DEK mediates AKT/mTOR signaling pathway promotes the proliferation, migration and EMT process of cholangiocarcinoma

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Research Article

Keywords: Cholangiocarcinoma, proto-oncogene DEK, Proliferation, Migration, Epithelial-mesenchymal transition, AKT/mTOR signaling pathway

Posted Date: April 15th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1552211/v1

Abstract

Objective: To explore the molecular mechanism of proto-oncogene DEK in promoting cell proliferation, migration and EMT process of cholangiocarcinoma, and to provide a theoretical basis for the diagnosis and treatment of cholangiocarcinoma.

Methods: The expression level of DEK in cholangiocarcinoma and its relationship with clinicopathological characteristics was analyzed by the UALCAN database, GEPIA database and the HPA database, respectively. The stable transfection cell line was constructed by lentiviral transfection, then the effect of DEK on the proliferation of cholangiocarcinoma cells were measured by MTT and colony formation assay. Wound scratch assay and Transwell assay observe the effect of DEK on the migration and invasion of cholangiocarcinoma cells. The effect of DEK on the expressions of epithelial-mesenchymal transition (EMT) related markers and AKT signaling pathway related proteins was assessed by Western Blot .

Results: Compared with normal tissues, DEK is highly expressed in cholangiocarcino

-ma tissues, and the positive expression of DEK is significantly correlated with clinical stage, pathological grade and lymph node metastasis. After upregulating DEK, the proliferation, migration, invasion and EMT process of cholangiocarcinoma cells were significantly increased, and silencing DEK has inhibited tumor cell proliferation and suppressed tumor metastasis. Western Blot showed that the phosphorylation level of AKT, 4EBP1, and S6 protein increased after upregulation of DEK, while silencing DEK had the opposite result.

Conclusion: DEK promotes the proliferation, migration, invasion and EMT process of cholangiocarcinoma cells through activating the AKT/mTOR signaling pathway.

Introduction

Cholangiocarcinoma (CCA) is a malignant tumor derived from bile duct epithelial cells, and ranks the second most common liver cancer after hepatocellular carcinoma[1]. It has the characteristics of insidious onset, high invasiveness, poor prognosis and high mortality. Surgical resection and liver transplantation for localized tumors are currently the more effective treatments for CCA. Until now, carcinoembryonic antigen and CA19-9 are save as the currently treatment clinical serum markers for cholangiocarcinoma, with limeted sensitivity and specificity. therefore, identification of effective early diagnosis molecular markers and molecular targets has extremely important clinical significance for cholangiocarcinoma treatment.

The proto-oncogene DEK is a highly conserved endogenous DNA-binding chromatin nuclear factor that encodes 375 amino acids [2]. DEK is described as part of the protein product of the DEK-NUP214 (originally called DEK-CAN) fusion oncogene, produced by a t(6;9)(p23;q34) translocation in a subgroup of acute myeloid leukemia patients [3.4]. DEK is one of only two known secreted nuclear chromatin

factors that lead to the regulation of many cellular processes, including hematopoietic regulation, DNA replication, gene transcription, and DNA repair [5–7]. So far, it has been reported that the DEK dysregulation is related to a great number of malignant tumors. such as, hepatocellular carcinoma, oropharyngeal squamous cell carcinoma, melanoma, ovarian cancer, pancreatic cancer, lung cancer, gastric adenocarcinoma, breast cancer [8–15]. Yi et al [16] showed that high expression of DEK was associated with poor prognosis in patients with esophageal squamous cell carcinoma. Zhou et al [13] found that the proto-oncogene DEK was highly expressed in lung cancer tissues and promoted the proliferation and migration of lung cancer cells. These findings suggest that DEK could be a potential biomarker and prognostic factor for tumor progression and therapy. However, the underlying molecular mechanisms of DEK in cholangiocarcinoma have not been fully understood. Thus, This article aims to investigate DEK expression and molecular pathways underlying CCA carcinogenesis involved in the proliferation and migration.

Materials And Methods

2.1 Cell lines and culture

Human normal bile duct epithelial cells HIBEpic and human cholangiocarcinoma cell lines HuCCT1, TFK1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) were obtained from ATCC. Cholangiocarcinoma cell lines RBE and QBC939 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured in RPMI-1640 medium containing 1% penicillin-streptomycin and 10% FBS. HuCCT1 was used for subsequent experiments.

2.2 Online database

UALCAN (http://ualcan.path. uab.edu/index.html) online database was used to analyze the relationship between DEK gene and Clinical pathology in CCA, and the level of DEK mRNA in CCA. DEK mRNA expression and overall survival were also evaluated using GEPIA online analysis software (http://gepia.cancer-pku.cn/). The Human Protein Atlas data (https://www.Proteinatlas.org/) was used to analyze the expression of DEK in human normal bile duct epithelium and cholangiocarcinoma tissues. We performed GSEA using normalized RNA-Seq data obtained from TCGA, GO terms and the KEGG pathways were analyzed to investigate possible biological functions of DEK. The correlation analysis for the TBC1D3 family members.

2.3 siRNA

Small interfering RNA (si-RNA) against DEK and corresponding negative control (si-NC) were purchased from Ribobio Biological Co., Ltd. (Guangzhou, China). The siRNA target sequences used 5'-TGTCCTCATTAAAGAAGAA-3. si-DEK sequences and si-NC sequences were transfected into cells using Lipofectamine3000 reagent. Transfection efficiency was determined by western blotting analysis.

2.4 Lentivirus transfection and stable cell clone establishment

The lentivirus in this study were constructed by Beijing Syngenta Technology Co., Ltd.(Beijing, China) based on the sequence of human DEK in GenBank (NM-003472). pHS-AVC-0466 (pLV-hef1a-mNeongreen-P2A-Puro-WPRE-CMV-DEK-3flag) vector was used for DEK overexpression. HuCCT1 cells were cloned into the pHS-B-0080 (pLV-hef1a-mNeongreen-P2A-Puro-WPRE-CMV-MCS-3flag) vector as a negative control. 5μg·ml-1polybrene (Sigma-Aldrich, Darmstadt, Germany) was used to enhance infection. HuCCT1 cells were seeded in 6-well culture plates, and the cells were confluent to about 50%, Lentiviruses were infected into HuCCT1 cells with an MOI 20 plus 2μl 5 mg/ml polybrene incubate for 8 h. After infection for 48 h, cells were selected for 2 weeks using puromycin (5 μg/ml, Solarbio，CA，USA) to produce a stable cell line for subsequent assays，The fluorescence intensity was observed under a fluorescence microscope, and the expression of DEK in HuCCT1 was detected by western blotting.

2.5 MTT assay

Transfected cells HuCCT1 were seeded at 5000 cells/well in 96-well plate in 100μL medium. After 24, 48, and 72 hours, add 1 mg/mL MTT working solution and incubate for 4 hours at 37℃ in a 5% CO_2 incubator. After 4h, add 100μL DMSO/well, shaking for 10min and measure the absorbance value at 490 nm on a full-wavelength spectrophotometer (TECAN-infinite M200 pro, Switzerland). Each independent experiment was performed three times.

2.6 Colony formation assay

Transfected cells were seeded at 500 cells/well in triplicate wells of a six-well plate and maintained in the appropriate medium containing 10% fetal bovine serum for 2 weeks. At 14 days, the cells were fixed with 4% paraformaldehyde for 30 min, and stained with 1.0% crystal violet for 20 min, washed and dried, photographed and counted.. Each independent experiment was performed three times.

2.7 Wound Scratch Assay

$2x10^6$ cells were seeded in 6-well culture plates and incubated for 24 hours. After 2 washes with cold PBS, a '1' type trace was drawn with a 200μL pipette. The migration distance was observed and photographed under the microscope at 0, 12, and 24 h, respectively. Cell motility was quantified by measuring the distance between the advancing edges of cells in three randomly selected microscope fields (x 200) at each time point.

2.8 Transwell assay

With or without 8μm pore size Matrigel coating (Corning), Transwell assays were carried out to detect the CCA cell migration and invasion. 3×10^4 HuCCT1 cells were added to the upper chamber with 1% serum RPMI-1640 medium and 700 μl RPMI-1640 medium containing 20% FBS was added to the lower chamber. After for 48 h, the cells were fixed with 4% for 30 min and then stained with 0.5% crystal violet solution for 30 min. The cells were washed three times with PBS and the cells on the top of upper chamber were removed gently with a cotton swab. The migration cells were observed in at least five to six

randomly selected fields per well under microscope. The data were analyzed by Image J software. At least three independent experiments were performed.

2.9 Western blotting

After the cells were collected, cell lysate was added to extract the total protein, and the protein concentration was determined by BCA method. 40μg of protein was taken and subjected to 10%~15% SDS-PAGE electrophoresis. After electrophoresis, transfer to PVDF membrane semi-dry. 5% skim milk powder was blocked at room temperature for 1 h, the primary antibodies were added to incubate at 4°Covernight, the secondary antibody was incubated at 37°Cfor 1 h, ECL luminescence was developed, and the gel imager was exposed to take pictures. Statistical analysis was performed using Image J software.

2.10 Statistics

Statistical analyses were performed using SPSS 22.0 software and GapdhPad Prism 8.0 software. All experimental data were expressed as the mean±SD of at least three independent experiments. One-way ANOVA was used to compare the means of multiple groups, and *t* test was used to compare the means between two groups. The *p* value < 0.05 was considered statistically significant

Results

DEK is highly expressed in various malignant tumors and correlated with histological grade and lymph node metastasis of cholangiocarcinoma

To investigate the differences of DEK expression between tumors and normal tissues in various types of cancers, the DEK mRNA levels were analyzed using UALCAN and GEPIA database. The results showed that the DEK expression was respectively higher in CESC (cervical squamous cell carcinoma and adenocarcinoma), CHOL (cholangiocarcinoma), COAD (colon adenocarcinoma), ESCA (esophageal carcinoma)GBM(glioblastoma multiforme), HVSC (head and neck squamous cell carcinoma), LIHC(hepatocellular carcinoma), LUSC (lung squamous cell carcinoma) and SARC (sarcoma) and other tumors. The expression differences were statistically significant (both $P < 0.05$), Figures 1A and 1B. In addition, The Human Protein Atlas database detected the expression of DEK in normal bile duct epithelial tissue and cholangiocarcinoma tissue, and divided into no expression, low expression and moderate expression, as shown in Figure 1C.These results indicate that DEK is up-regulated in CCA tissues, consistent with the results of UALCAN and GEPIA database. The UALCAN database was used to analyze the expression levels of DEK mRNA in 36 cases of cholangiocarcinoma tissues and 9 cases of normal bile duct epithelial tissues. The results showed that compared with normal bile duct epithelial tissues, DEK mRNA expression was significantly increased in cholangiocarcinoma tissues. See Figure 1D, the difference was statistically significant ($P < 0.05$). The relationship between DEK mRNA expression and clinicopathological features was further analyzed, and it was found that it was closely related to cholangiocarcinoma case grade, clinical stage and lymph node metastasis ($P < 0.05$). The results

showed that the expression level of DEK mRNA in clinical stage I, II and IV cholangiocarcinoma tissues was significantly higher than that in normal tissues , and the difference was statistically significant ($P < 0.05$). Compared with normal bile duct epithelial tissue, the expression of DEK was significantly different in G2 (15 cases), G3 (18 cases) and G4 (2) grade cholangiocarcinoma patients ($P < 0.05$). The expression in G1 (1 case) grade cholangiocarcinoma patients was not statistically significant. There were significant differences in the expression between G2 and G3, G2 and G4, G3 and G4 cholangiocarcinoma patients ($P < 0.05$), while G1 and G2, G3, and G4 were significantly different in their expression ($P < 0.05$). There was no significant difference in expression among patients with cholangiocarcinoma. Compared with normal bile duct epithelial tissue, the expression of DEK was significantly different in N0 group (26 cases) and N1 group (5 cases) (other grades were not provided by the database) ($P < 0.05$), as shown in Figure 1E. Survival analysis showed that DEK expression was not significantly associated with overall survival in patients with cholangiocarcinoma ($P > 0.05$), as shown in Figures 1F. The above results indicate that the high expression of DEK in cholangiocarcinoma tissue may be related to the clinical stage, pathological grade and lymph node metastasis of cholangiocarcinoma.

DEK promotes proliferation of CCA cells In Vitro

To further confirm the expression of DEK in normal bile duct epithelial cells and four cholangiocarcinoma cell lines, DEK was subjected to western blotting in CCA cell lines the protein levels of DEK in CCA cell lines were significantly up-regulated compared with normal bile duct epithelial cell HIBEpic (Figure 2A). The HuCCT1 cell line stably overexpressing DEK was constructed with lentivirus, and the transfection intensity was observed under a fluorescence microscope. The results showed that the lentivirus transfection intensity reached more than 95% (Figure 2B). The differential expression of DEK-silenced and overexpressed HuCCT1 cells was further detected by western blotting. The results showed that the expression level of DEK in si-DEK-HuCCT1 cells was significantly lower than that of the blank control group and the negative control group. significantly increased (Figure 2C), the above results indicated that DEK silencing and DEK overexpression cell lines were successfully established. MTT assays indicated that DEK overexpression or knockdown significantly promoted or inhibited cell growth in corresponding ACC cells respectively, and the percentage of inhibition rate reached 40%, while the proliferation ability of overexpressed DEK cells was significantly increased, reaching 50% (Figure 2D). Consistent with these results, Colony formation experiments showed that HuCCT cell proliferation was impaired in DEK knockdown group than those in control group (Figure 2E). These results suggest that DEK could increase the proliferation ability of cholangiocarcinoma cells.

DEK promotes Migration and Invasion of CCA cells In Vitro by facilitates EMT process

To explore whether DEK affects the migration and invasion of CCA cells, we performed the wound healing experiments and the transwell assays. As shown in Figures 3A and B, silenced of DEK significantly impaired the migration and invasion capability of HuCCT1.The migration and invasion abilities of HuCCT1 after DEK overexpression were significantly higher than those in the control group.These results suggest that DEK overexpression promotes migration and invasion of CCA cells.

Epithelial-mesenchymal transition (EMT) plays an important role in the occurrence and development of tumors. The dysregulation of the expression of the epithelial marker E-cadherin protein is a key step in the EMT process. To determine whether DEK is involved in the EMT process of cholangiocarcinoma, we used Western Blot Experimental detection of EMT-related markers. Western Blot results showed that, compared with the control group, the expression of epithelial marker E-cadherin decreased, while the expression levels of mesenchymal markers Vimentin and snail increased after DEK overexpression compared with the control group. After silencing the DEK gene, the results were reversed (Fig. 3C). These results suggest that DEK may promote the metastasis and invasion of cholangiocarcinoma cells through the EMT process.

DEK promotes cholangiocarcinoma progression through AKT/mTOR signaling pathway

To further understand the molecular mechanism of DEK's tumor-promoting effect in cholangiocarcinoma, we further investigated whether the proliferation-promoting effect of DEK is dependent on the AKT/mTOR signaling pathway. western blotting showed that the phosphorylation levels of AKT, S6, and 4EBP1 proteins ,the activated form of Akt, S6,4EBP1, was increased in overexpression-DEK CCA cells. while the phosphorylation levels of AKT signaling pathway-related proteins were significantly inhibited after silencing DEK (Figure 4A). These results indicate that DEK may be mediated by AKT/mTOR signaling pathway promotes cholangiocarcinoma progression. GO term and KEGG pathway analyses were performed to explore the potential biological functions of DEK. GSEA revealed significant differences (p-value < 0.050) in enrichment of GO terms and KEGG pathways in samples with high levels of DEK. We selected the highly enriched signaling pathways based on their normalized enrichment score (NES). As shown in Fig.4B, the biological processes and molecular functions strongly associated with DEK were cell cycle, cell proliferation and fatty acid metabolism. KEGG pathway analysis showed five pathways that had the strongest positive correlation with DEK expression: autophagy, cell cycle, adherens junction, actin cytoskeleton and glycosphingolipid biosynthesis globo series. GO annotation revealed five categories that were positively correlated with high levels of DEK: WNT signaling pathway, cell division,extent of cell growth, mesenchymal cell proliferation and negative regulation of notch signsling psthway . as shown in Fig.4C. The biological process of DEK was identified by overrepresentation enrichment analysis (ORA), which revealed that DEK expression was closely related to various signaling pathway.These results indicate that the pathways regulating cell cycle control and B cell receptor signaling pthway, which are critically important in CCA patients, were strongly associated with DEK expression.

Discussion

Cholangiocarcinoma is a refractory and high-risk malignancy with a low incidence, but with strong tumor heterogeneity, high invasiveness and poor prognosis. Recently, the incidence of cholangiocarcinoma has been increasing year by year, especially in South Korea, Shanghai and other places [17]. Because the early symptoms of cholangiocarcinoma are not obvious, most of them are in the middle and late stages when diagnosed. Surgery is the most important treatment option. However, studies have shown that the recurrence rate of cholangiocarcinoma after 3 years is as high as 85%. The five-year poor prognosis of

postoperative patients is less than 20% [18]. Therefore, it is urgent to further explore molecular markers and molecular prognostic factors for the early diagnosis of cholangiocarcinoma, and to clarify the possible molecular mechanisms, so as to provide a theoretical basis for the clinical diagnosis and molecular targeted therapy of cholangiocarcinoma.

The proto-oncogene DEK is a nuclear protein with a relative molecular mass of about 43KDa and contains 735 amino acids, of which all but 26 amino acids are included in the DEK-NUP214 fusion protein [4]. The roles of DEK and DEK-NUP214 proteins have been elucidated through global analysis of DNA binding and gene expression, as well as a variety of functional studies. Moreover, growing evidences of DEK in tumors have been reported. It has been widely implied that DEK is highly expressed in various malignant tumor tissues. OU et al [14] found that DEK was overexpressed in gastric cancer tissues and correlated with poor prognosis of patients. YANG et al. [19] constructed MCF cells stably transfected with shRNA-DEK and overexpressed DEK. It was found that the down-regulation of DEK expression level can inhibit the proliferation of triple-negative breast cancer cells, and overexpression of DEK promotes the proliferation of triple-negative breast cancer cells. Zheng [20] and other studies have confirmed that the up-regulation of miR-1204 can inhibit the expression of DEK, thereby inhibiting the proliferation of non-small cell lung cancer, blocking cell cycle distribution, and promoting cell apoptosis, suggesting that high DEK expression is expected to be an indicator of tumor development and prognosis. So further studies were carried out that the proliferation and migration of cholangiocarcinoma cells were significantly enhanced after DEK overexpression. What's more, the proliferation and migration of cholangiocarcinoma HuCCT1 cells were significantly inhibited after targeted silencing of DEK gene by RNA interference, which indicated that DEK gene could promote the proliferation and migration of cholangiocarcinoma cells. In addition, DEK is highly expressed in cholangiocarcinoma tissues. According to the survival data of UALCAN database and GEPIA database, there is no statistically significant difference in overall survival time between patients with different expression levels of DEK. At the same time, we further used The database analyzed the relationship between DEK expression and overall survival time of patients with other malignant tumors. High DEK expression was associated with low-grade glioma ($n = 275$), hepatocellular carcinoma ($n = 182$) and lung adenocarcinoma ($n = 239$), etc. The overall survival time of patients was negatively correlated ($p < 0.05$). We speculated that due to the small sample size in the database, the high expression of DEK may have no significance with the overall survival time of patients with cholangiocarcinoma.

Due to the important role of EMT in the regulation of metastasis in tumors. and the biological features and specific mechanism of DEK in cholangiocarcinoma are still unclear. We also detected the effects of DEK on EMT in CCA. Consequently, DEK was assessed to influence the expression of several EMT biomarkers in CCA. EMT is a kind of process that epithelial cells are depolarized and achieve the ability to migrate and invade. EMT is an indispensable process for tumor cells to move to adjacent cell layers from the original solid tumors, and can be concluded as a cellular process that enhances the cancer cells migration and invasion. Further experiments in this study showed that after silencing DEK, the expression of mesenchymal markers (vimentin, snail) was down-regulated and the epithelial marker (E-cadherin) was significantly increased. However, results were reversed after DEK overexpression. It is suggested that DEK

may promote metastasis of cholangiocarcinoma by EMT.Thus, DEK might also play a critical role in regulating EMT and further in promote the metastasis of CCA.

A large number of studies have found that excessive activation of the PI3K/Akt/mTOR signaling pathway can promote excessive cell proliferation, inhibit cell apoptosis, promote abnormal cell differentiation, and participate in processes such as autophagy, resulting in tumor formation and metastasis. Akt/mTOR signaling pathway is activated mainly through phosphorylation, and Akt activation is closely related to tumor cell proliferation, migration and invasion. Ribosomal S6 protein kinase and eukaryotic initiator 4E-binding protein 1 are the downstream key protein kinases of mTOR. After mTOR activation, S6 and 4EBP1 proteins can be phosphorylated, which can promote mRNA translation, thereby promoting a large number of cell proliferation and growth-related proteins expression, leading to the occurrence and development of various malignant tumor cells. The present study found that the phosphorylation expression levels of Akt, S6, and 4EBP1 were significantly down-regulated after down-regulation of DEK by RNA interference, which hindered Akt activation, thereby blocking Akt/mTOR signaling. After DEK overexpression, the phosphorylation levels of Akt, S6, and 4EBP1 were significantly increased, and the AKT/mTOR signaling pathway was activated. These results suggest that the AKT/mTOR signaling pathway may be one of the important mechanisms by which DEK promotes the proliferation of cholangiocarcinoma cells, but its specific mechanism needs further study. GO term and KEGG pathway analysis have revealed upregulated DEK to be primarily linked with cell cycle, cell division, fatty acid metabolism and pathways in cancer. Our work suggests that overexpression of DEK in CCA patients could alter cell cycle control, migration and invasion, NOTCH signaling and lipid metabolic in cancer cells. The Notch signaling pathway has been demonstrated to be one of the most frequently activated signaling pathways in cancer and its cascade is involved in cell cycle regulation [21] and hepatic inflammation [22]. Further studies could help verify which processes and pathways DEK functions through in CCA in vivo. Our results help to advance the understanding of the biological functions of DEK that make its overexpression so detrimental in CCA.

In summary, our study has demonstrated that DEK expression is significantly upregulated in cholangiocarcinoma cells. DEK abnormal expression may promote cell proliferation and tumorigenesis of CCA. Targeted silencing of DEK can block the AKT/mTOR signaling pathway and inhibit the biological process of cholangiocarcinoma cells, providing a theoretical basis for new therapeutic targets for cholangiocarcinoma. In addition, DEK has great value as potential tumor markers and therapeutic targets for cholangiocarcinoma. However, the precise molecular mechanism remains unanswered.

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Figures

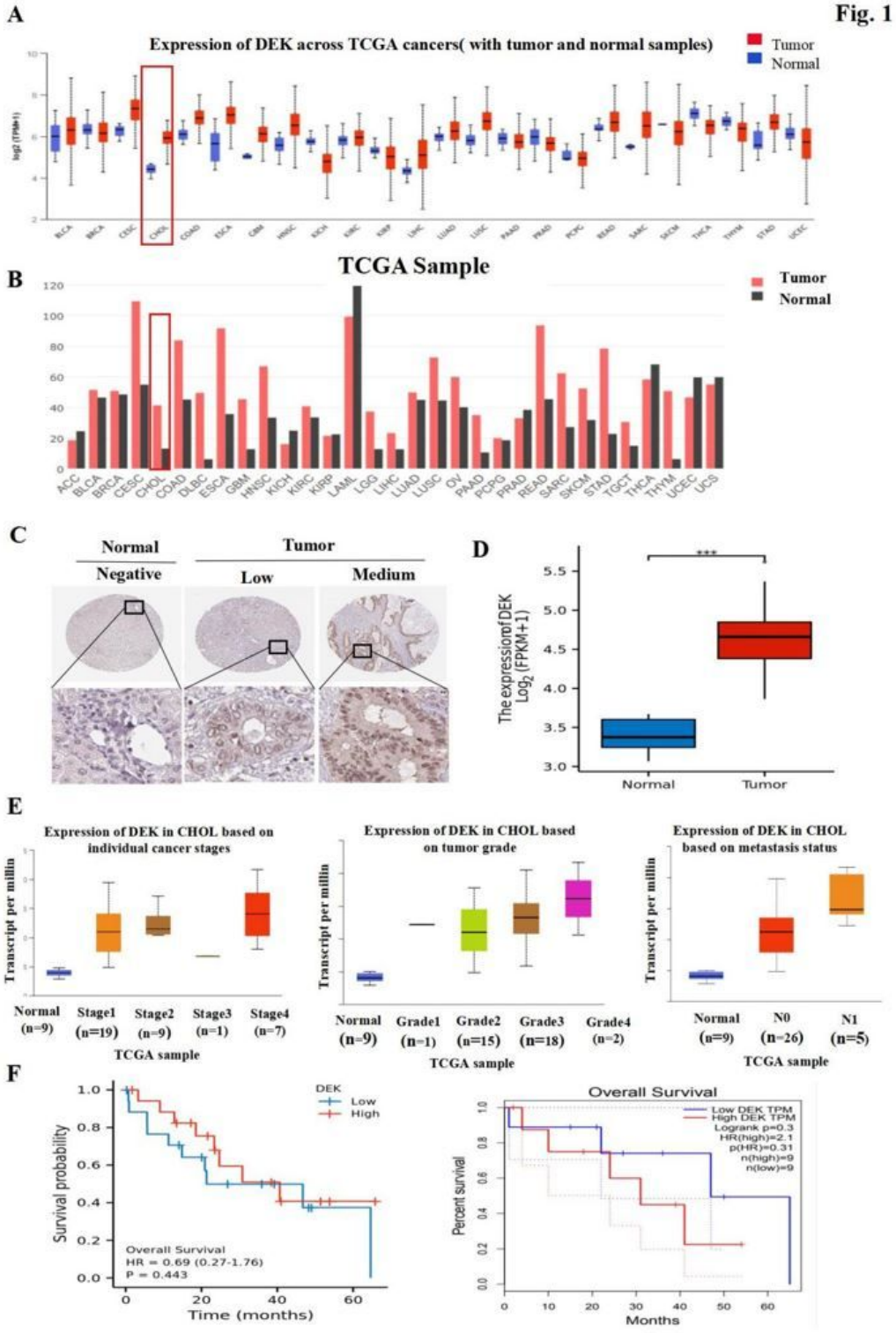

Figure 1

Expression of DEK in cholangiocarcinoma and correlated with histological grade and lymph node metastasis of cholangiocarcinoma

A and B. The expression trend of DEK in various malignant tumors from the UALCAN and GEPIA databases C. The protein expression of DEK in cholangiocarcinoma and normal bile duct epithelial

tissues were acquired from the Human Protein Atlas D. Expression of DEK mRNA in normal tissues and cholangiocarcinoma tissues E. Expression of DEK correlated significantly with clinical grade, pathological stage and N stage of lymph node metastasis F. The correlation between DEK expression and overall survival rate of patients with cholangiocarcinoma was analyzed using TCGA and GEPIA databases.

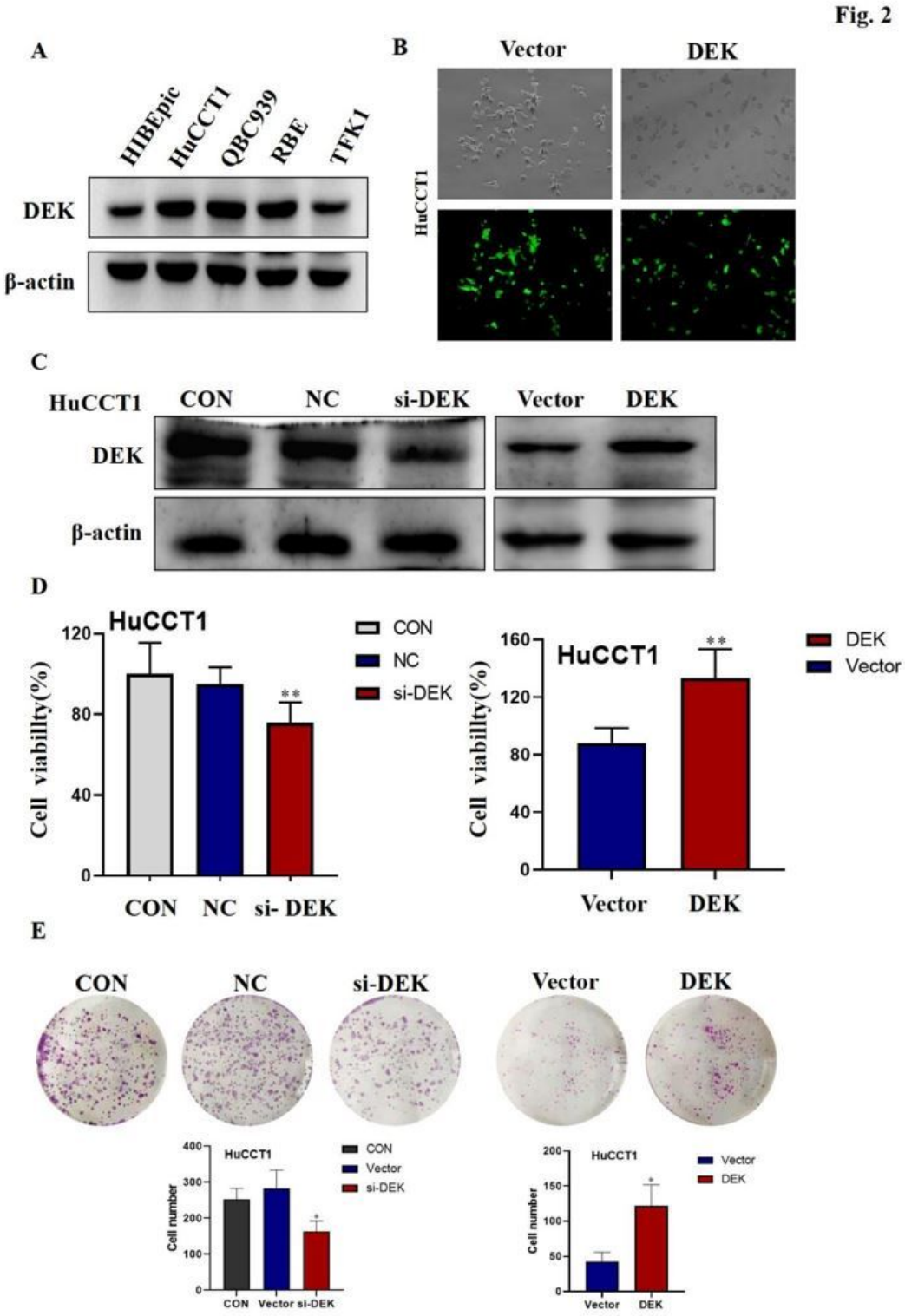

Figure 2

DEK promotes proliferation of CCA cells In Vitro

A. Expression levels of DEK protein in different cholangiocarcinoma cells and normal bile duct epithelial cell were investigated by Western blot B. Immunofluorescence was conducted to visualize the expression of DEK transfection intensity. C. DEK transfection rate confirmed using WB. D. Effect of DEK slicencing and overexpression on the proliferation of the HuCCT1 cell by MTT. E. Effect of DEK slicencing and overexpression on the colony formation ability of HuCCT1 cell by Colony formation assay.

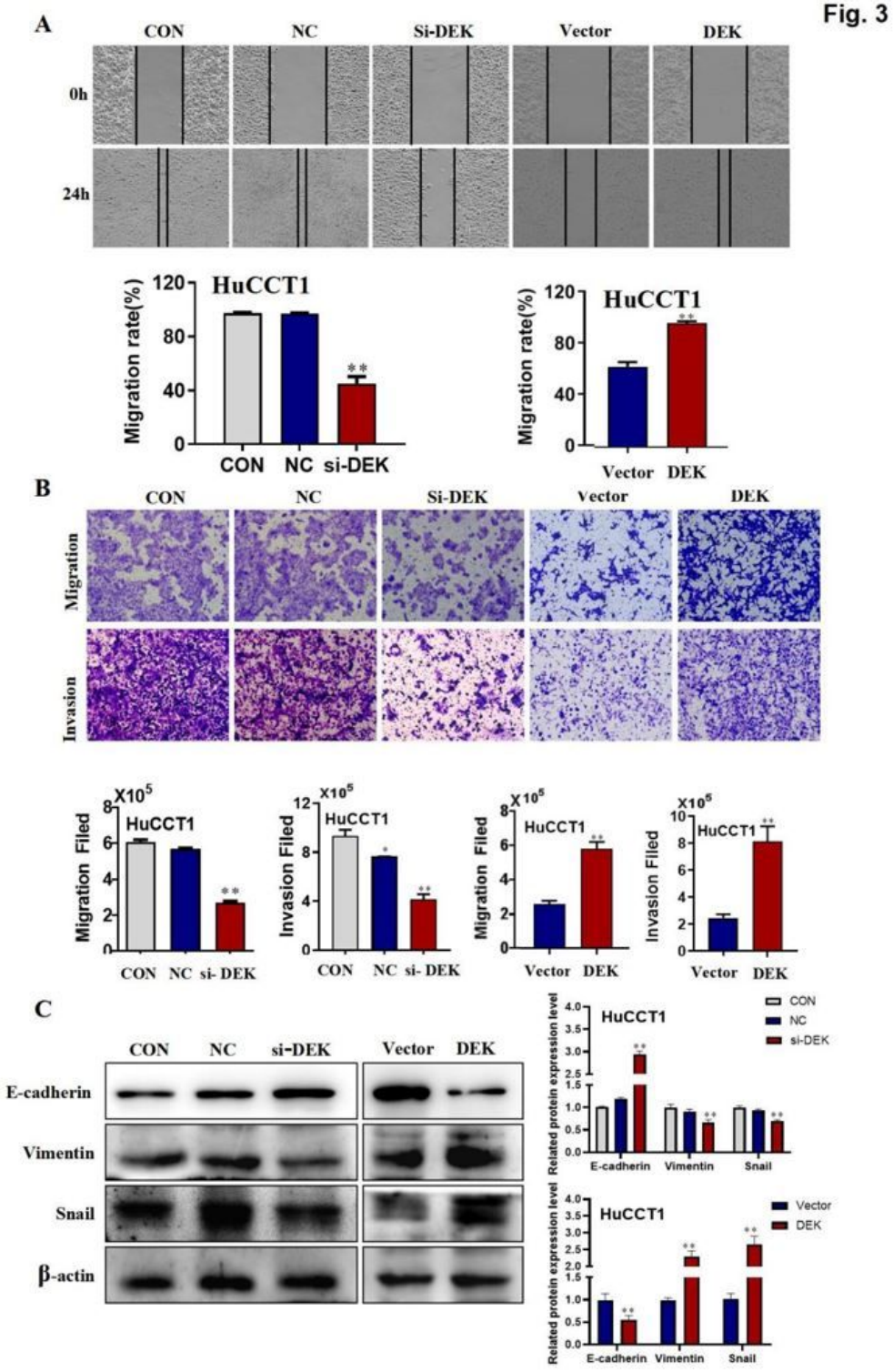

Fig. 3

Figure 3

DEK promotes Migration and Invasion of CCA cells In Vitro by facilitates EMT process

A. Effect of DEK on the lateral migration ability of HuCCT1 cells by Wound healing assay . Semi-quantitative analysis of cell migration area B. Effect of DEK on the longitudinal migration and invasion ability of HuCCT1 cells by Transwell assay. Statistical chart. C Effects of DEK silencing and overexpression on the expression of EMT-related markers E-cadherin, Vimentin and snail by Western Blot.

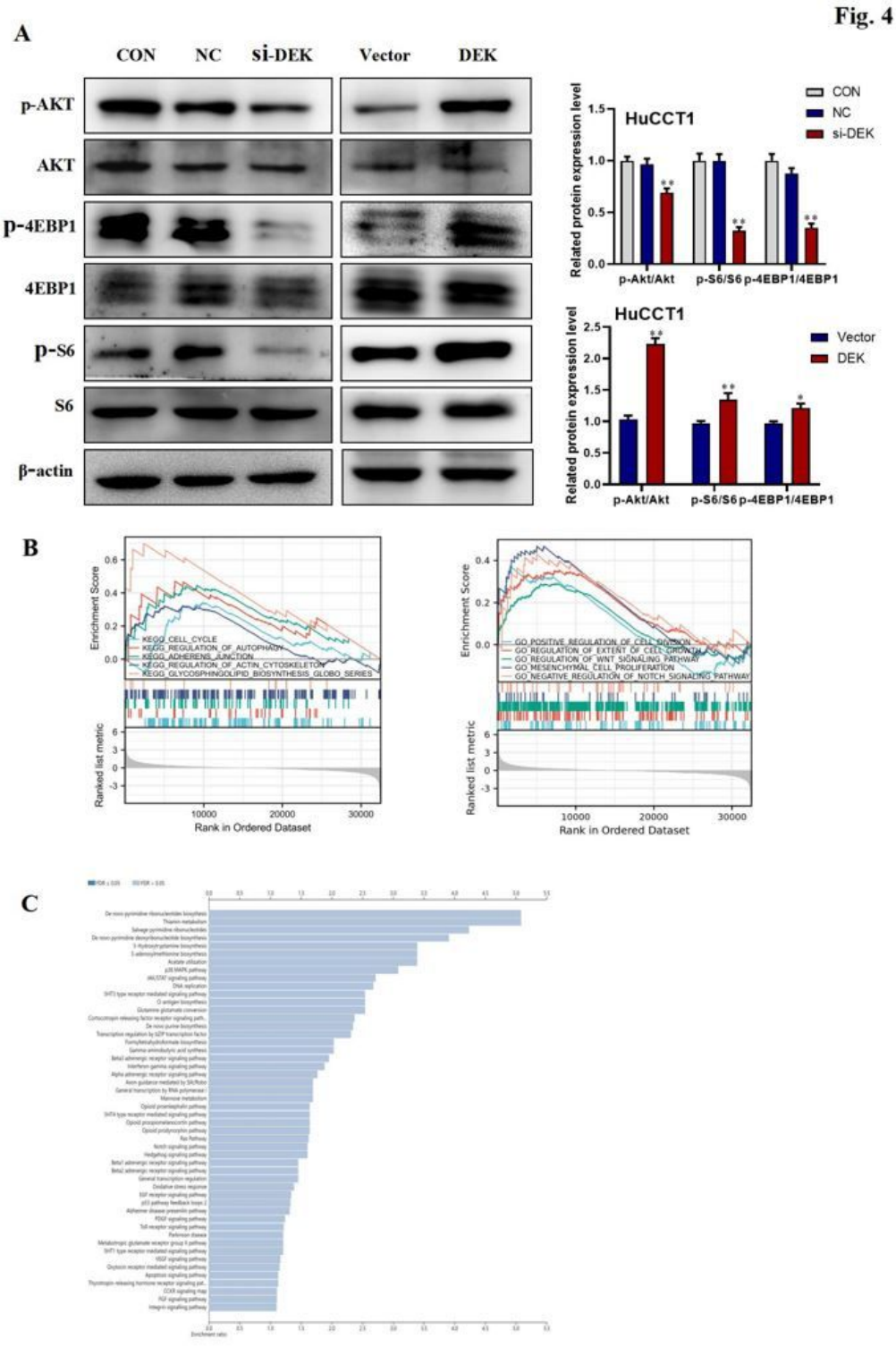

Figure 4

DEK promotes cholangiocarcinoma progression through AKT/mTOR signaling pathway

A. Effect of DEK silenced or overexpression on the expression levels of AKT signaling pathway-related proteins by Western Blot B. KEGG term and GO pathwayanalysis revealed five positively correlated groups correlated groups show C.The biological process of DEK was analyzed by LinkedOmics.