 doi:10.20944/preprints201706.0097.v1

Indirubin inhibits cell proliferation, migration, invasion and angiogenesis in tumor-derived endothelial cells

Zhuohong Li, Chaofu Zhu, Baiping An, Yu Chen, Xiuyun He, Lin Qian, Lan Lan, Shijie Li*

Department of Oncology, the Affiliated Hospital of Chengdu University of Traditional Chinese Medicine, Chengdu, Sichuan Province 610072, China

*Correspondence: Tel: +86-28-87765649 Fax: +86-28-87765649

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 20 June 2017 doi:10.20944/preprints201706.0097.v1

Abstract

Hepatocellular Carcinoma is one of the most predominant malignancies with high fatality rate and is rising at an alarming rate because it is quite resistant to radio- and chemotherapy. The proliferation, migration and activation of endothelial cells are involved in tumor occurrence and development. Indirubin is the major active anti-tumor ingredient of a traditional Chinese herbal medicine. In this research, Td-ECs were derived from human umbilical vein endothelial cells (HUVECs) by treating HUVECs with the conditioned medium of human liver cancer cell line HepG2. The effects of indirubin on cell proliferation, migration, invasion and angiogenesis of Td-ECs were assessed. Indirubin significantly inhibited Td-EC cell proliferation in a dose- and time-dependent manner. Indirubin also inhibited Td-EC migration and angiogenesis. However, indirubin's effects on HUVECs were weaker than on Td-ECs.

Keywords: Indirubin; Td-EC; proliferation; migration; invasion; angiogenesis

1. Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and the second most frequent cause of cancer-related death worldwide [1]. Tumor occurrence and development are very complicated processes. Cancer cells stimulate angiogenesis in an effort to maintain and allow tumor progression [2]. The proliferation, migration and activation of endothelial cells are involved in tumor angiogenesis [3]. Therefore, tumor-derived endothelial cells (Td-ECs) are considered to be ideal therapeutic targets in antiangiogenic therapies.

Current therapeutic modalities for HCC include curative options, such as surgical resection, liver transplantation, and local ablation, or palliative procedures, such as catheter-directed therapies and systemic therapy [4]. However, HCC mortality rate is very high because it is quite resistant to radio- and chemotherapy [5]. Thus, new treatment choices are critically required.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 20 June 2017 doi:10.20944/preprints201706.0097.v1

Traditional Chinese Medicine is a valuable resource that includes many biologically active components that are both safe and effective. Indirubin, extracted from indigo and an active ingredient of Danggui Longhui Wan, is the first Traditional Chinese Medicine extract shown to be effective in the treatment of chronic myelogenous leukemia [6]. However, the effects of indirubin on the Td-ECs have not been addressed.

In the present study, we investigated the effects of indirubin on the cell proliferation, migration, and angiogenesis of Td-ECs, in order to explore new approaches in cancer therapy.

2. Results

2.1. Td-EC induction and validation

To determine whether HUVECs were induced to become Td-ECs by HepG2 conditioned medium, TEM1 and TEM8 were detected by PCR. We found that TEM1 and TEM8 were expressed in Td-ECs but not in HUVECs (Figure 1A). Cell morphology showed that Td-ECs appeared with endothelial cell morphological features (Figure 1B-1D).

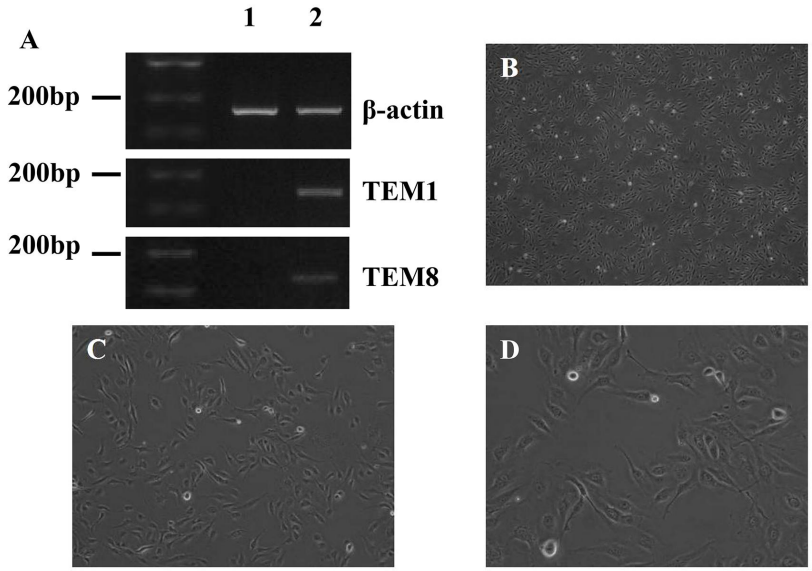

Figure 1. Td-EC detection and cell morphology. (A) TEM1 and TEM 8 gene expression of Td-ECs and HUVECs was detected by PCR. 1: HUVECs, 2: Td-ECs. The results were shown by

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 20 June 2017 doi:10.20944/preprints201706.0097.v1

agarose gel electrophoresis, β-actin served as internal control. Representative of Td-EC cell morphology at different magnification, 40×(B), 100×(C), 200×(D).

2.2. Effects of indirubin on cell proliferation of Td-ECs

We found that indirubin inhibited cell proliferation of HUVECs and Td-EC in time- and dose-dependent manners (Figure 2). Compared to untreated HUVECs and Td-ECs, indirubin at doses of 5 and 10 μM significantly inhibited Td-EC proliferation, whereas indirubin's effects on HUVECs were weaker than on Td-ECs (Figure 2).

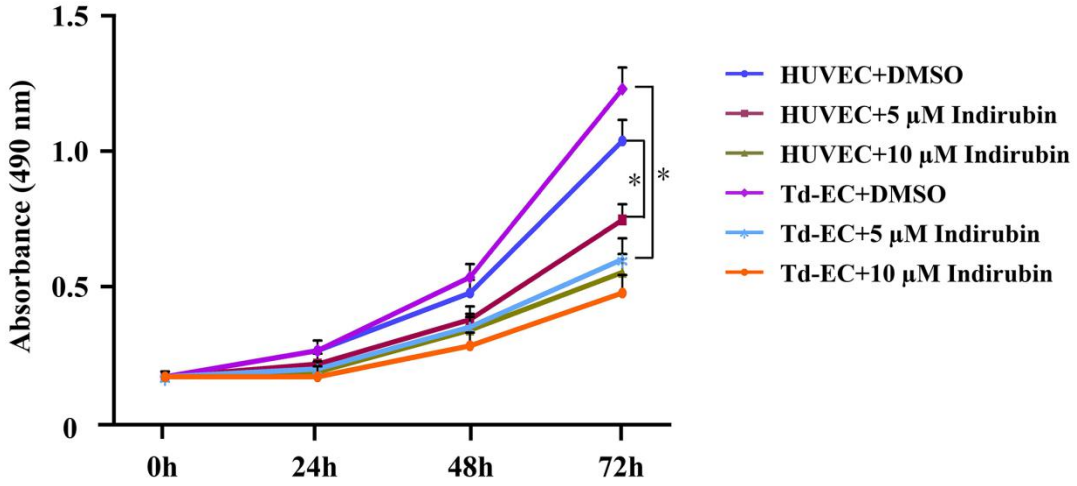

Figure 2. Effects of indirubin on cell proliferation. Ed-ECs and HUVECs were treated with indirubin at different concentrations (0, 5, 10 μM) for different time periods (24, 48h). DMSO was used as a negative control. All of the quantitative values are presented as the means±SEM. *P< 0.05.

2.3. Effects of indirubin on Td-EC migration

Compared to untreated HUVECs and Td-ECs, cell migration of HUVECs and Td-ECs treated with indirubin for 48h was suppressed in a dose-dependent manner (Figure 3). The migration ability of Td-ECs was stronger than HUVECs. However, the inhibitory effects of indirubin on Td-ECs were much stronger than on HUVECs (Figure 3).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 20 June 2017 doi:10.20944/preprints201706.0097.v1

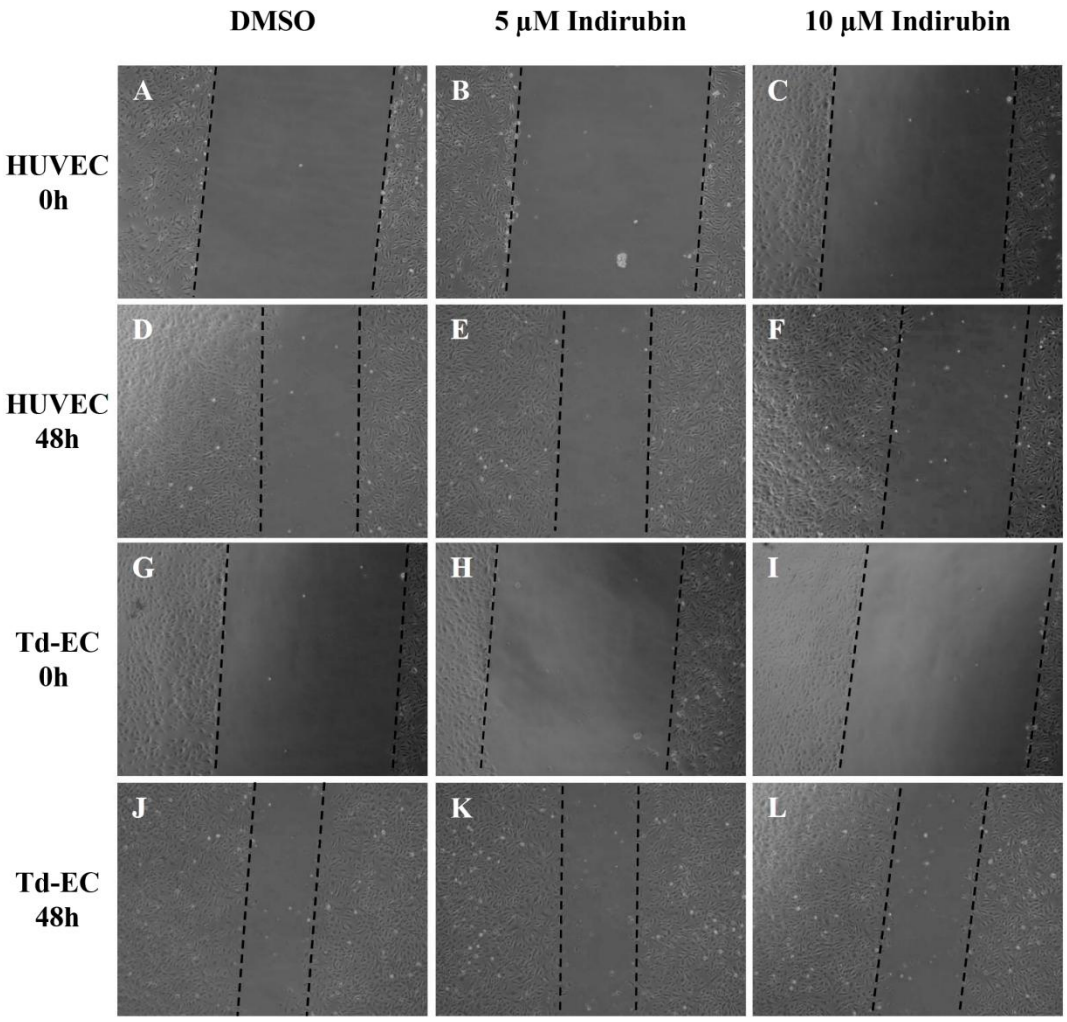

Figure 3. Effects of indirubin on Td-EC migration ability *in vitro*. Wounding healing assay detected indirubin's effects on HUVEC and Td-EC migration. DMSO was used as a negative control. Magnification 100×.

2.4. Effects of indirubin on Td-EC angiogenesis in vitro

Vascular tube formation ability in Td-ECs was markedly stronger than HUVECs (Figure 4A, C). The vascular tube formation ability in Td-ECs was significantly inhibited, but the inhibition was less obvious in HUVECs (Figure 4B, D，$P<0.05$).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 20 June 2017 doi:10.20944/preprints201706.0097.v1

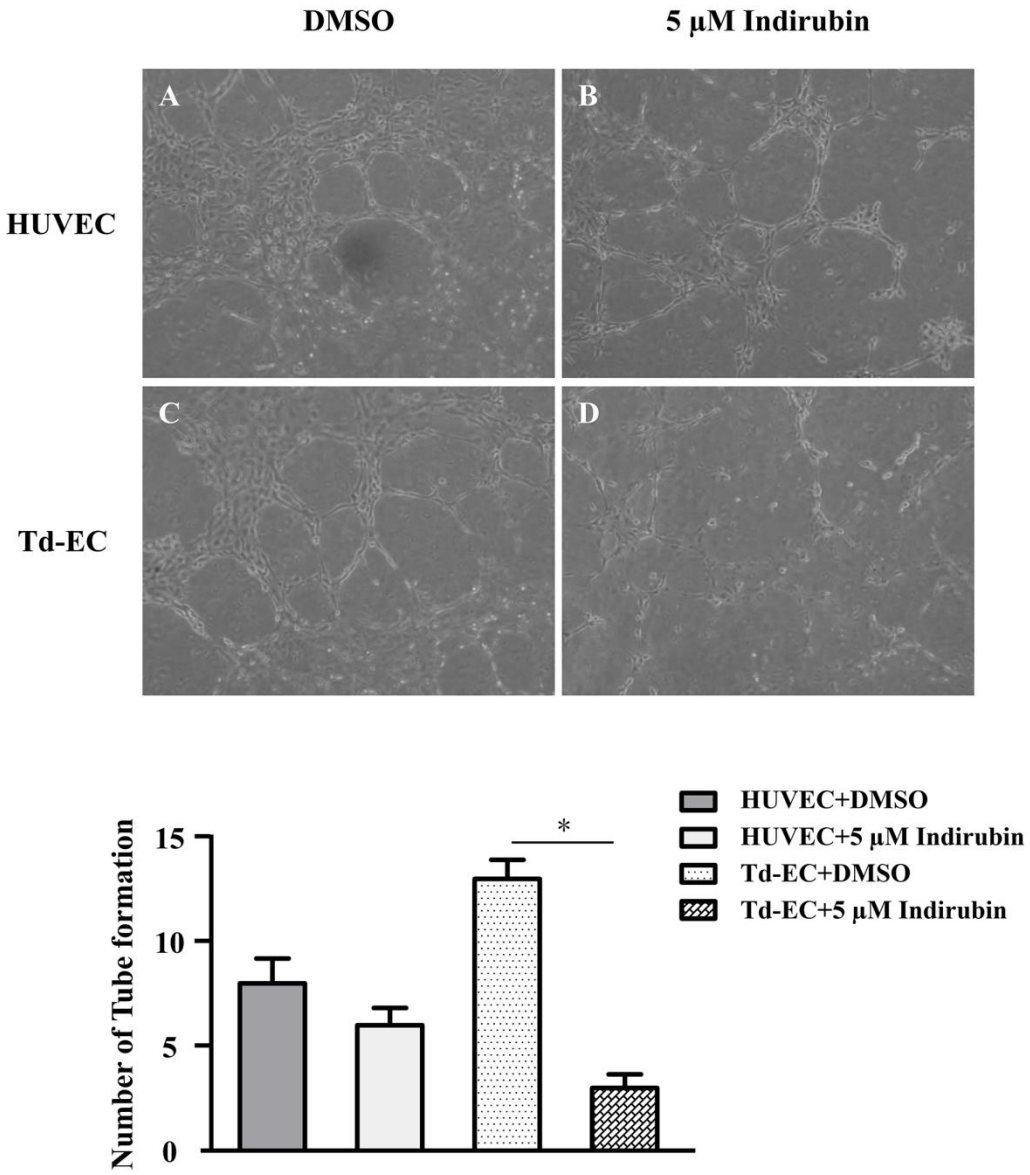

Figure 4. Effects of indirubin on Td-EC tube formation *in vitro*. Td-ECs and HUVECs were treated with indirubin. DMSO was used as a negative control. Magnification 100×. *P< 0.05.

2.5. Effects of indirubin on Td-EC invasion in vitro

The Transwell method showed that indirubin had no effects on HUVEC migration (Figure 5A, B), whereas the invasion cell number of Td-ECs was decreased significantly by indirubin treatment (Fig. 5C, D; P<0.05). The invasion cell number of Td-ECs was much more than HUVECs in the absence of indirubin treatment (Figure 5A, C).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 20 June 2017 doi:10.20944/preprints201706.0097.v1

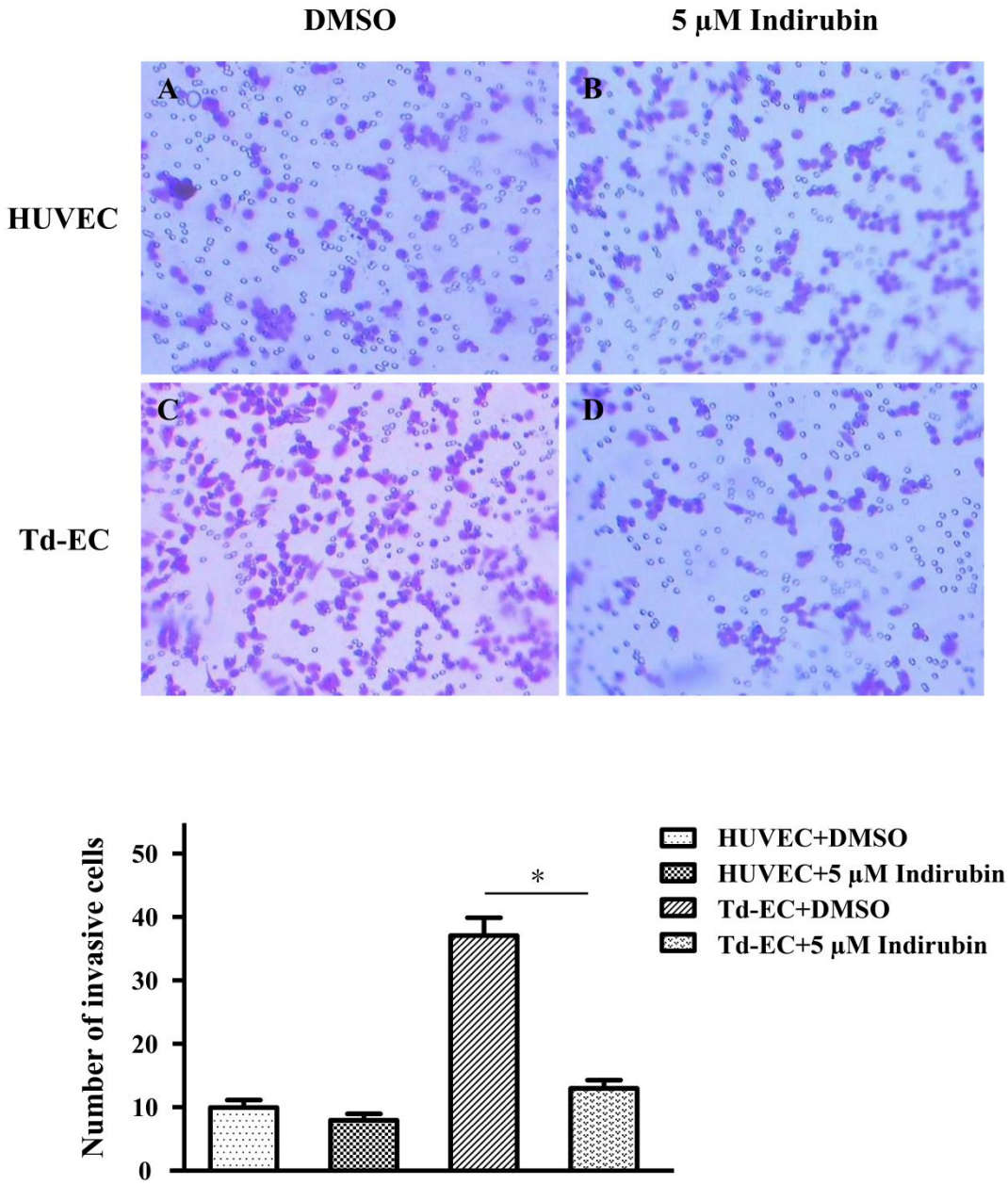

Figure 5. Effects of indirubin on cell migration ability *in vitro*. Td-ECs and HUVECs were treated with indirubin. DMSO was used as a negative control. Magnification 100 ×. *P< 0.05.

3. Discussion

Hepatocellular Carcinoma (HCC) is one of the most predominant malignancies with high fatality rate, leading to over 600, 000 deaths annually [7]. The treatment modalities for HCC include surgical resection, radiofrequency ablation and liver transplantation. Unfortunately, HCC frequently recurs [8]. Therefore, a growing

 doi:10.20944/preprints201706.0097.v1

number of researchers are seeking new treatments for HCC and focus on Traditional Chinese Medicine (TCM). TCM, a complete system of healing developed in ancient China, is receiving more and more attention in China and throughout the world in recent decades [9]. TCM is considered as a medical system for the prevention and treatment of diseases that focuses on the patient rather than the disease when compared to the Western medicine [10]. In this study, we focus on indirubin, the major active anti-tumor component of a traditional Chinese herbal medicine used for treatment of chronic myelogenous leukemia. However, there is little research about indirubin's effects on HCC.

In recent years, the stroma is known to maintain the tissue homeostasis and acts as a barrier toward tumor formation. However, when a cell starts to be malignant, its surrounding matrix changes in a way to support cancer development [11]. Thus, understanding the tumor microenvironment (TME) has become as important as the modulation of cancer cell progression itself [12]. The architecture of a typical TEM is composed of fibroblasts, myofibroblasts, endothelial cells, pericytes, adipose cells, immune and inflammatory cells, and the extracellular matrix (ECM) elements [13]. In this research, we pay close attention to Td-ECs which are surrounded by other cells such as pericytes that make them more stable and control the vessel diameter and elasticity as well [14]. Metastasis and angiogenesis are among the hallmarks of malignant behavior of cancer cells. Metastasis is responsible for the rapid progress and death by HCC. Angiogenesis and tumor progression are very closely linked to each other. Tumor cells are dependent on angiogenesis because their growth and expansion require oxygen and nutrients [15].

Human tumor endothelial marker 1 (TEM1), expressed in tumor endothelium but not in normal endothelium, was recently identified as a novel tumor endothelial cell surface marker potentially involved in angiogenesis [16]. TEM8 is considered as a novel extracellular tumor marker among the other cell surface TEMs. TEM8 expression pattern is tumor-specific and has not been detected in physiologic angiogenesis [17]. So, in this study, we chose TEM1 and TEM8 for Td-EC validation.

In our study, we found that indirubin inhibited cell proliferation, migration and

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 20 June 2017 doi:10.20944/preprints201706.0097.v1

angiogenesis of Td-ECs. All of these data suggest that indirubin may be a novel antiangiogenic treatment for HCC. There are many molecular mechanisms for Td-EC angiogenesis such as tumor hypoxia, epithelial-mesenchymal transition, cancer stem-like cells, and some transcription factors. More molecular mechanisms shall be identified in future indirubin studies.

4. Materials and Methods

4.1. Reagents

Indirubin (purity≥98%) was purchased from Shanghai Yuan Ye Biological Technology Co., Ltd. (Shanghai, China) and dissolved in dimethylsulfoxide (DMSO, Chengdu, China). PRMI1640 and fetal bovine serum (FBS) were purchased from Invitrogen (United States). 0.25% trypsin was purchased from HyClone (Logan, UT). Matrix was purchased from Becton Drive (BD, United States).

4.2. Cell culture

A human umbilical vein endothelial cell (HUVEC) line was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were grown in dulbecco's modified eagle medium (DMEM, Gibco, United States) containing 20% fetal bovine serum (FBS), 10 ng/L vascular endothelial growth factor (VEGF) and 1% antibiotics (100 units/mL penicillin, 100 mg/mL streptomycin) under standard conditions in a humidified incubator at 37℃ with 5% CO_2.

Human hepatocellular carcinoma cell line HepG2 cells were maintained in RPMI1640 medium with 10% FBS and 1% antibiotics under standard conditions. HepG2 cells were cultured in serum-free RPMI1640 for 48 hours when they reached 70% confluence. Then, the medium from the serum-starved HepG2 cells was collected, filtered by 0.22 μm filter membrane, and stored at -80℃ as the conditioned medium.

HUVECs were cultured with the complete medium with 50% of the HepG2 conditioned medium for 48 hours to become tumor-derived endothelial cells (Td-ECs).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 20 June 2017 doi:10.20944/preprints201706.0097.v1

The identity of Td-ECs were validated by checking tumor endothelial markers (TEMs) that are associated with tumor-specific angiogenesis and are potentially useful to distinguish between tumor and normal endothelium [18]. In this study, TEM1 and TEM8 were chosen for Td-EC detection.

4.3. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from Td-ECs and HUVECs using Trizol Reagent (Invitrogen), and cDNA was synthesized from total RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Leon-Rot, Germany). The conditions for PCR were 30 cycles of: denaturation (95°C/30 s), annealing (68°C/30 s), extension (72°C/1 min), and final extension (72°C/10 min) using Deam Taq PCR Master Mix (2X) (Thermo Scientific). TEM1 and TEM8 gene expression were detected by PCR with normalization to β-actin as an internal control. Each experiment was performed in triplicate. The sequences of the primers used in this study: forward primer 5′-ctgatgggtgaggtctggtt-3′ and reverse primer 5′-cattatcccaactgcccagc-3′ for TEM1; forward primer 5′-gtctcctcctggcagaactt-3′ and reverse primer 5′-gctgcaccactggaatgaaa-3′ for TEM8; forward primer 5′-cctctatgccaacacagtgc-3′ and reverse primer 5′-cctgcttgctgatccacatc-3′ for β-actin.

4.4. Cell proliferation assay

To test the effect of indirubin on cell proliferation, a quantitative colorimetric assay with 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumbromide (MTT) was applied. Briefly, HUVECs and Td-ECs were seeded into 96-well plates at 5×10^3 cells in 100 μL of medium per well. After incubation with 0, 5, 10 μmol/L of indirubin for 24, 48, 72 h, the cells were incubated with 0.5 mg/mL of MTT for 4 h at 37°C in darkness. After removing the culture medium, 100 μL of dimethylsulfoxide (DMSO) was added to dissolve formazan crystals within the cells. The absorbance was then measured using Synergy™ Microplate Reader (BioTek Instruments, Winooski, USA)

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 20 June 2017 doi:10.20944/preprints201706.0097.v1

at a wavelength of 490 nm. Three replicate wells were used for each analysis.

4.5. Cell migration assay

HUVECs and Td-ECs (4×10^5 cells/well) were plated to six-well plates till 90% confluence. A wound was created by manually scraping the cell monolayer with a p1000 pipette tip then cells were treated with 0, 5, and 10 μmol/L of indirubin for 48 h. The cells were washed once with 1 mL of DMEM, which was then replaced with 2 mL of the DMEM. Dishes were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells were observed 48 hrs later with an inverted fluorescence microscope (Leica DMI4000B, Germany) equipped with an Olympus Qcolor 3 digital camera. Cell migration was evaluated by measuring the distance that the cells migrated from the original wound margin. All images were processed with ImagePro Plus (Media Cybernetics, Rockville, USA). The experiments were repeated for three times.

4.6. In vitro tube formation assay

Serum-free culture medium and pre-cooled melted Matrigel (10 mg/mL, BD Biosciences) were mixed at a ratio of 2:1 (v/v). A 24-well plate was coated with the Matrigel mixture, which was then allowed to polymerize for 1 h at 37°C. HUVECs and Td-ECs (1.5×10^5 cells/well) were seeded on the surface of the Matrigel at 37°C for 12 h and tube formation was observed every 4 h. Changes in tube formation was observed after adding 5 μmol/L of indirubin for 48 h under a microscope and photographed at 100×.

4.7. In vitro cell invasion assay

Invasion of endothelial cells was tested using the Matrigel coated Transwell system (8 μm pore size and 6.5 mm diameter) in 24-well plates (Corning Costar, Cambridge, MA). The lower chamber was filled with 500 μL of DMEM medium with

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 20 June 2017 doi:10.20944/preprints201706.0097.v1

10% FBS. HUVECs and Td-ECs (1×10^5 cells/well) were seeded into the upper chamber in serum-free media overnight. Cells were then fixed with methanol, and stained with hematoxylin and eosin (Sigma). The cells on the upper filter surface were removed, and cell invasion was determined using a microscope, at 200 × magnification, by counting cells that had migrated to the lower filter side. Samples were assayed twice in triplicate.

4.8. Statistical analysis

All experiments were repeated at least three times and all numerical data were presented as mean ± standard error of mean (SEM). To evaluate the significant differences between two groups, the means were compared using Student's t-test. $P < 0.05$ was considered as statistical significant. These analyses were performed using SPSS 13.0 software (SPSS, Chicago, IL).

5. Conclusions

Our findings indicate that indirubin can inhibit Td-ECs proliferation, migration, and angiogenesis, suggesting that indirubin may be used in the anti-angiogenic treatment of human hepatocellular carcinoma.

Acknowledgments

The present study was supported by a Scientific and Technological Development Fund (#2013-0-yy-02) from the Affiliated Hospital of Chengdu University of TCM.

Author Contributions

Zhuohong Li and Shijie Li conceived and designed the experiments; Zhuohong Li, Lin Qian and Chaofu Zhu performed the experiments; Zhuohong Li and Baiping An analyzed the data and wrote the paper. Yu Chen and Xiuyun He revised the manuscript for important intellectual content. Shijie Li and Lan Lan initiated and

 doi:10.20944/preprints201706.0097.v1

supervised the study. All authors read and approved the final version of the manuscript.

Conflict of Interest

The authors have no conflict of interest to declare.

Abbreviations

Td-ECs	Tumor-derived Endothelial Cells
HUVECs	Human umbilical vein endothelial cells
HCC	Hepatocellular carcinoma
TEMs	Tumor endothelial markers
TCM	Traditional Chinese Medicine
TME	Tumor microenvironment
ECM	extracellular matrix

References

1. Ferlay, J.; Soerjomataram, I.; Dikshit, R.; Eser, S.; Mathers, C.; Rebelo, M.; Parkin, DM.; Forman, D.; Bray, F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer.* **2015**, 136, E359-386.
2. Shojaei, F. Anti-angiogenesis therapy in cancer: current challenges and future perspectives. *Cancer Lett.* **2012,** 320, 130-137.
3. Wei, Y.; Yang, Q.; Zhang, Y.; Zhao, T.; Liu, X.; Zhong, J.; Ma, J.; Chen, Y.; Zhao, C.; Li J. Plumbagin restrains hepatocellular carcinoma angiogenesis by suppressing the migration and invasion of tumor-derived vascular endothelial cells. *Oncotarget.* **2017,** 8, 15230-15241.
4. Tomita, Y.; Dorward, H.; Yool, A.J.; Smith, E.; Townsend, A.R.; Price, T.J.; Hardingham, J.E. Role of Aquaporin 1 Signalling in Cancer Development

and Progression. *Int J Mol Sci.* **2017**, 18, pii: E299.

5. Wahid, B.; Ali, A.; Rafique, S.; Idrees, M. New Insights into the Epigenetics of Hepatocellular Carcinoma. *Biomed Res Int.* **2017**, 1609575.
6. Xiao, Z.; Hao, Y.; Liu, B.; Qian, L. Indirubin and meisoindigo in the treatment of chronic myelogenous leukemia in China. *Leuk Lymphoma.* **2002,** 43, 1763-1768.
7. Chen, W.; Zheng, R.; Baade, P.D.; Zhang, S.; Zeng, H.; Bray, F.; Jemal, A.; Yu, X.Q.; He, J. Cancer statistics in China, 2015. *CA Cancer J Clin.* **2016**;66, 115-132.
8. Grandhi, M.S.; Kim, A.K.; Ronnekleiv-Kelly, S.M.; Kamel, I.R.; Ghasebeh, M.A.; Pawlik, T.M. Hepatocellular carcinoma: from diagnosis to treatment. *Surg Oncol.* **2016**, 25, 74-85.
9. Yu, F.; Takahashi, T.; Moriya, J.; Kawaura, K.; Yamakawa, J.; Kusaka, K.; Itoh, T.; Morimoto, S.; Yamaguchi, N.; Kanda, T. Traditional Chinese medicine and Kampo: a review from the distant past for the future. *J Int Med Res.* **2006**, 34, 231-239.
10. Sánchez-Vidaña, D.I.; Rajwani, R.; Wong, M.S. The Use of Omic Technologies Applied to Traditional Chinese Medicine Research. *Evid Based Complement Alternat Med.* **2017**; 2017: 6359730.
11. Shimoda, M.; Mellody, K.T.; Orimo, A. Carcinoma-Associated Fibroblasts Are a Rate-Limiting Determinant for Tumour Progression. *Semin Cell Dev Biol.* **2010**, 21, 19-25.
12. Pavlova, N.N.; Thompson, C.B. The Emerging Hallmarks of Cancer Metabolism. *Cell Metab.* **2016**, 23, 27-47.
13. Chen, F.; Zhuang, X.; Lin, L.; Yu, P.; Wang, Y.; Shi, Y.; Hu, G.; Sun, Y. New horizons in tumor microenvironment biology: challenges and opportunities. *BMC Med.* **2015**,13, 45.
14. Dudley, A.C. Tumor endothelial cells. *Cold Spring Harb Perspect Med.* **2012**, 2, a006536.
15. Chen, H.F.; Wu, K.J. Endothelial Transdifferentiation of Tumor Cells

 doi:10.20944/preprints201706.0097.v1

Triggered by the Twist1-Jagged1-KLF4 Axis: Relationship between Cancer Stemness and Angiogenesis. *Stem Cells Int*. **2016**, 2016, 6439864.

16. Opavsky, R.; Haviernik, P.; Jurkovicova, D.; Garin, M.T.; Copeland, N.G.; Gilbert, D.J.; Jenkins, N.A.; Bies, J.; Garfield, S.; Pastorekova, S.; et al. Molecular characterization of the mouse Tem1/endosialin gene regulated by cell density in vitro and expressed in normal tissues in vivo. *J Biol Chem.* **2001**, 276, 38795-38807.
17. Rmali, K.A.; Puntis, M.C.; Jiang, W.G. TEM-8 and tubule formation in endothelial cells, its potential role of its vW/TM domains. *Biochem Biophys Res Commun.* **2005**, 334, 231-238.
18. Pietrzyk, Ł. Biomarkers Discovery for Colorectal Cancer: A Review on Tumor Endothelial Markers as Perspective Candidates. *Dis Markers.* **2016**, 2016, 4912405.