Abstract. Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. It has been reported that HCC has a poor prognosis. In the majority of cases, once metastatic, HCC is incurable. To identify an effective treatment for HCC, it is important to understand the underlying molecular mechanisms of HCC-associated occurrence, proliferation, metastasis and carcinogenesis. In the present study, the role of Up-frameshift 1 (UPF1), a potential tumor suppressor, was investigated in the HCC cell lines. The expression levels of UPF1 in an HCC cell line were examined by reverse transcription-quantitative polymerase chain reaction. The expression levels of 19 key proteins in numerous signaling pathways were detected via protein array analysis in the presence of UPF1 overexpression. The present study further investigated the effects of UPF1 expression levels on the epithelial-mesenchymal transition (EMT) process by targeting E-cadherin, N-cadherin, Vimentin and Twist. The results of the present study revealed that UPF1 was significantly downregulated in an HCC cell line. The majority of the proteins exhibited upregulated expression levels in the presence of UPF1 overexpression in the HCC cell line, Huh-7. Key proteins, including cluster of differentiation (CD)31 (platelet endothelial cell adhesion molecule-1), Vimentin, CD44, PCNA, Ki-67, N-Cadherin, Survivin, P53, Met and retinoblastoma exhibited a significant association with UPF1. Furthermore, western blotting indicated that the expression levels of N-cadherin, Vimentin and Twist were notably upregulated while UPF1 was overexpressed; however, E-cadherin was downregulated and opposing observations were reported with protein array analysis. In summary, E-cadherin expression levels were regulated by the manifold, and UPF1, a potential tumor suppressor, may promote the EMT process in Huh-7 HCC cells. The findings of the present study suggested that UPF1 expression levels affected the EMT process by targeting E-cadherin, N-cadherin, Vimentin and Twist.

Introduction

Hepatocellular carcinoma (HCC) is a primary liver cancer with high mortality (1,2) and is one of the most common malignancies worldwide, particularly in Asia, Africa and Southern Europe (3). Numerous advanced methods and techniques have been recently developed and applied for the treatment of cancer, including chemotherapy, targeted therapy and immunotherapy from which therapeutic outcomes have been observed; however, the majority of HCC cases remain incurable once metastatic and poor prognosis is exhibited (4). To develop an effective treatment against HCC, understanding the underlying molecular mechanisms of the occurrence, proliferation, metastasis and carcinogenesis associated with HCC is required.

Epithelial-mesenchymal transition (EMT) is a biological process in which epithelial cells transform and exhibit a mesenchymal phenotype via a specific program (5). The EMT process serves an essential role in embryonic development, chronic inflammation, tissue reconstruction, cancer metastasis and a variety of fibrotic diseases; EMT also possesses unique characteristics of gene regulation, such as that for the downregulation of E-cadherin (6). In addition, it has been demonstrated that EMT exhibits a significant effect on malignant tumors, whereby epithelial cells transform to obtain migration and invasion abilities associated with malignancy (7). Thus, it is important to investigate the molecular mechanism underlying the EMT process and understand its pathological significance in tumor occurrence, development and metastasis.

A previous study demonstrated that Up-frameshift 1 (UPF1), a potential tumor suppressor, not only serves a key role in RNA degradation pathways, but exhibits significant effects...
on cell proliferation and differentiation by promoting these potentials of undifferentiated cells (8). UPF1 also promotes the decay of mRNAs encoding several other proteins that oppose the proliferative and undifferentiated cell state (9). Our previous study reported that UPF1 was downregulated and its gene was commonly mutated in pancreatic adenosquamous carcinoma (ASC), and to the best of our knowledge, it was the first to report UPF1 gene mutations in nonsense-mediated mRNA decay in ASC (10). However, a limited number of studies have reported the mechanism by which the expression levels of UPF1 affect HCC.

In the present study, it was demonstrated that UPF1 was significantly downregulated in HCC cell lines compared with in normal hepatocytes. Based on this finding, the expression levels of 19 key proteins associated with numerous signaling pathways were investigated in the presence of UPF1 overexpression (OE). In addition, the effects of UPF1 expression on the EMT process were analyzed by targeting the protein expression of E-cadherin, N-cadherin, Vimentin and Twist-related protein 1 (Twist). The findings of the present study may contribute to the development of HCC treatment in the future.

Materials and methods

Cell culture. The Huh-7 HCC cell line, HL-7702 normal hepatocytes and 293T normal cells were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in Minimum Essential medium with 10% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C. HL-7702 normal hepatocytes and 293T normal cells were cryopreserved and thawed prior to analysis.

Polymerase chain reaction (PCR). DNA was extracted from 293T cells following transfection with the UPF1 lentiviral vector. The UPF1 gene was amplified using primers designed by Primer Premier 5.0 software (PREMIER Biosoft International, Palo Alto, CA, USA) and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The primer sequences were as follows: UPF1 forward, 5'-AACACTATCAACCGGGAGC-3' and reverse, 5'-GCTAGCTCATTTGTCGTCATC-3'; GAPDH forward, 5'-TGAATTCACAGCGACACCACCA-3' and reverse, 5'-CACCCTGGTGCTGATAGGCCAA-3'. The PCR reaction system contained 13.25 µl double-distilled water, 5 µl 10X PCR buffer (Mg²⁺ Plus), 2 µl deoxynucleotide triphosphates, 2 µl PCR template, 1 µl forward primer, 1 µl reverse primer and 0.25 µl rTaq DNA polymerase (all from Takara Bio, Inc., Otsu, Japan), in a total volume of 25 µl. The PCR thermocycling conditions were as follows: 95˚C for 5 min, followed by 35 cycles of 95˚C for 30 sec, 55˚C for 30 sec and 72˚C for 1 min; and 72˚C for 10 min. The PCR products were separated by 1.5% agarose gel electrophoresis. The positive control was GAPDH.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from cells using SuperFectTR containing TRIzol (Takara Bio, Inc., Otsu, Japan). cDNA was obtained following RT using the M-MLV kit (Promega Corporation), according to the manufacturer's protocols. qPCR was performed on Step-one Plus system (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols of the SYBR-Green kit (Takara Biotechnology Co., Ltd., Dalian, China). The thermocycling conditions were as follows: Denaturation at 95˚C for 10 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 20 sec. GAPDH was used as an internal control, and the 2-ΔΔCT method (11) was used to normalize expression levels against GAPDH. Each sample was analyzed in triplicate. The primers used in the present study were presented in Table I.

Lentiviral vector construction and transduction. UPF1 (GenBank: NM_002911; https://www.ncbi.nlm.nih.gov/genbank/) was cloned into GV358 vector (Ubi-MCS-3FLAG-SV40-EGFP-IRE-PUROMycin; Shanghai Genechem Co., Ltd., Shanghai, China) at restriction site AgeI. A total of 4x10⁵ 293T cells were transfected with 1 µg plasmid using the FuGene HD transfection reagent (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocols. Nontransduced cells (CON) and negative control cells transfected with the empty vector (NC) were used as the controls. Green fluorescent protein was used as a reporter, to measure transfection efficiency by confocal microscopy (magnification, x100). Four randomly chosen microscopic fields were analyzed. The lentiviral supernatant was harvested 48-72 h after transfection and used to infect Huh-7 cells at a viral titer of 2x10⁴ TU/ml. The expression levels of UPF1 were confirmed by RT-qPCR and western blotting (described below).

Western blotting. Protein was extracted from cells using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) and protein concentration was determined by bicinchoninic acid assay. Cellular proteins (20 µg) from each sample were electrophoresed by SDS-PAGE (5% stacking and 10% separating gels). Then, the proteins were transferred onto polyvinylidene fluoride membranes (Merck KGaA, Darmstadt, Germany) and blocked with 5% skim milk in Tris-buffered saline and Tween-20 for 1 h at room temperature. The membranes were incubated with primary antibodies overnight at 4˚C. Subsequently, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1.5 h at room temperature. All antibodies used in the present study were listed in Table II. Protein bands were visualized using Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.). The positive control was the standard protein SURVIVIN-3FLAG-GFP (Shanghai Genechem Co., Ltd.).

Protein antibody array assay. The PathScan® Cancer Phenotype Antibody Array kit (Cell Signaling Technology, Inc., Danvers, MA, USA) was utilized to investigate alterations in the expression of key proteins associated with various signaling pathways in the presence of UPF1 overexpression. A total of 2x10⁶ cells were lysed using cell lysis buffer (cat. no. 7018; Cell Signaling Technology, Inc.) on ice, and then incubated with a slide for 2 h at room temperature or 4˚C overnight. Following washing, the slide was incubated sequentially with detection antibody cocktail, horseradish peroxidase-linked streptavidin, and LumiGLO® and peroxide.
reagents. Images were obtained using a chemiluminescence scanner (ChemiScope 5300; Clinx Science Instruments Co., Ltd., Shanghai, China).

Statistical analysis. Data are presented as the mean ± standard deviation from at least three independent experiments. A Student's t-test, \( \chi^2 \) test or Fisher's exact test were used for the comparisons between groups using SPSS 22.0 software (IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

**UPF1 is significantly downregulated in an HCC cell line.** The expression levels of UPF1 were determined within HCC (Huh-7) and normal hepatocyte (HL-7702) cell lines. A significant downregulation of UPF1 in the HCC cell line was observed compared with in HL-7702 cells, as presented in Fig. 1. The expression levels of UPF1 in the HCC cell line was ~60% lower than that of normal hepatocytes (P<0.01).

**Successful transduction of the lentiviral vector constructs and UPF1 overexpression.** Following transduction, the packaging cell line, 293T, exhibited notable green fluorescence (Fig. 2A). This indicated that the lentivirus was successfully transduced and that the UPF1 target gene was overexpressed. Expression of the target sequence following total DNA extraction and subsequent PCR was observed. Furthermore, a band near 125 kDa was detected on the gel, whose location is consistent with the target protein observed via western blotting (Fig. 2B). The positive control (PC) was encoded by a scrambled sequence, independent of the UPF1 sequence.

**UPF1 OE exhibits no effect on the levels of protein phosphorylation.** Numerous studies have demonstrated that the phosphorylation of key proteins is involved in tumorigenesis (12,13). To investigate the association between UPF1 OE and the extent of phosphorylation, the levels of phosphorylated UPF1 protein (P-UPF1) following lentiviral transduction of Huh-7 HCC and 293T normal cells were determined. Prior to transduction, no target band was observed for P-UPF1 for Huh-7 cells; however, a weak target band was observed under the UPF1 OE condition in Huh-7 cells (Fig. 3A). Following over-exposure several nonspecific bands were observed; therefore, the P-UPF1 band in Huh-7 cells may also be considered nonspecific (Fig. 3A). To investigate the effects of lentiviral transduction on the HCC cell line, another set of experiments were conducted to transduce the HCC cell line for UPF1 OE. As presented in Fig. 3B, UPF1 and P-UPF1 were detected in Huh-7 cell. Compared with in the CON and NC cell groups, UPF1 was notably upregulated in the OE group. However, the multiple bands observed for P-UPF1 may be nonspecific.

Table I. Primer sequences employed in the present study.

| Genes        | Forward primer (5'-3') | Reverse primer (5'-3') | Length (bp) |
|--------------|------------------------|------------------------|-------------|
| UPF1         | CCTTGTGAGGGCAAAATGCAA  | TGAAGCCCGAGGAGAGACGT   | 113         |
| GV358-UPF1   | GAGGATCCCCCGGTACGGTGCGACTGA | TCCCTGTAGTCATACCATACTG | 3398        |
| GAPDH        | TGACTTCAACAGGCACACCCA  | CACCCGTTACGTAGCCAAA    | 121         |

Table II. Antibodies employed in the present study.

| Antibody (cat. no.)         | Dilution               | Host species | Supplier                                           |
|-----------------------------|------------------------|--------------|---------------------------------------------------|
| UPF1 (ab109363)             | 1:10,000-50,000        | Rabbit       | Abcam (Cambridge, UK)                             |
| P-UPF1 (07-1016)            | 1:500                  | Rabbit       | Merck KGaA                                       |
| GAPDH (sc-32233)            | 1:2,000                | Mouse        | Santa Cruz Biotechnology, Inc. (Dallas, TX, USA)  |
| FLAG (F1804)                | 1:3,000                | Mouse        | Sigma-Aldrich; Merck KGaA                         |
| E-cadherin (14472)          | 1:1,000                | Mouse        | Cell Signaling Technology, Inc.                   |
| N-cadherin (13116)          | 1:1,000                | Rabbit       | Cell Signaling Technology, Inc.                   |
| Vimentin (5741)             | 1:1,000                | Rabbit       | Cell Signaling Technology, Inc.                   |
| Twist (ab50581)            | 0.5-1.0 µg/ml         | Rabbit       | Abcam                                             |
| Goat anti-mouse IgG-HRP (sc-2005) | 1:5,000              | Goat         | Santa Cruz Biotechnology, Inc.                   |
| Goat anti-rabbit IgG-HRP (sc-2004) | 1:5,000              | Goat         | Santa Cruz Biotechnology, Inc.                   |

HRP, horseradish peroxidase; IgG, immunoglobulin G; P-UPF1, phosphorylated Up-frameshift 1; Twist, Twist-related protein 1.
UPF1 is involved in the regulation of several key proteins of numerous signaling pathways. The PathScan Cancer Phenotype Antibody Array kit was utilized to investigate the effects of UPF1 on the expression of numerous signaling pathways in Huh-7 HCC cells. All analyzed genes were listed in Table III; the results of chemiluminescent detection and grayscale analysis were presented in Fig. 4A. Compared with the NC groups, the expression levels of the majority of proteins were upregulated when UPF1 was overexpressed. The protein expression levels of cluster of differentiation (CD)31 (platelet endothelial cell adhesion molecule-1, PECAM-1), Vimentin, CD44, proliferating cell nuclear antigen (PCNA), Ki-67, N-Cadherin, retinoblastoma (Rb), Survivin, P53 and Met were significantly upregulated in their respective signal pathways (Fig. 4B; P<0.05). These findings suggested that the expression levels of UPF1 exerted significant effects on numerous signaling pathways.

UPF1 serves an important role in EMT process. EMT is an important biological process for the development and progression of malignant tumors, in which epithelial cells are transformed to obtain malignant migration and invasive abilities (14,15). In the present study, 4 proteins (E-cadherin, N-cadherin, Vimentin and Twist) were selected as targets to investigate whether the expression levels of UPF1 can affect the EMT process in Huh-7 HCC cells. Western blotting revealed that compared with in the CON and NC groups, E-cadherin and N-cadherin were downregulated when UPF1 was overexpressed; however, that of Vimentin and Twist were notably upregulated (Fig. 5).

Overexpression of UPF1 affects the expression of antioncogene P53. P53 gene is a tumor suppressor gene (16,17). In the present study, the expression levels of P53 were investigated in the presence of UPF1 OE in Huh-7 HCC cell (Fig. 6). The results revealed that the expression of P53 was notably unaffected in the OE experimental group when UPF1 was overexpressed.

Discussion

HCC is the third leading cause of cancer-associated mortality worldwide (18). Several studies have demonstrated that tumorigenesis is a process associated with numerous factors (19-21). In the present study, the association between UPF1 expression and the EMT process underlying the tumorigenesis of HCC was investigated.

The expression levels of UPF1 in two cell lines were determined in the present study. RT-qPCR revealed that the expression levels of UPF1 in HCC cell lines were lower than that in normal hepatocytes, which was consistent with a recent report (22). This finding suggested a potentially important role of UPF1 in tumorigenesis. In addition, the phosphorylation posttranslational modification level of UPF1 was also evaluated in the presence and absence of UPF1 OE. Interestingly, no notable difference was observed in the OE group when UPF1 was overexpressed. Studies have reported that phosphorylation is one of the main factors associated with tumorigenesis (23-25); the present study reported notable association between the phosphorylation and overexpression of UPF1 in HCC.

To further understand the effects of UPF1 expression on the EMT process, a protein assay was conducted to determine whether UPF1 OE affected the expression of key proteins of several signaling pathways. The majority of the proteins analyzed in the present study were upregulated in the presence of UPF1 OE. Among these proteins, CD31/PECAM-1, Vimentin, CD44, PCNA, Ki-67, N-cadherin, Survivin, p53, Met and Rb exhibited a significant association with UPF1 OE. Additionally, the expression levels of E-cadherin, of N-Cadherin and Vimentin were upregulated, which has been associated with the EMT process. Based on these findings, the present study investigated how UPF1 affects the EMT process. As aforementioned, the process of EMT serves an important role in embryonic development, chronic inflammation, tissue reconstruction, cancer metastasis and variety of fibrotic diseases, and possesses unique characteristics of gene regulation, such as that for E-cadherin downregulation (26,27). Western blotting
demonstrated the expression levels of N-cadherin, Vimentin and Twist were upregulated under the UPF1 OE condition, which was consistent with the findings of PathScan analysis of Huh-7 HCC cells. In addition, when UPF1 was overexpressed, the expression of E-cadherin was downregulated in the present study, which was contradictory to the results of PathScan analysis. These observations suggested that UPF1 did not serve its role to suppress the expression of N-cadherin, Vimentin and Twist when overexpressed; the expression of E-cadherin may be regulated by the manifold. In a variety of human tumors, the role of E-cadherin

### Table III. Protein array analysis results.

| Target               | Site     | Modification     | Average Gray Value | Standard deviation | P-value | Upregulated/downregulated (%) |
|----------------------|----------|------------------|--------------------|--------------------|---------|-------------------------------|
|                      | NC       | OE               | NC                 | OE                 |         |                               |
| Positive control     | N/A      | N/A              | N/A                | N/A                | N/A     | N/A                           |
| CD31 (PECAM-1)       | N/A      | N/A              | 8.00               | 11.18              | 2.27    | 1.57 0.0181 39.79             |
| EpCAM                | N/A      | N/A              | 33.52              | 37.78              | 9.04    | 3.17 0.3159 12.73             |
| Vimentin             | N/A      | N/A              | 14.38              | 19.92              | 3.21    | 3.37 0.0155 38.47             |
| CD44                 | N/A      | N/A              | 8.05               | 10.70              | 1.99    | 1.44 0.0247 32.92             |
| CD45                 | N/A      | N/A              | 9.80               | 11.08              | 2.23    | 0.84 0.2169 13.10             |
| PCNA                 | N/A      | N/A              | 26.75              | 37.73              | 8.83    | 7.33 0.0410 41.06             |
| Ki-67                | N/A      | N/A              | 41.90              | 55.62              | 5.60    | 6.16 0.0024 32.74             |
| p27 Kip1             | N/A      | N/A              | 23.88              | 24.57              | 3.47    | 2.51 0.7043 2.86              |
| E-Cadherin           | N/A      | N/A              | 47.90              | 56.78              | 8.21    | 6.69 0.0670 18.55             |
| N-Cadherin           | N/A      | N/A              | 46.78              | 63.45              | 6.81    | 2.41 0.0012 35.63             |
| VE-Cadherin          | N/A      | N/A              | 12.58              | 14.13              | 2.62    | 2.55 0.3235 12.32             |
| MUC1                 | N/A      | N/A              | 13.28              | 17.88              | 3.54    | 3.79 0.0550 34.63             |
| Rb                   | Ser807/811| Phosphorylation | 69.27              | 76.88              | 4.64    | 1.27 0.0089 11.00             |
| HIF-1a               | Total    | N/A              | 13.18              | 16.32              | 2.93    | 2.83 0.0890 23.77             |
| Survivin             | Total    | N/A              | 17.25              | 24.10              | 4.65    | 3.49 0.0163 39.71             |
| p53                  | Total    | N/A              | 27.05              | 33.82              | 3.48    | 3.86 0.0097 25.02             |
| HER2/ErbB2           | Total    | N/A              | 6.50               | 6.75               | 1.73    | 1.69 0.8051 3.85              |
| Met                  | Total    | N/A              | 15.07              | 19.08              | 3.52    | 2.55 0.0473 26.66             |
| EGF Receptor         | Total    | N/A              | 18.18              | 23.02              | 5.32    | 2.48 0.0716 26.58             |

Values in bold indicate proteins that were significantly different in OE vs. NC (P<0.05). CD, cluster of differentiation; EGF, epidermal growth factor; EpCAM, epithelial cell adhesion molecule; HER2/ErbB2, human epidermal growth factor receptor 2; HIF-1α, hypoxia inducible factor-1α; PECAM-1, platelet endothelial cell adhesion molecule-1; PCNA, proliferating cell nuclear antigen; MUC1, mucin 1; N/A, not applicable; NC, negative control; OE, overexpression; Rb, retinoblastoma protein; VE-cadherin, vascular endothelial cadherin.

Figure 3. UPF1 OE exhibits no notable effects on the phosphorylation of UPF1. (A) Phosphorylation analysis of cells prior to lentiviral transduction. (B) Detection of UPF1 expression and phosphorylation following lentiviral transduction of the UPF1 gene. NC, negative control; CON, untransduced cells; OE, overexpression; P-UPF1, phosphorylated-Up-frameshift 1.

In the present study, it was found that under the condition of UPF1 OE, the expression of N-cadherin, Vimentin and Twist were upregulated, which was consistent with the findings of PathScan analysis of Huh-7 HCC cells. However, when UPF1 was overexpressed, the expression of E-cadherin was downregulated, which was contradictory to the results of PathScan analysis. These observations suggested that UPF1 did not serve its role to suppress the expression of N-cadherin, Vimentin and Twist when overexpressed; the expression of E-cadherin may be regulated by the manifold.
may be affected by gene mutations, resulting in mutant proteins, abnormal post-translational modification (phosphorylation and glycosylation) and increased protein hydrolysis (28). Conversely, the results of the present study indicated that higher expression levels of Twist associated with the overexpression of UPF1 may lead to the downregulation of E-cadherin and upregulation of N-cadherin, which is consistent with recent findings (29,30). At present, the molecular mechanism of UPF1 underlying the EMT process is notably complex and is not fully understood; however, the results of the present study suggested the possibility that the expression levels of UPF1 affect the EMT process by targeting E-cadherin, N-cadherin, Vimentin and Twist proteins.

Furthermore, the expression levels of P53 were determined when UPF1 was overexpressed in the present study. P53 has been reported as a tumor suppressor gene, and its inactivation may promote tumor formation (31). The results of the present study demonstrated that UPF1 OE did not affect the expression levels of P53 in Huh-7 HCC cell.

Several studies have demonstrated low expression levels of UPF1 in HCC cells and UPF1-suppressed tumorigenesis (32). Conversely, the results of RT-qPCR and western blotting in the present study revealed that UPF1 OE promoted the expression of numerous key proteins in several signaling pathways, including N-cadherin, Vimentin and Twist, contrary to the characteristics of UPF1; the expression of E-cadherin may be regulated by the manifold. The results of the present study indicated that UPF1 may be a potential target for regulating the EMT process (32).

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Availability of data and materials

The datasets used or analyzed during the present study are available from the corresponding authors on reasonable request.

Authors' contributions

YL, QW and FS designed the study and drafted the manuscript. YL, TZ, YC and ZZ performed the experiments. SQ, SQ,
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The authors declare that they have no competing interests.

The references are correct and complete.

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