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Original article

HIF1A and VEGF regulate each other by competing endogenous RNA mechanism and involve in the pathogenesis of peritoneal fibrosis

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ABSTRACT

Background: Peritoneal fibrosis is a major intractable complication of long-term peritoneal dialysis, and would eventually lead to peritoneal ultrafiltration failure and the termination of peritoneal dialysis. Hypoxia-inducible factor 1-alpha (HIF1A) has been reported to regulate vascular endothelial growth factor (VEGF) and involves in peritoneal fibrosis, but the exact molecular regulation mechanism remains unknown.

Methods: HIF1A and VEGF protein levels were measured in 42 peritoneal patients using enzyme linked immunosorbent assay. Bioinformatics, reverse transcription-polymerase chain reaction, correlation analysis, RNA interference, gene over-expression and luciferase assays were performed to clarify the competing endogenous RNA (ceRNA) regulation between HIF1A and VEGF.

Results: Both HIF1A and VEGF levels were elevated in the peritoneal effluent of peritoneal dialysis patients with ultrafiltration problems, and were positively correlated with each other at protein level and mRNA level. Bioinformatics analysis identified 8 common targeted miRNAs for HIF1A and VEGF, including miR-17-5p, 20a, 20b, 93, 106a, 106b, 199a-5p and 203. MiR-17-5p was proved to be present in patients' peritoneal effluent and selected for further studies. HIF1A mRNA and VEGF mRNA could regulate each other, and miR-17-5p was required in the regulations. Down/up regulation of HIF1A mRNA and VEGF mRNA resulted in up/down regulation of miR-17-5p. Furthermore, down/up regulation of miR-17-5p was associated with up/down regulation of HIF1A mRNA and VEGF mRNA. Luciferase assay indicated that HIF1A and VEGF regulated each other through 3′UTR.

Conclusion: HIF1A and VEGF could regulate each other in peritoneal mesothelial cell in the mediation of miR-17-5p and 3′UTR, indicating HIF1A and VEGF might regulate each other through competing endogenous RNA mechanism in the development of peritoneal fibrosis.

1. Introduction

Peritoneal dialysis (PD), as an established method of home dialysis, is the optimal renal replacement therapy for patients with end-stage kidney disease [1]. However, long-term PD would lead to functional and structural changes of peritoneal membrane, which is notoriously well known as peritoneal fibrosis [2–4]. Peritoneal fibrosis is characterized by injury of mesothelial cells, submesothelial thickening and alterations in the structure and number of blood vessels [5,6]. Several factors have been reported to be involved in the pathogenesis of peritoneal fibrosis, including uremia, bioincompatible dialysate solutions, high concentration, hyperosmotic glucose, glucose degradation products, acidic pH, peritonitis and chronic inflammation [4,6–9]. Peritoneal fibrosis is associated with impairment of peritoneal function [10], which will gradually lead to ultrafiltration (UF) dysfunction, encapsulating peritoneal sclerosis, peritoneal function failure and discontinuation of PD [2,9,11,12]. However, the exact mechanism of peritoneal fibrosis remains unknown [12].

Many factors had been reported to induce peritoneal fibrosis. Angiogenesis is an important event in the pathological process of peritoneal fibrosis [2,12]. Long-term exposure to PD induces angiogenesis in peritoneum, and inhibition of angiogenesis could alleviate peritoneal fibrosis [12]. Vascular endothelial growth factor (VEGF) is involved in the fibrosis and angiogenesis of peritoneal membrane,

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through the transforming growth factor-β (TGF-β)-VEGF pathway in mesothelial cells and fibroblast [1,13]. VEGF, as an inducer of angiogenesis, could stimulate the formation of new capillaries, lead to angiogenesis and peritoneal fibrosis in peritoneal membrane [2,14]. And the inhibition of VEGF has also been reported to ameliorate pathological angiogenesis [2].

Hypoxia was reported to be associated with a series of responses in peritoneal tissue, including angiogenesis, fibrosis and submesothelial thickening, mainly by the regulation of hypoxia-inducible factor 1-alpha (HIF1A) [11,15]. Studies have also identified an association between hypoxia and fibrosis in liver cirrhosis, lung and kidney [16,17]. But the acting mechanism of HIF1A in peritoneal fibrosis is still unknown. There is extensive evidence which showing that HIF1A could induce VEGF expression in hypoxic condition [18,19]. Inhibition of HIF1A with rapamycin leads to decrease of angiogenesis and VEGF [11]. In our previous study, we found that low molecular weight heparin (LMWH) might alleviate peritoneal fibrosis through inhibition of HIF1A and VEGF [20]. However, the exact regulating mechanism between HIF1A and VEGF expression is poorly understood.

Accumulating evidence suggests that diverse noncoding RNAs (ncRNA), including mRNA, rRNA, tRNA, microRNA, circular RNA, sncRAN and lncRNA, are involved in the progression of a wide variety of diseases [21–23]. MicroRNA (miRNA) is a type of endogenous small noncoding RNA which inhibits mRNA by binding to their miRNA response elements (MREs), and then affects their stability and translation. Several miRNAs have been reported to modulate fibrosis in kidney and peritoneal membrane [24]. By competing for common miRNAs, different mRNAs can crosstalk with each other in the mediation of miRNAs, and this interaction is known as competing endogenous RNA (ceRNA) regulation mechanism [25]. It has been reported that HIF1A and VEGF could regulate each other through ceRNA mechanism in the pathogenesis of diabetic retina [26]. However, gene interaction is complex and usually organ specific. It is unclear whether HIF1 A and VEGF could regulate each other through ceRNA mechanism in peritoneal fibrosis. According to the bioinformatic analysis, HIF1A and VEGF were found to have multiple common miRNAs. Therefore, in this study, we are going to explore whether HIF1A and VEGF could regulate each other by ceRNA mechanism in the pathogenesis of peritoneal fibrosis.

2. Material and methods

2.1. Patient population

Fourty-two PD patients who had undertaken dialysis for more than one month in our unit from Jan 2015 to December 2017 were included. All patients received standard peritoneal equilibration test (PET) and dialysis adequacy assessment around 4 weeks after they were stable on PD. We collected 11 consecutive new PD patients, and 31 prevalent PD patients who had clinical UF problem and needed PET. Peritoneal dialysis effluents were collected from overnight dwelled dialysis samples of PD patients. This study was approved by the Committee on Ethics of Biomedical Research, Changhai Hospital, Shanghai, China. Written informed consent was obtained from all included patients.

2.2. Enzyme linked immunosorbent assay (ELISA)

The concentrations of HIF1A and VEGF protein in peritoneal dialysis effluents, were quantified using the human HIF1A ELISA Kit (Bioswamp, HM10162) and the VEGF ELISA Kit (NeoBioscience, EHC108.48), according to the manufacturer's instructions. Absorbance was read using a microplate reader (Multiscan MK3, Thermo, San Jose, CA, USA) under the wavelengths of 450 nm. Standard HIF1A and VEGF samples were used to create a standard curve. All samples were assessed twice.

2.3. Prediction software of target miRNA

Both of the miRanda and TargetScan software, which could be accessed from the websites "http://www.miRNA.org/miRNA/getDownloads.do" and "http://www.targetscan.org/vert_50/", were used to predict common targeted miRNAs of HIF1A and VEGF mRNA.

2.4. Cell culture

Human peritoneal mesothelial cell line (HMrSV5) was purchased from Tongpai Biotechnology Company (Shanghai, China). They were maintained in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified incubator with a constant air flow of 5% CO_2.

2.5. Transfection

Human-specific HIF1A siRNA lentivirus (shHIF1A), HIF1A overexpression lentivirus (oeHIF1A), VEGF siRNA lentivirus (shVEGF), VEGF overexpression lentivirus (oeVEGF), mimics control lentivirus and inhibitor control lentivirus were purchased from GeneCopoeia Inc (Rockville, MD, USA). Lentivirus transfection was performed according to the manufacturer's recommendations. Briefly, 48 h after 293 T cells were co-transfected with shHIF1A or oeHIF1A lentivirus and two helper plasmids, pCMVΔR8.92 and pVSVG-I (Shanghai Preii, China) using Lipofectamin2000 (Invitrogen). Culture media containing packaged lentivirus was harvested and concentrated. For cell infection, a concentration of 5×10^4/well HMrSV5 cells were incubated with shHIF1A or oeHIF1A with a multiplicity of infection (MOI) of 50 for 24 h. The culture medium was replaced every 24 h. Gene expression modulation efficiency was validated by real-time PCR and western blot analysis at day 5 post transduction. Hypoxia environment was simulated by adding a layer of mineral oil over the nitrogen pretreated culture media. After covering the cells for 16 h, the hypoxia culture medium was replaced by fresh medium.

The miR-17-5p mimics and miR-17-5p inhibitor oligonucleotides (anti miR-17-5p) liposome were synthesized by GenePharma (Shanghai, China, Table 1). The transfection of anti miR-17-5p and miR-17-5p mimics was performed with the same method.

2.6. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from HMrSV5 cells and peritoneal dialysis effluents using PureLink RNA Mini Kit (Ambion, USA). Complementary DNA (cDNA) was reversely transcribed using SuperScript™III First-Strand Synthesis SuperMix for (Invitrogen, USA), according to the manufacturers' recommendations. The gained cDNA was used for RT-PCR, and every sample was repeatedly measured in triplicate. PCR was performed using SYBR GreenER™ qPCR SuperMix Universal (Invitrogen, USA). All data were normalized to an constantly expressed endogenous RNA (β-actin expression for mRNA, or U6 for miRNA). Primers used in RT-PCR were shown in Table 1.

2.7. Western blot analysis

Samples were homogenized in lysis buffer, and the lysate was centrifuged. Total protein was firstly subjected to SDS-PAGE, and then transferred to PVDF membranes. Samples were incubated together with primary antibodies: HIF1A (Affinity, AF1009), VEGF (Affinity, AF5131) and β-actin (Boster, BM0627). And then secondary HRP-tagged antibodies were added to the membranes, and signals were visualized, quantified and normalized to the corresponding β-actin signals.

Table 1
List of primers and other oligonucleotides used in this study.

Gene	Forward	Reverse
qPCR		
HIF1A	ATACCAACAGTAACCAACCT	TGAATAATACCACTAACAAC
VEGF	CATGGTCCTGCTGGAGTTCGTG	CGTCGCCGTCCAGCTCGACCAG
miR-17-5p	ACACTCCAGCTGGGCAAAGTGCTTACAGTGC	TGGTGTCGTGGAGTCG
β-actin	GTGGACATCCGCAAAGAC	AAAGGGTGTAACGCAACTA
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
MicroRNA reverse transcription		
miR-17-5p	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCTACCTGC	CAAAGUGCUUACAGUGCAGGUAG
Oligonucleotides		
shHIF1A	GUGAUGAAAGAAUUACCGAAUTT	
shVEGF	AUGUGAAUGCAGACCAAAGAA	
Mimics control	UCACAACCUCCUAGAAAGAGUAGA	
Inhibitor control	UUGUACUACAAAAGUACUG	
miR-17-5p mimics	CAAAGUGCUUACAGUGCAGGUAG	
miR-17-5p inhibitor	ACUACCUGCACUGUAAGCACUUUG	

Table 2
The clinical characteristics of the 42 included peritoneal dialysis patients.

Parameters	Group 1: new PD patients	Group 2: PD patients without UF problem[a]	Group 3: PD patients with UF problem[a]	P
Number of patients	n = 11	n = 21	n = 10	–
Age (year)	55.4 ± 9.0	54.4 ± 14.9	45.3 ± 15.7	0.180
Gender (Male/Female)	5/6	11/10	6/4	0.801
Diagnosis				0.723
Glomerulonephritis	4(36.4%)	11(52.4%)	5(50.0%)	
Diabetic nephropathy	2(18.2%)	4(19.0%)	2(20.0%)	
Hypertensive nephrosclerosis	4(36.4%)	3(14.3%)	1(10.0%)	
Polycystic kidney	1(9.1%)	2(9.5%)	1(10.0%)	
Interstitial nephritis	0(0%)	1(4.8%)	0(0%)	
Gouty nephropathy	0(0%)	0(0%)	1(10.0%)	
Body mass index (kg/m^2)	23.7 ± 3.0	22.1 ± 2.5	21.4 ± 2.3	0.139
Dialysis duration (month)	3.3 ± 0.5	55.7 ± 33.4	89.8 ± 43.7	< **0.001**
Blood urea nitrogen (mmol/L)	18.4 ± 6.8	16.0 ± 5.1	17.5 ± 5.3	0.126
Serum creatinine (μmol/L)	639 ± 212	979 ± 238	1052 ± 228	< **0.001**
Urine volume (mL)	1154 ± 425	410 ± 150	0 ± 0	< **0.001**
Net ultrafiltration (mL)	1050 ± 479	893 ± 358	258 ± 125	< **0.001**
Glomerular filtration rate ($mL/min/1.73\ m^2$)	5.1 ± 2.1	1.4 ± 2.8	0 ± 0	< **0.001**
White blood cells ($10^9/L$)	6.6 ± 2.4	7.2 ± 2.4	6.4 ± 2.4	0.627
Platelets ($10^9/L$)	218 ± 57	208 ± 87	173 ± 79	0.388
Hemoglobin (g/L)	107 ± 38	109 ± 20	106 ± 23	0.935
Total bilirubin (μmol/L)	2.2 ± 1.1	2.4 ± 1.1	2.3 ± 0.5	0.263
Albumin (g/L)	35.2 ± 6.3	35.9 ± 5.8	33.9 ± 5.4	0.670
Prealbumin (mg/L)	298 ± 56	320 ± 74	298 ± 90	0.658
Uric acid (mmol/L)	0.67 ± 0.63	0.41 ± 0.07	0.40 ± 0.05	0.298
Total cholesterol (mmol/L)	4.92 ± 1.31	4.35 ± 0.69	4.11 ± 1.59	0.238
Triacylglycerol (mmol/L)	2.25 ± 0.89	1.50 ± 0.77	1.22 ± 0.35	0.092
LDL (mmol/L)	2.71 ± 0.96	2.40 ± 0.63	2.44 ± 0.98	0.608
HDL (mmol/L)	1.16 ± 0.35	1.26 ± 0.34	1.10 ± 0.33	0.517
D/P creatinine	0.62 ± 0.11	0.65 ± 0.10	0.66 ± 0.10	0.586
Total Kt/V	2.21 ± 0.79	2.03 ± 0.55	1.91 ± 0.40	0.511
PTH (pg/mL)	308 ± 137	407 ± 155	528 ± 216	0.238
CRP (mg/L)	2.43 ± 1.15	5.04 ± 1.86	6.96 ± 2.61	0.472
Ferritin (μg/L)	243 ± 126	392 ± 137	394 ± 154	0.561

[a] UF: ultrafiltration; LDL: low density lipoprotein; HDL: high density lipoprotein; D/P creatinine: dialysate-to-plasma concentration ratio of creatinine; PTH: parathyroid hormone.

2.8. Measurement of miRNAs

Total RNA was extracted from HMrSV5 cells using PureLink RNA Mini Kit (Ambion, USA), and small RNA was purified using miRNA Purification Kit (CW0627S, Biotech, China). cDNA was reverse transcribed using miRNA cDNA Synthesis Kit (CW2141S, Biotech, China), and quantified using miRNA qPCR Assay Kit (CW2142S, Biotech, China). Primers of miR-17-5p were shown in Table 1. U6 was used as a control for normalization.

2.9. Luciferase assays

Luciferase assay was performed to explore whether HIF1A and VEGF could regulate each other by binding to their counterpart's 3′UTR. The HIF1A 3′UTR and VEGF 3′UTR were obtained by RT-PCR amplification. psiCHECK-2 and HIF1A 3′UTR, VEGF 3′UTR were co-transfected with HIF1A shRNA with Lipofectamine® 2000 according to the manufacturer's instructions. About 72 h after transfection, both firefly and renilla luciferase activities were measured using the Dual-Luciferase reporter system (Promega) [27].

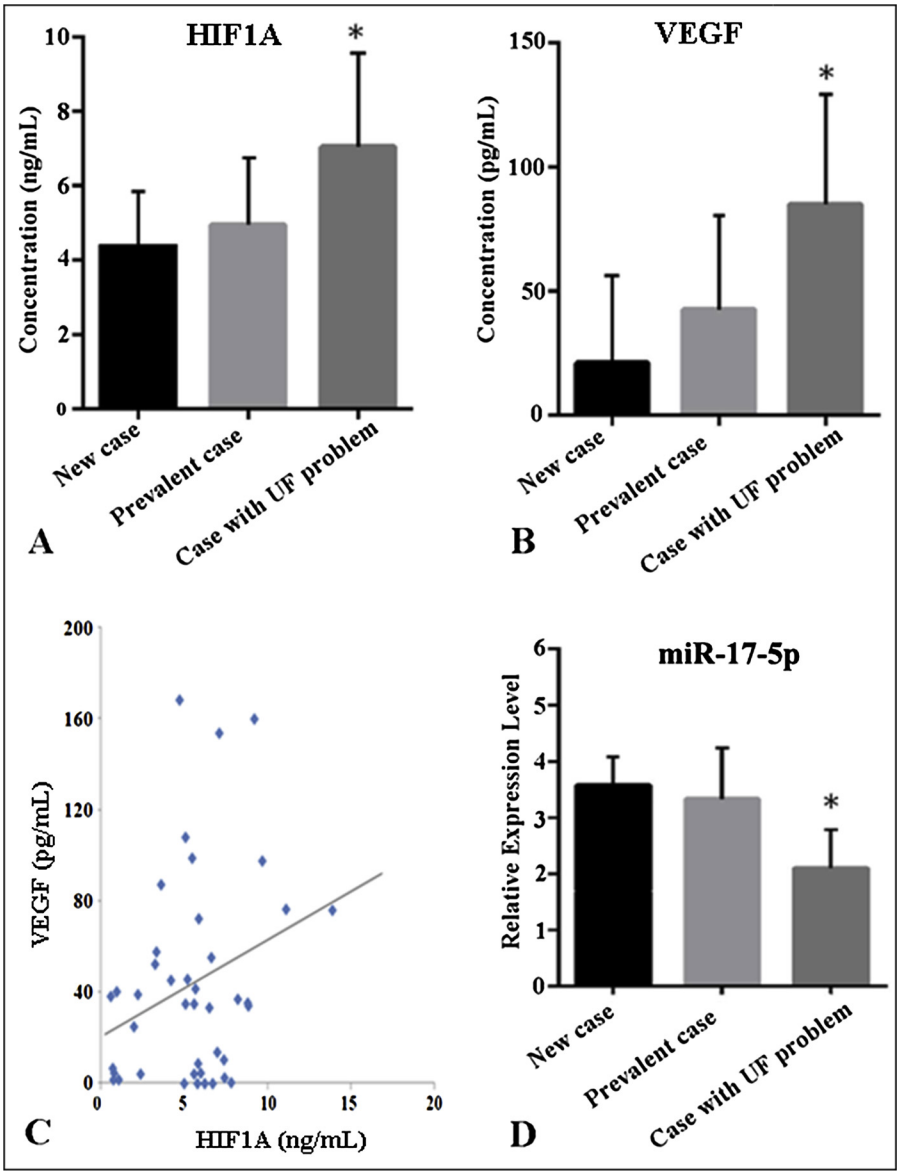

Fig. 1. Comparisons of HIF1 A and VEGF protein, and miRNA levels in peritoneal dialysis effluents of 42 PD patients. Group 1: new PD patients (n = 11); Group 2: prevalent PD patients without UF problem (n = 21); Group 3: prevalent PD patients with UF problem (n = 10). A: HIF1 A protein; B: VEGF protein; C: HIF1 A and VEGF protein levels were positively correlated with each other (r = 0.284, P = 0.039); D: miR-17-5p. (*P < 0.05 vs. New case, using Kruskal-Wallis test).

2.10. Statistical analysis

All data were analyzed with the SPSS 22.0 statistical package (Chicago, USA). Student's t test, ANOVA analysis and nonparametric analysis were used to detect the differences of continuous parameters as appropriate. Correlation of two continuous parameters was calculated by Pearson correlation coefficient (r). P < 0.05 (two sided) was considered as statistically significant.

3. Results

3.1. Correlation between HIF1A and VEGF protein levels in the peritoneal dialysis effluent of PD patients

Of the included 42 PD patients; 11 were new PD patients with dialysis duration less than one year (new PD patients, Group 1) and 31 were prevalent PD patients. Among the 31 prevalent patients, 21 had no objective UF problem by PET (prevalent patients without UF problem, Group 2), while the other 10 had documented UF problem by PET (prevalent patients with UF problem, Group 3). Their clinical characteristics, biochemical, peritoneal transport, and dialysis adequacy parameters were summarized in Table 2. As expected, Group 3 had longer dialysis duration and higher serum creatinine; lower urine volume, net UF and glomerular filtration rate (Table 2). No significant differences were identified in other parameters among the three groups.

Levels of HIF1 A and VEGF protein were detected by ELISA in 42 PD patients. Compared with Group 1, Group 3 had significant higher levels of HIF1 A (Fig. 1A, P = 0.035) and VEGF (Fig. 1B, P = 0.019). HIF1 A and VEGF protein levels were positively correlated with each other (r = 0.284, P = 0.039, Fig. 1C).

3.2. Bioinformatics analysis

Bioinformatics prediction using miRanda showed that VEGF mRNA and HIF1 A mRNA had 25 possible common target miRNAs. Prediction with TargetScan indicated that HIF1 A and VEGF mRNA had 12 possible common target miRNAs. Incorporating the prediction results of two software, there were 8 common miRNAs between HIF1 A and

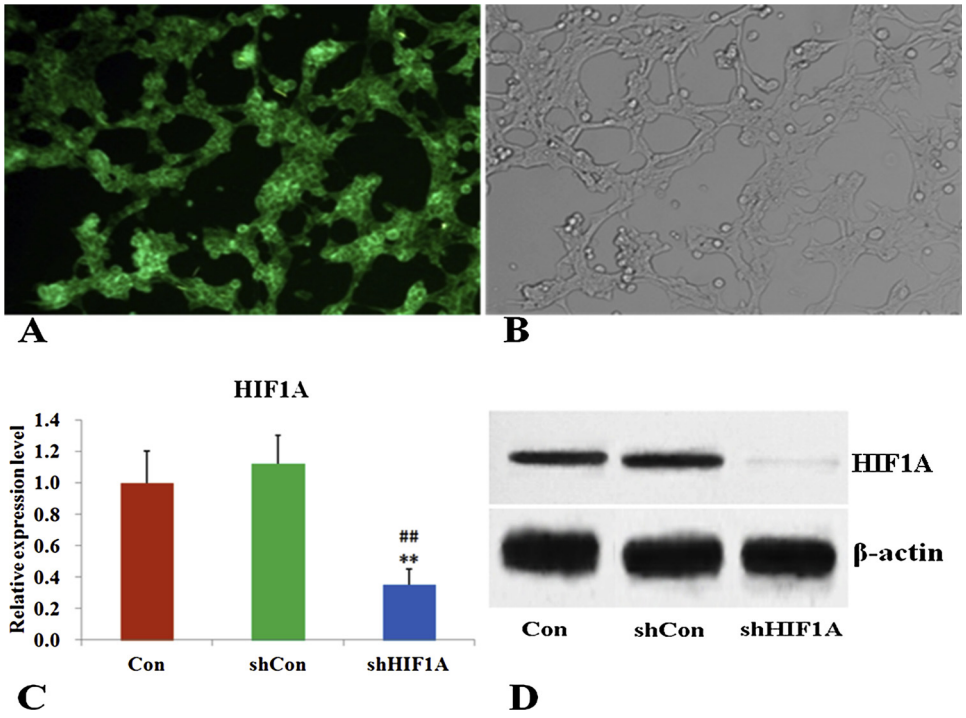

Fig. 2. Knockdown of HIF1 A in HMrSV5 cells (n = 3 per group). A: under fluorescence microscope; B: under bright microscope; C: RT-PCR; D: Western blot. (Con: no siRNA lentivirus; shCon: nonsilencing siRNA lentivirus; shHIF1 A: anti-HIF1 A siRNA lentivirus; ** P < 0.01 vs. Con; ##P < 0.01 vs. shCon).

VEGF, including miR-17-5p, 20a, 20b, 93, 106a, 106b, 199a-5p and 203. Our RT-PCR results proved the presence of miR-17-5p, miR-20a and miR-20b in the peritoneal dialysis effluents of PD patients. Furthermore, miR-17-5p had been reported in peritoneal tissues and peritoneal mesothelial cell lines in the literature [28]. So, miR-17-5p was selected for further study.

3.3. Relationship between expression levels of HIF1A mRNA, VEGF mRNA and miR-17-5p in the peritoneal dialysis effluents of 42 PD patients

Expression levels of HIF1 A mRNA, VEGF mRNA and miR-17-5p were assessed by RT-PCR in the peritoneal dialysis effluents of 42 PD patients. Compared with new cases, cases with UF problem had significant lower levels of miR-17-5p (Fig. 1D, P = 0.010). Correlation analysis demonstrated that HIF1 A and VEGF mRNA were positively correlated with each other (r = 0.652, P < 0.001); HIF1 A mRNA was inversely correlated with miR-17-5p (r = −0.385, P < 0.001); VEGF mRNA was inversely correlated with miR-17-5p (r = −0.436, P < 0.001).

3.4. Knockdown and overexpression of HIF1A and VEGF in HMrSV5 cells

After being cultured in hypoxia medium for 16 h, the levels of HIF1 A and VEGF secreted by HMrSV5 cells increased significantly. And then, anti-HIF1 A siRNA lentivirus (shHIF1 A) was used to suppress HIF1 A. At the highest infection efficiency, GFP was identified in more than 95% of HMrSV5 cells (Fig. 2A, B). After infection for 5 days, HIF1 A mRNA in HMrSV5 cells infected with siHIF1 A decreased significantly (Fig. 2C). Western blot also confirmed that HIF1 A protein decreased significantly (Fig. 2D). Similarly, HIF1 A was upregulated with oeHIF1 A lentivirus particles. And the expression of VEGF was inhibited and over-expressed using the same methods.

3.5. HIF1A and VEGF regulate each other in the mediation of miR-17-5p

Silencing of HIF1 A decreased VEGF mRNA level significantly (Fig. 3A). After knockdown of miR-17-5p, the downregulation of VEGF was attenuated greatly (Fig. 3B). In the contrary, overexpression of HIF1 A increased VEGF mRNA level significantly (Fig. 3C). After knockdown of miR-17-5p, the upregulation of VEGF was attenuated obviously (Fig. 3D).

Similarly, silencing of VEGF decreased HIF1 A mRNA level significantly (Fig. 3E). After knockdown of miR-17-5p, the downregulation of HIF1 A was attenuated greatly (Fig. 3F). In the contrary, overexpression of VEGF increased HIF1 A mRNA level significantly (Fig. 3G). After knockdown of miR-17-5p, the upregulation of HIF1 A was attenuated obviously (Fig. 3H).

3.6. The regulation of HIF1A mRNA and VEGF mRNA on miR-17-5p in HMrSV5 cells

Suppression of HIF1 A mRNA by shHIF1 A led to significant increase of miR-17-5p in HMrSV5 cells (Fig. 4A); while overexpression of HIF1A mRNA caused significant decrease of miR-17-5p (Fig. 4B). Similarly, inhibition of VEGF mRNA led to significant increase of miR-17-5p (Fig. 4C); while overexpression of VEGF mRNA resulted in significant decrease of miR-17-5p (Fig. 4D).

3.7. The regulation of miR-17-5p on HIF1A mRNA and VEGF mRNA

In order to clarify the impact of miR-17-5p on HIF1 A and VEGF mRNA, miR-17-5p was silenced and overexpressed with “anti miR-17-5p” and “miR-17-5p mimics”, respectively. Our study revealed that silencing of miR-17-5p in HMrSV5 was related with increase of HIF1 A mRNA (Fig. 4E) and VEGF mRNA (Fig. 4F). The overexpression of miR-17-5p was associated with decrease of HIF1 A mRNA (Fig. 4G) and VEGF mRNA (Fig. 4H).

3.8. Luciferase assay

To explore whether the regulation HIF1 A on VEGF is dependent upon 3′UTR, a chimeric luciferase plasmid combined with VEGF 3′UTR

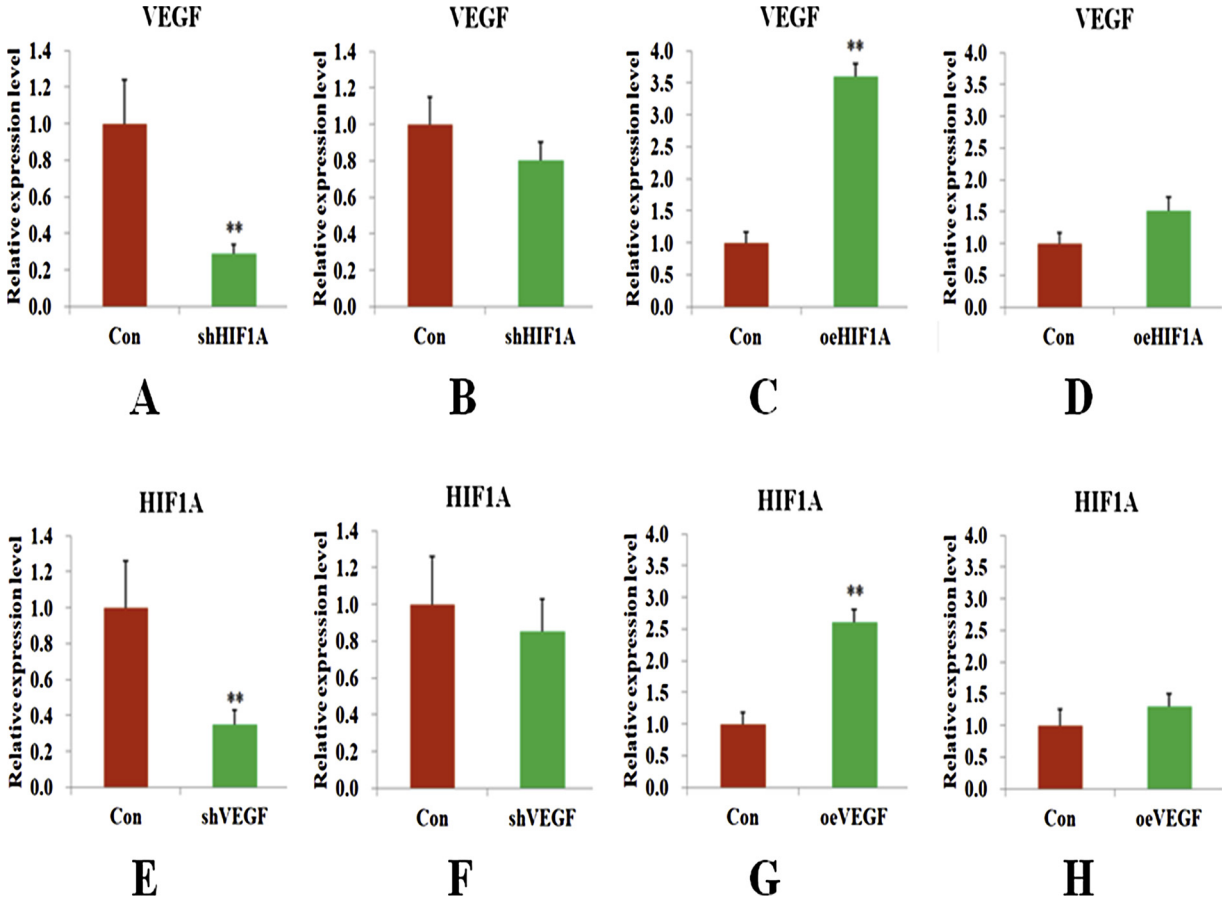

Fig. 3. HIF1 A and VEGF regulate each other in the mediation of miR-17-5p (n = 3 per group). In HMrSV5 cells, silencing of HIF1 A mRNA decreased VEGF mRNA level (A); after knockdown of miR-17-5p, the decrease of VEGF was attenuated (B). Overexpression of HIF1 A mRNA increased VEGF mRNA level (C); after knockdown of miR-17-5p, the increase of VEGF was attenuated (D); silencing of VEGF mRNA decreased HIF1 A mRNA level (E); after knockdown of miR-17-5p, the decrease of HIF1 A was attenuated (F). Overexpression of VEGF mRNA increased HIF1 A mRNA level (G); after knockdown of miR-17-5p, the increase of HIF1 A was attenuated (H). (Con: no siRNA lentivirus; shHIF1 A: anti-HIF1 A siRNA lentivirus; oeHIF1 A: HIF1 A overexpression lentivirus; shVEGF: anti-VEGF siRNA lentivirus; oeVEGF: VEGF overexpression lentivirus; ** $P < 0.01$ vs. Con).

was constructed. Luciferase assay revealed that luciferase activity decreased significantly in the "VEGF 3′UTR + shHIF1 A" group, indicating that HIF1 A regulates VEGF expression through VEGF 3′UTR (Fig. 4I). In the contrary, we also found that luciferase activity decreased significantly in the "HIF1 A 3′UTR + shVEGF" group, indicating that VEGF regulates HIF1 A expression through HIF1 A 3′UTR (Fig. 4J).

4. Discussion

Our results showed that both HIF1 A and VEGF were elevated in the peritoneal effluent of PD patients with UF problems, and they were positively correlated with each other in mRNA level and protein level. Bioinformatics analysis identified 8 common targeted miRNAs for HIF1 A and VEGF, including miR-17-5p, 20a, 20b, 93, 106a, 106b, 199a-5p, 203. The first 3 miRNAs were proved to be present in the peritoneal effluent of PD patients, and miR-17-5p was selected for further cellular tests. MiR-17-5p levels were inversely correlated with HIF1 A mRNA and VEGF mRNA. HIF1 A mRNA and VEGF mRNA could regulate each other, and the modulation was attenuated after miR-17-5p silencing, which indicated that miR-17-5p was required in the mutual regulation between HIF1 A and VEGF. Suppression/overexpression of HIF1 A mRNA and VEGF mRNA resulted in increase/decrease of miR-17-5p. Furthermore, inhibition/overexpression of miR-17-5p was associated with increase/decrease of HIF1 A and VEGF mRNA. Luciferase assay demonstrated that HIF1 A regulated VEGF expression via VEGF 3′UTR, and VEGF regulated HIF1 A expression via HIF1 A 3′UTR. Taken together, our results indicated that HIF1 A and VEGF might regulate each other through the ceRNA regulation mechanism, and involved in the pathogenesis of peritoneal fibrosis.

Peritoneal fibrosis is a major intractable complication of long-term PD, and would eventually lead to UF failure and termination of PD. So, clarification of its pathogenesis is critical to preserve peritoneal membrane integrity and extend PD duration [29]. Extracellular miRNAs had been detected in peritoneal dialysis effluent and was positively associated with the severity of peritoneal fibrosis [29]. In a study of rat model, miRNA screening found 8 decreased miRNAs (miR-31, miR-93, miR-100, miR-152, miR-497, miR-192, miR-194 and miR-200b) and one increased miRNA (miR-122) in the peritoneal fibrosis group [30]. In another study with peritoneal fibrosis rat model, miRNA screening identified 6 significantly increased (> 2 fold) miRNAs (miR-142-3p, miR-21-5p, miR-221-3p, miR-223-3p, miR-34a-5p and miR-327) in peritoneal tissue [24]. And the levels of miR-221-3p and miR-34a-5p were significantly correlated with peritoneal functions in PD patients [24]. The miR-17-5p level in dialysis effluent was found to be correlated with UF volume [28]. Decrease of miR-29b was found to be associated with peritoneal fibrosis in PD mice, and ectopic overexpression of miR-29b alleviated peritoneal fibrosis and UF dysfunction, by blocking TGF-β/Smad3 signal pathway [31]. Inhibition of miR-21-5p in peritoneal fibrosis mouse model led to attenuation of peritoneal thickening and better peritoneal functions [24]. In addition, miR-199a and miR-214 have also been reported to be involved in the damage and fibrosis of peritoneum in PD [32]. That is to say, miRNAs play an

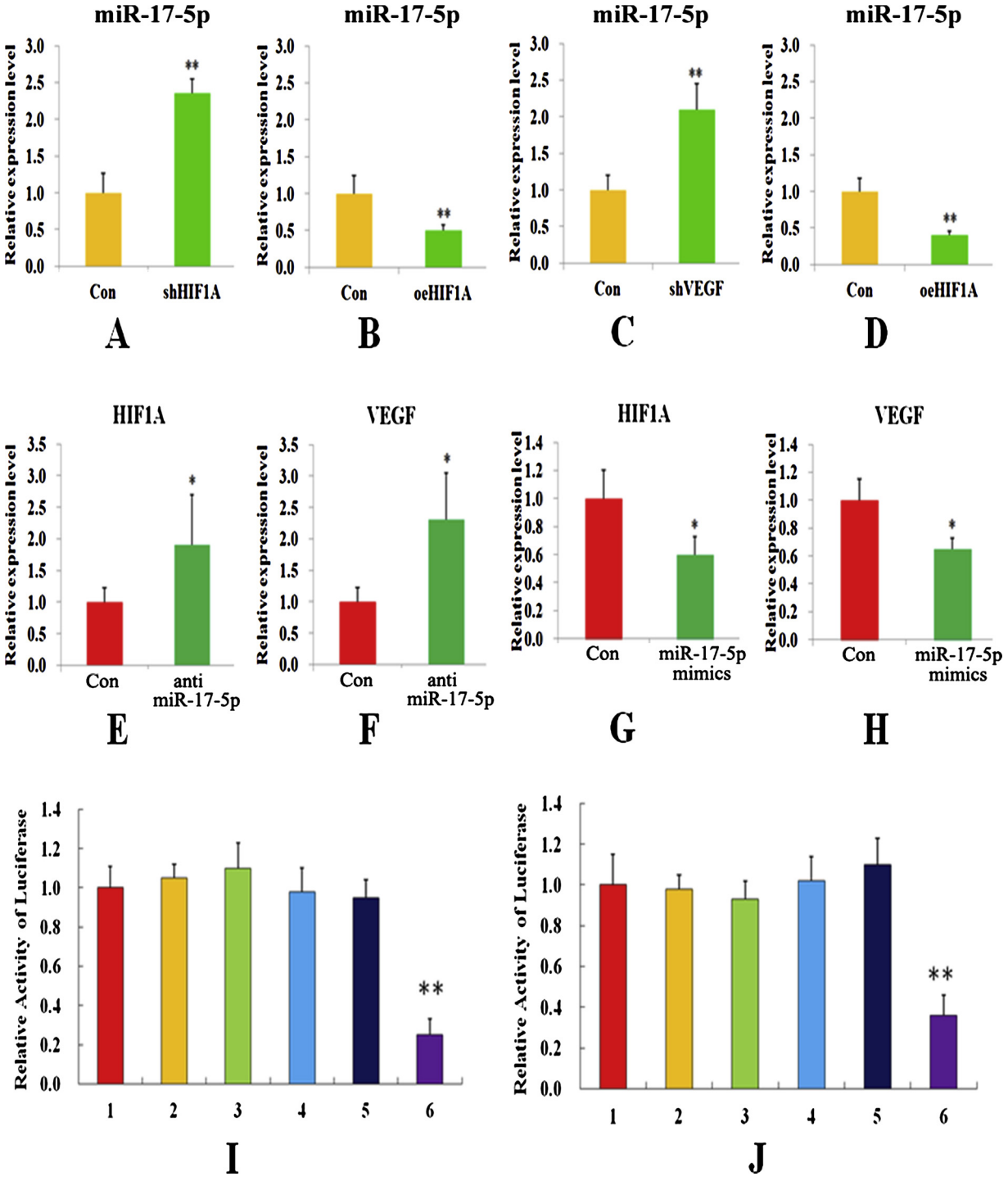

Fig. 4. The mutual regulation between HIF1 A and VEGF is dependent on miR-17-5p and 3′UTR (n = 3 per group). Suppression of HIF1 A mRNA led to significant increase of miR-17-5p (A); overexpression of HIF1 A mRNA caused significant decrease of miR-17-5p (B); inhibition of VEGF mRNA led to significant increase of miR-17-5p (C); overexpression of VEGF mRNA resulted in significant decrease of miR-17-5p (D). Silencing of miR-17-5p resulted in increase of HIF1 A mRNA (E) and VEGF mRNA (F), and overexpression of miR-17-5p resulted in decrease of HIF1 A mRNA (G) and VEGF mRNA (H). I: Luciferase assays showed luciferase activity decreased significantly in the "VEGF 3′UTR + shHIF1 A" group, indicating that HIF1 A regulate VEGF expression through VEGF 3′UTR (1: Con; 2: shCon; 3: shHIF1 A; 4: VEGF 3′UTR + Con; 5: VEGF 3′UTR + shCon; 6: VEGF 3′UTR + shHIF1 A). *J*: Luciferase assays showed luciferase activity decreased significantly in the "HIF1 A 3′UTR + shVEGF" group, indicating that VEGF regulate HIF1 A expression through HIF1 A 3′UTR (1: Con; 2: shCon; 3: shVEGF; 4: HIF1 A 3′UTR + Con; 5: HIF1 A 3′UTR + shCon; 6: HIF1 A 3′UTR + shVEGF). (Con: no siRNA lentivirus; shCon: nonsilencing siRNA lentivirus; shHIF1 A: anti-HIF1 A siRNA lentivirus; oeHIF1 A: HIF1 A overexpression lentivirus; anti miR-17-5p: anti miR-17-5p siRNA lentivirus; shVEGF: anti-VEGF siRNA lentivirus; oeVEGF: VEGF overexpression lentivirus; miR-17-5p mimics: miR-17-5p overexpression lentivirus; * $P < 0.05$ vs. Con; ** $P < 0.01$ vs. Con).

important role in development of peritoneal fibrosis.

Hypoxia has been found to play an important role in the development of peritoneal fibrosis by activating HIF1 A, and then increasing VEGF production and epithelial-to-mesenchymal transition (EMT) of peritoneal mesothelial cells [15]. Inhibition of VEGF was found to increase UF volume, reduce mass transfer of glucose and decrease number of new blood capillaries [33]. The VEGF level in patients' PD effluent was reported to be correlated with the dialysate-to-plasma ratio of creatinine. VEGF mRNA in the peritoneal tissues was correlated with vessel number and peritoneal thickness in UF failure patients [13]. In a word, HIF1 A could regulate VEGF, and then involve in peritoneal fibrosis. However, the molecular mechanism between HIF1 A and VEGF in peritoneal fibrosis is still unknown. Our study showed HIF1 A and VEGF were correlated with each other in PD patients in protein level and mRNA level. Based on the ceRNA theory [25], if ceRNA regulation mechanism existed between two genes, their mRNA levels would be positively associated with each other. So, HIF1 A might regulate VEGF via ceRNA regulation. The further bioinformatic prediction indicated that they had 8 common targeted miRNAs, and three of them were proved to be present in dialysis effluent of PD patients. In the further advanced experiments, we demonstrated that HIF1 A and VEGF could regulate each other in the mediation of miR-17-5p and 3′UTR. Our results obtained from this study proved that HIF1 A and VEGF might regulate each other via ceRNA regulation mechanism in peritoneal fibrosis.

Ling et al had proved that the ceRNA regulation between VEGF and HIF1 A had contributed to diabetic retina [26]. The regulation mechanism and its corresponding biological effects vary greatly in different tissues and disease entities. And it is unclear whether such ceRNA interaction has a role in peritoneal fibrosis. As the best of our knowledge, no previous literature has reported the ceRNA mechanism in peritoneal fibrosis. In this study, we had reported the first evidence that HIF1 A and VEGF might regulate each other through the ceRNA regulation mechanism and involved in the pathogenesis of peritoneal fibrosis.

Better clarification of the molecular mechanism involving in peritoneal fibrosis, would be helpful in developing new strategy for the prevention and management of UF failure in PD patients. The inhibition of both HIF1 A and VEGF using miR-17-5p is a potential therapeutic target for the treatment of peritoneal fibrosis. However, miR-17-5p also has multiple targeted mRNAs. Overexpression of miRNA may lead to decrease of HIF1 A and VEGF, and have some beneficial effects [34]; while at the same time, it may also inhibit other targets and lead to some inverse effects [26]. Fluctuation of miRNAs levels have been reported in different disease entities [26], which should be evaluated completely before put into practice.

This is a preliminary study on the ceRNA regulation mechanism between HIF1 A and VEGF in the development of peritoneal fibrosis. Inevitably, it had several limitations. Firstly, we have investigated thoroughly the regulation between HIF1 A and VEGF with series experiments, but we haven't studied the exact binding site of VEGF and HIF1 A. Secondly, we haven't studied the biological effects of ceRNA regulation, although we had some clinical clues that increase of HIF1 A and VEGF were related with peritoneal dysfunction in PD patients. Thirdly, all our evidences came from cellular experiments and dialysis effluents of PD patients. No study on the peritoneal fibrosis animal models had been performed. Fourthly, HIF1 A and VEGF have several common targeted miRNAs, but we only studied the effect of one miRNA (miR-17-5p). The biological effects of other common miRNAs may be different. Even with the above mentioned limitations, our study still proved that HIF1 A and VEGF could regulate each other via miR-17-5p in peritoneal mesothelial cells.

5. Conclusion

Our study demonstrates that HIF1 A and VEGF could regulate each other in peritoneal mesothelial cell, in the mediation of miR-17-5p and 3′UTR, which indicating HIF1 A and VEGF might regulate each other through the ceRNA regulation mechanism. It facilitates us to understand the molecular mechanism of peritoneal fibrosis pathogenesis and to devise new strategies of treating/preventing peritoneal fibrosis in peritoneal dialysis patients.

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Author contributions statement

1) Li J, Li SX, and Gao XH: performing experiments, collecting and analyzing of data, drafting manuscript;

2) Zhao LF, Du J, and Wang TY: collection and analysis of data and manuscript preparation;

3) Wang L and Zhang J: critical review of manuscript;

4) Dong R, Guo ZY: concept, general supervision and manuscript revisions.

Conflict of interest

The authors have no conflicts of interest to disclose.

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