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Silencing of Kangai 1 C-terminal interacting tetraspanin suppresses progression of cholangiocarcinoma

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ABSTRACT

Cholangiocarcinoma (CC) is the second most common primary hepatic malignancy. CC treatment options are very limited especially for patients with distant metastasis. Kangai 1 C-terminal interacting tetraspanin (KITENIN) is highly expressed in numerous cancers, but the role of KITENIN in CC remains unknown. Here, we have investigated for the first time the function of KITENIN in human CC cell lines (TFK-1, SZ-1), tissues and a CC mouse model (Alb-Cre/LSL-KRAS^{G12D}/p53$^{L/L}$). KITENIN was expressed in 92.2% of human CC tissues, in murine CC samples and also in human CC cell lines. Knockdown of KITENIN by small interfering RNA (siRNA) effectively reduced proliferation, migration, invasion and colony formation in both intra- and extra-hepatic CC cells. The expression of epithelial-mesenchymal transition (EMT) markers like N-cadherin, Vimentin, Snail and Slug were suppressed in KITENIN knockdown CC cells. Our results indicate that KITENIN is crucial for cholangiocarcinogenesis and it might become a potential therapeutic target for human CC treatment.

1. Introduction

Cholangiocarcinoma (CC) is a deadly disease with a five-year survival rate lower than 30% [1,2]. Surgery is the only curative therapy for early stage CC patients, but many patients present at diagnosis with advanced tumor stage [1,2]. Currently, palliative treatment with gemcitabine and cisplatin is the standard of care [3]. However, median overall survival under treatment is 11.7 months and more drugs are urgently needed to improve the overall survival and provide new systemic therapeutic options.

KAI1/CD82, a 40–75-kDa transmembrane glycoprotein of the tetraspanin family, was identified as a suppressor of prostate-specific metastasis [4]. KAI1 COOH-terminal interacting tetraspanin (KITENIN) was found to be a binding protein of KAI1/CD82. KAI1/CD82 is associated with the progression and poor prognosis of hepatocellular carcinoma, prostate cancer, non-small lung cancer, ovarian cancer, and bladder carcinoma [5–9]. In addition, KITENIN induces invasion and migration in different cancer models and KITENIN down-regulation by small interfering RNA (siRNA) has been shown to inhibit tumor growth in colorectal cancer, squamous cell cancer and hepatocellular carcinoma [10–17]. Although, the mechanism how KITENIN involves in cancer progression and metastasis is still unclear, however, current data has shown that KITENIN interacts with different signalling pathway such as EMT, stemness markers, MAPK/ERK/AP1, ErbB4-CYT-2 and promotes cancer cells growth and metastasis [11,18,19].

The epithelial–mesenchymal transition (EMT) is a transforming process of epithelial cells to mesenchymal stem cells by losing their cell polarity and enhancing on migratory and invasive capability [20–22].

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EMT marker is a protein family involving in this process. It plays a crucial role in carcinogenesis and serves as a marker of metastasis property and prognosis for cancer patients [20–22]. In CC, EMT markers are involved not only in CC progression but also taken part in CC chemotherapy response [22]. Furthermore, EMT overexpression plays as a poor prognosis factor in CC patients [23]. They promote CC development and induce metastasis. Most of CC patients are diagnosed in the late stage, therefore they are not suitable for curative surgery and treatment options are chemo- or radio-therapy which currently have a limited effect on CC patients in advance stage [22]. Thus, the rate of 5-year survival is low in CC patients with metastasis [22,24]. In addition, EMT is involved in CC resistance to chemotherapy, however particular mechanisms of this circumstance are still not yet clear [25]. Recent studies showed that KITENIN contributed to the development and metastasis of some tumor types and EMT was also involved in this process [11,18,19]. Hence, to understand whether KITENIN plays a role on CC progression and metastasis might provide indispensable information for CC treatment in the future.

Various engineered animal tumor models have been developed and became useful tools for different research fields including CC [26–28]. However, these models have limitations such as long latent period or development of prominent hepatocellular carcinoma [26–28]. Recently, a new murine engineered CC model was developed by using the combination of Albumine-Cre, LSL- $KRAS^{G12D}$ and $p53^{L/L}$ [29]. This mouse model can develop specific CC in a short period of time and therefore, serve as an efficient model for CC research [29].

KITENIN expression itself acts as a biomarker for poor prognosis and metastases, but the role of KITENIN in cholangiocarcinogenesis is unknown. Our study aimed to evaluate the role of KITENIN in murine and human CC. We have demonstrated that KITENIN acted as an oncogene in CC and that silencing KITENIN could impair the progression of CC and KITENIN therefore might become a novel promising target for future therapeutic strategies.

2. Materials and methods

2.1. Cell lines, cell culture and CC murine tissues

Extra-hepatic (TFK-1), intra-hepatic CC (SZ-1) cells, pancreatic cancer cells (Panc1) and human fibroblast (a gift from Prof. Klaus Schulze-Osthoff) were cultured as previously described [30–32]. Briefly, the cells were cultured in RPMI 1640 + GlutaMAX medium (Gibco, Thermo Fisher Scientific Inc., USA) supplemented with 10% Fetal Bovine Serum (FBS, Biochrom, Germany) and 100 U/ml penicillin/streptomycin (Invitrogen, Germany). The cells were kept for culture in an incubator maintained at 37 °C and 5% CO_2. CC murine tissues were collected from genetically engineered mice (Alb-Cre/LSL-$KRAS^{G12D}$/$p53^{L/L}$, n = 5). The tissues were fixed in formalin and embedded in paraffin (FFEP). FFEP tissue blocks were cut to 5 µm sections and placed on histological glass slides for further processing (Hematoxylin/Eosin staining and immunohistochemistry).

2.2. Small interfering RNA preparation and in vitro treatment

KITENIN siRNA (siKITENIN) was purchased from Qiagen (Germany); control-siRNA was purchased from Santa Cruze (USA); SilentFect transfection reagent was purchased from Biorad (USA) and prepared using manufacturer's instructions. CC cells were treated with either 80 nM siRNA control or siKITENIN in media added the transfection reagent. After 24 h, the media with siRNA and the transfection reagent was removed and replaced by fresh media and processed further for different experiments.

2.3. Cell proliferation, migration, invasion and colony formation assay

All these experiments were performed as described in previous studies [30,31,33]. Briefly, for cell proliferation assay, cells were seeded at a density of 1000 cells/well in 96-well plate and then treated with either 80 nM control siRNA or siKITENIN. After 24 h, the media was refreshed and the cells were kept in culture until 96 h. This was followed by incubation with WST-1 reagents (Roche Diagnostics, Mannheim, Germany) for 2 h, after which the optical density was detected in the microplate reader at 490 nm and 650 nm.

For migration assay, 2×10^5 cells were seeded in 6-well plate in media supplemented 10% FBS and 24 h later the cells were treated with either 80 nM siRNA control or siKITENIN. After 24 h, the media was replaced with media supplemented 2% FBS. The cells were maintained for 24–48 h and a wound was created in the cell monolayer in each well using a sterile 200 µl pipette tip. The media was renewed to exclude float cells. The wells with wounds were imaged at 0 h and 48 h by using a Leica DMI 6000 B microscope (Leica, Wetzlar). The wound areas were then measured in ImageJ software (National Institutes of Health, USA) and migration index was calculated using the following formula:

$$\text{Migration Index} = \frac{\text{Area of the wound at 0h} - \text{Area of the wound at 48h}}{\text{Area of the wound at 0h}} \times 100$$

For invasion assay, 6-well BD BioCoat™ Matrigel™ Invasion Chamber (BD Biosciences, Bedford, UK) was used following manufacture's instruction. The cells were added in the upper chamber at a density of 5×10^5 cells in 2 ml serum free media supplemented with either 80 nM siRNA control or siKITENIN and transfection reagent. The cells were kept in culture for 48 h, and the media in upper chambers and wells was changed every day. Afterward, the non-invaded cells were removed and the invaded cells were fixed with methanol and stained with 1% toluidine blue. Then, the invaded cells were photographed under microscope and analysed by ImageJ software. Invasion index was calculated based on invaded cells by following formula:

$$\text{Invasion index} = \frac{\%\ \text{Invasion test cells}}{\%\text{Invasion control cells}}$$

For colony formation assay, 1000 cells were seeded in 6-well plates, followed by treatment with either 80 nM siRNA control or siKITENIN. After 24 h, the media was refreshed and the cells were kept for culture in the incubator for 1 week. The cells were then stained with 1% toluidine blue and photographed. The number and size of colonies were analysed using ImageJ 1.47.

2.4. Transforming growth factor beta-1 (TGFß1) treatment

Cells (TFK-1, SZ-1 and Panc1) were seeded in 6-well plates in media supplemented 10% FBS and 24 h later the cells were treated with PBS or 5 ng/ml or 10 ng/ml TGFß1 (100–21, PeproTech, Inc., Germany). Images were taken at 24, 48, 72 and 96 h to observe the cell morphology. After 96 h, the cells were harvested and lysed to isolate proteins for western blotting.

2.5. Western blot and antibodies

Western blot was performed as described in previous studies [30–33]. The following antibodies were used in this study: KITENIN (HPA025235, diluted 1:1000, Atlas Antibodies, Sweden), E-cadherin (24E10, diluted 1:1000, Cell signalling, USA), N-cadherin (EPR1792Y, diluted 1:1000, Merck Millipore Corporation, USA), Slug (C19G7, diluted 1:1000, Cell signalling, USA), Snail (C15D3 #3879, diluted 1:1000, Cell signalling, USA), Vimentin (sc-7557-R, diluted 1:500, Santa Cruz Biotechnology, USA), beta actin (AC-74, diluted 1:10.000, Sigma-Aldrich Corporation, USA).

2.6. Immunofluorescence

Immunofluorescence was performed following standard protocol.