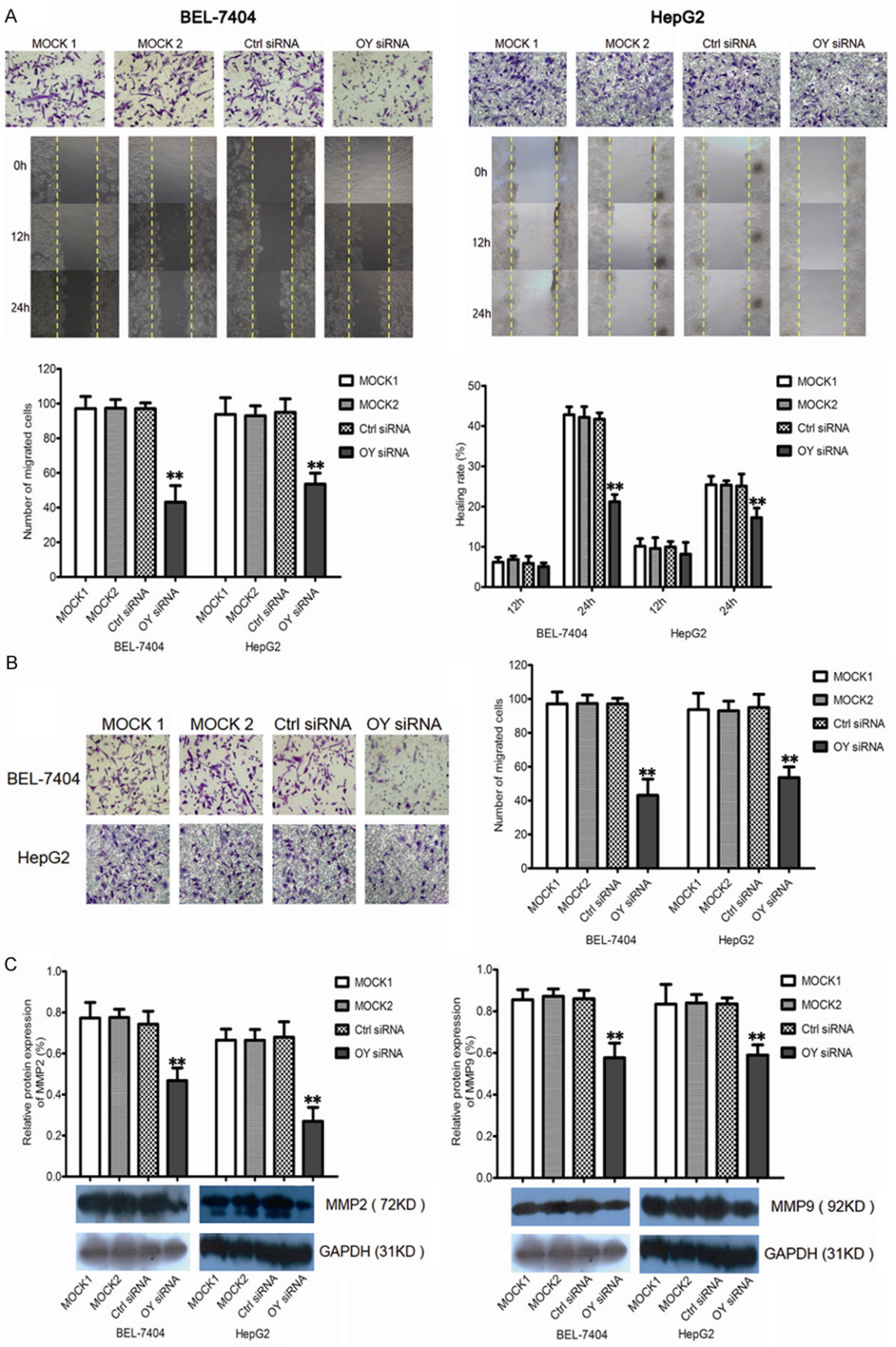
A
BEL-7404
HepG2
MOCK 1
MOCK 2
Ctrl siRNA
OY siRNA
0h
12h
24h
Number of migrated cells
Healing rate (%)
MOCK1
MOCK2
Ctrl siRNA
OY siRNA
BEL-7404
HepG2
B
C
Relative protein expression of MMP2 (%)
Relative protein expression of MMP9 (%)
MMP2 (72KD)
MMP9 (92KD)
GAPDH (31KD)

Figure 6. OY-TES-1 knockdown inhibited motor capability. A. Wound healing assay and Transwell migration assay. B. Matrigel invasion assay. C. Western blot analysis of MMP2 and MMP9 expression levels. The columns show the mean for three separate experiments; bars, sd. $^{**}P < 0.01$.

Discussion

In this study we performed the analysis of OY-TES-1 function in HCC. OY-TES-1, as one of CT antigens, was expressed in a wide range of tumor types including liver carcinoma, but restrictively expressed in normal tissues other than testis [10, 11]. Activation of OY-TES-1 suggests that its specific expression may contribute to the development and progression of malignances including HCC. Therefore, we assessed the function of OY-TES-1 and potential significance in HCC with the RNAi method. The OY-TES-1 siRNA sequence designed according to the OY-TES-1 coding sequence. The effectiveness of OY-TES-1 siRNA was confirmed in previous research [16]. We established three control groups in our research, and confirmed that the transfection reagents and unrelated siRNA have no cell toxicity and effect to cell biological behavior.

Knock-down of OY-TES-1 resulted in a significant growth-suppressive effect, manifested by cell viability and cell cycle arrest in the G_0/G_1 phase. It is widely accepted that altered expression of genes that drive uncontrolled cell cycle progression is among the requisite events during tumorigenesis [19]. The progression of the cell cycle is regulated by many factors, including cyclins, CDKs (cyclin-dependent kinases), and CDKs inhibitors [20]. According a previous report based on a bioinformatic analysis combined with RNAi and oligonucleotide microarray, it was predicted that downregulation of OY-TES-1 was accompanied by an increase in cyclin D2 and cyclin D3, which are able to improve G_1-S transition [21, 22]. The other hand, WEE1, as a negative regulator of G_2-M transition [23], also increased. That report inferred that OY-TES-1 downregulation will accelerate the cell cycle by improve G_1-S transition and block G_2-M transition [24]. As the prediction was discrepant with our research found, we decide choose other cell cycle involved genes to detection. Cyclin D1 plays distinct roles in cell cycle progression through the G_1 phase [25], its over-expression disrupts normal cell cycle controls and might play an important role in the development of a subset of human HCC [26], and its down-expression correlates with G_0/G_1 cell cycle arrest [27]. In the other hand, cyclin E, a cell cycle regulator at the late G_1 stage contributing to G_1 progression and chromosomal instability, is also an oncoprotein of HCC. cyclin E may involve information and progression and patient's outcome in the HCC [28-30]. In current study, we only found that the expression of cyclin E reduced in HCC cell lines when OY-TES-1 was down-regulated, while cyclin D1 remained intact. There may be possibility that down-regulation of OY-TES-1 in HCC cells might impair cyclin E synthesis ultimately leading to slowed cell growth and cell cycle block. We thereby propose that OY-TES-1 might be an upstream modulator of cyclin E. Low levels of G_1-checkpoint proteins such as cyclin E and other unknown regulators might attenuate the oncogenic phenotype of HCC cell lines. However, it remains unclear for the underlying mechanisms.

Apoptosis and proliferation are opposing processes in tumor growth, and tumor progression is thought to be determined by the balance with apoptosis and proliferation. To certify whether the observed cell growth suppression by OY-TES-1 down-regulation was due to the induction of cell apoptosis, the cell apoptosis was then tested by Flow cytometry. Suppressed expression of OY-TES-1 in HCC cell lines resulted in a significance increase of apoptotic cell population as compared to controls, which was in agreement with increased expression of caspase-3, one of the apoptosis markers in caspase protease family [31, 32]. Whether other caspase protease family, such as caspase-9, Bcl-2 and Bax [33-35], involved in apoptosis induced by OY-TES-1 siRNA is not well understood and needs to be further investigated.

Metastasis is a major cause of death in cancer. Here, we first demonstrate the down-regulation of OY-TES-1 may contribute to attenuation of metastasis capacity of HCC cell lines via at least two aspects: the promotion of cell migration and invasiveness in vitro. It was well-known that one of molecular basis related to the migration and invasiveness in tumor cells was MMP. MMP is a family of related zinc-dependent proteinase that degrades extracellular matrix (ECM) with nearly 20 members reported

[36, 37]. Among them, MMP2 and MMP9 play the most important role in tumor metastasis and invasiveness. Alone this line, we tested MMP2 and MMP9 in OY-TES-1 down-regulated HCC cell lines clearly indicating that OY-TES-1 is involved in the regulation of both migration and invasiveness through decreased expression of MMP2 and MMP9. There were evidences that MMP9 not only promotes invasiveness, but also affects proliferation and induces apoptosis in various tumor cells [38, 39]. Therefore, we infer that weakened expression of MMP9 may also involve in arresting the cell growth and increasing apoptosis in HCC cells by OY-TES-1 down-regulation.

In summary, our results demonstrated that blocking the expression of OY-TES-1 with siRNA decreased cell growth, induced the block of cell cycle and apoptosis, and prevented migration and invasion in HCC cells in vitro. These findings imply that OY-TES-1 gene functions as an oncogene in HCC, and may shed light on the gene therapy of OY-TES-1 in HCC.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (No. 81360371), Natural Science Foundation of Guangxi (No. 2014GXNSFAA118089, No. 2014GXNSFAA118231, and No. 2012GXNSFAA053114) and Innovative project for Postgraduate of Guangxi Educational Bureau (No. YCSZ2014103).

Disclosure of conflict of interest

None.

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