Tripartite motif 16 inhibits hepatocellular carcinoma cell migration and invasion

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Abstract. Tripartite motif 16 (TRIM16), a member of the RING B-box-coiled-coil (RBCC)/tripartite motif (TRIM) protein family, has been demonstrated to have significant effects on tumor migration by previous studies, but its specific contribution to hepatocellular carcinoma (HCC) is currently unknown. The aim of this study was to evaluate the prognostic value of TRIM16 and investigate its functional roles in HCC. The expression of TRIM16 in HCC patient samples were examined using qRT-PCR and western blotting. HCC cell lines with either TRIM16 overexpression or knockdown were established. The effect of TRIM16 on HCC cell migration and invasion was investigated using these cells. Compared with paired normal liver tissues in clinical cancer samples, we found that the expression of TRIM16 was significantly downregulated in HCC lesions. We also found knockdown of TRIM16 promoted epithelial-mesenchymal transition (EMT) in a manner correlated with HCC metastasis in vitro and in vivo. Mechanistically, TRIM16 inhibited ZEB2 expression, which in turn inhibited transcription of the pivotal ZEB2 target gene E-cadherin. RNA interference-mediated silencing of ZEB2 attenuated shTRIM16-enhanced cell migration and invasion. In conclusion, our findings define TRIM16 as an inhibitor of EMT and metastasis in HCC that predicts poor clinical outcomes.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common and generally incurable malignancies, which represent the third-leading cause of cancer-related deaths worldwide (1,2). The high mortality is due to late stage detection of this cancer when most of the therapies available are not effective (3). The disease is progressive and death usually occurs within 10 months of initial diagnosis (3). Most HCC related deaths are due to advanced metastatic disease, resulting from lymphatic, blood, or contiguous local spread, highlighting the need for a better understanding of this disease pathogenesis (4).

Characterized by a RING B-box-coiled-coil protein domain architecture, the tripartite motif (TRIM) families of proteins are involved in the pathogenic mechanism of various cancers, acting as either oncogenes or tumor suppressors (5). TRIM16 is highly expressed in basal keratinocytes and increases differentiation markers in keratinocytes, but is downregulated during the proliferative phase of wound healing (6). In a previous study, it was found that the expression of TRIM16 was significantly downregulated in SCC lesions. We also found knockdown of TRIM16 promoted epithelial-mesenchymal transition (EMT) in a manner associated with SCC metastasis in vitro and in vivo. Mechanistically, TRIM16 inhibited ZEB2 expression, which in turn inhibited transcription of the pivotal ZEB2 target gene E-cadherin. RNA interference-mediated silencing of ZEB2 attenuated shTRIM16-enhanced cell migration and invasion. In conclusion, our findings define TRIM16 as an inhibitor of EMT and metastasis in SCC that predicts poor clinical outcomes.

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clinical outcomes, collectively indicating that TRIM16 represents a novel therapeutic target in HCC.

**Materials and methods**

**Chemicals and antibodies.** Lipofectamine transfection and TRIzol reagents were purchased from Invitrogen (Grand Island, NY, USA). MG132 was purchased from Selleckchem (Houston, TX, USA). Antibodies against TRIM16, E-cadherin, N-cadherin, vimentin, HA, Myc, snail, slug, ZEB1, ZEB2 and β-actin antibodies were from Cell Signaling Technology (Danvers, MA, USA). Anti-α-catenin antibody and Matrigel were from BD (Franklin Lakes, NJ, USA). Unless otherwise noted, all other chemicals were from Sigma (St. Louis, MO, USA).

**Cell lines and cell culture.** Liver cancer cell lines HepG2, SMMC-7721, HCCLM3, MHCC97H, and HEK 293 Phoenix amphi-packaging cells were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Chinese Academy of Sciences; liver cancer cell lines were routinely cultured as previously described (9). Cell lines were maintained at 37°C in an atmosphere containing 5% CO₂, unless otherwise noted. Media used for cell culture included Dulbecco's modified Eagle's medium or RPMI-1640 supplemented with 10% fetal bovine serum.

**Patients and specimens.** Sixty-one tumor and para-cancerous tissues which, were used for qRT-PCR and western blot analysis, were randomly collected from HCC patients who underwent curative resection with informed consent between 2011 and 2014 at the Department of Laparoscopic Surgery, First Affiliated Hospital of Dalian Medical University. Study protocols were approved by the Hospital Ethics Committee of Dalian Medical University, and written informed consent was obtained from patients based on the Declaration of Helsinki.

**Establishment of TRIM16 stable expression and TRIM16, ZEB2 knockdown cell lines.** pBabe retroviral construct containing human TRIM16 cDNA and pSuper with shRNA against human TRIM16 were prepared as described previously (10). The generation of retrovirus supernatants and transfection of cancer cells were conducted as described previously (11). Infected cells were selected by adding 2 µg/ml puromycin to the culture medium for 48 h and then maintained in complete medium with 0.5 µg/ml puromycin. Empty retroviral-infected stable cell lines were also produced by the above protocols. siRNA against ZEB2 expressed in pSuper vector were prepared as described previously (11). The generation of retrovirus supernatants and transfection of cancer cells were conducted as described above except infected cells were selected by adding 400 µg/ml of G418. The expression of TRIM16 and ZEB2 was confirmed by qRT-PCR and western blot analysis.

**Cell invasion and motility assay.** Invasion assay was performed using Matrigel (BD)-coated Transwell inserts (Costar, Manassas, VA, USA) containing polycarbonate filters with 8-µm pores as detailed previously (12). According to the manufacturer's instructions, the inserts were coated with 50 µl of 1 mg/ml Matrigel matrix. Cells (2x10⁵) in 200 µl of serum-free medium were plated in the upper chamber, whereas 600 µl of medium with 10% fetal bovine serum were added to lower chamber. After 24-h incubation, cells that migrated to the lower surface of the membrane were fixed and stained. For each membrane, five random fields were counted at x10 magnification. Motility assays were similar to Matrigel invasion assay except that the Transwell insert was not coated with Matrigel.

**Confocal immunofluorescence microscopy.** Cell lines were plated on culture slides for 24 h. In addition, the cells were rinsed with phosphate-buffered saline fixed with 4% paraformaldehyde in PBS. Next, cell membrane was permeabilized using 0.5% Triton X-100. These cells were then blocked for 30 min in 10% BSA in PBS and incubated with primary antibodies in 10% BSA overnight at 4°C. After three washes in PBS, the slides were incubated for 1 h in the dark with FITC-conjugated secondary antibodies. Slides were stained with DAPI for 5 min to visualize the nuclei, and examined using a confocal imaging system (LSM 780) (Carl Zeiss, Jena, Germany).

**Western blot analysis.** Standard methods were used for western blot analysis. Cell lysates were prepared by extraction with lysis buffer. Proteins (10 µg) were separated by SDS-PAGE under reducing conditions and blotted onto a polyvinylidene difluoride membrane. Membranes were probed with specific antibodies. Blots were washed and probed with respective secondary peroxidase-conjugated antibodies, and the bands visualized by chemiluminescence.

**qRT-PCR.** Total RNA was extracted using TRIzol reagent and cDNA was synthesized using SuperScript II reverse transcriptase. qRT-PCR and data collection were performed with an ABI PRISM 7900HT sequence detection system. The primers used in this study are listed in Table I.

**In vivo tumor metastasis.** Different cells were resuspended in PBS at a concentration of 1x10⁷ cells/ml in metastasis assays. Cell suspension (0.1 ml) was injected into tail veins of nude mice. The mice were sacrificed by CO₂ 60 days after inoculation.

**Statistical analysis.** Data are presented as means ± standard deviation (SD). Data were analyzed by SPSS/Win11.0 software (SPSS, Inc., Chicago, IL, USA) in t-test (unpaired, two-tailed), and results were considered significant at P<0.05.

**Results**

**TRIM16 is downregulated and correlated with distant metastasis in HCCs.** To investigate whether TRIM16 might be involved in HCC, the mRNA expression level of TRIM16 in HCC tissues and their matched normal adjacent tissues was determined by qRT-PCR in 61 samples (Fig. 1A). As compared with normal tissues, HCC specimens showed lower expression of TRIM16 (Fig. 1B). We then analyzed TRIM16 expression in HCCs without or with metastasis property; we found that TRIM16 mRNA lower expression was significantly correlated with metastasis property in HCC tissues (Fig. 1C). We examined TRIM16 protein expression in the same HCC samples by western blot analysis (Fig. 1D). We observed that
the level of TRIM16-positive cells was markedly lower in HCC tissues than the level in the normal liver tissues (Fig. 1E). Most importantly, TRIM16 lower expression was consistently significantly correlated to distant metastasis in these HCC samples (Fig. 1F). As showed in Fig. 2, expression level of TRIM16 protein (Fig. 2A) and mRNA (Fig. 2B) in invasive

Figure 1. Expression of TRIM16 is downregulated in HCC tissues. (A) TRIM16 mRNA expression was analyzed by quantitative RT-PCR in 61 HCCs and adjacent tissues. (B) Comparison of the expression levels of TRIM16 mRNA in HCCs and adjacent tissues. (C) Comparison of the expression levels of TRIM16 mRNA in non-metastatic and metastatic HCC tissues. (D) TRIM16 protein expression was analyzed by western blotting in 61 HCCs and adjacent tissues. (E) Comparison of the expression levels of TRIM16 protein in non-metastatic and metastatic HCC tissues. **P<0.01 is based on the Student’s t-test. The results are from three independent experiments. Error bars, SD.
Let al: TRIM16 FUNCTION IN HCC

HCC cell lines was lower than that in the no-invasive HCC cell lines. These data demonstrated that the downregulation of TRIM16 might be relevant to invasive property of HCC.

Suppression of TRIM16 promotes HCC cell migration and invasion in vitro. To investigate the effect of TRIM16 knockdown on hepatoma carcinoma cell migration and invasion, HepG2 human hepatoma cell line, was first infected with shTRIM16 or the control. We used western blot and qRT-PCR analyses to detect transfection efficiency. Compared with the control cells, the HepG2 cells, transfected with the TRIM16 shRNA plasmid, displayed significantly decreased TRIM16 expression at the protein and mRNA levels (Fig. 3A and B). Boyden chamber assay was first used to assess the influence of TRIM16 on HCC cell migration to detect the effect of TRIM16 on HCC cells. As indicated by the Transwell assay and Matrigel assay, knockdown of TRIM16 expression significantly inhibited HCC cell migration and invasion (Fig. 3C and D). These results further demonstrated that silencing TRIM16 promotes HCC cell migration and invasion in vitro.

Ectopic TRIM16 expression inhibits HCC cell migration and invasion in vitro. We also used MHCC97H cells to establish a stable cell line that constitutively overexpressed TRIM16. The transfection efficiency was confirmed using western blot and qRT-PCR analyses. As shown in Fig. 4A and B, the MHCC97H cells that had been transfected with the TRIM16 expression plasmid displayed significantly lower TRIM16 mRNA expression levels compared with the control cells, with fold change of 2.0 in qRT-PCR. Ectopic expression of TRIM16 downregulated the expression of invasiveness-related genes such as integrin αv, integrin β5, and fibronectin. As indicated by the Transwell assay and Matrigel assay, ectopic expression of TRIM16 significantly inhibited HCC cell migration and invasion (Fig. 4C and D). These results further demonstrated that silencing TRIM16 promotes HCC cell migration and invasion in vitro.

Table I. Primer sequences used for qRT-PCR.

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<tr>
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Figure 2. Expression level of TRIM16 was measured in HCC cell lines. (A) Expression level of TRIM16 protein was measured by western blotting in non-invasive HCC cell lines (HepG2 and SMMC-7721) and invasive HCC cell lines (MHCC97H and HCCLM3). (B) Comparison of the relative expression levels of TRIM16 mRNA between non-invasive and invasive HCC cell lines. "P<0.01 based on the Student's t-test. The results are from three independent experiments. Error bars, SD.
increased TRIM16 expression both at the protein and mRNA levels compared with the vector cells. In order to measure the effect of TRIM16 on HCC cell migration and invasion, the effect of TRIM16 on HCC cell migration was assessed by the Boyden chamber assay. The overexpression of TRIM16 markedly reduced the migratory (Fig. 4C) and invasive (Fig. 4D) capacity of MHCC97H cells. These results indicate that ectopic TRIM16 expression inhibits HCC cell migration and invasion in vitro.

Silencing TRIM16 promotes HCC cell distant metastasis in vivo. We then investigated the functional relevance of TRIM16 for metastasis in vivo. HepG-2-shTRIM16 #2 and its control cells were injected into nude mice through the tail vein. We observed that silencing TRIM16 not only significantly increased the number of mice with distant metastasis (Fig. 5A), but also markedly increased the number of metastatic tumors in the lungs of each mouse (Fig. 5B and C). Therefore, the in vivo results further demonstrate the critical role of TRIM16 in HCC metastasis.

TRIM16 inhibits EMT behavior in HCC cells. During the establishment of these cell lines, we observed that HepG-2-shTRIM16 cells exhibited fibroblastic morphology,
compared to their respective control cells (data not shown). This observation was further confirmed by western blot and immunofluorescence analyses of epithelial and mesenchymal molecular marker expression. We showed that TRIM16 knockdown decreased the levels of epithelial markers (E-cadherin and α-catenin) and increased the levels of mesenchymal markers (N-cadherin and vimentin) (Figs. 6A and 7A). Moreover, expression levels of mRNA correlated with the corresponding protein levels (Fig. 6B), suggesting that TRIM16 affected the expression of epithelial and mesenchymal markers at the transcript level. Conversely, MHCC97H-TRIM16 cells reverted to a mesenchymal phenotype as compared to their respective control cells (data not shown). Consistent with this, ectopic TRIM16 expression resulted in an increase in expression of epithelial markers, and a decrease in expression of mesenchymal markers (Figs. 6C and D and 7B).

TRIM16 downregulates ZEB2 expression through interaction. To better understand the mechanisms by which TRIM16 engaged in the EMT program, we assessed the expression of the EMT regulating genes, such as Snail, Slug, ZEB1 and ZEB2, on HepG2-shTRIM16, MHCC97H-TRIM16 and their control cells. The results shown that HepG2-shTRIM16 cells exhibited greatly increased ZEB2 expression at protein

Figure 4. Ectopic TRIM16 expression inhibits HCC cell migration and invasion in vitro. (A) The levels of TRIM16 protein was verified by western blotting in MHCC97H-TRIM16s and the control cells. (B) The levels of TRIM16 mRNA was verified by qRT-PCR in MHCC97H-TRIM16 and the control cells. (C) MHCC97H-TRIM16 and its control cells were subjected to Transwell migration. quantification of migrated cells through the membrane and invad cells through Matrigel invasion assays. quantification of migrated cells through the membrane and invad cells through Matrigel invasion assays. (D) MHCC97H-TRIM16 and the control cells were subjected to Matrigel invasion assays. quantification of migrated cells through the membrane and invad cells through Matrigel invasion assays. **P<0.01 based on the Student’s t-test. The results are from three independent experiments. Error bars, SD. Scale bar, 50 nm.

Figure 4. Ectopic TRIM16 expression inhibits HCC cell migration and invasion in vitro. (A) The levels of TRIM16 protein was verified by western blotting in MHCC97H-TRIM16s and the control cells. (B) The levels of TRIM16 mRNA was verified by qRT-PCR in MHCC97H-TRIM16 and the control cells. (C) MHCC97H-TRIM16 and its control cells were subjected to Transwell migration. quantification of migrated cells through the membrane and invad cells through Matrigel invasion assays. quantification of migrated cells through the membrane and invad cells through Matrigel invasion assays. (D) MHCC97H-TRIM16 and the control cells were subjected to Matrigel invasion assays. quantification of migrated cells through the membrane and invad cells through Matrigel invasion assays. **P<0.01 based on the Student’s t-test. The results are from three independent experiments. Error bars, SD. Scale bar, 50 nm.
level (Fig. 8A), whereas, ectopic TRIM16 in MHCC97H cells markedly decreased ZEB2 expression at protein levels (Fig. 8C). While ZEB2 mRNA level had no significant change in either TRIM16 knockdown or ectopic expression (Fig. 8B and D), indicating that the regulation function of TRIM16 on ZEB2 expression only took place on the post-transcriptional level.

To understand how TRIM16 exerts its effects on ZEB2, 293T cells were co-transfected with HA-tagged TRIM16 and Myc-tagged ZEB2 constructs. Immunoprecipitation and subsequent immunoblot analyses revealed that Myc-tagged ZEB2 co-immunoprecipitated with HA-tagged TRIM16 (Fig. 9A), confirming a physical interaction between the two proteins. The physical interaction between TRIM16 and ZEB2 suggested that TRIM16 maybe target ZEB2 for ubiquitination and subsequent degradation. Next, in order to investigate if the regulation of TRIM16 on ZEB2 protein is dependent on the ubiquitin-proteasome pathway, we combined the treatment of proteasome inhibitor MG132 with ectopic TRIM16 treatment. We found that ZEB2 degradation caused by TRIM16 ectopic expression was completely abrogated by proteasome inhibition (Fig. 9B), demonstrating that the regulation of ZEB2 expression by TRIM16 is indeed mediated via proteasome-dependent pathway.

ZEB2 is a mediator for shTRIM16-induced migration and invasion behavior in HCC cells. We transfected HepG2-shTRIM16 cells with ZEB2 siRNAs to silence ZEB2 gene expression to investigate whether TRIM16-induced metastatic capacity was mediated by ZEB2 expression (Fig. 10A and B). It was found that knockdown of ZEB2 in HepG2-shTRIM16 cells were accompanied by the reduction of migratory and invasive capacities (Fig. 10C). Collectively, these results show that ZEB2 mediates shTRIM16-induced migration and invasion in HCC cells.

Discussion

Hepatocellular carcinoma (HCC) is a highly lethal malignancy with increasing incidence globally (1). Recent studies have indicated that early frequent metastasis has critical roles in the development and progression of HCC (13). Therefore, identifying novel molecules that regulate HCC metastasis will promote the development of anti-metastasis strategies. Mounting evidence shows that in epithelial cancers, including HCC, induction of EMT is a major event that provides mobility to cancer cells in order to generate metastases (14).

EMT is a process where cells undergo a morphological switch from the epithelial phenotype to mesenchymal phenotype. In this process, epithelial cells not only lose defined cell-cell/cell-substratum contacts and their structural polarity, but also they become spindle shaped (15). On the molecular level, EMT is defined as the loss of cell-cell adhesion molecules, downregulation of epithelial differentiation markers and transcriptional induction of mesenchymal markers (13). It has been shown that EMT is associated with cancer progression and cancer-related death. Moreover, EMT has been recognized to play pivotal roles in several diverse processes during embryonic development, chronic inflammation and fibrosis, as well as tumor progression. Numerous observations support the concept that the EMT process plays a role in the progression of tumors, including HCCs (16).

In this study, the clinical significance of TRIM16 in HCC and the mechanistic role of TRIM16 in inhibiting HCC cell metastasis were first delineated to our knowledge. We found that TRIM16 down-expression in HCC cells induced EMT, migration and invasion in vitro and enhanced metastatic capacity in vivo. In contrast, ectopic TRIM16 expression reversed these events in other aggressive and invasive HCC cells. It was also shown that a mechanistic link exists between TRIM16 and migration through TRIM16-mediated downregulation of ZEB2, which may subsequently lead to transcriptional downregulation of E-cadherin expression. Moreover, knockdown of ZEB2 attenuated shTRIM16 induced migration and invasion. Considering all these results,
Figure 6. TRIM16 regulates the transition between epithelial and mesenchymal phenotypes in HCC cells. (A) Expression of epithelial and mesenchymal marker was analyzed by western blotting in HepG2-shTRIM16 #2 and the control cells. (B) Expression of epithelial and mesenchymal marker was analyzed by qRT-CPR in HepG2-shTRIM16 #2 and the control cells. (C) Expression of epithelial and mesenchymal marker was analyzed by western blotting in MHCC97H-TRIM16 and the control cells. (D) Expression of epithelial and mesenchymal marker was analyzed by qRT-CPR in MHCC97H-TRIM16 and the control cells. *P<0.01 based on the Student’s t-test. The results are from three independent experiments. Error bars, SD.

Figure 7. Expression of epithelial and mesenchymal marker was analyzed by immunofluorescence stains. (A) Expression of E-cadherin and N-cadherin were analyzed by immunofluorescence stains in HepG2-shTRIM16 #2 and the control cells. (B) Expression of E-cadherin and N-cadherin was analyzed by immunofluorescence stains in MHCC97H-TRIM16 and the control cells.
Figure 8. Expression of EMT-inducer. (A) Expression of Snail, Slug, ZEB1 and ZEB2 were analyzed by western blotting in HepG2-shTRIM16 #2 and the control cells. (B) Expression of Snail, Slug, ZEB1 and ZEB2 were analyzed by qRT-PCR in HepG2-shTRIM16 #2 and the control cells. (C) Expression of Snail, Slug, ZEB1 and ZEB2 was analyzed by western blotting in MHCC97H-TRIM16 and the control cells. (D) Expression of Snail, Slug, ZEB1 and ZEB2 was analyzed by qRT-PCR in MHCC97H-TRIM16 and the control cells.

Figure 9. TRIM16 downregulates ZEB2 expression through interaction. (A) HA-TRIM16 is specifically co-immunoprecipitated with Myc-ZEB2. (B) Western blot analysis shows that MG132 abrogated ZEB2 degradation caused by TRIM16 ectopic expression in MHCC97H cells. No significant difference of ZEB2 expression was found in MG132 treated MHCC97H cells compared with control DMSO treated BT549 cells. **,## P<0.01 is based on the Student’s t-test.
we propose a model for TRIM16 regulation of EMT and metastasis through downregulation of ZEB2 in HCC cells.

The tripartite motif or TRIM family of proteins were originally described by its multi-domain design of three structurally distinct motifs, the RING finger zinc-binding domain, a B-box zinc-binding domain and the coiled-coil domain (17). TRIM16 is also known as the estrogen-responsive B-box protein due to its original discovery as an estrogen responsive protein in human mammary epithelial cells (17). TRIM16 has been shown to suppress tumor progression through regulatory pathways involved in growth inhibition, migration, differentiation and apoptosis. Recently research has demonstrated that TRIM16 can heterodimerize with other TRIM proteins and has E3 ubiquitin ligase activity (6). These data strongly support a role for TRIM16 as a tumor suppressor gene. However, the exact mechanisms of TRIM16 involvement in HCC remain unclear. Our study points to a novel function of TRIM16 in HCC metastasis through inhibiting essential characteristics of metastatic disease in HCC: EMT. First, HCC cells expressing low level of TRIM16 displayed an EMT phenotype, including

Figure 10. ZEB2 is a mediator for shTRIM16-induced migration and invasion behavior in HCC cells. (A) The level of ZEB2 protein was verified by western blotting. (B) The level of ZEB2 mRNA was verified by qTR-PCR. (C) HepG2-shTRIM16 #2 siZEB2s and their control vector cells were subjected to Transwell migration (top), and Matrigel invasion assays (bottom), quantification of migrated cells through the membrane and invaded cells through Matrigel of each cell line is shown as proportion of their vector controls. **P<0.01 based on the Student’s t-test. The results are from three independent experiments. Error bars, SD. Scale bar, 50 nm.
the associated stimulatory effects on in vitro migration and invasion. Interestingly, our results indicate that TRIM16 not only inhibits EMT, but ectopic expression of TRIM16 also leads to MET. All of these characteristics that are induced by shTRIM16 in vitro culminated to increased number of distant metastases in vivo. These empirical findings provide a mechanistic framework to explain the clinical observations that HCC patients with low levels of TRIM16 in tissue samples have a greater chance of distant metastasis.

The roles of several transcription factors as EMT regulators have been extensively reported. In our effort to elucidate the mechanism how TRIM16 inhibits metastasis in HCC cells, we identified ZEB2 as an effective mediator of shTRIM16 inducing these phenomena. The mechanistic connection between TRIM16 and ZEB2 was previously unknown. In this study, we found that modulation of TRIM16 expression altered ZEB2 expression. Thus, in conclusion, TRIM16 inhibits migration and invasion in vitro and metastasis in vivo by downregulating ZEB2 expression.

Metastasis and EMT are essential for HCC cells to disseminate from adjacent tissues and seed new tumors in distant sites (18). Our results demonstrated that TRIM16 regulated these two essential characteristics of metastatic disease and TRIM16-induced processes are reversible with the suppression of TRIM16 expression, providing us an optimal therapeutic option to manipulate TRIM16 levels in clinical HCC practice.

Acknowledgements

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