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TRIM26 functions as a novel tumor suppressor of hepatocellular carcinoma and its downregulation contributes to worse prognosis

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ABSTRACT

Hepatocellular carcinoma (HCC) is the one of the most common malignancies worldwide and its prognosis is extremely poor. Tripartite motif (TRIM) proteins play crucial roles in cancer cell biology but the function of tripartite motif 26 (TRIM26) has not been investigated. We demonstrated that low expression level of TRIM26 in tumor samples was significantly correlated with worse prognosis in HCC patients. We also demonstrated its expression level was associated with several clinicopathologic features such as AFP level and T stage of HCC patients. Furthermore, we validated that TRIM26 was significantly downregulated in HCC tissue compared with normal liver tissue. To further clarify the functional role of TRIM26 in HCC, We confirmed that TRIM26 silencing can promote cancer cell proliferation, colony forming, migration and invasion in vitro with HCC cell lines HepG2 and Bel-7402. Then we utilized bioinformatic tool to predict gene influenced by TRIM26, showing TRIM26 could modulate gene sets about cancer cell metabolism. In conclusion, we proved that TRIM26 is a novel tumor suppressor modulating multiple metabolism-related pathways in HCC. To our best knowledge, this is the first study to investigate the function of TRIM26 in cancer biology. Our findings provide useful insight into the mechanism of HCC origin and progression. Moreover, TRIM26 may represent a novel therapeutic target for HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most commonly diagnosed malignancies, especially in China. Discovering dysregulated oncogene and suppressor is essential to clarify the potential mechanism of HCC and offers targets and markers for HCC diagnosis, treatment and prognosis prediction [1].

Tripartite motif (TRIM) proteins have evolutionary conserved domain structures and are considered as key regulators of cell biology. TRIM members contain multi-domain ubiquitin E3 ligases characterized by the presence of the N-terminal tripartite motif (including three zinc-binding domains, a RING, a Box type 1 and a B-Box type 2, and a coiled-coil region) [2]. Growing evidence showed that the TRIM proteins affect cell proliferation, differentiation, innate immunity, apoptosis regulation, cell migration and so on [2–5]. In recent years, the TRIM protein family has been reported for their roles in cancers. For example, TRIM16, TRIM3, TRIM28, TRIM40 and so on [6–9].

The gene of TRIM26 localizes to the major histocompatibility complex (MHC) class I region on chromosome 6 and the protein mainly localizes to cytoplasmic bodies [10]. Limited reports showed that it has ubiquitin ligase activity [11], plays roles in aspirin-exacerbated respiratory disease [12], anti- HIV-1 infection [13]

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and schizophrenia [14]. However, the detailed function of this protein is not well defined, and to date, it's role in tumor remains unclear.

In this work, by using the data from Gene Expression Omnibus (GEO), we found that the prognosis in HCC patients who had higher expression of TRIM26 in tumor could be better compared to the low expression patients. We also found it was significantly decreased in HCC tissues by immunohistochemical staining. Data from functional assays supported the notion that TRIM26 acted as a tumor suppressor in HCC by regulating cancer cell proliferation, colony forming ability, migration and invasion. A Gene Sets Enrichment Analysis (GSEA) was performed, showing TRIM26 modulated multiple metabolism-related pathways. For all we know, this is the first report showing TRIM26 works as a tumor suppressor.

2. Materials and methods

2.1. Cell culture, culture conditions and antibodies

Eight hepatocellular carcinoma cell lines were obtained from our lab conservation. Immortal normal liver epithelial cell (L02) was obtained from the Chinese Academy of Sciences Committee Type Culture Collection cell bank. The cells were cultured in DMEM medium with 10% FBS and 100 U penicillin/streptomycin at 37 °C in an atmosphere of 5% CO_2. Rabbit polyclonal anti-TRIM26 antibody was purchased from Biosynthesis Biotechnology (Beijing, China). Mouse monoclonal anti-β-actin antibody was purchased from Santa Cruz Biotechnology (Dallas, TX). Anti-goat and anti-mouse HRP-conjugated secondary antibodies were obtained from Promega Biotechnology (Dallas, TX).

2.2. Patients and histological and immunohistochemical (IHC) staining

All the cancer samples and normal tissues were retrieved from the Department of Pathology, Renmin Hospital of Wuhan University, Wuhan, China. The paraffin-embedded tissues were first stained with hematoxylin and eosin (HE) for histological examination. Subsequently, sections were subjected to antigen retrieval by heating the slides in a microwave at 100 °C for 10 min in 0.1 M citric acid buffer (pH = 6.0), and then incubated with corresponding antibodies at 4 °C overnight. After secondary antibody incubation at room temperature for 1 h, the slides were developed in 0.05% diaminobenzidine containing 0.01% hydrogen peroxidase. For negative controls, specific antibodies were replaced with normal goat serum by co-incubation at 4 °C overnight preceding the immunohistochemical staining procedure.

2.3. siRNA transfection

siRNAs were designed and purchased commercially (Genepharma, Shanghai, China) as follows:

siRNA sequence	Sense (5′–3′)	Anti-sense (5′–3′)
TRIM26 siRNA-1	CCGGAGAAUUCUCAGAUAATT	UUAUCUGAGAAUUCUCCGGTT
TRIM26 siRNA-2	GAGAGAAGCUGCACUACUATT	UAGUAGUGCAGCUUCUCUCTT
TRIM26 siRNA-3	GCAAAGGGAGAAGCUGAUATT	UAUCAGCUUCUCCCUUUGCTT
Scramble siRNA	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

siRNA were transfected into HepG2 and Bel-7402 cells using Lipofectamine 2000 (Invitrogen) in the absence of serum, with cells being 50–60% confluent. After 48 h, the media was replaced and cells were cultured for another 24 h. Then the cells were harvested for analysis of efficiency of knockdown.

2.4. Western analysis

Briefly, protein extracts from HCC cells were equally loaded on 10% SDS-PAGE, electrophoresed, and transferred on to nitrocellulose membrane (Millipore, Billerica, MA). After blocking with 5% non-fat milk in TBS containing 0.1% Tween-20, the membranes were incubated with the indicated primary antibodies and followed by secondary antibodies. Then the signals were detected by chemiluminescence phototope-HRP kit (Pierce Biotechnology, Rockford, USA) according to manufacturer's instructions.

2.5. RNA isolation and real-time PCR analysis

Total RNA was isolated from HCC cells using Trizol (Invitrogen) according to the manufacturer's instructions. Then, 2 μg of total RNA from each samples was subjected to cDNA synthesis. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analyses were performed in triplicate with the SYBR Green PCR Master Mix (TaKaRa, Otsu, Shiga, Japan) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. Relative expression levels of target genes = $2^{-\triangle\triangle Ct}$. The sequences of the primers are listed as follow:

Primers	Forward sequence (5′–3′)	Reverse sequence (5′–3′)
TRIM26	TGCACTACTACTGTGAGGACG	TCCTTAGGGTACTCAGGTGGT
β-actin	CATTAAGGAGAAGCTGTGCT	GTTGAAGGTAGTTTCGTGGA

2.6. Cell proliferation assay

Briefly, cells from each group were plated onto 96-well plates containing complete medium on Day 0 and allowed to attach overnight. Then, 10 μL CCK-8 reagent was added to each well and cultured at 37 °C and 5% CO_2 for 1 h from Day 1 to Day 5. Then the absorbance was measured with the multifunctional microplate reader at 450 nm. The growth curve was constructed with time as abscissa and absorbance as ordinate.

2.7. Plate colony formation assay

Cells treated by TRIM26-siRNA or scramble control were routinely seeded in 6-well plates (1000 cells/well). The cells were then incubated for 2 weeks terminated when visible clones were formed. (The medium was changed at the day 7). After that, cells were washed with PBS for three times, then fixed with 4% paraformaldehyde for 15 min, and stained with 0.1% crystal violet solution for 30 min. After these, the colonies were carefully washed with PBS until the background is clear. At last, the number of colonies was calculated.

2.8. In vitro migration and invasion assays

In vitro cell migration and invasion assays were performed using transwell chambers 8 μm filters (Corning). Cells were trypsinized and resuspended in serum-free medium, and 200 μl of the cell suspension (5×10^4 cells) was added to the upper chamber. The chamber was then cultivated in 5% CO_2 at 37 °C for 48 h. After that, the cells in the upper chamber were removed with a cotton swabs, and the attached cells in the lower section were stained with 0.1% crystal violet.

2.9. Gene set enrichment analysis (GSEA)

JAVA program for GSEA (http://www.broadinstitute.org/gsea) was utilized to analyze the potential genes influenced by TRIM26 high expression. HCC patient gene profiling data (GSE14520 [15]) was obtained from Gene Expression Omnibus (GEO) site. The patients were divided into two groups according to their TRIM26 expression level (top 50%: high vs. bottom 50%: low) and GSEA was carried out to assess the effects of TRIM26 expression level on various biological gene sets. Significantly gene sets were confirmed, which produced nominal P value <0.05 and false discovery rates (FDR) <0.25.

2.10. Statistical analysis

Overall survival and recurrence-free survival analyses were carried out using Kaplan–Meier method and results were statistically analyzed by log-rank test. Chi-square significance test was applied to analyze the correlation between gene expression and the clinicopathologic features. We performed three independent experiments in cellular studies to acquire the averaged replicates, and results were evaluated by the two-tailed, unpaired Student's t-test. Results are shown as mean values with 95% confidence intervals. Error bars represents standard error. Differences were considered significant with a value of P <0.05.

3. Results

3.1. The expression levels of TRIM26 in HCC patients correlates with several clinicopathologic characteristics and HCC patients' survival

In order to confirm the correlation between the expression level of TRIM26 and clinicopathologic factors in HCC, we downloaded the clinical information from GSE14520 and analyzed them statistically. Then the samples pooled in the dataset were classified into two groups according to the expression level of TRIM26 in tumor tissue and χ^2 test was used. As shown (Table 1), lower TRIM26 expression was closely associated with higher AFP ($P = 0.0018$), AJCC T stage ($P = 0.0246$), and CLIP stage ($P = 0.0019$). Current analysis also revealed that lower TRIM26 expression significantly correlated with high predicted risk metastasis gene signature ($P < 0.0001$), which strongly indicated

Table 1
Correlations of TRIM26 with clinicopathological features of HCC (GSE14520).

Characteristics	No. of patient	TRIM26 expression		Chi-square value	*P* value
		High	Low		
Age					
≤55	166	82	84	0.08	0.7818
>55	76	39	37		
Gender					
Male	211	106	105	0.04	0.8475
Female	31	15	16		
AFP, ng/mL					
≤200	128	76	52	9.74	0.0018
>200	110	43	67		
ALT, U/L					
≤50	142	71	71	0.00	1.0000
>50	100	50	50		
Cirrhosis					
Yes	223	110	113	0.51	0.4734
No	19	11	8		
Tumor size, d/cm					
<5	153	78	75	0.24	0.6267
≥5	88	42	46		
Tumor number					
Solitary	190	100	90	2.45	0.1146
Multiple	52	21	31		
AJCC T stage					
T1	96	58	38	7.27	0.0246
T2	78	39	39		
T3	51	19	32		
BCLC stage					
0	20	10	10	5.88	0.1176
A	152	86	66		
B	24	10	14		
C	29	10	19		
CLIP stage					
0	98	63	35	12.50	0.0019
1	79	36	43		
2, 3, 4, 5	48	17	31		
PRMS classification					
High	121	39	82	30.56	<0.0001
Low	121	82	39		

Data are presented as number.
AJCC, American Joint Committee on Cancer; BCLC, Barcelona Clinic Liver Cancer; CLIP, Cancer Liver Italian Program; PRMS, Predicted risk Metastasis Signature.

that TRIM26 could influence the expression profile of metastasis-related gene. Importantly, Kaplan–Meier survival analysis showed HCC patients with tumors displaying low TRIM26 expression levels had significantly shorter overall survival (OS) and recurrence-free survival (RFS) compared to those with high TRIM26 expression tumors (Fig. 1A, B; $P = 0.0265$ and 0.0308 respectively). These results strongly suggested that TRIM26 functioned as a tumor suppressor in HCC and could represent a potential new prognostic factor for HCC after curative hepatectomy.

3.2. TRIM26 is downregulated in liver cancer samples and HCC cell lines

To further investigate the role of TRIM26 in liver cancer, we respectively examined TRIM26 expression by immunohistochemistry in 70 human liver cancer tissues (61 cases of HCC and 9 cases of cholangiocellular carcinoma (CC)) and 10 normal hepatic tissues. We demonstrated that TRIM26 mainly localized in cytoplasma, which is consistent with previous report [10]. The percent of the samples of strongly positive in normal liver tissue (60%, 6/10) was significantly higher than that in cancer tissues (15.7%, 11/70) ($P = 0.0014$, Yates' corrected chi-square analysis). Among 10 normal tissues, 6 samples (60%) were strongly TRIM26-positive, 3 samples (30%) were moderately positive and only 1 sample (10%) was weakly positive (Fig. 1C and D). In contrast, 5 out of 9 cases (56%) of CC tissues were weakly positive, 3 (33%) were moderately positive and only 1 case (11%) was strongly positive (Fig. 1E and F); in HCC, 20 samples (33%) were weakly positive, 31 samples (51%) were moderately positive and only 10 cases (16%) were strongly positive (Fig. 1G and H). The results implied that the expression of TRIM26 was decreased in liver cancers including HCC and CC. What's more, we examined the expression level of TRIM26 in different HCC cell lines. Western-blot showed that TRIM26 protein expression was significantly lower in most HCC cell lines compared to immortalized normal liver cell line L02 (Fig. 2A). These results indicated that TRIM26 expression was significantly reduced in liver cancer tissues and the reduction of TRIM26 in HCC cells would promote the malignant behavior of cancer cell.

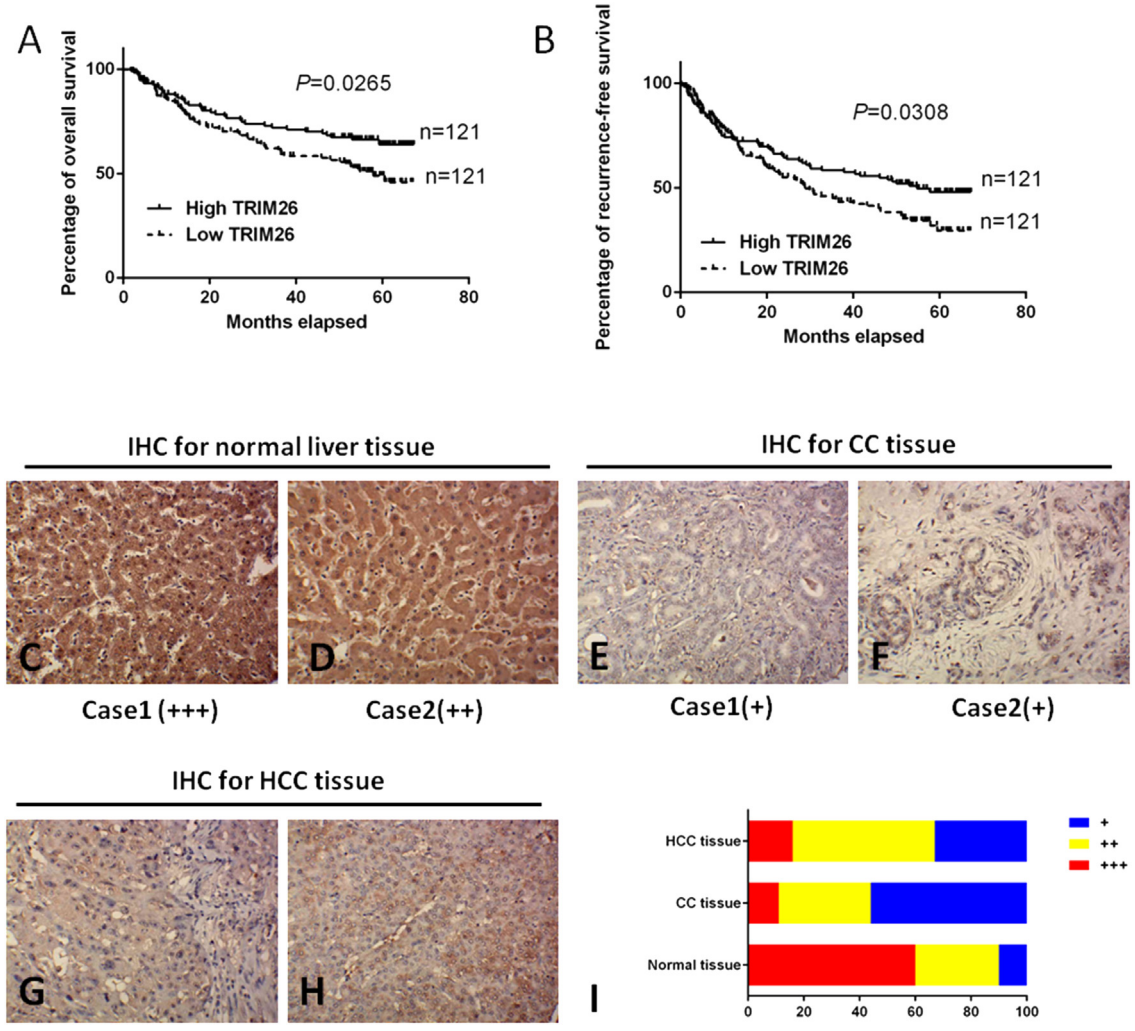

Fig. 1. Reduced expression of TRIM26 is associated with poor prognosis of HCC patients and it is reduced in cancerous liver tissues. (A) Low TRIM26 mRNA levels reduce overall survival of HCC patients in dataset GSE14520. (B) Low TRIM26 mRNA levels reduce recurrence-free survival of HCC patients in dataset GSE14520. The *P* values in the graphs were obtained from a long-rank test. (C, D) Representative photographs of the strongly positive staining (+++, C) and moderately positive staining (++, D) of TRIM26 protein in normal liver tissues are shown respectively. (E, F) Representative photographs of two cases of weakly positive staining (+) of TRIM26 protein in cholangiocellular carcinoma tissues are shown respectively. (G, H) Representative photographs of two cases of weakly positive staining (+) of TRIM26 protein in hepatocellular carcinoma tissues are shown respectively. (I) Distributions of TRIM26 staining grades (+, ++ and +++) in normal liver tissue, CC tissue and HCC tissue. (magnification, ×200).

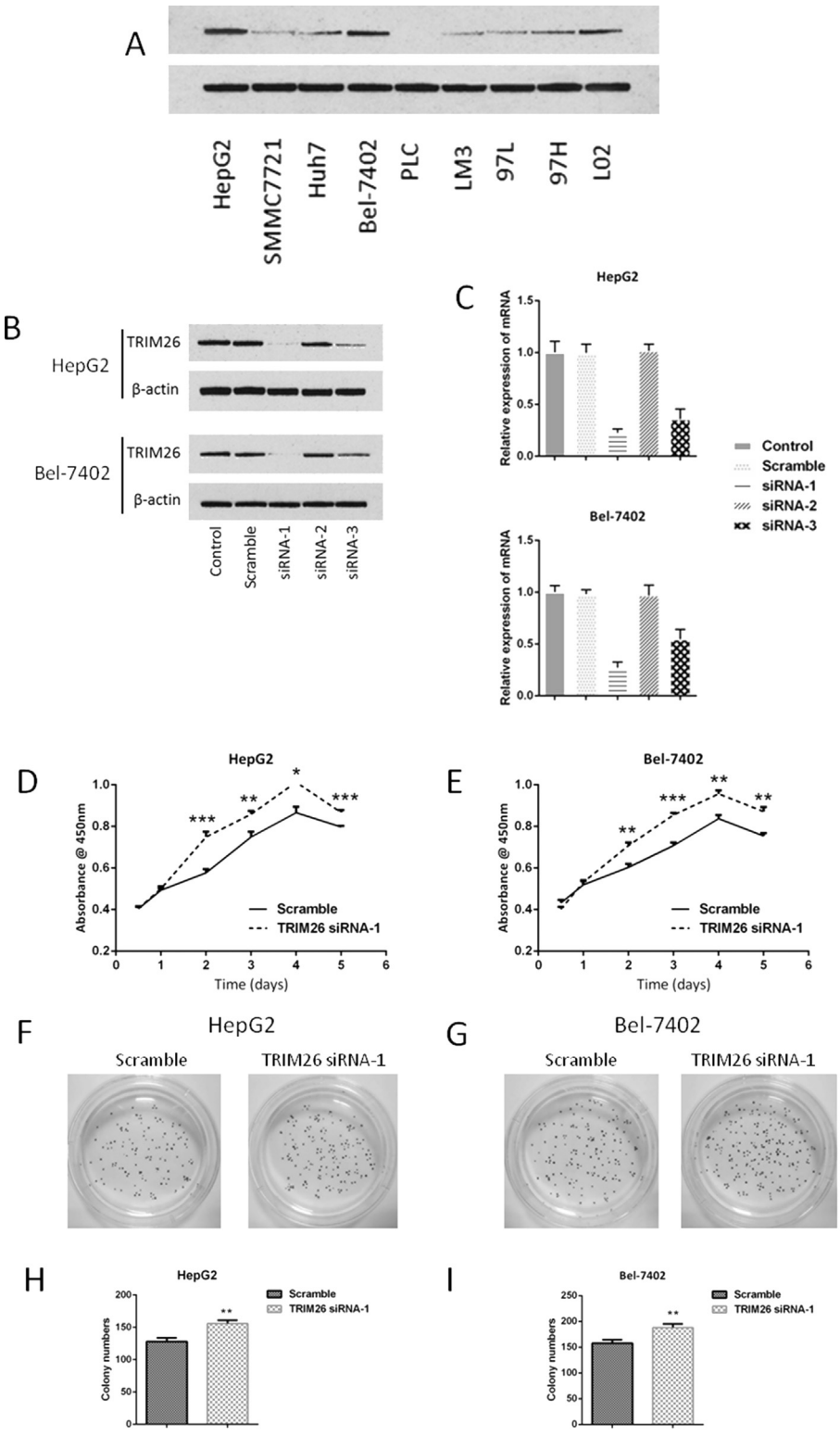

Fig. 2. Expression of TRIM26 protein in HCC cell lines and knockdown of TRIM26 by TRIM26 siRNA-1 promotes proliferation and colony forming ability in HCC cells in vitro. (A) Western blotting analysis of TRIM26 expression in immortalized normal liver cell L02 and 8 HCC cell lines. (B) Silencing of TRIM26 in HepG2 (top) and Bel-7402 (bottom) cell lines by siRNA targeting TRIM26 was confirmed by western blotting; β-actin was used as a loading control. (C) Silencing of TRIM26 in HepG2 (top) and Bel-7402 (bottom) cell lines by siRNA targeting TRIM26 was confirmed by real-time PCR. (D, E) Effect of TRIM26 silencing by siRNA-1 on the proliferation on HepG2 cells (D) and Bel-7402 cells (E) was determined by CCK-8 assay. (F, G) Effect of TRIM26 knockdown by siRNA-1 on colony numbers was determined by colony formation assay in HepG2 cells (F) and Bel-7402 cells (G). (H, I) Quantitative analysis of colony formation numbers is shown for HepG2 cells (H) and Bel-7402 cells (I). The bars represent the mean values of triplicate tests (mean ± SD). *, **, *** represents $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively.

3.3. TRIM26 enhances proliferation and colony forming ability HCC cells

Then we detected the cytological effect of TRIM26. Three siRNAs were designed to suppress the expression of TRIM26 in HepG2 and Bel-7402 cells. We carried out the qRT-PCR and Western-blot (Fig. 2B and C) to verify the knockdown efficiency, and then the siRNA-1 and siRNA-3 was chosen for the following experiments. The optimal time of silencing was at 48 h after transfection (data not shown). Firstly, cell proliferation assay and colony forming assay were chosen to perform upon TRIM26 silencing. We used CCK-8 assay to compare the cell proliferation rate of TRIM26 knockdown cells with the control groups. As shown (Fig. 2D and E; Supplementary Fig. 1A and B), the growth rates of two TRIM26 knockdown cell lines were both upregulated versus those of control groups, suggesting that TRIM26 silencing resulted in cellular events about enhancing proliferation signals. For the purpose of confirming the effect of TRIM26 on tumorigenesis, colony forming assay was also conducted in the two cell lines mentioned above and found that the colony numbers in the TRIM26 knockdown group were significantly more than the control groups in both cell lines (Fig. 2F–G; Supplementary Fig. 1C–F). This result pointed out that TRIM26 downregulation would strengthen the survival ability of single cancer cell.

3.4. TRIM26 down-regulated promotes migration and invasion of HCC cells

We then investigated the potential role of TRIM26 in regulating the ability of migration and invasion in HCC cells. Through the transwell assays we found that lower levels of TRIM26 expression strongly exhibited increased rate of migration after 48 h, compared with the control group in both cell lines (Fig. 3A, B, E, F; Supplementary Fig. 2A, B, E, F). Likewise, transwell assay with matrigel showed that inhibition of TRIM26 would enhance the invasion ability of HCC cells (Fig. 3C, D, G, H; Supplementary Fig. 2C, D, E, H). These results were coherent with that TRIM26 expression was inversely associated with metastasis potential gene signature (Table 1). This study reinforced that TRIM26 suppresses the metastatic ability of HCC cells.

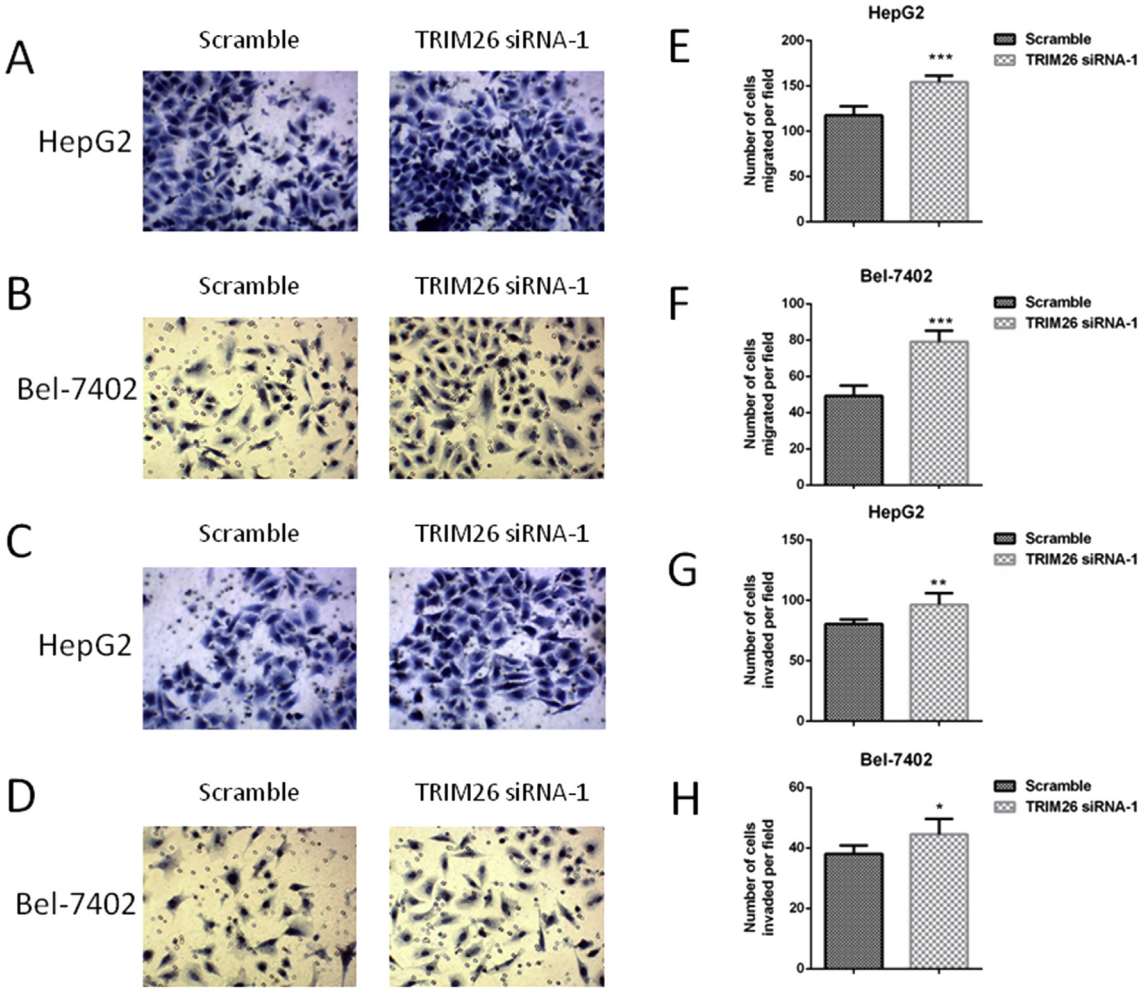

Fig. 3. Knockdown of TRIM26 by TRIM26 siRNA-1 promotes migration and invasion ability in HCC cells in vitro. (A, B) Cell migration was assessed by transwell assay in HepG2 cells (A) and Bel-7402 cells (B) after the cells were treated with 100 nM TRIM26 siRNA-1 for 48 h. The cells which migrated into the bottom surface of the filters were stained. (C, D) Cell invasion was assessed by transwell assay with matrigel in HepG2 cells (C) and Bel-7402 cells (D) after the cells were treated with 100 nM TRIM26 siRNA-1 for 48 h. The cells which migrated into the bottom surface of the filters were stained. (E–H) Quantitative analysis of migrated or invaded cell numbers is shown for HepG2 cells (E, G) and Bel-7402 cells (F, H). The bars represent the mean values of six independent tests (mean ± SD). *, **, *** represents $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively.

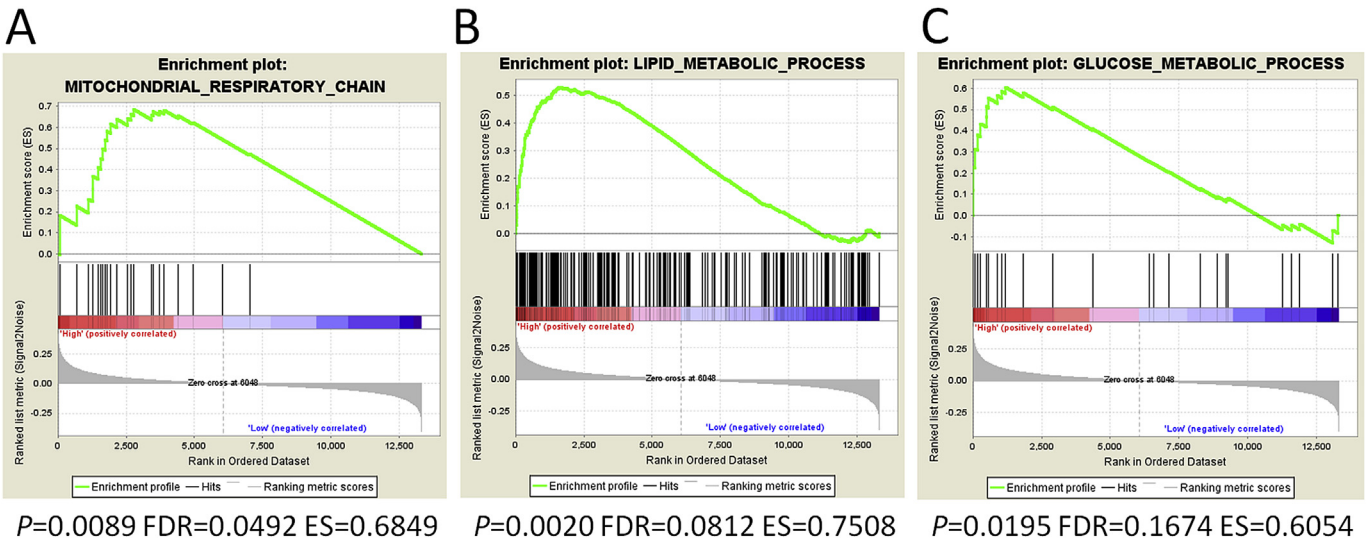

Fig. 4. Gene set enrichment analysis shows biological process modulated by TRIM26. GSEA analysis of GO terms showed TRIM26 might regulate gene sets associated with mitochondrial respiratory chain (A), lipid metabolic process (B) and glucose metabolic process (C).

3.5. TRIM26 may modulate metabolic biological processes of HCC cells

We speculated that TRIM26 may influence some crucial signal pathways or biological process in cancer biology based on its negative effect of cell's malignant phenotype, so we conducted a GSEA. As shown (Fig. 4A–C; Supplementary Table S1), the results from GSEA using HCC patient gene profiling data (GSE14520) showing that gene set differences in TRIM26 high vs. low patients indicated that TRIM26 regulates gene sets mainly associated with cell metabolism, such as mitochondrial respiratory chain, lipid metabolic process, glucose metabolic process and so on. We concluded that TRIM26 regulates of cell metabolism and its reduction may result in the metabolic reprogramming of cancer cell.

4. Discussion

Proteins in the TRIM family exert various physiological functions in innate immunity, development differentiation, host cell antiviral defense and so on [16,17]. Recently their abilities in cancer biology are emerging. Many members of the TRIM family are revealed to be important regulators of carcinogenesis by targeting their various substrates including cell cycle regulators, checkpoint molecules, apoptosis-related factors and tumor-promoting or inhibitory factors [18,19]. For example, TRIM15 in colon cancer [20], TRIM62 in breast cancer [21] and TRIM3 in glioma [7]. TRIM proteins also play important roles in HCC. As a result of genomic deletion and gene hypermethylation, TRIM35 is downregulated in HCC tissues and the expression level of TRIM35 is negatively correlated with the tumor grade, tumor size, and serum AFP level of patients [22]; germline inactivation of TRIM24 in mice leads to the development of HCC via disrupting RA-signaling in hepatocytes [23].

In this study, we aim to clarify the function of TRIM26, a TRIM protein not been well characterized, in cancer. To our best knowledge, the present work is the first to analyze the expression of TRIM26 in tumor tissue by histopathology. We revealed that low expression of TRIM26 is frequent in HCC tissue. Consistent with our expectations, we demonstrated that its low expression was associated with higher AFP level, AJCC T stage, and CLIP stage of HCC patients. We further defined TRIM26 as a novel tumor suppressor of HCC by demonstrating that silencing of TRIM26 promoted HCC cell proliferation, colony forming, migration and invasion. It should be noted that the downregulation of TRIM26 is more frequent and obvious in CC tissues. CC is the second most common liver malignancy after HCC, characterized by resistance to conventional radiotherapy and chemotherapy [24]. The prognosis of CC is even worse than HCC, so it is imperative to clarify the molecular mechanisms of CC origin and progression. Our findings provide a valuable clue that TRIM26 may also be tumor suppressor in CC.

Intriguingly, the bioinformatic analysis shows that TRIM26 is involved in the regulation of cancer cell metabolism of HCC. Metabolic reprogramming is the hallmark of cancer cell and is crucial for tumor initiation, growth and metastasis [25]. This often includes increased glycolytic metabolism (Warburg effect) and lipogenesis relative to abnormal mitochondrial electron transport [26,27]. Cancer cells predominantly produce energy by a high rate of glycolysis in the cytosol, instead of a comparatively low rate of aerobic respiration characterized by oxidation of pyruvate in mitochondria in most normal cells [26]. The lipogenesis signaling is also continually activated (called *de novo* lipogenesis), and this is essential for the cells to obtain enough lipid for membrane synthesis during proliferation [27]. Accompanied by metabolic reprogramming, there is signaling transduction dysfunction, change of gene expression profile and change of sensitivity to chemotherapeutics [28]. The key regulators of cancer cell metabolism are consequently considered promising therapeutic targets. For example, uncoupling protein 2 (UCP2), which is crucial for cancer cells to keep Warburg effect, can inhibit the activation of the mitochondrial permeability transition and reduce the release of proapoptotic factors to enhance the chemoresistance of cells [29]; ATP-citrate lyase (ACLY) in many tumors including HCC is upregulated and it can catalyze the generation of acetyl-CoA from citrate, providing vital block for lipogenesis [30](Supplementary Fig. 3). Our results indicates that TRIM26 may be crucial for maintaining normal metabolic functions of mitochondrion, and the reduction of its expression or defects of its function will result in the cancer-like metabolic signature. But the detailed molecular mechanism remains to be clarified in the further, probably directly or indirectly regulating downstream USP2, ACLY or other pivotal molecules in metabolism.

In conclusion, we identified TRIM26 as a tumor suppressor of HCC associated with clinical prognosis. We also observed that the knockdown of TRIM26 could directly increase cell proliferation and promote cell metastasis. Bioinformatic analysis showed TRIM26 might function as a key regulator of cancer cell metabolism. It may function as a potential biomarker and therapeutic target for HCC. The follow studies should focus on trying to explain the underlying mechanism of its inhibitory effects on HCC and investigating the role of TRIM26 in more types of cancers.

Conflict of interest

The authors declare that they have no competing interest.

Author's contribution

Conceived and designed the experiments: Ding Qianshan.

Performed the experiments: Wang Yi, He Du, Yang Liang, Dai Jinfen, Kang Jian.

GSEA analysis: Ding Qianshan.

Contributed funds/reagents/materials: He De, Wen Bo. Wrote the paper: Ding Qianshan, Zhang Qian, He Weiyang.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.117.

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