Original Article

FOXP3 expression in FOXP3$^+$ tumor cells promotes hepatocellular cells metastasis

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Background: Forkhead transcription factor 3 (FOXP3) is a key molecule for the development of regulatory T cell. Recent studies showed that FOXP3 was also expressed in tumor cells. This study is designed to identify the expression of FOXP3 and its pathogenesis in hepatocellular carcinoma (HCC).

Methods: Through immunohistochemistry, RNA extraction and Real-Time Quantitative PCR, western blot analysis, transwell cell migration assay and invasion assays, and *in vivo* experiment, we detected FOXP3 expression in HCC and analyzed the expression of tumor metastasis-related genes in HCC cells using female BALB/c-Nude mice.

Results: The results showed that FOXP3 was expressed in partial HCC tissues samples and cell lines. The distant metastasis rate was remarkably higher in the FOXP3 positive HCCs than that in the negative group. The positive rate of FOXP3 expression in the metastatic HCC was higher than that in primary HCC, and the expression level of FOXP3 was found to increase as the enhancement in the metastatic potential of the cell lines. Furthermore, in HCC cell lines, FOXP3 overexpression can promote the cell metastasis and invasion by regulating MMP-1. The *in vivo* experiment showed that the proliferation ability of HepG2 cells in nude mice increased significantly after FOXP3 was overexpressed, and the incidence rate of lung metastasis in MHCC97L cells was significantly deceased after knocking down the FOXP3 expression.

Conclusions: Our findings support that partial HCC tissues and cell lines expressing FOXP3 can promote the metastasis by regulating MMP-1.

Keywords: Hepatocellular carcinoma (HCC); forkhead transcription factor 3 (FOXP3); MMP-1; tumor metastasis

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common and aggressive cancers worldwide (1). Despite improvements in the diagnosis and treatment of HCC, the overall survival (OS) time of patients with HCC remains poor (2). Forkhead transcription factor 3 (FOXP3) is reported as a suppressor gene of HCC. Previous study has demonstrated that high-density of the FOXP3$^+$ Treg infiltration is associated with tumor aggressiveness and poor clinical outcome in HCC (3,4).

However, the expression of FOXP3 in HCC has not been investigated deeply, thus a more in-depth research is required to assess the role of FOXP3 in HCC.

FOXP3 has been identified as a key gene that is well known to be the hallmark of immune suppressive T regulatory cells (Tregs), especially thymically derived natural Tregs (5-8). FOXP3- positive Tregs have been reported to increase in peripheral blood and tumors in patients with various types of cancers, including ovarian cancer (9,10), breast cancer (11-13), HCC (14), and other tumors (15). In addition to their potential value in predicting disease progression and relapse, FOXP3 has been studied as a therapeutic target and it has been reported that vaccination to eradicate FOXP3- expressing cells enhances tumor immunity (16). Therefore, FOXP3 is an important suppressor gene in tumors and it is necessary to explore the expression of FOXP3 in tumors.

Although Tregs are the major cells that expressing FOXP3, recent studies have demonstrated that the tumor cells themselves can also express FOXP3 (17). The expression of FOXP3 in tumor cells has also been recently reported in pancreatic cancer (18), melanoma (19), breast cancer (20-23), oral squamous cell carcinoma (24) and other tumor cell lines. This finding that FOXP3 is expressed in tumor cells implies that T-cell function may be modulated not only by FOXP3 in Tregs but also by tumor-associated FOXP3. Merlo *et al.* found that FOXP3 expression was associated with overall and distant metastasis free survival but no relevance to local relapse. Andrea *et al.* suggested that FOXP3 expression might be related to the metastatic potential of the tumor rather than suppressing a specific immune response. However, it has not been clarified whether FOXP3 expression in tumor cells affects prognosis, and the mechanism by which expression of FOXP3 in tumor cells affects endogenous tumor-specific immunity. Here, using both *in vivo* and the tumorigenicity assay in nude mice, study revealed FOXP3 was expressed in HCC cell lines and was further identified as an independent predictor for better prognosis in HCC patients.

This study was designed to further investigate the expression of FOXP3 in HCC and its pathogenesis mechanism. We detected the expression levels of FOXP3 and MMP-1 in HCC cell lines with different metastatic potentials. In addition, the cell migration and invasion ability after FOXP3 overexpression or knockdown were also explored. We present the following article in accordance with the ARRIVE reporting checklist (available at http://dx.doi.org/10.21037/tcr-20-1875).

Methods

Cell lines and animals

MHCC97H and MHCC97L were provided by Academy of Military Medical Sciences (Beijing, China), and BEL7404, BEL7402, Huh7, HepG2 and Hep3B were purchased from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). All of these cell lines were cultured in DMEM (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA) at 37 ℃ in a humidified, 5% CO_2 atmosphere. Female BALB/c-Nude mice (6–8 weeks old, weighing 15–18 g, Vital River Laboratories, Beijing) were housed in specific pathogen-free conditions. This study was implemented after the approval of Medical Ethics Committee of Peking University People's Hospital (2019PHE018). All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985).

HCC patients and specimens

A total of 179 liver cancer patients admitted in Peking University People's Hospital (PKUPH) and Guilin Medical University (GLMU) affiliated hospital from November 2001 to April 2018 were enrolled in this study and tissue samples included 12 normal liver tissues and tumor tissues were obtained from these patients. Clinical information including age, gender, family history, alpha-fetoprotein (AFP) levels, and hepatitis B or hepatitis C infection status were recorded in detail in the database. Primary tumour size, histologic tumour type, histologic grade, lymph node (LN) stage and distant metastasis were routinely assessed according to the TNM staging criteria proposed by the International Union against Cancer (UICC) and American Joint Committee on Cancer (AJCC). This study was implemented after the approval of Ethics Committee of Peking University People's Hospital (2019PHE018). Written informed consent was obtained from each patient prior to tissue sample collection in accordance with the Declaration of Helsinki (as revised in 2013).

Immunohistochemistry (IHC)

Tissue sections cut from formalin-fixed and paraffin-embedded blocks were deparaffinized with xylene and then rehydrated through three decreasing concentrations

of alcohol. Endogenous peroxidase was blocked by 3% hydrogen peroxide for 10 min. Antigen retrieval was performed by microwaving the samples in 10 mM Tris Base 1 mM EDTA (pH 9.0) buffer. Non-specific binding was blocked by 5% bovine serum albumin (BSA, Genview, USA) for 20 min at room temperature, sections were then incubated with monoclonal mouse anti-human FOXP3 (1:100 dilution, clone 236A/E7, Abcam, Cambridge, UK) at 4 ℃ overnight. The negative control was performed by replacing the primary antibody with phosphate buffered saline (PBS). After samples were washed with PBS (pH 7.4), sections were incubated with ChenMate™ EnVision + /HRP anti-rabbit/mouse reagent (GeneTech, Shanghai, China) for 30 min at room temperature. The sections were stained with DAB and rinsed gently with distilled water according to manufacturer's protocol. After counterstaining with hematoxylin, sections were dehydrated through three increasing concentrations of alcohol to xylene and mounted. The stained sections were scored independently by two pathologists.

RNA extraction, reverse-transcription (RT) PCR and real-time quantitative PCR (qPCR)

The total RNA was isolated with TRIzol (Invitrogen, USA) according to the manufacturer's protocols and the concentration was determined by SmartSpec™ Plus spectrophotometer (Bio-Rad, USA), and then was reverse transcripted to construct cDNA library with high-capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA). Quantitative real-time RT-PCR was performed by a LightCycler® 480 (Roche Applied Science, Swiss Confederation) using human FOXP3 primers (forward: 5'-CACAACATGCGACCCCCTTTCACC-3', reverse: 5'-AGGTTGTGGCGGATGGCGTTCTTC-3') and human MMP-1 primers (forward: 5'-ACACATCTGACCTACAGGATTGA-3', reverse: 5'-GTGTGACATTACTCCAGAGTTGG-3') combined with SYBR Green Master Mix (Applied Biosystems, USA). GAPDH was used as the endogenous control (primers of GAPDH: forward: 5'-CCACATCGCTCAGACACAT-3', reverse: 5'-GGCAACAATATCCACTTTACCAGAGT-3'). All experiments were done in triplicate.

Western blot analysis

Cells were lysed in ice-cold lysis buffer (Pierce, USA). Protein concentration was determined using the BCA™ Protein Assay Reagent (Pierce, USA). An aliquot of the lysate was mixed with an equal volume of 2× loading buffer and boiled for 5 min. Total protein (50 μg) was electrophoresed on a 15% SDS-PAGE and transferred onto Nitrocelluose membrane (Invitrogen, USA). Membranes were then blocked with 5% skimmed milk in TBS containing 0.1% Tween 20 (TBST). Target proteins were detected using primary antibodies for FOXP3 (1:250 dilution, clone 236A/E7, Abcam, Cambridge, UK), MMP-1 (1:200 dilution, clone 3B6, Santa Cruz Biotechnology, California, USA), and GAPDH (1:2,000, dilution, Cell Signaling Technology). Membranes were incubated with primary antibody overnight at 4 ℃, washed, and incubated with anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, California, USA) diluted 1:2,000 for 1.5 h at room temperature. Membranes were washed with 1× TBS-Tween 20 (0.1%) and incubated with SuperSignal® West chemiluminescent substrate (Pierce, USA) for 3 min and then exposed. Images were acquired using a Kodak X-Omat BT film processor (Kodak, USA) and data were analyzed with Bandscan (Glyko Biomedical Limited, Canada) software.

Plasmid construction and transient transfection

The cDNAs encoding for FOXP3 full length (fl) protein isoforms were synthesized by reverse transcription-polymerase chain reaction (RT-PCR), using total RNA isolated from HepG2. The synthesized FOXP3 cDNAs were then cloned into the pcDNA3 vector to generate the plasmid (pReceiver M02-Foxp3, p-Fc) for expressing FOXP3 protein. The FOXP3 shRNA expressing vectors were constructed by introducing U6 RNA polymerase III promoter and EGFP expression cassette into a vector of pDC316-EGFP-U6 (p-Fsh). Hairpin shRNA sequence of FOXP3 (FOXP3: 5'-GGGACCAAGAAGTGAGGTTTC TCAAGAGGAAACCTCACTTCTTGGTCCC-3') were cloned into the pDC316-EGFP-U6 shRNA expressing vectors by restriction sites of Hind III and BamH I. The MMP-1 shRNA expressing vectors were constructed by introducing U6 RNA polymerase III promoter and EGFP expression cassette into a vector of pDC316-EGFP-U6 (p-Msh). Hairpin shRNA sequence of MMP-1 were cloned into the pDC316-EGFP-U6 shRNA expressing vectors. The plasmids (p-Fc, p-Fsh and p-Msh) was transiently transfected by liposomes (Bio-Rad TransFectin, Bio-Rad, Hercules, CA, USA) into the hepatoma cell lines for the

FOXP3 and MMP-1 mRNA and protein expression.

Expression profiling of FOXP3 over-expressed HepG2

This experimental system was totally based on Affymetrix DNA Microarray Guide. Total RNA of FOXP3 over-expressed HepG2 was isolated with TRIzol (Invitrogen, USA) according to the manufacturer's protocols and purified by RNeasy mini Kit (Qiagen, Germany), then Affymetrix one-cycle cDNA Synthesis Kit (Affymetrix, USA) was used to synthesis cDNA, then purified by Affymetrix GeneChip Sample Cleanup Module (Affymetrix, USA). Hybridization, elution and staining of chip were followed untill synthesis (GeneChip IVT Labeling Kit, Affymetrix, USA), purification (Genechip Sample Cleanup Module, Affymetrix, USA) and fragmentation of cRNA were done. And the data was analyzed after scanning chip.

Cell migration and invasion assay

The transwell cell migration assay was performed in polyethylene terephthalate (PET)-based migration chambers (24-well, Coring). Invasion assays were performed with Cell Invasion Assay Kit (24-well, Chemicon). 300 μL of warm serum free DMEM was added to the interior of the inserts to rehydrate the ECM layer for 2 hours at room temperature. Cells (5.0×10^4) suspended in 300 μL of serum free DMEM were seeded in each insert. 500 μL of DMEM containing 10% fetal bovine serum as a chemoattractant was added into the lower chamber. After 72 h incubation, no invading cells on the upper side of the membrane were removed with a cotton swab, whereas the cells that had crossed ECMatrix™ to the underside of the membrane were stained with cell stain solution (Chemicon) for 20 minutes at room temperature. Inserts were rinsed in a beaker of water several times and then allowed to air dry. Cell numbers were counted by photographing the membrane in ten separate fields through the microscope. Data were expressed as the mean value of cells in ten fields based on three independent experiments.

Lentiviral construction and live-animal imaging

The pLenti6.3-FOXP3-fl-IRES-EGFP plasmid and pLenti6-GFP-FOXP3-shRNA plasmid were constructed and co-transfected into 293T cells with packaging plasmid mix (Invitrogen, USA) to package lentivirus. FOXP3-overexpressing (F-O) cells, FOXP3-knockout (F-K) cells and control cells were generated by Lenti6.3-FOXP3-fl, Lenti6-FOXP3-shRNA and Lenti6.3-con, and selected by growth in the presence of puromycin (5 μg/mL) for luciferase and blasticidin (3 μg/mL) for FOXP3. Tumor growth/metastasis model *in vivo* was established by respectively injecting F-O HepG2 cells (3×10^6 cells per mouse) subcutaneously, F-K MHCC97L cells (2×10^6 cells per mouse) intravenously and control cells with luciferase (luc) reporter into female nude mice (BALB/c-Nude; 6–8 weeks old). Mice were anesthetized with 2% isoflurane and injected with 100 μL of 15 mg/mL D-luciferin weekly, and placed in the chamber of the IVIS® Imaging System (Caliper Life Sciences, Hopkinton, MA) and imaged for the whole 30 mins after injection. Images were analyzed using the Living Image software, version 4.2. Signal intensity was quantified in defined regions of interest as photon count rate per body unit body area per unit solid angle subtended by the detector (units of photons/sec/cm^2/sr).

Statistical analysis

Data analysis was performed using SPSS 16.0 software. The *t*-test was employed to verify the differences in the measurement among the different groups. The chi-square test was used to compare the correlation of the ranked data between the two categorical variables. $P<0.05$ was considered as a significant difference.

Results

FOXP3 expression in HCC cell lines and tumor tissues of HCC patients

The results of RT-PCR and Western blot determined the expression of FOXP3 in HCC cell lines including BEL7404, BEL7402, MHCC97H, Huh7 and HepG2, $CD4^+$ T-cells were used as control. The expression level of FOXP3 in MHCC97H cells was higher than other cell lines (*Figure 1A*,*B*). The results of IHC revealed that FOXP3 was expressed in the nuclei of tumor cells in 19% (34/179) cases, and $FOXP3^+$ Treg was detected in 87% (155/179) HCC cases. FOXP3 protein was not detected in normal liver tissues (*Figure 1C*).

The positive expression of FOXP3 is related to the distal metastasis in HCC

The cases were divided into two groups: $FOXP3^+$ group (34 cases) and $FOXP3^-$ group (145 cases). The Kaplan-Meier analysis did not reveal a significant difference in the

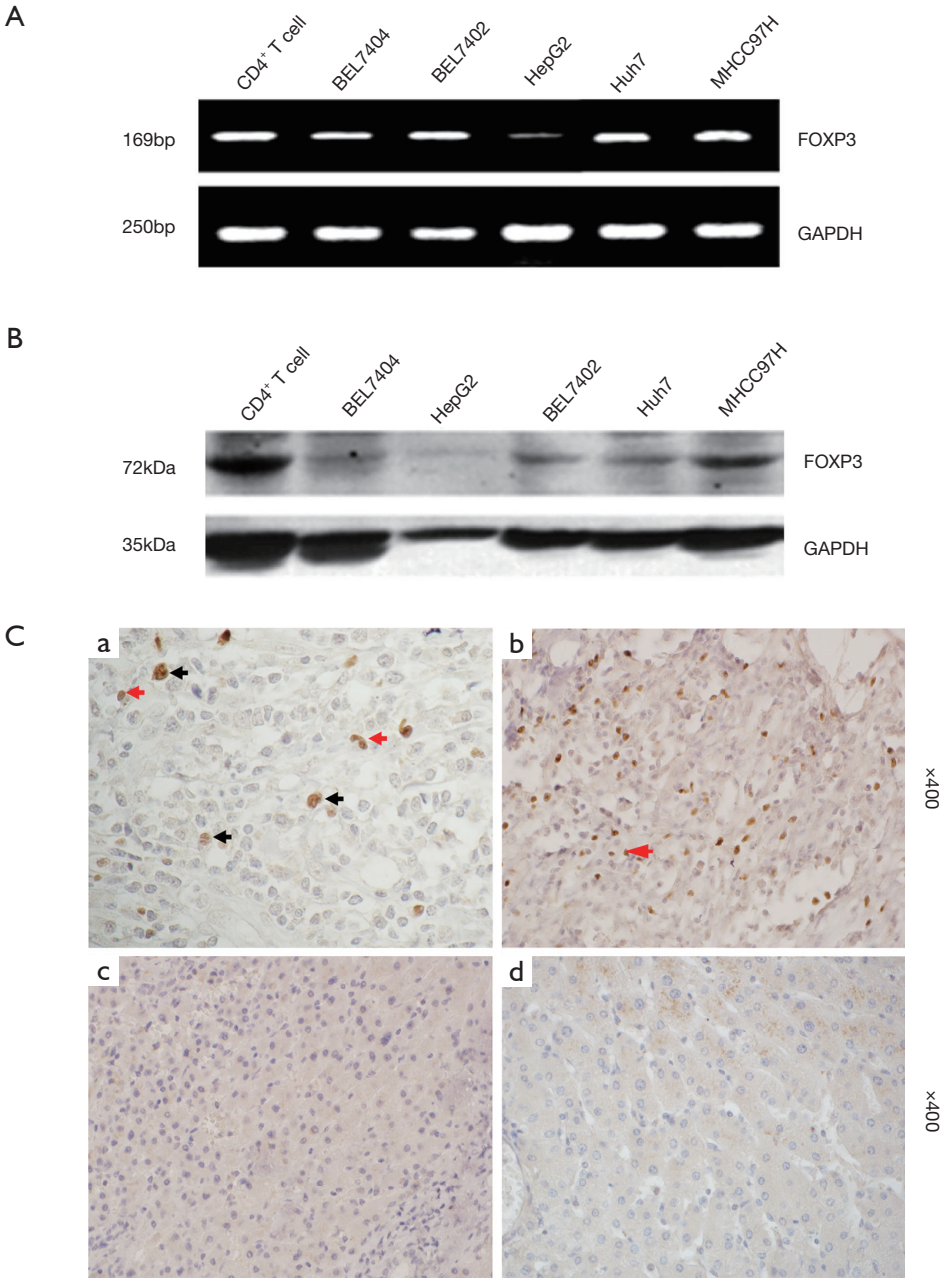

Figure 1 The expression of FOXP3 in HCC cell lines and tumor tissues of HCC patients. (A) Detection of FOXP3 mRNA expression in HCC cell lines by RT-PCR; (B) Detection of FOXP3 protein expression in HCC cell lines by Western blot. (C) The IHC results of (a) expression of FOXP3 in HCC cells (black arrow, ×400), (b) the presence of FOXP3+ Treg cells infiltration in HCC tissues (red arrow, ×400), (c) normal liver tissues did not express FOXP3, (d) negative control (n=179). FOXP3, forkhead transcription factor 3; HCC, hepatocellular carcinoma.

overall survival between two groups. The stratified analysis of the clinical data of patients in two groups showed that the distal metastasis rate in the FOXP3$^+$ group was higher than that in the FOXP3$^-$ group (35% *vs.* 10%, P=0.013) (*Table 1*). To evaluate the relationship between the expression of FOXP3 and the metastasis of HCC, the expression of FOXP3 was assessed in 9 HCC tissue samples, including carcinoma *in situ* and metastatic carcinoma in the same patients (chest wall, lymph node, spleen, abdominal cavity, greater omentum, left mandible, pelvic cavity, and intestinal wall). The results showed that 2/9 (22%) cases of primary carcinoma exhibited the positive expression of FOXP3 in tumor cells and in 6/9 (67%) cases of metastatic carcinoma (*Figure 2*).

FOXP3 may influence the cell migration and invasion by regulating MMP-1 in HCC

Western blot was used to detect the expression of the FOXP3 protein in HCC cell lines with different metastatic potentials (Huh7, HepG2, MHCC97L, and MHCC97H), the expression level of FOXP3 was found to increase as the enhancement in the metastatic potential of the cell lines (*Figure 3A*). After FOXP3 cDNA plasmid (p-Fc) was transduced into the HepG2 cells with low metastatic potential, the results of the gene expression profiles showed that the expression level of MMP-1 in cells was increased with the overexpression of FOXP3 (*Table 2*). Real-time PCR employed for the detection of the expression levels of MMP-1 in three HCC cell lines (HepG2, MHCC97L, and MHCC97H) demonstrated that the mRNA expression level of MMP-1 increased with the improvement in the metastatic potential of the cell lines (*Figure 3B*).

In order to further analyze the relationship between FOXP3 and MMP-1 in HCC cells, we applied *in vitro* interference of low metastatic potential to the HCC cell line HepG2, and MHCC97H with high metastatic potential via FOXP3 shRNA plasmid (p-Fsh), FOXP3 cDNA plasmid (p-Fc), and MMP-1 shRNA plasmid (p-Msh). The effects of FOXP3 overexpression or knockdown on the abilities of migration and invasion were analyzed. After overexpressing FOXP3 in the HepG2 cells, the expression level of MMP-1 protein in the cells was increased (*Figure 4*), the migration (P=0.0032) and invasion (P=0.0119) abilities of HepG2 cells were also observed to have an increase (*Figure 5A,B,C,D*). As for knocking down FOXP3 in the MHCC97H cells, the expression of MMP-1 protein in the cells was also decreased but did not significantly (*Figure 4B*), and the migration (P=0.0248) and invasion (P=0.0378) abilities of MHCC97H cells was also significantly decreased (*Figure 5E,F,G,H*). The tumorigenicity assay in nude mice and small animal imaging technology was used to detect the overexpression of FOXP3 in HepG2 cells. The *in vivo* tumorigenicity and metastatic abilities of MHCC97L cells were evaluated in the cells with knocked down expression of FOXP3. The results showed that after FOXP3 was overexpressed, the proliferation ability of HepG2 cells in nude mice increased significantly (P=0.0004) (*Figure 6A*). As a result of the knocked-down expression, the incidence rate of lung metastasis in MHCC97L cells was 33% while that in the control group was 67% (*Figure 6B*).

Discussion

It is reported that FOXP3 plays an important role in Tregs in HCC invasion. Our study detected the expression of FOXP3 in hepatoma cells and found it was associated with HCC distal metastasis. In addition, this study suggested FOXP3 in HCC cells promoted tumor cell migration and invasion via up-regulating MMP-1.

It has been reported that epigenetic control of FOXP3 in intratumoral T-cells regulates growth of HCC (25), but there is few studies assessing the FOXP3 expression in HCC cells and the role in tumor metastasis have been evaluated. Previous studies have reported that the intensity of FOXP3 expression in tumor cells is negatively correlated with the survival rate of patients, and it is also an independent predictor of distant tumor metastasis (26). Yang *et al.* found that overexpression of FOXP3 significantly induced the proliferation, migration and invasion of NSCLC cells, and the *in vivo* studies also confirmed that FOXP3 promotes tumor growth and metastasis (27). In some other types of cancers (28-31), FOXP3 has also been shown to promote cancer growth, migration and invasion. In our research, we found a similar phenomenon in HCC that FOXP3-positive patients exhibited a higher risk of distant metastasis than FOXP3-negative patients. In addition, the positive rate of FOXP3 in metastasis tumors was 67%, which was much higher than that in the primary tumors (22%). *In vitro*, the protein expression levels of FOXP3 in HCC cell lines (Huh7, HepG2, MHCC97L, and MHCC97H) increased with the enhancement in the metastatic potentials of cell lines. Moreover, the *in vivo* experiments further proved the above results, that is, the proliferation ability of HepG2

Table 1 Correlation analysis of FOXP3 and clinicopathological parameters in HCC patients

Pathological/clinical parameter	FOXP3 positive		FOXP3 negative		Significance (P)
	n	%	n	%	
Age, years					0.292
<40	3	9	33	23	
40–50	12	35	50	34	
51–60	11	32	35	24	
>60	8	24	27	19	
Gender					0.917
Male	30	88	127	88	
Female	4	12	18	12	
Tumor size					0.265
≤2	3	9	11	8	
>2, ≤5	10	29	31	21	
>5, ≤10	7	21	54	37	
>10, ≤20	10	29	42	29	
>20	4	12	7	5	
No. of tumor nodule					0.563
1	20	59	93	64	
≥2	14	41	52	36	
Tumor grade					0.541
I	5	15	28	19	
II	8	24	20	14	
III	20	59	91	63	
IV	1	3	6	4	
Lymph node metastasis					0.424
Absent	32	94	130	90	
Present	2	6	15	10	
Portal vein tumor thrombus					0.242
Absent	23	68	112	77	
Present	11	32	33	23	
Distal metastasis					0.013
Absent	22	65	131	90	
Present	12	35	14	10	
Recurrence					0.242
Absent	23	68	112	77	
Present	11	32	33	23	

FOXP3, forkhead transcription factor 3; HCC, hepatocellular carcinoma.

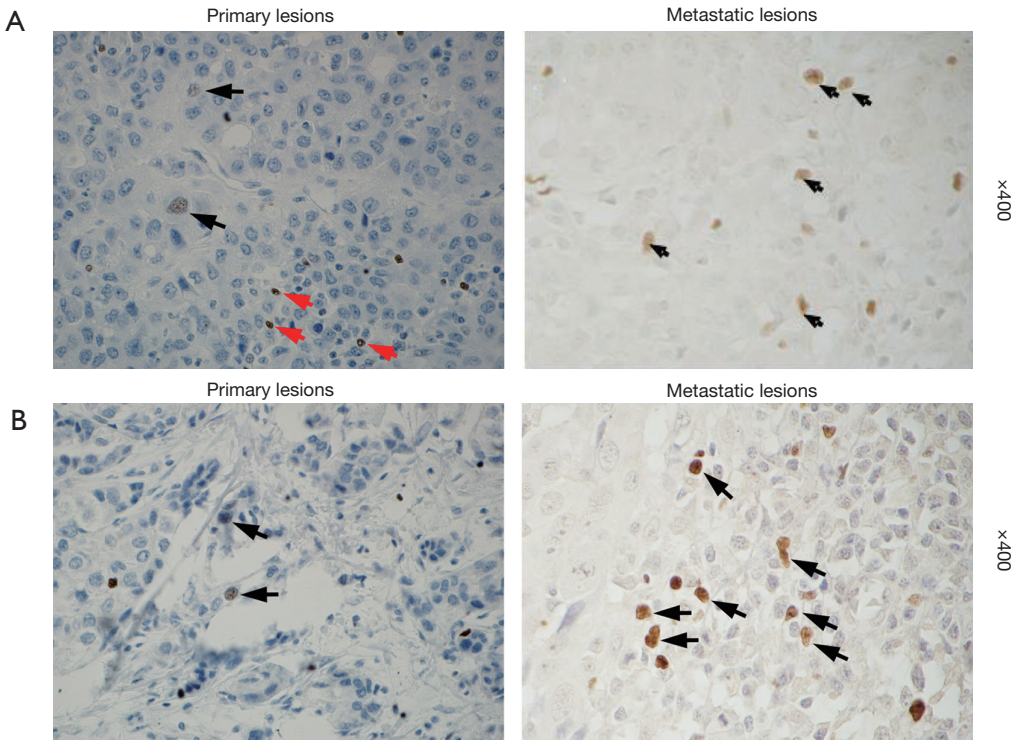

Figure 2 The relationship between FOXP3 and the metastasis of HCC in 9 HCC tissue samples. (A) Expression of FOXP3 in tumor cells (black arrow) and Treg cells (red arrow) of primary and metastatic HCC from case 1 (×400); (B) the presence of FOXP3$^+$ tumor cells and Treg cells in tissues of primary and metastatic HCC from case 2 (×400). FOXP3, forkhead transcription factor 3; HCC, hepatocellular carcinoma.

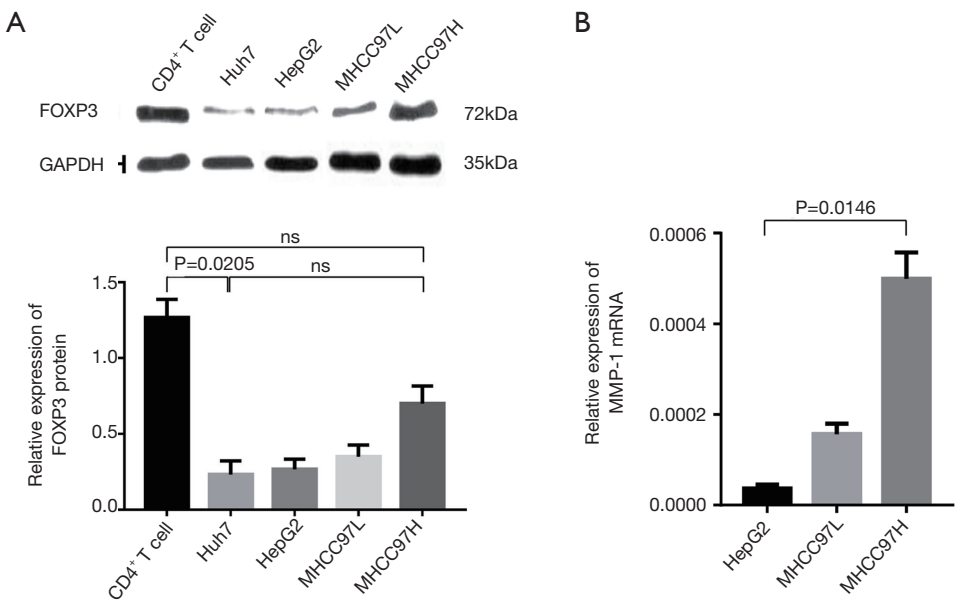

Figure 3 The expression levels of FOXP3 and MMP-1 in HCC cell lines with different metastatic potentials. (A) Expression levels of FOXP3 in the four HCC cell lines (Huh7, HepG2, MHCC97L, and MHCC97H) detected by Western blot; (B) expression levels of MMP-1 mRNA in the three HCC cell lines (HepG2, MHCC97L, and MHCC97H) detected by real-time PCR. FOXP3, forkhead transcription factor 3; MMP-1, matrix metalloproteinase-1; HCC, hepatocellular carcinoma.

Table 2 Microarray-based gene expression profiles after FOXP3 overexpression in HepG2 cells

Gene	Ratio2	Ratio
SF1	2.0269	1.4879
FOXP3	63.599	84.2395
MMP-1	2.3047	2.1619
CTAG1B	4.5483	4.0921
LUZP1	2.618	2.8296

SF1, steroidogenic factor 1; *FOXP3*, forkhead box protein 3; MMP-1, matrix metalloproteinases-1; *CTAG1B*, cancer/testis antigen 1b: LUZP1, leucine zipper protein 1.

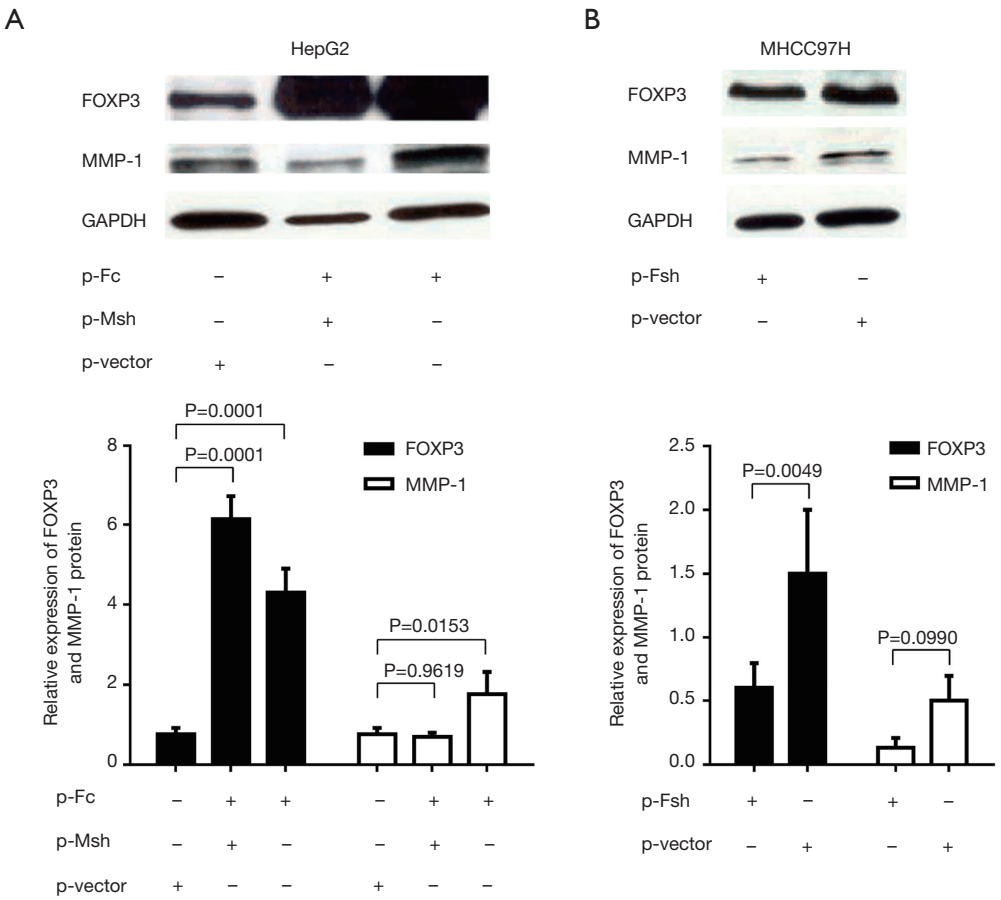

Figure 4 The expression levels of FOXP3 and MMP-1 in HepG2 and MHCC97H. (A) After FOXP3 overexpressed, or MMP-1 knocked down together in HepG2 cells, the expression levels of FOXP3 and MMP-1 were detected by western blot; (B) after the expression of FOXP3 was knocked down in MHCC97H cells, the expression levels of FOXP3 and MMP-1 were detected by western blot. FOXP3, forkhead transcription factor 3; MMP-1, matrix metalloproteinase-1.

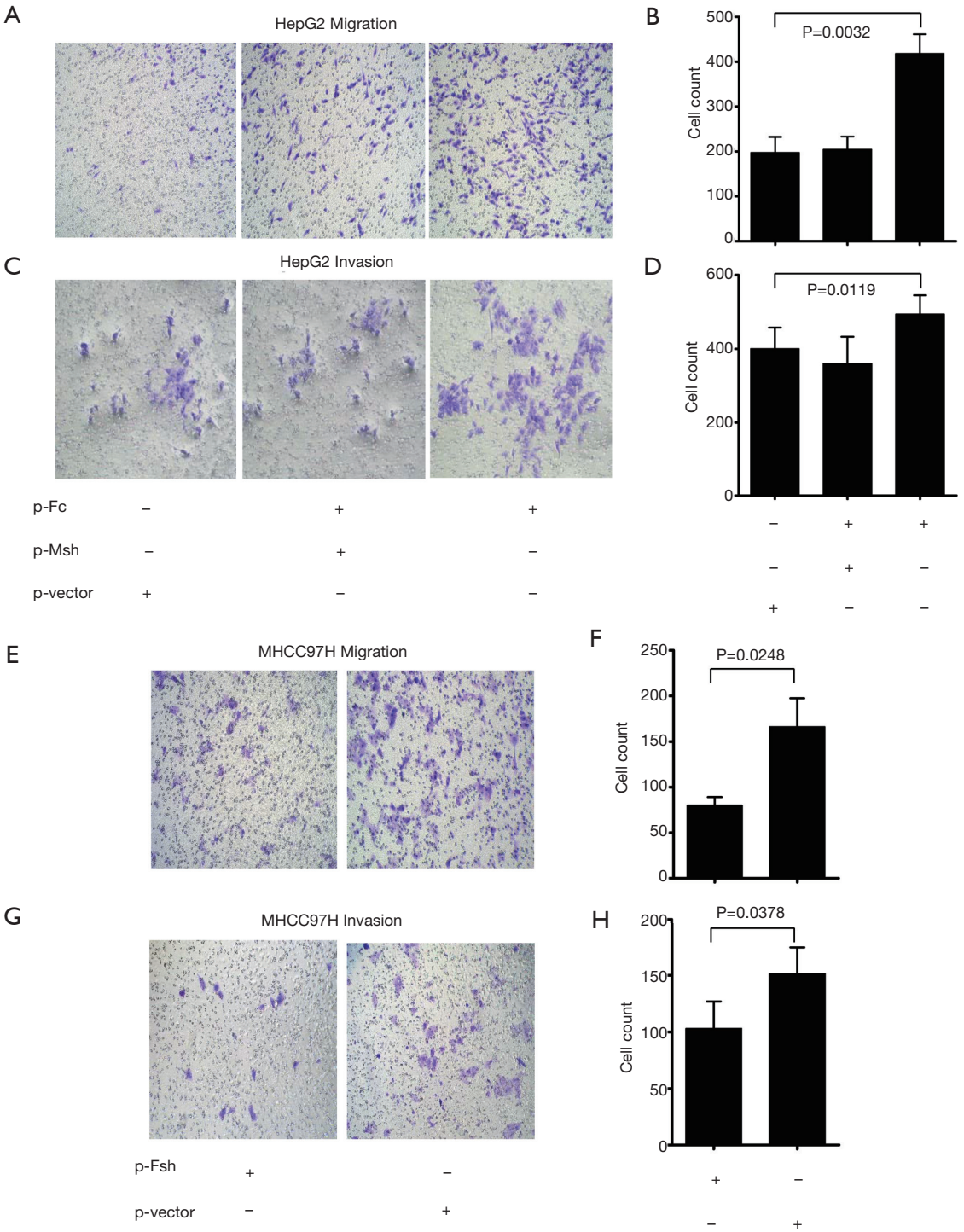

Figure 5 The migration and invasion abilities of HepG2 and MHCC97H. (A and B) After FOXP3 overexpressed, or MMP-1 knocked down together in HepG2 cells, the number of migrating cells was detected by transwell assay (×200). (C and D) After FOXP3 overexpressed, or MMP-1 knocked down together in HepG2 cells, the number of invasive cells were detected by invasion assay (×200). (E and F) After the expression of FOXP3 was knocked down in MHCC97H cells, the number of migrating cells was detected by transwell assay (×200). (G and H) After the expression of FOXP3 was knocked down in MHCC97H cells, the number of invasive cells was detected by invasion assay (×200). FOXP3, forkhead transcription factor 3; MMP-1, matrix metalloproteinase-1.

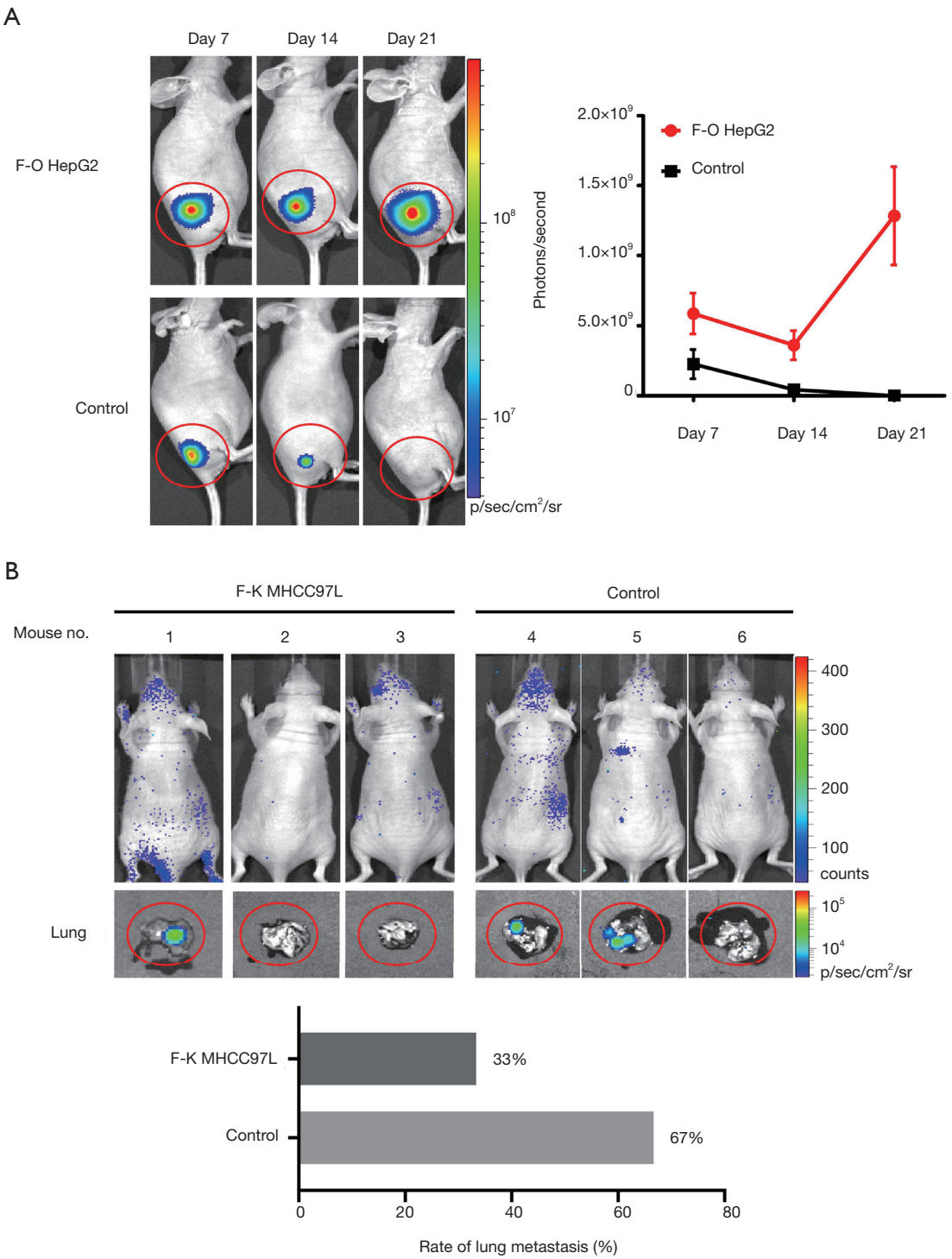

Figure 6 Tumor formation and metastatic abilities of HepG2 and MHCC97L in nude mice. (A) After FOXP3 was overexpressed in HepG2 cells, the tumor formation ability was evaluated in nude mice in day 7, 14, 21; (B) after the expression of FOXP3 was knocked down in MHCC97L cells, the incidence rate of lung metastasis was evaluated in nude mice in day 21. FOXP3, forkhead transcription factor 3; F-O HepG2, FOXP3 overexpressed HepG2 cells; F-K MHCC97L, FOXP3 knocked down MHCC97L cells; photon, photon number.

cells overexpressing FOXP3 was significantly improved, while the incidence of lung metastasis in MHCC97L cells that knocked down FOXP3 was significantly reduced. Therefore, we believed that the expression of FOXP3 in HCC cells might be related to the metastasis of HCC.

MMPs have been studied for a long time in cancer biology, and accumulated evidence has showed that MMPs are related to cancer progression and prognosis (32). It is known that MMP-1 plays a critical role in cancer metastasis, and more and more evidence shows that elevated MMP-1 expression was observed in the border of solid tumors (such as oral cancer and breast cancer) (33,34). To further explore the metastasis of HCC, the FOXP3 overexpression, or plasmid knockdown and microarray analysis of gene expression profiles were applied to analyze the expression of tumor metastasis-related genes in HCC cells. The results showed that overexpression of FOXP3 in HepG2 cells upregulated the expression of MMP-1. Furthermore, the migration and invasion ability of HCC cells were observed to increase after knocking down FOXP3, supported by the findings that overexpressed MMP-1 was associated with an elevated metastasis capacity, invasion and migration ability of the HCC cells (35,36). Base on the result, we concluded that FOXP3 in HCC cells promotes the metastasis of HCC through the regulation of the expression of MMP-1. To the best of knowledge, there is no such report illustrating the contributions of FOXP3 and MMP-1 in HCC metastasis with the combinations of both *in vitro* and *in vivo* studies.

In conclusion, our current research results indicated that the immunotherapy of HCC directed against FOXP3 gene may become a new direction for HCC treatment. In the future study, we aspire to collect a large number of samples from HCC patients and categorize them into different groups according to their disease backgrounds to explore the mechanisms of FOXP3 underlying the pathogenesis of HCC.

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was implemented after the approval of Medical Ethics Committee of Peking University People's Hospital (2019PHE018). Written informed consent was obtained from each patient prior to tissue sample collection in accordance with the Declaration of Helsinki (as revised in 2013). All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985).

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