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Research Article

Implications of TRAP1 Modulate Cell Invasion and Epithelial–Mesenchymal Transition in Liver Cancer: Evidence from *in vitro* HepG2 Study

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Abstract

Background: Although the list of biomarkers is still growing, the molecular mechanisms associated with liver cancer development and progress are still uncertain. We recently confirmed the high expression of molecular chaperone TRAP1 in HepG2 cells. In this context, the aim of this study is to investigate the effects of gain or loss function of TRAP1 on cell invasion in HepG2 cells, and to evaluate whether TRAP1 regulate cell invasion by controlling EMT related gene expression.

Methods: The TRAP1 overexpression and knockdown HepG2 cells were constructed by lentivirus method. To investigate the effects of TRAP1 on cell function, cell proliferation was measured by MTT method. Cell apoptosis was detected by flow cytometry. The migration and invasion ability were measured by wound healing assay and transwell migration assay. mRNA and protein expression of TRAP1 and EMT related genes were performed by qRT-PCR and western blot, respectively.

Results: TRAP1 overexpression and knockdown cells were successfully established in two recombined cell lines, we studied the general effects of TRAP1 and its association to EMT pathway. The results indicated that TRAP1 promoted cell malignant phenotypes, which including cell proliferation, apoptosis and migration. Besides, TRAP1 inhibited the epithelial markers expression and increased mesenchymal markers expression in both mRNA and protein levels.

Conclusions: These results may provide novel approaches in liver cancer with increasing TRAP1 expression, which could be associated with EMT pathway, with potential future intervention in liver cancer invasion and metastasis.

Keywords: TRAP1; Epithelial–mesenchymal transition; Liver cancer; HepG2 cells

Introduction

The development of liver cancer is considered a multi-gene, multi-path and multi-stage related disease [1], specific molecular mechanisms driving liver cancer invasion and metastasis are critical to find novel targets and finally achieve cure for liver cancer. Among the phenomenon and processes in liver cancer, the epithelial–mesenchymal transition (EMT) has been suggested to play a fundamental role in tumor development [2-3].

EMT is a mechanism that drives epithelial cells to differentiate into mesenchymal-like or mesenchymal phenotypes, depending on the way in which they are differentiated. EMT-induced changes in epithelial plasticity can be demonstrated by the down-regulated epithelial markers, such as E-cadherin, the adhesion component, and the up-regulation of the expression of mesenchymal proteins such as vimentin, N-cadherin, α-SMA, snail (sna1) and slug (sna2)

[4-5]. EMT appears to play a key role in pathological processes including cancer development and metastases of liver cancer [2,6,7]. Thus, EMT regulators may serve as prognostic biomarkers and potential therapeutic strategies in the systemic treatment of liver cancer.

Tumor necrosis factor receptor-associated protein-1 (TRAP1) is a member of heat shock protein 90 family. It plays a role in embryogenesis, tissue development, and pathological processes including cancer progression and metastasis [8-10]. In fact, TRAP1 functions in the processes of local cancer progression towards metastatic spread disease and lower TRAP1 expression was correlated to increased overall survival in cancer patients [11-13]. In particular, TRAP1 can act as a molecular chaperone in the regulation of cancer cell energy metabolism and it is vital factor in abnormal proliferation, drug tolerance and activation of anti-apoptotic signaling pathways in tumor cells [14-15]. Recent study indicated that TRAP1 downregulation in human ovarian cancer enhances invasion and EMT [16-18]. Thus, TRAP1 may be a novel and efficient therapeutic target in EMT. We recently demonstrated that hepatocellular carcinoma cells, HepG2, with medium invasive ability expressed high levels of TRAP1 [19-21].

In this context, we hypothesized that TRAP1 may play a role in liver cancer invasion via EMT. To verify this hypothesis, the aim of this study was to investigate the effects of gain or loss function of TRAP1 on cell invasion in HepG2 cell line in vitro, and evaluate whether TRAP1 regulate cell invasion by controlling EMT related gene expression.

Materials and Methods

Cells and reagents

Human hepatoma cell line HepG2 and Huh7; DMEM high glucose medium (Gibco); trypsin (Life Technologies); fetal bovine serum (Sera Pro); apoptosis detection kit (BD); Matrigel glue (Corning); Transwell Permeable Supports (Corning); mouse anti-β-actin monoclonal antibody and HRP-labeled goat anti-mouse IgG (Beijing Zhongshan Jinqiao), TRAP1 rabbit anti-human polyclonal antibody and HRP-labeled rabbit anti-mouse IgG (Cell Signaling Technology); Highly sensitive chemiluminescence detection kit (Shanghai Qihai Futai Co., Ltd.); TRizol and DEPC (Invitrogen); RT-PCR kit (TOYOBO), QPCR kit (Tiangen); MTT and dimethyl sulfoxide (DMSO) (Sigma); DAPI (Sigma); 4% paraformaldehyde (Solarbio); Exa Fluor 594-conjugate Goat anti-Rabbit IgG (H+Lp) (Proteintech); Triton X-100 (Solarbio).

Cell culture

HepG2 and Huh7 (purchased from Guangzhou Saiku Bio) was cultured in a DMEM high-sugar medium containing 10% inactivated fetal bovine serum at 37 ℃, saturated humidity, and 5% CO_2, and passaged every 2 to 3 days. Logarithmic growth phase cells were used for experiments.

Cell transfection

TRAP1 transient silencing (KD) was performed using 75 nM siRNAs target sequence, sense strand, 5′-CGGUCCCUGUACUCAGAAATT-3′, antisense strand, 5′-CGG UCC CUG UAC UCAG AAA TT-3′. Cells were transfected with a similar amount of control siRNA as control groups (KD NC). Transient transfections of siRNAs were performed using FlexiTube Transfection Reagent (Qiagen Cat. No. 1027420) according to the manufacturer's protocol. Transient overexpression (OE) of the pCMV6-TRAP1 was performed using the Neon transfection system (Invitrogen). The control groups of overexpression (OE NC) were established with a similar amount of non-targeting empty plasmid. The HepG2 and Huh7 cells were plated into 24-well plates, and evenly adhered to the bottom of the plate after incubated overnight at 37 ℃ with shaking. Cells were in a sub confluent state on the second day. The virus was prepared and slowly melted on the ice, then we discarded the original culture medium, and add 250 μl volume of the culture medium (the culture medium is mixed with the virus stock solution and the fresh culture solution of Polybrene). Inoculation at 37 ℃ for 4 h, 4 h after the completion of the culture solution to a normal volume of 500 μl. 24 h after infection, fresh whole culture medium was replaced and culture was continued at 37 ℃. After 48 hours of transfection, the fresh complete growth culture medium containing Puromycin was used for maintenance screening culture. After confirmed at mRNA and protein expression levels, they were used in subsequent experiments.

Measurement of cell proliferation

Cells in logarithmic growth phase were seeded in 96-well plates at a cell density of 1×10^4/well. After incubation for 0 h, 24 h, 48 h, 72 h, and 96 h, 20 μl of MTT solution (5 mg/ml) was added to each well and further incubated for 4 h. The supernatant was abandoned, and 100 μl of DMSO was added. After shaking the plates for 5 min to dissolve the formed formazan, absorbance A values of each well were read at 490 nm using a microplate reader. Five replicate wells were set in each group and the experiment was repeated three times. The growth curve of the cells is plotted based on absorbance and time.

Cell apoptosis assay

HepG2 or Huh7 cells were seeded in a 6-well plate and grown to 80% confluence. Adherent cells were collected by EDTA-free trypsin digestion. After centrifugation at 1000 rpm for 3 min, cells were washed twice with PBS. About $1\sim5\times10^5$ cells per well were collected. Cells were resuspended with 100 ul of Binding Buffer. 5 ul of Annexin V-FITC/ Propidium Iodide was added and mixed well Samples were analyzed with BD FACSAria III flow cytometer within one hour.

Wound healing assay

HepG2 or Huh7 cells were seeded in 6-well plates at a cell seeding density of 5×10^5 /well. After incubation at 37 ℃ for 24 h, cells were scratched with a pipette tip was scratched, and washed with PBS for 3~5 times to completely remove the exfoliated cells. Subsequently, 2 ml of basal medium was added and cells were cultured in 37℃, 5% CO_2 incubator. Representative images of wound healing were taken at 0h, 24h, 48h, 72h and 96h after the wound scratched. The images were processed with ImageJ software to measure the scratch area.

Cell invasion assay

HepG2 or Huh7 cells were cultured in DMEM supplemented with 10% fetal bovine serum for 12 h. The upper transwell chambers were pretreated by adding 300 µl of basal medium containing Matrigel and incubating for 1 h before discarding the culture. 500 µl of medium containing 15% fetal bovine serum was added to the lower chambers. The cells were harvested and resuspended in serum-free medium containing 0.05% ~ 0.2% BSA and seeded on to the upper chambers at a density of 2×10^4 cells/well. After 36 h of incubation, the upper chambers were carefully removed. The cells attached to the upper chambers were wiped with a wet cotton swab and the cells on the surface of the lower chambers were fixed with methanol for 20 min at room temperature, stained with crystal violet, washed three times with PBS. Five fields of view were randomly counted, and two parallel wells were set up in each group of cells. This experiment was repeated three times.

Quantitative *Real-time PCR (q*RT-PCR)

In the logarithmic growth phase, HepG2 cells were digested with trypsin, and total RNA was extracted by adding Trizol. The cDNA was synthesized by reverse transcription and detected by real-time PCR. Primer design was performed using primer premier 5.0 (Table 1), and ABI7500 instrument was used for detection. The amplification conditions were: 55 °C for 10 min, 95 °C for 30 s, 95 °C for 30 s, 55.0 °C for 30 s, 72 °C for 1 min, and 60 °C for 1 min for a total of 40 cycles. GAPDH was used as an internal reference, and the results were analyzed by the $2^{-\Delta\Delta Ct}$ method, and the experiment was repeated three times.

Gene	Primers	Sequence(5'－3')
TRAP1	F	CAGGGTTCCACTTCCAAACA
	R	TGGAGATCAGCTCCCGTATAA
GAPDH	F	GGAGGAGTGGGTGTCGCTGT
	R	GTGGACCTGACCTGCCGTCT
Vimentin	F	GACGCCATCAACACCGAGTT
	R	CTTTGTCGTTGGTTAGCTGGT
E-Cadherin	F	CGAGAGCTACACGTTCACGG
	R	GGGTGTCGAGGGAAAAATAGG
AGO2	F	CGTGCCTGCTGGAATGTTTC
	R	CCATCCGTGAGGCCTGTATC
UBC	F	GGGGCACCTCCTATGAGAGAT
	R	TCCCTCCGCAGTTTCTTCCA
VEGF	F	CTACCTCCACCATGCCAAGT
	R	AGCTGCGCTGATAGACATCC
ErbB2	F	TGCAGGGAAACCTGGAACTC
	R	ACAGGGGTGGTATTGTTCAGC
Snail	F	TCGGAAGCCTAACTACAGCGA
	R	AGATGAGCATTGGCAGCGAG

Table 1: TRAP1 and GAPDH Primer Sequences.

Western blot analysis

Logarithmic growth phase HepG2 cells were digested with trypsin, lysed with lysate, and total protein was extracted. Samples were processed to 10% SDS-PAGE electrophoresis to separate the proteins and transferred to the PVDF membrane. The membrane was blocked with 5% fetal bovine serum albumin for 1 hour and incubated with β-actin or TRAP1 primary antibodies overnight at 4 °C. After rinsed 3 times with TBST; the membrane was incubated with secondary antibody at 37 °C for 1h. The bound antibodies were detected with ECL illuminates, and the intensity of the protein was measured by Image J software for gray value analysis.

Statistical analysis

Results Statistical analysis was performed using SPSS 22.0 software. The measurement data were expressed as mean ± sd. The comparison was performed by t-test. The statistically significant difference was $P < 0.05$.

Results

TRAP1 enhanced the cell proliferation in HepG2 and Huh7 cells

The results of qPCR and WB showed that TRAP1 overexpression (OE) and knockdown (KD) cells were established in Huh7 (upper) and HepG2 (lower), as showed in Figure 1 A and Figure 1 B. The results of MTT assay showed that comparing with the wild-type (WT) cells, TRAP1 overexpression (OE) significantly enhanced cell proliferation and knockdown of TRAP1 (KD) significantly inhibited cell proliferation, especially on day 5. The proliferation rate of OE was 1.320 and 1.236 times higher than that of WT or NC (P<0.001), and 1.977 and 1.677 times higher than that of KD in HepG2 and Huh7 cells which shown in Figure 1C.

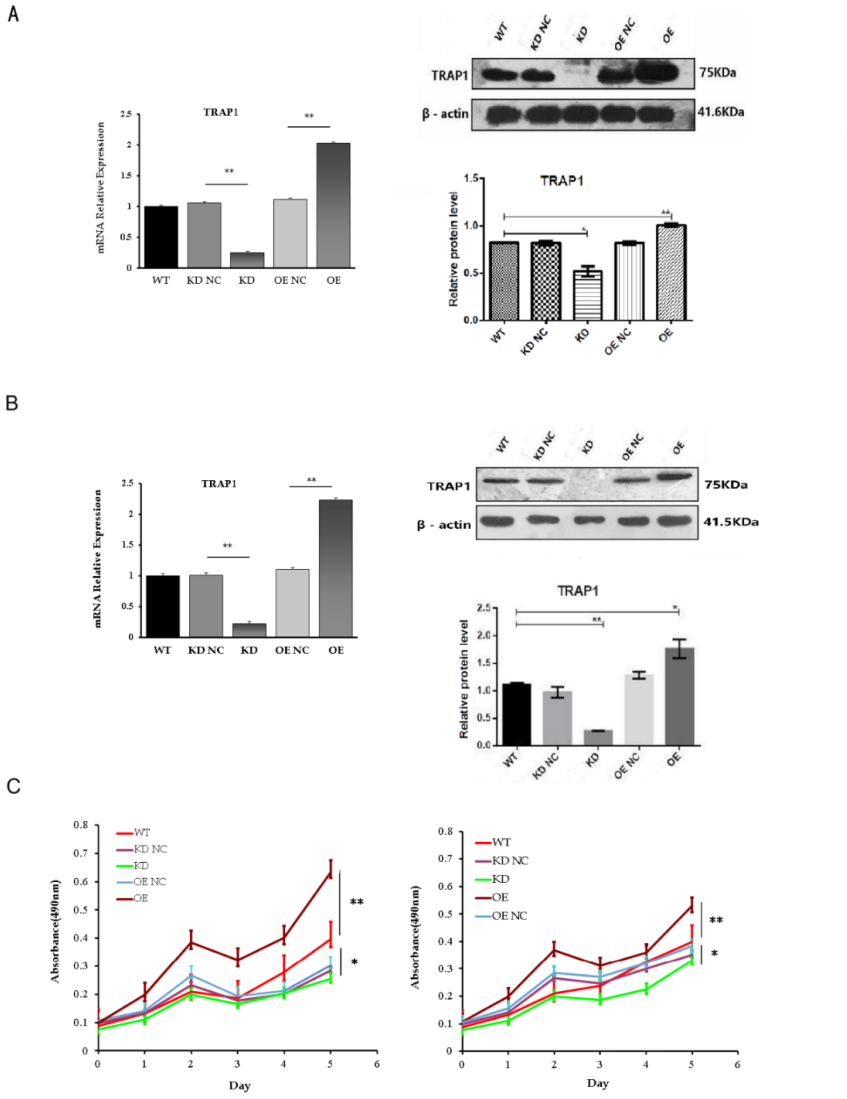


Figure 1: TRAP1 affected the cell proliferation in different groups of Huh7 (upper) and HepG2 (lower) cells. (A and B) Huh7and HepG2 cells were stably transfected with TRAP1 shRNA (OE) and knocked out TRAP1 gene (KD) separately, and established OENC and KDNC groups with both of empty plasmid control (NC), western blotting and real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) were used to quantify the expression of TRAP1 after lentiviral interference. (C) A microplate reader was applied to detect the absorbance value at 490 nm wavelength and growth curve of each group cells was plotted to visualize and analysis (left HepG2; right Huh7). *p<0.05, **p<0.01

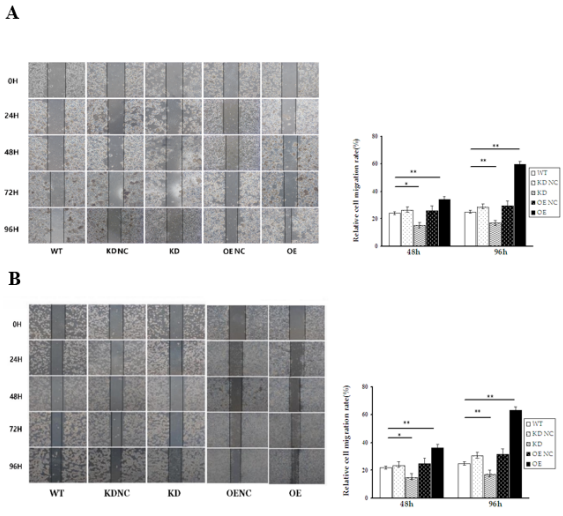


Figure 2: Comparison of the migration ability of transfected cells by scratch assay. Images of cell scratching were taken at 0, 24, 48, 72, 96 hours and the cell scratch area were analyzed with ImageJ in Figure 2 (upper HepG2, lower Huh7). $^{**}p<0.001$.

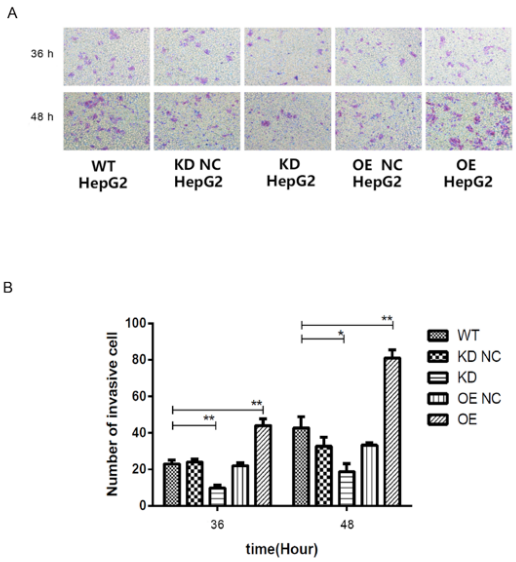


Figure 3: The migration ability of HepG2 cells detected by Transwell invasion. Cell migration ability was observed in five randomly selected fields of cell passed through the membrane with inverted microscope, and plotted to analyze the TRAP1 effect on the cell transmembrane migration ability after 36 and 48 h. ($p<0.001$).

TRAP1 promoted cell migration activity

Wound healing percentage was calculated by the following equation: (scratch area of 0h – scratch area of 48h or 96h) / scratch area of 0h) %. The healing percentage was 24.26 ± 0.1565(%), 26.13 ± 0.1674(%), 13.27 ± 2.050(%), 26.47 ± 0.5561(%) and 36.68 ± 1.714(%), for WT, KDNC, KD, OENC and OE HepG2 cells, and 21.33 ± 0.1125(%), 22.43 ± 0.1455(%), 15.62 ± 2.104(%), 25.21 ± 0.1522(%) and 37.16 ± 1.434(%), for WT, KDNC, KD, OENC and OE Huh7 cells respectively, after 48h incubation, 31.10 ± 1.093(%), 35.23 ± 1.176(%), 21.36 ± 1.045(%), 35.61 ± 1.123(%) and 61.41 ± 1.137(%) in HepG2 cells and 31.87 ± 1.412(%), 36.56 ± 1.243(%), 28.23 ± 1.115(%), 36.89 ± 1.332(%) and 62.21 ± 1.137(%) in Huh7 cells after 96h incubation. The percentage of cell wound healing

at 48h and 96h was statistically significant ($P < 0.001$) comparing to that of 0h, and the results are shown in Figure 2 (A, HepG2, B, Huh7). The results indicated that the migration ability of HepG2 and Huh7 cells may be related to the expression of TRAP1.

TRAP1 prompted the cell invasion ability in HepG2 cells

Using Transwell chamber coated with extracellular matrix to detect cell invasion ability. After an incubation of 36h, the percent of cells that invaded through were 23.00 ± 1.528(%), 23.42 ± 1.332(%), 9.667 ± 1.202(%), 22.84 ± 1.212(%) and 44.00 ± 2.646(%); for WT, KDNC, KD, OENC and OE HepG2 cells, respectively. After an incubation of 48h, the corresponding percentages were 42.67 ± 4.372 (%), 33.24 ± 3.042 (%), 18.67 ± 3.180 (%), 34.26± 2.542 (%) and 80.85 ± 3.215(%), respectively. The cell invasion activity in 36h and 48h were statistically significant ($P<0.001$). The results showed that TRAP1 overexpression promoted the cell invasive ability, and the invasion ability of HepG2 cells may be positively correlated with the expression of TRAP1. The results are shown in Figure 3.

TRAP1 inhibited the cell apoptosis in HepG2 cells

Cells apoptosis was detected by flow cytometry expressed with apoptosis rate (%). The experiment was independently repeated for three times. A highest rate, 14.180 ± 0.2082(%) of apoptosis was observed in down-regulated TRAP1 (KD) and lowest rate, 3.333 ± 0.1453(%) of apoptosis in overexpressed TRAP1 (OE) cells compared with the apoptosis rate in wild-type HepG2 cells 6.872 ± 0.1253(%), There was a statistically significant difference in the apoptosis rate among the three groups as shown in Figure 4 ($P < 0.001$).

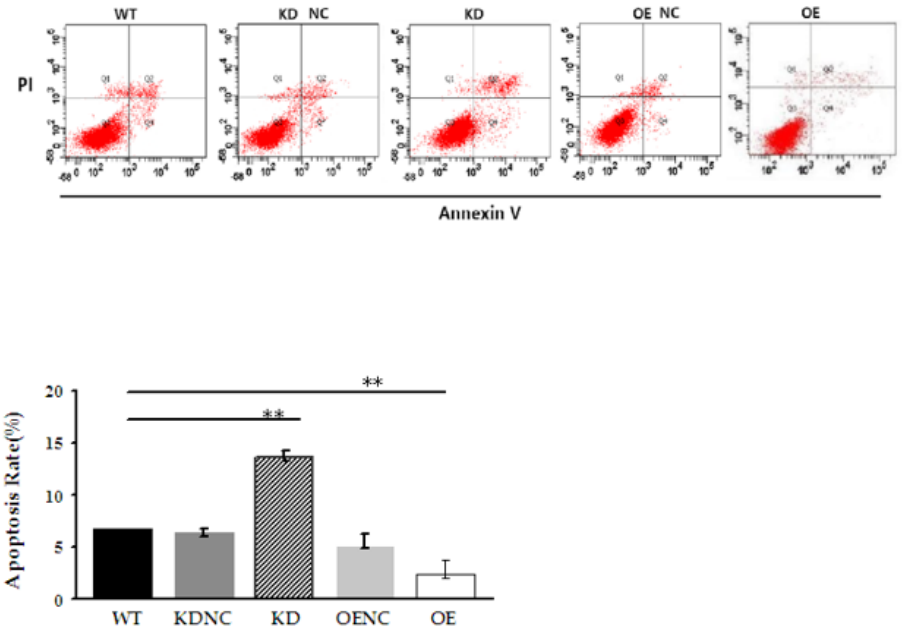


Figure 4: Flow cytometry to detect the effect of TRAP1 on apoptosis of transfected HepG2 cells. Apoptotic cells were calculated on the right half of the panel. The cell apoptosis rate was 6.08±0.12%, 5.96±0.54%, 14.63±0.63%, 4.23±0.56%, 2.93±0.66% for WT, KDNC, KD, OENC and OE respectively. $^{**}p<0.001$.

TRAP1 regulated EMT-related gene expression in HepG2 cells

EMT-related genes were analyzed by western blot. The results showed that the protein levels of E-cadherin were inversely correlated with the changes in the expression of TRAP1. The protein levels of E-Cadherin in cells overexpressed with TRAP1 were significantly lower than that of WT or NC HepG2 cells ($P<0.01$), while knockdown of TRAP1 caused an elevated E-Cadherin expression in HepG2 cells ($P<0.01$). The protein levels of Vimentin changed in a different trend, that the highest protein level was observed in cells overexpressed with TRAP1 ($P<0.01$). As shown in Figure 5

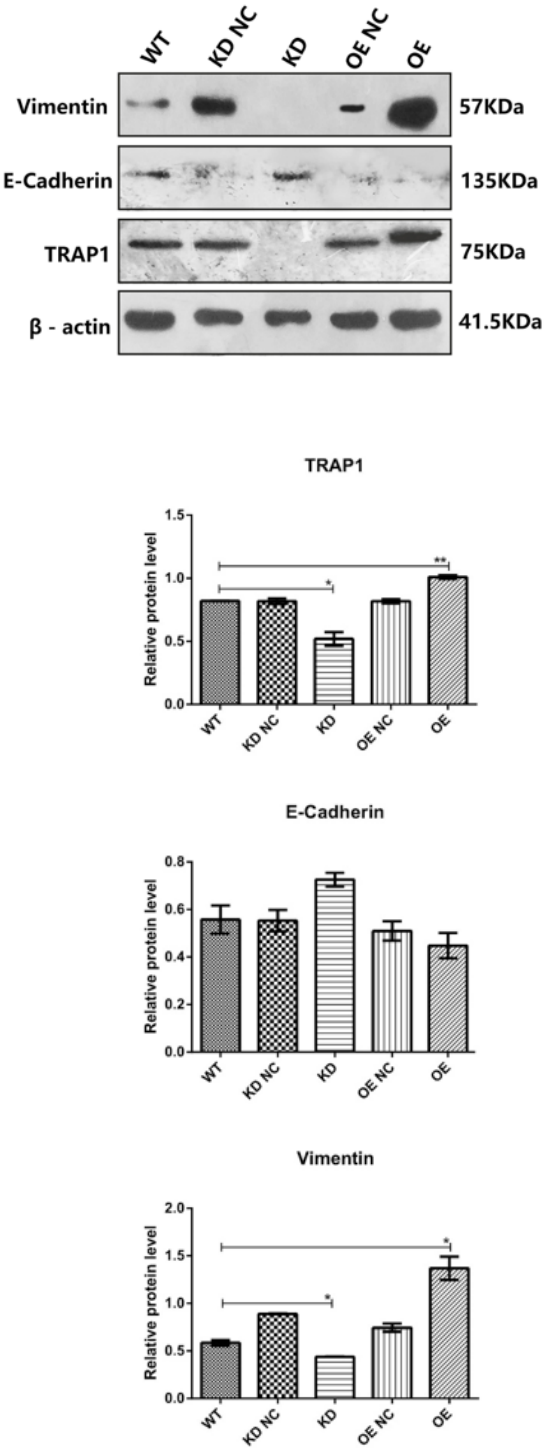
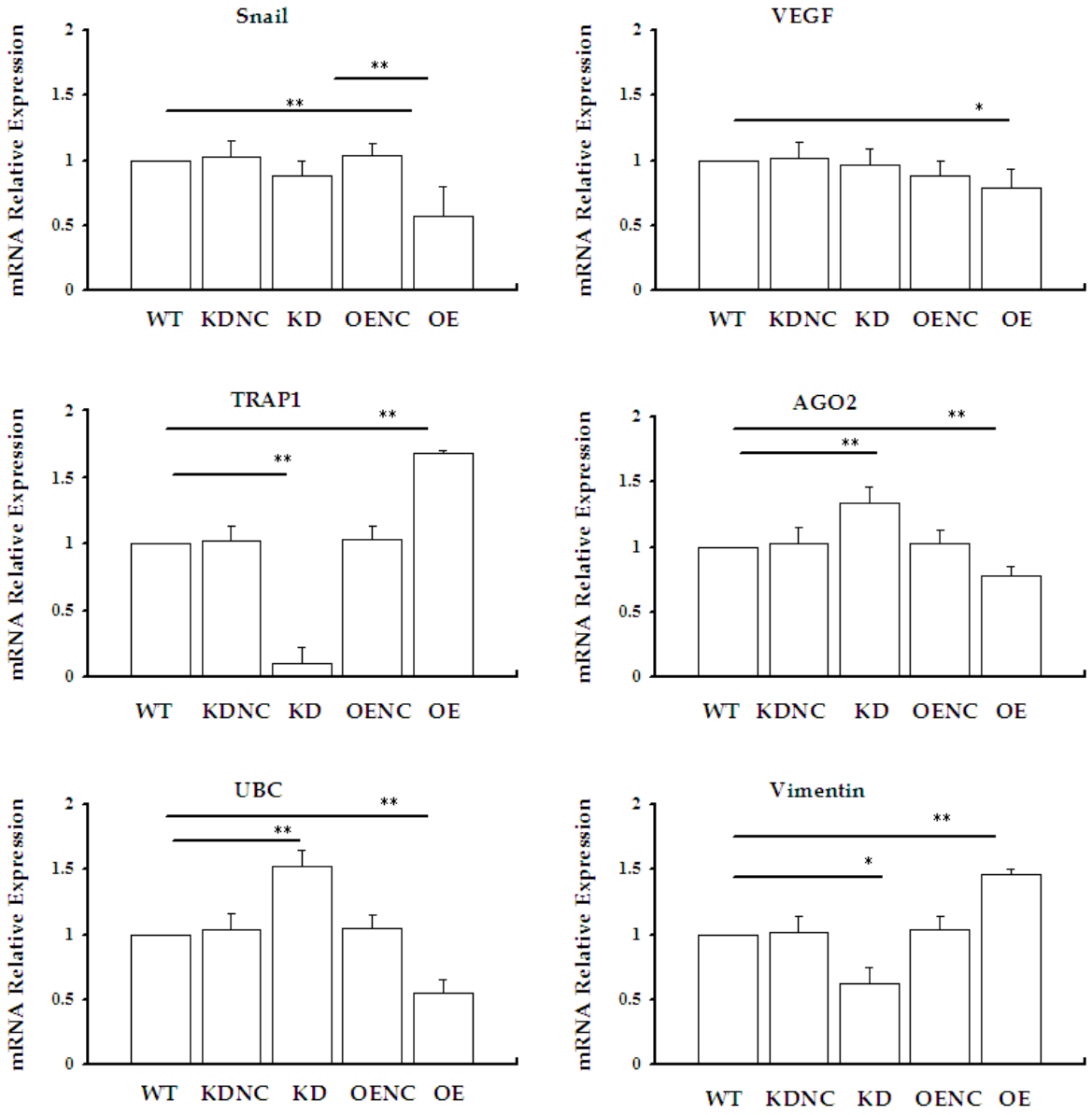


Figure 5: EMT-related gene expression in WB was associated with TRAP1 in HepG2 cells. Anti-Vimentin and E-cadherin antibody were applied to detect the protein expression of Vimentin and E-cadherin in WB. ** $p<0.01$.

TRAP1 affected tumor related gene expression between different signal transduction pathways

A crosstalk of different signal transduction pathways, including SNAIL, ErbB2Ago2, VEGF gene which related to the angiogenesis, oxidative stress damage, apoptosis, and metabolism, were evaluated with real-PCR on the mRNA level . TRAP1 affected expression levels of these gene (Figure 6).



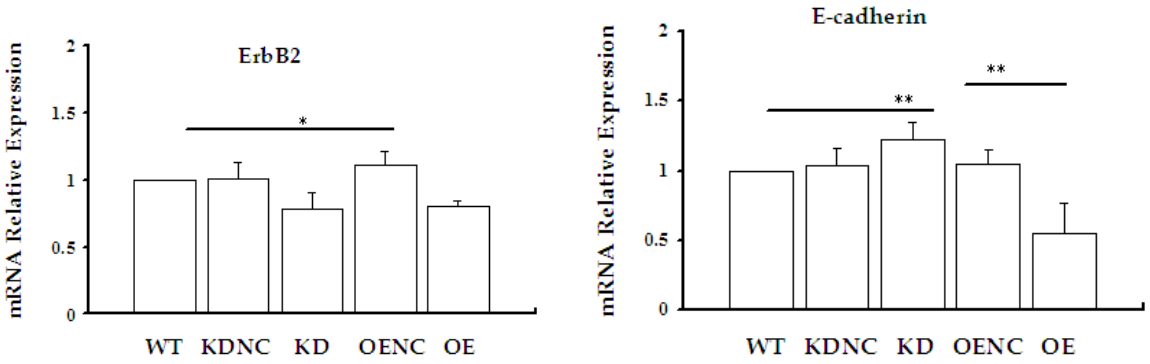


Figure 6: TRAP1 affected mRNA levels of tumor related gene expression between different signal transduction pathways was done by qRT-PCR.

Statistical Analysis

All P-values were two sided, and $p<0.05$ was considered to indicate a statistically significant difference. The experimental data were expressed as the mean ± standard deviation, and analysis of variance with an LSD t-test was used to assess the relationship between two groups. Statistical analyses were performed using the SPSS v20.0 software (SPSS, Inc., Chicago, IL, USA).

Discussion

Liver cancer is one of the most common malignant tumors worldwide. Progresses in investigating the specific molecular mechanisms driving liver cancer invasion are critical in finding new target and finally achieving the goal of completely cure of liver cancer. In this study, we used HepG2 cells to study the function of TRAP1 in cell invasion and evaluated its association with EMT process.

TRAP1 is mainly expressed on the inner mitochondrial membrane and plays an important role in maintaining mitochondrial integrity and function, regulating mitochondrial apoptosis, and participating in the regulation of mitochondrial respiration and energy metabolism [22-24]. TRAP1 has been considered uniformly oncogenic in a variety of cancer types. Previous studies have shown that the expression of TRAP1 is up-regulated in various cancers including colon cancer, breast cancer, prostate cancer and lung cancer [23] and the abnormal expression is closely related to the occurrence and development of tumors. Intervention of TRAP1 function can lead to the death of tumor cells, but has no effect on normal cell types. Overexpression of TRAP1 gene in ovarian cancer cell line MCF-7 causes inhibition of mitochondrial aerobic respiration, shift of tumor cell energy metabolism, and alterations in tumor malignant biological behavior [24]. TRAP1 is overexpressed in epithelial ovarian cancer, particularly in poorly differentiated types, and the overexpression is associated with advanced PT, FIGO stage, lymph node metastasis, and distant metastasis, resulting in an increase in its malignant phenotype [25]. Overexpression of TRAP1 is involved in local infiltration of colorectal cancer, contributes to disease progression and worse specific survival. In non-small cell lung cancer, up-regulation of TRAP1 expression leads to an increased risk of recurrence of the disease [26-27]. Some data suggest that although cell viability was suppressed, metastasis was increased and resistance to normal conditions impairing cell migration was induced in prostate and colorectal cancer patients with high TRAP1 expression. In the present study, we firstly explored the general effects of TRAP1 on HepG2 cells and found that TRAP1 promoted the malignant phenotypes of HepG2 by regulating cell proliferation, apoptosis and migration. Our results showed that the expression of TRAP1 was high in HepG2 cells and TRAP1 played an oncogenic role in liver carcinogenesis.

Migration of tumor cells is considered a prerequisite for tumor invasion and metastasis, and the latter is a complex phenomenon involves a variety of environmental conditions and regulatory mechanisms, such as EMT. During the EMT process, the resting epithelial cells of the primary tumor are transformed into mesenchymal cells with high migration ability, offering extremely aggressive phenotype. The epithelial cells undergo EMT changed from an epithelial morphology to a more fibroblastic morphology and from relatively non-motile cell to highly motile cells that able to secrete components of the extracellular matrix. The loss or decrease of E-cadherin is a fundamental event in EMT and the levels of E-cadherin is considered a potential biomarker for EMT [28-30]. Besides of the down-regulation of E-cadherin, the increase of Vimentin in the cytoskeletal component is associated with enhanced migration and invasion ability of tumor cells, and thus also a commonly used biomarker for the occurrence of EMT [31,32]. We found that TRAP1 expression correlated with changes in the expression of the above genes that commonly associated to

EMT. TRAP1 suppressed the expression of epithelial markers and promoted the expression of mesenchymal markers in both mRNA and protein levels [33,34]. These data indicated that TRAP1 regulated cell invasion partly through EMT in HepG2 cells.

In summary, our results suggest that TRAP1 can stimulate liver cancer cells invasion by regulation EMT process. TRAP1 may be a potential candidate biomarker for liver cancer. These results provide relevant theoretical basis for identifying potential therapeutic opportunities in diagnosis and future treatment of liver cancer.

Disclosure

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and material: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests for this research.

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Authors' Contributions: Conceptualization and Methodology, Peipei Yi and Liu Yang; Software and Formal Analysis, Yingqi Zhao; Data Curation; Honglin Zou; Writing – Original Draft Preparation, Hongling Zou; Writing – Review & Editing, Project Administration and Funding Acquisition, Yuanming Lu.

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