RNF216 Promotes Occurrence and Progression of Cholangiocarcinoma via Regulation of the DIAPH3 Ubiquitination

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Research

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Abstract

Background

Cholangiocarcinoma is a relatively uncommon malignant tumor with high mortality. However, the molecular underpinnings behind malignant progression of cholangiocarcinoma are incompletely understood. Here we demonstrate that RNF216 plays a suppressive role in cholangiocarcinoma occurrence and metastasis.

Methods

IHC and Western blot analysis were performed to examine the expression pattern of RNF216 and DIAPH3 in the clinical CRC cholangiocarcinoma. The relationship between RNF216 and DIAPH3 was then validated using in western blot analysis. The mechanism of RNF216-mediated ubiquitination modification of DIAPH3 was analyzed via Co-IP analysis. Gain- or loss-of-function approaches were manipulated to evaluate the modulatory effects of RNF216 and DIAPH3 on cell growth and metastasis. The mediatory effects of RNF216 and DIAPH3 on cancerogenesis were validated in vivo.

Results

Clinical data indicated that expression levels of RNF216 were associated with favorable clinical outcomes. RNF216 was downregulated in cholangiocarcinoma and inhibited cell proliferation and colony formation in vitro and xenograft tumorigenicity in vivo. Moreover, RNF216 suppressed Invasion and migration of cholangiocarcinoma. Mechanistic investigations further showed that RNF216 was involved in the ubiquitination of DIAPH3, a member of formin family related to assembly of actin cytoskeleton. RNF216 elicits tumor suppressor role by promoting degradation of DIAPH3. Importantly, expression of DIAPH3 rescued RNF216-mediated suppression of proliferation, cell migration, and invasion.

Conclusion

Our findings uncover a suppressive role for RNF216 in cholangiocarcinoma proliferation and metastatic progression and provide novel insight into that RNF216 is a potential biomarker or serves as a therapeutic target for cholangiocarcinoma.

Background

Cholangiocarcinoma is the most frequent malignancy of the biliary tract(1). The morbidity

of cholangiocarcinoma is second only to hepatocellular carcinoma (HCC) in hepatic malignancy(2). cholangiocarcinoma is categorized as intrahepatic (iCCA), perihilar (pCCA), or distal (dCCA) by anatomical location(3). Surgical resection remains the central curative treatment for all three disease subtypes(2, 4–6). Patients with cholangiocarcinoma lack of clinical symptoms in the early stage, most cases are diagnosed at advanced stages(7, 8). Therefore, the early diagnosis of cholangiocarcinoma

needs further development. In the past years, the incidence rate of cholangiocarcinoma has gradually increased(9–11). Development of strategies for early diagnosis and effective treatment is the top priority.

Ring finger protein 216 (RNF216) belongs to the RING family of E3 ubiquitin ligases, which are involved in cellular protein degradation(12). Multiple loss-of-function mutations in RNF216 have recently been identified in patients with Gordon Holmes syndrome (GHS)(13–16). RNF216 is also involved in the immune response. RNF216 interacts with BECN1 to inhibit autophagy and impact macrophage response in infection(17, 18). Moreover, RNF216 involves ubiquitination and proteolytic degradation of several toll-like receptor (TLR), such as TLR4 and TLR9, which restricts TLR signaling intensity and duration(17–21). Tumor necrosis factor receptor-associated factor 3 (TRAF3) is also the target of RNF216 for degradation, the process regulates RNA virus infection(22). Thus, RNF216 plays an essential role in regulating innate immunity. It has been reported that RNF216, as a downstream of miR-520b, plays an inhibitory role in ovarian cancer growth(23). However, the gene function of RNF216 in mammalian species especially in cancer remains unknown.

Formins forms a family including 15 proteins in human, which is characterized by the presence of two formin homology domains(24). Diaphanous formins known as DIAPH 1, 2 and 3 (DIAPH 1–3) in mammals, which are a subgroup of the formin family related to *Drosophila* diaphanous(25, 26). The diaphanous homologue DIAPH3 play a key regulatory role for actin cytoskeleton(27). DIAPH3 interacted with microtubules, and its deficiency altered microtubules dynamics, decreasing polarized force generation, contractility, and response to substrate stiffness(28–30). DIAPH3 has been reported to be up-regulated in a variety of tumors, including lung cancer, liver cancer, prostatic cancer and breast cancer(31–35). The function of Mammalian DIAPH3 involve in assembly of actin cytoskeleton that underlies tumor cell migration and invasion(32). Moreover, DIAPH3 promotes the tumorigenesis of lung adenocarcinoma by interacting with STK38 thus activating ERK signaling(34). DIAPH3 was identified as binding protein of HSP90, which activates the beta-catenin/TCF signaling and disrupts the interaction between GSK3beta and HSP90(35). DIAPH3 silencing destabilized microtubules and evoked amoeboid properties, which increase invasion and promoted metastasis in mice(36). Therefore, DIAPH3 may be a target for tumor therapy.

Materials And Methods

Reagents

The reagents used in this study are listed in Table S1.

Cell Culture

Two human cholangiocarcinoma cell lines (RBE and HUCCT1) were obtained from the American type culture collection (ATCC, Manassas, VA, USA) and were authenticated by monitoring cell vitality, mycoplasma contamination, and short tandem repeat profiling. Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum in a 5% CO₂ incubator at 37 °C.

Plasmid Construction

Molecular cloning was performed by standard protocols. All construct sequences were verified by DNA sequencing. The detailed information concerning expression constructs and the primers used for molecular cloning is provided in Supplementary Tables S2 and S3.

Reverse Transcription-Quantitative PCR

Total RNA was extracted using TRIzol agent (Invitrogen, Waltham, MA, USA) and subjected to cDNA synthesis using PrimeScript RT Master Mix (Takara, Shiga, Japan). qPCR was performed using iQ SYBR Green Master kit (Roche, Shanghai, China) following the manufacturer's instructions. All data were normalized to the housekeeping gene β -actin, and quantitative measures were obtained using the comparative CT method.

Colony formation survival and CCK-8 assays

A total of 1×10^4 cells were seeded into 6-well in triplicates for plate colony formation survival assay or 5×10^3 cells were seeded into 96-well in triplicates for CCK-8 assay. For colony formation assays, cells were fixed after two weeks by methanol, stained with 0.2% crystal violet solution then photographing. Colonies consisting of > 50 cells were counted. For CCK-8 assays, 10 μ L CCK-8 solution (Sigma-Aldrich, St. Louis, MO, USA) was added to each well every 7 days after seeding. The plates were incubated in an incubator for 3 h, and then absorbance at 450 nm was determined.

Co-Immunoprecipitation Assay and Immunoblotting

For immunoblotting analysis, modified RIPA buffer (50 mM Tris-HCl, pH7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, and 1 mM EDTA) supplemented with protease inhibitors and phosphatase inhibitors (Bimake, Houston, USA) was used to lyse cells. BCA protein assay reagent (Yeasten, Shanghai, China) was used to detect protein concentrations. Cellular extracts were resolved through SDS-PAGE, transferred to PVDF membranes (Millipore, Billerica, USA), then incubated with the indicated primary antibodies. Enhanced chemiluminescent substrate kit (Yeasten) was used to analyze corresponding antibody specific signals. Table S4 lists the antibodies used. To immunoprecipitate endogenous proteins, cell extracts were incubated with primary antibodies or control IgG in a rotating incubator overnight at 4 °C, followed by incubation with protein A/G magnetic beads (Sigma-Aldrich, St. Louis, MO, USA) for another 3 h. The immunoprecipitates were washed three times with lysis buffer and analyzed by immunoblotting.

Xenograft Tumorigenicity Assay

A total of 3×10^6 cells in 300 μ L PBS were injected subcutaneously into the flank region of 6-week-old BALB/c female nude mice (Shanghai SLAC Laboratory Animal, Shanghai, China) for subcutaneous inoculation. The tumors were measured every 7 days after injection and the tumor volume was calculated by the formula $(\text{length} \times \text{width}^2)/2$. The mice were sacrificed 5.5 weeks after inoculation.

Immunohistochemistry

Immunohistochemistry (IHC) staining was carried out using EnVision Detection Systems Peroxidase/DAB (DAKO, Shanghai, China) following the manufacturer's recommendations, using an antibody against RNF216 (ab25961, 1:500, Abcam), DIAPH3 (ab227276, 1:500, Abcam) and Ki67 (ab15580, 1:500, Abcam). Interpretation of the IHC results was performed by two independent pathologists who were blinded to the clinicopathological information. By recording the percentage of positive staining (0 = negative, 1 $\leq$ 10%, 2 = 10–50%, 3 $\geq$ 50%) and staining intensity (0 = no, 1 = weak, 2 = moderate, 3 = strong) for each sample, immunoreactivity score (IRS) (0–9) was calculated by multiplying positive staining percentage with staining intensity. Low and high expression were defined according to the median IRS.

Wound Healing Assay

Cells were seeded in culture inserts (SPL Life Science, Gyeonggi-do, Korea) at 2×10^4 on 6-well plates. After 24 h of incubation, the wound was created by 1 ml tips. Images were taken at the indicated time points and the wound closure ratios were calculated.

Migration and invasion assays

Transwell chambers were used to perform migration and invasion assays in the absence (migration) and presence (invasion) of growth factor-reduced Matrigel (Corning, NY, USA). Briefly, 2.5×10^4 cells in FBS-free medium were plated in the top chamber. Growth medium containing 10% FBS was used as a chemoattractant in the lower chamber. After indicated times, migrated and invaded cells were fixed and stained with 0.1% crystal violet. Cells were

counted under an inverted microscope at 100 $\times$ magnification.

CHX assay

Cells were treated with 100 μ g/mL cycloheximide (CHX) and then harvested at indicated time points for immunoblotting analysis. The densitometry of Western blots was quantified using ImageJ software.

Statistical analysis

All data are presented as the mean $\pm$ standard deviation from at least three independent experiments. Overall survival curves were plotted using the Kaplan–Meier method and compared using the log-rank test. All statistical analyses were performed using SPSS software. A value of $p < 0.05$ was considered statistically significant.

Results

Identification of RNF216 Associated with Overall Survival (OS) and Disease Free Survival (DFS) in Cholangiocarcinoma

To identify the potential genes involved in tumorigenesis and predict survival in cholangiocarcinoma, we analyzed the genes that affect overall survival (OS) and disease-free survival (DFS) in cholangiocarcinoma from TCGA using GEPIA online website (<http://gepia2.cancer-pku.cn>). Among these screened genes, we have paid attention to RNF216, which is a member of RING family of E3 ubiquitin ligases. The regulation and specific mechanism of RNF216 on tumor remain unknown. The database analysis indicated that patients with high RNF216 expression had better OS (Fig. 1A) and DFS (Fig. 1B). To study further the clinical significance of RNF216 expression in patients with cholangiocarcinoma, we analyzed RNF216 expression levels by Immunohistochemistry (IHC) on 39 cholangiocarcinoma samples from the Fifth Affiliated Hospital of Wenzhou Medical University. The results also demonstrated that high expression levels of RNF216 are associated with better prognosis of patients with cholangiocarcinoma in OS as well as DFS (Fig. 1C and 1D), supporting the notion that RNF216 may be a tumor suppressor in cholangiocarcinoma. According to the score, representative IHC images are shown in Fig. 1E. Semiquantitative analysis showed that high expression of RNF216 was observed in 51.3% (20/39) of patients, while 48.7% (19/39) of patients had RNF216 low expression (Supplementary Figure S1A). RNF216 expression was found to be correlated with tumor size (Supplementary Figure S1). We next examined the expression levels of RNF216 in 39 cases cholangiocarcinoma tissues and 9 cases adjacent normal tissues by IHC. Results showed that average RNF216 IHC score was deregulated in cholangiocarcinoma samples as compared with their normal counterparts (Fig. 1F and 1G).

RNF216 enhances cell proliferation in vitro and tumor growth in vivo

To investigate the impact of RNF216 on malignant phenotypes of cholangiocarcinoma cells, we tested the mRNA level of RNF216 in HCC9810, huh28, Tfk1, RBE and HUCCT cells by real-time PCR. We observed that the mRNA level of RNF216 in HUCCT cells is higher than other cell lines. The expression of RNF216 in RBE is Medium level among those cell lines (Supplementary Figure S1). We stably expressed RNF216 in RBE cells by lentiviral infection (Fig. 2A). We used shRNA to knockdown the expression level of RNF216 in RBE and HUCCT cell lines (Fig. 2B and 2C). Cell proliferation assays using CCK-8 kit revealed that overexpression of RNF216 inhibited cell proliferation in RBE cells (Fig. 2D). Colony growth assays indicated that expression of RNF216 decreased colony formation of RBE cells (Fig. 2E and 2F). In contrast, knockdown of RNF216 in RBE and HUCCT cells using shRNF216 increased cell viability (Fig. 2G and 2H) and clonogenicity (Fig. 2I-2K).

To investigate whether RNF216 could inhibit tumorigenic capacity of cholangiocarcinoma in vivo, HUCCT cells stably expressing shNC and shRNF216 were subcutaneously injected into flank region of 6-week-old BALB/c nude mice. Consistent with in vitro results, xenograft tumors expressing shRNF216 grew faster than those expressing empty vector (Fig. 2L-2M). Together, these results suggest that RNF216 suppress cholangiocarcinoma cell proliferation in vitro and tumor growth in vivo.

RNF216 promotes cholangiocarcinoma cell migration, invasion, and metastasis

Distant metastasis is main reason for death in tumor patients. Therefore, in addition to the effect of RNF216 on tumor growth, we intend to explore whether RNF216 influence tumor metastasis. The ability to invade surrounding tissues and metastasize to distant organs is an important hallmark of cholangiocarcinoma cells(2), we next examined whether RNF216 affects migratory and invasive properties of cholangiocarcinoma cells in vitro. Wound-healing assays showed that expression of RNF216 in RBE cells decreased wound closure rate compared to their control cells (Fig. 3A and 3B). However, knockdown of RNF216 by shRNF216 in RBE and HUCCT cells enhanced wound closure rate (Fig. 3C-3F). These results were further confirmed by Boyden's chamber migration assays. knockdown of RNF216 in HUCCT cells enhanced their migratory capacity (Fig. 3G-3H). Moreover, RBE cells stably expressing RNF216 showed a less degree of invasion through Matrigel-coated invasion chambers (Fig. 3G and 3H). In contrast, knockdown of RNF216 in RBE and HUCCT cells enhanced their migratory and invasive capacity (Fig. 3I-3L).

RNF216 interacts with DIAPH3 and promotes its polyubiquitination

An integrated bioinformatics platform for investigating the human E3 ubiquitin ligase substrate interaction network (<http://ubibrowser.ncpsb.org/ubibrowser/>) was used to predict the substrate of RNF216. We paid attention to that DIAPH3 may serve as a substrate for RNF216. we next examined whether RNF216 interacts with DIAPH3. The interaction between RNF216 and DIAPH3 at the endogenous protein levels was validated in RBE and HUCCT cells by co-immunoprecipitation with an anti-RNF216 antibody and anti-DIAPH3 (Fig. 4B). We next examined whether RNF216 expression correlates with DIAPH3 in cholangiocarcinoma cells. We observed that knockdown of RNF216 in RBE and HUCCT cells was responsible for the increased expression level of DIAPH3 (Fig. 4C and Supplementary Figure S3). The data indicates RNF216 expression was inversely correlated with DIAPH3 in cholangiocarcinoma cell. There is no correlation between RNF216 and DIAPH3 at mRNA level (Fig. 4D-4E). Considering RNF216 is a member of RING family of E3 ubiquitin ligases, we speculated that RNF216 regulation of DIAPH3 occurs at post-transcriptional level. In agreement with these observations, RNF216-mediated downregulation of DIAPH3 in RBE and HUCCT cells were effectively restored after treatment with 10 μ M of proteasome inhibitor MG-132 for 6 h (Fig. 4F-4G), and knockdown of RNF216 in RBE and HUCCT cells enhanced the half-life of DIAPH3 protein (Fig. 4H-4I Supplementary Figure S4B-S4B). These results suggest that RNF216 targets DIAPH3 protein for proteasomal degradation. The sequential IP and immunoblotting analysis showed a significant increase of polyubiquitinated DIAPH3 protein in RNF216 transfected HEK293T cells (Fig. 4J). Overexpression of RNF216 increased in RBE cells (Fig. 4K), whereas knockdown of RNF216 decreased the ubiquitination levels of endogenous DIAPH3 (Fig. 4L).

RNF216 suppresses EMT and Erk by targeting DIAPH3 for proteasomal degradation

To examine the clinical relevance of our findings, we first evaluated the expression levels of RNF216 and DIAPH3 in 6 pairs of primary cholangiocarcinoma and matched adjacent noncancerous tissues by immunoblotting. The results showed that the protein level of RNF216 in cholangiocarcinoma tissues was lower than adjacent noncancerous tissues (Fig. 5A). On the contrary, the level of DIAPH3 in cholangiocarcinoma tissues was higher than adjacent noncancerous tissues (Fig. 5A). And we also observed that there was a negative correlation in expression levels between RNF216 and DIAPH3 in those samples, this is consistent with our previous conclusion (Fig. 5A). DIAPH3 was reported to promote cells growth and metastasis in other tumors including lung adenocarcinoma, hepatocellular carcinoma, triple-negative breast cancer and prostate cancer. Therefore, we examined the function of DIAPH3 in cholangiocarcinoma. xenograft tumors expressing DIAPH3 grew faster than those expressing empty vector (Fig. 5B-5D). At the same time, we also verified expression correlation of Ki67 and DIAPH3 in 39 clinical samples by immunohistochemistry. The results showed that the expression level of DIAPH3 was positively correlated with Ki67 expression (Fig. 5E-5F). It is suggested that DIAPH3 plays a central role in promoting the growth of cholangiocarcinoma. We then intended to explore whether DIAPH3 as a downstream of RNF216 affects the prognosis of patients with cholangiocarcinoma. The results confirmed that patients with high expression of DIAPH3 had significantly lower overall survival and disease-free survival (Fig. 5F-5G). DIAPH3 has been reported to promotes the expression of ERK and mesenchymal markers of EMT(34, 35). We speculated RNF216 regulate the expression of these molecules through its target protein DIAPH3. The results suggested that ERK and p-ERK were upregulated after RNF216 knockdown (Fig. 5I). Mesenchymal markers of EMT, N-cadherin, Vimentin and Snail, were also upregulated through knockdown for RNF216 (Fig. 5J). Moreover, we found that the expression of ERK, p-ERK and mesenchymal markers of EMT recovered significantly after re-overexpression of DIAPH3 in RNF216 knockdown cells (Fig. 5K-5L). Together, these results indicate that RNF216 regulates expression of ERK and mesenchymal markers of EMT through ubiquitinating its target protein DIAPH3.

RNF216 inhibited growth and metastasis of cholangiocarcinoma through DIAPH3

We next examined whether DIAPH3 regulates the growth and metastasis of cholangiocarcinoma through ubiquitinating DIAPH3. Cell proliferation assays using CCK-8 kit revealed that increased cell proliferation caused by knockdown of RNF216 rescued by knockdown of DIAPH3 (Fig. 6A). The same results were observed in the colony growth assays. Knockdown of RNF216 promoted cell growth, while knockdown of DIAPH3 significantly inhibit cell growth (Fig. 6B-6C). Moreover, Wound-healing assays showed that knockdown of RNF216 in HUCCT cells increased wound closure rate compared to their control cells. The increased wound closure rate was disappeared after knockdown of DIAPH3 (Fig. 6D-6E). Knockdown of RNF216 also inhibited the metastasis and invasion of cells, and inhibit DIAPH3 could significantly inhibit the metastasis and invasion of HUCCT cells (Fig. 6F-6G). The increased metastasis and invasion also were impaired by knockdown of DIAPH3. These results suggested that RNF216 regulates the growth and metastasis of cholangiocarcinoma through ubiquitination and dgradation of DIAPH3.

In summary, findings presented here showed that RNF216 plays both anti-tumorigenic and anti-metastatic roles in cholangiocarcinoma progression. RNF216 exerts its tumorigenic- and metastasis-suppressive functions through ubiquitin-dependent degradation of DIAPH3. Downregulated DIAPH3 decreased the protein level of ERK and Mesenchymal markers of EMT, N-cadherin, Vimentin and Snail. These new findings provide mechanistic insights into the functional role for RNF216 in regulating cholangiocarcinoma development and progression and are clinically relevant.

Dicussion

Cholangiocarcinoma is a rare malignancy and accounts for 2% of all malignancies. Incidence is on the increase in the world (37). Patients with cholangiocarcinoma have rapidly rising incidence and mortality(38). Early diagnosis of cholangiocarcinoma needs to explore the available biomarkers. In this study, we discovered that compared with normal biliary tissues, the expression of RNF216 dramatically decreased in cholangiocarcinoma and was associated with better prognosis of patients with cholangiocarcinoma. These results indicate that RNF216 may serve as a novel biomarker for cholangiocarcinoma.

We also found that DIAPH3, as a substrate of RNF216, also plays a central role in promoting the growth and metastasis of cholangiocarcinoma. Although DIAPH3 has been reported to be involved in the growth and metastasis of a variety of tumors(34, 35, 39–41), its role in cholangiocarcinoma is not explicit. We elaborated the regulatory upstream of DIAPH3. RNF216 promotes degradation of DIAPH3 through ubiquitin. The expression level of RNF216 decrease in cholangiocarcinoma. Thus, the expression of DIAPH3 was increased in cholangiocarcinoma and promoting tumorigenesis and metastatic of cholangiocarcinoma. Moreover, these findings uncover the impact of RNF216 and DIAPH3 on the prognosis of patients with cholangiocarcinoma and the correlation of their expression levels in clinical samples.

RING-in-between-RING (RBR) E3 ligases are one family of E3 ligases, which is characterized by the unique RING-HECT hybrid mechanism to function with E2s to transfer ubiquitin to target proteins for degradation(42). Accumulated evidence has indicated that RBR E3 ligases promotes the degradation of tumor promoters or suppressors thus exerting their physiological functions in various types of cancers. Ring finger protein 216 (RNF216) known as Triad3 has been reported to target beclin1 (BECN1), which is a critical factor in autophagy. RNF216 modulates ubiquitination and degradation of BECN1 thus leading to inhibition of autophagy in macrophages. A study showed that RNF216 expression is increased in human colorectal cancer (CRC) tissues and correlated with CRC progression(17). Moreover, other evidence showed that RNF216 enhances cell proliferation and motility via promotion of BECN1 degradation in CRC cells(17, 18). The evidence for regulation on autophagy of RNF216 suggests that RNF216 could be an available target for treating inflammatory diseases(18, 43, 44). However, the correlation between autophagy and tumor still required to be deeper determined. Evidences show that autophagy promotes tumorigenesis and conducts the survival of tumor in adverse environment, such as radiotherapy and chemotherapy(45–47). Another part of the evidence suggests that autophagy promotes

the cell death of tumor cells thus inhibition of the proliferation for tumor(48, 49). Moreover, high expression of miR-520b in ovarian cancer promoted cell growth through RNF216(23). Consistently, RNF216 expression is decreased in ovarian cancer tissues, whereas expression of miR-520b is high. Undoubtedly, the biological role of RNF216 in tumorigenesis is required to be deeper determined in the future.

DIAPH3 has been reported to be up-regulated in a variety of tumors, promoting tumor growth and metastasis(34, 35, 40, 50). Moreover, DIAPH3 knockout in breast and prostate cancer increase the sensitivity of tumor cells to paclitaxel(29). Thus, it would be of high clinical relevance to analyze if the expression of DIAPH3 might be a predictor of response to taxanes. The isoform 1 of DIAPH (DIAPH1) has also been reported as a target for tumor therapy(51–53). DIAPH1 plays an important role in MTs dependent early adhesion of colon cancer cells(51, 54). In response to extracellular stimulation, the actin nucleation activity of DIAPH1 drives invasion by promoting the formation of invasive foot(51). DIAPH3 has been also shown to be essential for stabilizing interphase MTs(39).

Conclusion

We observed that RNF216 inhibits cholangiocarcinoma tumor cell growth in vitro and in vivo through ubiquitination for DIAPH3 in this study. we also demonstrated that low expression of RNF216 is associated with poor prognosis of patients with cholangiocarcinoma. Our findings provide novel insight into that RNF216 is a potential biomarker and therapeutic target for cholangiocarcinoma.

Abbreviations

NC: negative control; sh-NC: NC shRNA; sh-RNF216: shRNA targeting RNF216; sh-EGLN3: shRNA targeting EGLN3; RT-qPCR: Reverse transcription quantitative polymerase chain reaction; HEK: Human embryo kidney; Co-IP: Immunoprecipitation; Co-IP: Co-immunoprecipitation; ANOVA: analysis of variance; FBS: fetalbovine serum; DMEM: Dulbecco's modified Eagle's medium.

Declarations

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Author contributions

Jiansong Ji and Xiaoxi Fan conceived-designed experiment and wrote the manuscript. Jianfei Tu and Weiqian Chen performed experiments. Jianfei Tu, Weiqian Chen, Miaomiao Meng and Siyu Zhao analyzed data. Jianfei Tu and Weiqian Chen prepared the figures. All authors reviewed the manuscript.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the ethics committee of Affiliated Lishui Hospital of Zhejiang University. The informed consent was obtained from each participant. All animal experiments were approved by Animal Care and Use Committee of Affiliated Lishui Hospital of Zhejiang University. Extensive efforts were made to ensure minimal suffering of the animals used during the study.

Conflict of interest statement

The authors declare no potential conflicts of interest.

Consent for publication

Not applicable.

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Figures

Figure 1

Figure 1

Identification of RNF216 Associated with Overall Survival (OS) and Disease Free Survival (DFS) in Cholangiocarcinoma (A-B) Kaplan-Meier curves of OS (A) and DFS (B) for 36 cholangiocarcinoma patients from TCGA using GEPIA online website with high or low RNF216 expression. (C-D) Kaplan-Meier curves of OS (C) and DFS (D) for 39 cholangiocarcinoma patients with high or low RNF216 expression. (E) Representative IHC images of RNF216 expression are shown. Scale bars, 50 μm . (F) 39 cases cholangiocarcinoma tissues and 9 cases adjacent normal tissues were subjected to IHC analysis with the indicated antibodies. Representative IHC images of RNF216 expression are shown. Scale bars, 10X 200 μm , 4X 100 μm . ***, $p < 0.001$.

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Figure 2

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RNF216 suppresses cholangiocarcinoma cell proliferation in vitro and tumor growth in vivo (A) RBE cells stably expressing pCDH and Flag-RNF216 were analyzed by immunoblotting. (B-C) RBE (B) and HUCCT (C) cells stably expressing shNC, shRNF216 #1 and shRNF216 #2 were analyzed by immunoblotting. (D-F) RBE cells stably expressing pCDH and Flag-RNF216 was subjected to cell proliferation assays by CCK-8 (E) and colony growth assays (E-F). (G-K) RBE and HUCCT cells stably expressing shNC, shRNF216 #1 and shRNF216 #2 were subjected to cell proliferation assays by CCK-8 (G-H) and colony growth assays (I-K). (L-N) HUCCT cells stably expressing shNC and shRNF216 were injected into flank region of 6-week-old female BALB/c nude mice (n=6). After 5.5 weeks of injected, xenograft tumors were harvested. Tumor growth curves (M), photographs of harvested tumors (L), and tumor weight (N) are shown.

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Figure 3

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RNF216 suppresses cholangiocarcinoma cell migration and invasion. (A-B) RBE cells stably expressing pCDH and Flag-RNF216 were subjected to wound-healing assays. (C-F) RBE (C-D) and HUCCT (E-F) cells stably expressing shNC and shRNF216 #1 were subjected to wound-healing assays. (G-H) RBE cells stably expressing pCDH and RNF216 were subjected to Boyden's chamber migration assays (left) and Matrigel-coated invasion assays (right). (I-L) RBE (I-J) and HUCCT (K-L) cells stably expressing shNC and shRNF216 #1 were subjected to Boyden's chamber migration assays (left) and Matrigel-coated invasion assays (right).

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Figure 4

Figure 4

RNF216 interacts with DIAPH3 and promotes its polyubiquitination (A) Ubibrowser was used to predict the substrate of RNF216. (B) Lysates from RBE and HUCCT cells were immunoprecipitated with control IgG, an anti-RNF216 or an anti-DIAPH3 antibody, followed by immunoblotting analysis. (C) RBE cells stably expressing shNC, shRNF216 #1 and shRNF216 #2 were subjected to analyzed by immunoblotting using indicated antibody. (D-E) The DIAPH3 mRNA level of HUCCT (D) and RBE (E) cells stably expressing shNC, shRNF216 #1 and shRNF216 #2 were subjected to analyzed by real-time PCR. (F-G) RBE (F) and HUCCT (G) Cells stably expressing pCDH and Flag-RNF216 were treated with DMSO or 10 μ M MG-132 for 6 h and analyzed by immunoblotting. (H-I) Cells stably expressing shNC and shRNF216 were treated with 100 μ g/mL of CHX for the indicated times and then analyzed by immunoblotting (H). Relative expression levels of DIAPH3 to Vinculin were shown in I. (J) HEK293T cells were transfected with pCDH, Flag-RNF216, Flag-DIAPH3 and V5-Ubiquitin alone or in combination. IP and immunoblotting analysis were performed with the indicated antibodies after 48 h of transfection. Cells were treated with 20 μ M MG-132 for 6 h before harvest. (K) RBE cells were transfected with pCDH and Flag-RNF216. IP and immunoblotting analysis were performed with the indicated antibodies after 48 h of transfection. Cells were treated with 20 μ M MG-132 for 6 h before harvest. (L) RBE cells were transfected with shNC and shRNF216. IP and immunoblotting analysis were performed with the indicated antibodies after 48 h of transfection. Cells were treated with 20 μ M MG-132 for 6 h before harvest.

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Figure 5

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RNF216 suppresses EMT and Erk by targeting DIAPH3 for proteasomal degradation (A) Lysates from 6 pairs of human breast tumor tissues (T) and adjacent noncancerous normal tissues (N) were subjected to immunoblotting analysis with the indicated antibodies. The expression levels of DIAPH3 and RNF216 were normalized to those of Vinculin. (B-D) HUCCT cells stably expressing pCDH and DIAPH3 were injected into flank region of 6-week-old female BALB/c nude mice (n=6). After 5.5 weeks of injected, xenograft tumors were harvested. photographs of harvested tumors (B), Tumor growth curves (C) and tumor weight (D) are shown. (E) 39 clinical samples were subjected to IHC analysis with the indicated antibodies. Representative images are shown. Scale bars, 20 μ m. (F) Correlation analysis of expression levels between Ki67 and DIAPH3. (G-H) Kaplan-Meier curves of OS (G) and DFS (H) for 36 cholangiocarcinoma patients from TCGA using GEPIA online website with high or low DIAPH3 expression. (I-J) HUCCT cells stably expressing shNC, shRNF216 #1 and shRNF216 #2 were subjected to analyzed by immunoblotting using indicated antibody. (K-L) HUCCT cells stably expressing shNC, shRNF216 #1, shRNF216 #2, shDIAPH3 alone or in combination were subjected to analyzed by immunoblotting using indicated antibody.

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Figure 6

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RNF216 suppresses cholangiocarcinoma cell migration and invasion through promoting degradation of DIAPH3 (A-C) HUCCT cells stably expressing shNC, shRNF216#1, pCDH, shDIAPH3 alone or in combination was subjected to cell proliferation assays by CCK-8 (A) and colony growth assays (B-C). (D-E) RBE cells stably expressing shNC, shRNF216#1, pCDH, shDIAPH3 alone or in combination were subjected to wound-healing assays. (F-G) RBE cells stably expressing shNC, shRNF216#1, pCDH, shDIAPH3 alone or in combination were subjected to Boyden's chamber migration assays (left) and Matrigel-coated invasion assays (right).

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