Abstract. Certain types of cancer exhibit downregulated expression of zonula occludens-1 (ZO-1), which serves an important function in tumor progression; however, the underlying molecular mechanisms that lead to this downregulation in cancer remain unclear. In the present study, the expression of ZO-1 in liver cancer (LC) tissues was investigated. Western blot and reverse transcription-quantitative polymerase chain reaction assays were used to detect the expression of ZO-1 protein and mRNA in LC tissues and paired adjacent non-tumorous tissues. The results indicated that, compared with non-tumorous tissues, the expression of ZO-1 was significantly downregulated at the protein (P<0.001) and mRNA (P=0.006) levels in LC tissue samples. In addition, various cellular and molecular methods were applied, including MTT, colony formation, flow cytometry and Transwell assays. The results indicated that overexpression of ZO-1 inhibited cell viability, proliferation and migration, and induced G₀/G₁ phase arrest in vitro.

Introduction
Liver cancer (LC) is the sixth most common type of cancer, ranking as high as third for cancer-associated mortality globally (1), and is particularly prevalent in Asia (2). It has been proposed that the incidence and mortality rates of LC has been increasing (3). Owing to the high prevalence of hepatitis B virus (HBV) infection in Chinese populations, HBV-associated liver cirrhosis or LC has become a major disease burden in China (4), accounting for between 75 and 90% of malignant tumors in adult livers (5). Early detection of LC allows for curative or palliative treatment with surgical treatments such as liver resection and liver transplantation (6). However, owing to a lack of detectable early symptoms, insidious onset and its high recurrence rate following surgery, there is a relatively low reported 5-year survival rate (7,8). It is therefore important to develop novel methods to prevent cancer recurrence and improve the prognosis for patients with LC. Although an increasing number of molecular biomarkers with high sensitivity and specificity for LC have been reported, none has so far justified its routine use in clinical practice (9). Furthermore, to the best of our knowledge, there has been no previous investigation of the potential function of zonula occludens-1 (ZO-1) in LC.

ZO-1s are members of the membrane-associated guanylate kinase (MAGUK) protein family, including ZO-1 (10), ZO-2 (11) and ZO-3 (12). ZO-1 is a 220-kDa scaffolding protein which contains various domains (an Src homology 3 domain, three PDZ domains, a proline-rich region and a guanylate kinase domain) that allow its interaction with specialized sites of plasma membrane as well as with other proteins (13,14). ZO-1 is associated directly with actin filaments, anchoring tight junction transmembrane proteins to the actin cytoskeleton (15,16). ZO-1 is a characteristic factor of tight junctions, which has also been demonstrated in epithelial (E)-cadherin junctions (17-19). In addition, it has a scaffolding function, serving an increasingly vital function in signal transduction by clustering critical membrane proteins (20). Deletions or mutations in the ZO-1 gene led to overgrowth, suggesting that ZO-1 may function as a tumor suppressor (13). For example, insulin-like growth factor I receptor (IGF-IR) induces E-cadherin-mediated cell-cell adhesion by upregulating ZO-1 in breast cancer cells. On the other hand, the expression of IGF-IR and ZO-1 increased growth, and survival of the primary tumor may decrease the occurrence of metastasis (21). Decreased ZO-1 expression has been identified to be associated with increased invasiveness in breast cancer (22), colorectal cancer (23) and gastrointestinal

Decreased expression of ZO-1 is associated with tumor metastases in liver cancer

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tumors (24). Furthermore, it is reported that ZO-1 is involved in tumor invasion associated with epithelial-mesenchymal transition processes (25).

In the present study, ZO-1 expression in LC tissue samples was investigated. In addition, the effect of expression of ZO-1 on LC cell viability, proliferation and migration were also investigated. Furthermore, the effects of ZO-1 on the LC cell cycle were also determined in vitro. Taken together, the results of the present study indicated that the ZO-1 gene may act as a tumor suppressor in LC, and serve an important function in LC development and progression.

Materials and methods

Cell culture and transfection. HepG2 cells (an LC cell line) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.), 100 µg/ml penicillin and 100 µg/ml streptomycin. Cells were incubated at 37˚C in a humidified atmosphere containing 5% CO₂. For overexpression of ZO-1, the coding sequence of ZO-1 was amplified and subcloned into the pcDNA3.1 (+) vector (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Cells were transfected with a negative control vector or a ZO-1-expressing plasmid using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.).

Patients and tissue specimens. Fresh LC and surrounding non-tumor tissue samples were obtained from 30 randomly selected patients with LC, including 18 males and 12 females (age range, 40-60 years), all of whom had undergone surgical resection at Liaocheng People's Hospital (Liaocheng, China) between January 2014 and January 2015. The tumor tissues and their adjacent normal liver tissues, which were located >5 cm from the LC, were collected and maintained at -80˚C for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis. None of the patients had received adjuvant therapies before surgery. All of the tissues were sampled and then verified by pathological examination. The histopathological type and stage of LC were determined according to the criteria of the World Health Organization classification (26). Tumor differentiation was assessed according to the Edmonson and Steiner grading system (27). All LC tissues were collected following approval by the Ethics Committee of Liaocheng People's Hospital.

Western blot analysis. Fresh LC tissues and the surrounding non-tumor liver tissues were treated with lysis buffer containing protease inhibitors (Promega Corporation, Madison, WI, USA). Following centrifugation at 20,000 x g at 4˚C for 20 min, the supernatant was collected for determination of total protein concentration using the DC protein assay method (Bio-Rad Laboratories, Inc., Hercules, CA, USA) to maintain equal loads (20 µg/lane). Then protein samples were electrophoretically separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes, which were then blocked at room temperature for 1 h with 5% non-fat dried milk in Tris-buffered saline containing Tween-20 (TBST; 50 mm Tris/HCl, 100 mm NaCl and 0.1% Tween-20, pH 7.4). Subsequently, membranes were incubated with a polyclonal goat anti-human ZO-1 antibody (1:500; catalog no. sc-33725; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight. Following three washes with TBST for 5 min, the membranes were further incubated with IRDye800-conjugated anti-goat immunoglobulin G secondary antibody (1:5,000; catalog no. P/N 925-32210; Rockland Immunocchemicals, Inc., Limerick, PA, USA) for 2 h at room temperature. Anti-β-actin antibody (1:2,000; catalog no. sc-7031; Santa Cruz Biotechnology, Inc.) was used as a loading control. Finally, membranes were scanned using an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA) and analyzed using PDQuest software (version 7.2.0; Bio-Rad Laboratories, Inc.).

RT-qPCR. TRIzol® reagent (Thermo Fisher Scientific, Inc.) was used to extract RNA from paired LC samples. cDNA was synthesized from total RNA using an Omniscript RT kit (Qiagen, Inc., Valencia, CA, USA), according to the manufacturer's protocol. Subsequently, qPCR was used to determine the mRNA level of ZO-1, which was performed using a Mastercycler Ep Realplex instrument (Eppendorf, Hamburg, Germany). Reaction volumes of 25 µl included 2 µl cDNA, 12 µl 2xFast EvaGreen™ qPCR Master mix (Biotium Inc., Freemont, CA, USA), 1 µl primers (10 mM) and 10 µl RNase/DNase-free water. Cycling parameters were as follows: Hot start at 95˚C for 10 min; 40 cycles of amplification/quantification at 95˚C for 10 sec, 60˚C for 30 sec and 72˚C for 30 sec during which time fluorescence was determined. Melting curve analysis was performed using continuous fluorescence acquisition between 65 and 97˚C. These cycling parameters generated single amplicons for the two primer sets used according to the presence of a single melt peak. The relative expression level for each target gene was normalized using the Cq value of GAPDH (internal reference) using the 2^(-ΔΔCq) relative quantification method (28). Primer sequences were as follows: GAPDH forward, 5'-TGG GCA AAC AGA CCA AGC-3' and reverse, 5'-TTT CTT CCA CAG GGC TTT G-3'; and ZO-1 forward, 5'-TAT TAT GGC ACA TCA GCA CG-3' and reverse, 5'-TTT CTT CCA CAG GGC TTT G-3'.

Cell proliferation assay. An MTT assay was used to detect the effect of ZO-1 on cellular proliferation. In total, 5x10⁴ cells were plated in each well of a 96-well plate. Following incubation for 24 h, 20 µl MTT (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added, prior to incubation at 37˚C for another 4 h in a 5% CO₂ incubator. Following removal of the supernatants, the formazan crystals were dissolved in 100 µl/well dimethylsulfoxide. A multilabel plate reader (PerkinElmer, Inc., Waltham, MA, USA) to determine the absorbance of each sample at 490 nm. Three independent experiments were performed.

Colony formation assay. Cells were seeded in a 6-well plate at a density of 1x10⁵ cells/well. Following culture for 2 weeks, cells were fixed with 4% paraformaldehyde for 20 min and then enumerated following staining with 1% crystal violet. Three independent experiments were performed.
Cell cycle analysis. Cell cycle distribution was analyzed using flow cytometry. A total of 48 h after transfection of cells with a negative control vector or a ZO-1 overexpressing plasmid, cells were trypsinized, rinsed with PBS, fixed with 70% ethanol at 4˚C overnight, and treated with RNase A (0.02 mg/ml) in the dark at room temperature for 30 min. Cells were resuspended in 0.05 mg/ml propidium iodide and analyzed using flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). DNA histograms were analyzed using ModFit LT (version 2.0; Verity Software House, Inc., Topsham, ME, USA). For each sample, >10^4 events were recorded.

Migration assays. A Transwell chamber assay (EMD Millipore, Billerica, MA, USA) was used to determine cell migration. Cells (1x10^5 cells/well) were suspended in 100 µl serum-free DMEM. Subsequently, the upper chamber of the inserts was added, and then DMEM containing 10% FBS was added to the lower chamber as the chemotactic factor. Following 24 h incubation at 37˚C, the cells that migrated were fixed and stained at room temperature for 30 min with a dye solution which contained 0.2% crystal violet and 20% methanol. The number of migrated cells was determined under an inverted microscope (IX71; Olympus Corporation, Tokyo, Japan) at x200 magnification in random fields in each well.

Statistical analysis. Statistical analyses were performed using SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA). One-way analysis of variance followed by a post hoc Dunnett’s test was used to analyze the comparison of the means for three groups. Student’s t-test was used to evaluate the differences between two groups. Results are presented as the mean ± standard error of the mean. P<0.05 was considered to indicate a statistically significant difference.

Results

ZO-1 expression is downregulated in LC tissues. Western blotting was used to detect the protein levels of 18 randomly selected pairs of LC and their matched adjacent liver tissues. Fig. 1A presents four representative cases of the western blot result. The relative quantity of ZO-1 protein expression was normalized to the β-actin in the same samples. Compared with their adjacent normal liver tissues, the expression of ZO-1 protein was downregulated in the LC tissues (13/18), and the mean ZO-1 protein level in LC tissues was significantly decreased compared with in their adjacent normal liver tissues (P<0.001; Fig. 1B). These results were further confirmed by determining mRNA levels by RT-qPCR, which was used to determine the mRNA level of ZO-1 in 30 paired LC cancerous and matched adjacent normal liver tissues. The results indicated that the expression of the ZO-1 mRNA level was significantly lower in 23/30 (76.7%) LC tissues compared with the adjacent non-tumor tissues (Fig. 1C). The mean mRNA expression level of ZO-1 was significantly decreased in LC tissues compared with that in their corresponding normal liver tissues (P=0.006; Fig. 1D).

Overexpression of ZO-1 inhibits LC cell viability and proliferation in vitro. Since ZO-1 was significantly decreased in LC tissues, it was investigated whether overexpression of ZO-1 affected cell viability and proliferation of LC cells. The effects of ZO-1 on LC cell viability and proliferation were further evaluated using MTT and colony formation assays, respectively. The results indicated that overexpression of ZO-1 significantly inhibited the viability of HepG2 cells, and markedly decreased the number of colonies compared with the control and negative control vector cells (Fig. 2).
Upregulation of ZO-1 decreases LC cell cycle and migration in vitro. To investigate the potential mechanism responsible for the effects of ZO-1 on the proliferation of LC cells, the cell cycle was analyzed in HepG2 cells transfected with ZO-1-overexpressing plasmid or negative control plasmid using flow cytometry. In cell cycle analysis, a significant increase in the G0/G1 phase and decrease in the S-G2 phase was identified (Fig. 3).

Furthermore, the potential effect of ZO-1 on cell migration was investigated using Transwell assays. HepG2 cells were transfected with ZO-1-overexpressing or control plasmid and seeded in the Transwell chamber. Overexpression of ZO-1 significantly decreased the migratory capacity of HepG2 cells (Fig. 4).

Discussion

LC is one of the most prevalent tumors globally and the third leading cause of cancer-associated mortality (29,30). Worldwide, ~750,000 new cases of LC are diagnosed each year. Population-based analysis indicated that the incidence rate continues to parallel the death rate, which indicates that
the majority of individuals who develop LC succumb to this disease (31). Although tumor resection and liver transplantation are effective treatments for selected patients with LC, the prognosis of LC remains poor because the disease is often at a fairly advanced stage at the time of diagnosis (32). Surgical treatment is not applicable for patients at advanced tumor stages (33). LC is involved in multiple gene alterations including tumor suppressor inactivation, oncogene activation and apoptosis-associated gene dysregulation (34). Therefore, there is an urgent requirement to identify a sensitive and specific biomarker for the detection of liver cancer at the curative stage.

ZO-1 serves as a scaffolding protein that links the transmembrane tight junction proteins to cytoplasmic proteins and the actin cytoskeleton (15,35). As a member of the MAGUK family of putative signaling proteins, ZO-1 may be involved in signal transduction, and ZO-1 has been identified to bind a target of Ras: AF6 (36). Previous studies indicated that epidermal growth factor and vascular endothelial growth factor are able to increase ZO-1 tyrosine phosphorylation, modulate its subcellular localization, and consequently lead to increased permeability (37-39). ZO-1 serves an important function in maintaining tight junction integrity, which is disrupted in a number of invasive cancers and intestinal diseases (40). Consequently, studies have demonstrated that ZO-1 downregulation is involved in tumor development and progression (41,42).

To the best of our knowledge, the present study is the first to investigate the expression of ZO-1 and its function in LC progression. Using RT-qPCR and western blot analysis, it was identified that the expression of ZO-1 was decreased at the mRNA and protein levels in the majority of patients with LC. Furthermore, it was also identified that overexpression of ZO-1 significantly inhibited cell viability and migration of LC cells in vitro. In addition, upregulation of ZO-1 induced cell cycle arrest. These results suggested that ZO-1 could also serve a tumor suppressor function in LC, and that abnormal ZO-1 expression may be associated with tumor progression and metastasis of LC. Further investigation into the potential molecular mechanism underlying the effects of ZO-1 are required. The mechanisms which contributed to ZO-1 downregulation in LC also require further investigation.

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Availability of data and materials

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

Authors' contributions

XZ, YQ and CN designed the study. XZ, YQ, LW, HZ and FT conducted the experiments, performed the data analysis and wrote the manuscript. CN and YQ analyzed the data and revised the manuscript. All authors discussed the results and reviewed the manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from each patient and the present study was approved by the Ethics Committee of Liaocheng People's Hospital (Shandong, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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