NKD1 targeting PCM1 regulates the therapeutic sensitivity of HHT on colorectal cancer cells

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Research Article

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Abstract

Colorectal cancer (CRC) is the most common primary malignant tumor with a significantly higher incidence in the worldwide. Homoharringtonine (HHT) often used to treatment of acute leukemia. Recent research revealed it could be used for solid cancer therapy. However, the regulatory target and mechanism of HHT in CRC progression remain elusive. This study proved that HHT suppressed cell proliferation and promoted cell cycle arrest and apoptosis. Transcriptome sequence indicated that NKD1 was the target of HHT in CRC. HHT could suppress NKD1 expression in a concentration and time dependent manner. NKD1 was overexpressed in CRC tissues and depletion of NKD1 enhanced the therapeutic effect of HHT on CRC in vitro and vivo. Furthermore, proteomic analysis revealed that PCM1 involved in the process of cell proliferation and cell cycle regulated by NKD1. NKD1 interacts with PCM1, and NKD1 promotes the ubiquitination degradation of PCM1. Moreover, overexpression of PCM1 can effectively reverse the promoting effect of NKD1 interference on cell cycle arrest and apoptosis. These results suggested that the NKD1/PCM1 axis participated in mediating the therapeutic sensitivity of HHT to CRC. Our findings provide evidence for clinical application of NKD1-targeted therapy in improving HHT sensitivity for CRC treatment.

Introduction

Colorectal cancer (CRC) is one of the common malignant cancer associated with high risk of incidence and mortality worldwide [1]. Although significant progress has been made in the diagnosis and treatment of CRC, the prognosis of patients with advanced stage still poor [2]. It has been reported that recurrence, metastasis and drug resistance are the leading causes of poor outcome with CRC patients [3]. Due to the pathological mechanism remain elusive, it is difficult to predict the tumorigenesis of CRC. Therefore, it is of great value to develop potential targeted drugs and therapeutic biomarkers to improve the clinical outcome of CRC patients.

Homoharringtonine (HHT) is a plant alkaloid which also known as a protein synthesis inhibitor in the treatment of acute and chronic myeloid leukemia [4, 5]. Mechanistic studies have shown that HHT prevents aminocacy binding to the ribosomal subunit during the process of protein elongation, thereby disrupting the transcription of oncogenes and ultimately leading to the apoptosis of leukemia cells [6, 7]. Recently, it has been reported that HHT exerts anticancer properties in several solid malignancies. For example, Guo et al [8] reported that HHT exerted anti-neoplastic activity in the lung cancer by regulating TMEM16A activity. Liu et al [9] proved that HHT induced PSMD11 protein synthesis through activating MEK1/ERK1/2 signaling pathway in pancreatic cancer. Zhu et al [10] proved that HHT inhibited hepatocellular carcinoma cells proliferation and migration by regulating EphB4-mediated β-catenin loss. Our previous research found that HHT suppressed breast cancer cells growth and promoted apoptosis through miR-18a-3p-AKT-mTOR signaling pathway [11]. Besides, Shi et al [12] found that HHT could decrease LoVo cell growth by inhibiting EphB4 and its downstream signaling. Qu et al [13] also demonstrated HHT inhibited cell proliferation and promoted apoptosis in CRC by activating
PI3K/AKT/mTOR signaling pathway. Therefore, it is necessary to investigate the detail of HHT pharmacological targets and its molecular mechanism in the tumor process.

Naked cuticle homolog 1 (NKD1), located on chromosome 16q12.1, is an antagonist of the canonical Wnt signaling pathway. NKD1 influences cells proliferation by preventing the nuclear accumulation of β-catenin [14]. NKD1 is widely expressed in multiple tissues as well as plays a critical role in various tumors. Downregulation of NKD1 increased the invasive ability of nonsmall-cell lung cancer and correlates with a poor prognosis [15]. Knockdown of NKD1 was associated with poor prognosis in invasive breast ductal carcinoma [16]. The lower expression of NKD1 in HCC tissues was associated with extra hepatic metastasis and histological differentiation [17]. Meanwhile, Wang et al [18] found that NKD1 may be an important colorectal cancer biomarker and acts as a curative target for treatment of CRC. However, the functions and specific mechanisms of NKD1 in CRC progression remain poorly understood.

In this study, we aimed to elucidate the antitumor properties and molecular mechanisms of HHT. We demonstrated that HHT inhibits CRC cells growth and proliferation through inducing the cell cycle arrest and apoptosis. NKD1 was upregulated in CRC tissues and silencing NKD1 substantially increased HHT-induced apoptosis and improved the efficiency of HHT to CRC in vitro and in vivo. Further research revealed that NKD1 could interact with PCM1 in the nucleus and promoted its degradation through the ubiquitin-proteasome pathway. Overexpression of PCM1 can reverse the promoting effect of NKD1 on cell cycle arrest and apoptosis. Our results provide evidence that NKD1 targeting PCM1 regulates the therapeutic sensitivity of HHT on CRC.

Materials And Methods

Cell culture and treatment

Human colorectal cancer cell lines HT29 and SW620 were purchased from the Shanghai Institute of Cell Biology at the Chinese Academy of Sciences, and maintained in McCoy’s 5A modified medium (Gibco, USA) and Leibovitz’s L-15 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Australia) and 1% antibiotics (penicillin and streptomycin). Cells were cultured in a humidified incubator containing 5% CO2 atmosphere at 37°C. Homoharringtonine was obtained from Minsheng pharmaceutical Co. Ltd (Zhejiang, China). The cells were treated with different concentration of HHT injection (1.5µM, 3µM, 6µM) to detect the cell cytotoxicity. Lipofectamine 3000 transfection reagent (Invitrogen, USA) was used to transfect the SW620 cells with the PCM1 plasmid according to manufacturer’s protocols.

Cell viability and Colony formation assays

For the CCK8 assay, HT29 and SW620 cells (2×10^3 cells/well) were seeded into 96-well plates and incubated overnight at 37°C. The cells were treated with HHT at the indicated concentrations for another 24h, 48h, 72h. Then, 10µL CCK8 solution (KeyGEN BioTECH, China) was added to each well and further
incubated for an additional 2h at 37°C according to the manufacturer's instruction. The cell viability was assessed by measurement of the absorbance at 450 nm with a microplate reader. For the colony formation assay, SW620 cells transfected with NKD1 or PCM1 were seeded into six-well plates and cultured for 2 weeks. Then, the cells were washed with cold PBS before being fixed with 100% methanol and stained with 0.5% crystal violet solution for 10 min. The number of colonies were counted using ImageJ software from representative areas. All experiments were performed in triplicate.

5-Ethynyl-2’-deoxyuridine (EdU) staining assay

Cell proliferation ability and DNA synthesis were determined using the EdU staining assay. Briefly, HT29 and SW620 cells were treated with different concentrations of HHT for 48 h, and then cells in logarithmic growth phase were seeded on the cover slips (NEST, USA), and added with 10 uM EdU solution (Abcam, USA) to each well for incubation 2h at 37°C. Subsequently, the cells were fixed with 4% paraformaldehyde for 20 min and treated with 2M HCl for 30 min at room temperature. After washed with cold PBS buffer three times, each well was supplemented with penetrant with 0.5% Triton X-100 (Solarbio, China) and blocked with 10% goat serum (Solarbio, China) for 1h. The nucleus was visualized with DAPI reaction solution (Sigma, USA) for 20min in the dark and mounted with fluorescence quenching agent (Solarbio, China). EdU positive cells were finally photographed and counted under a fluorescence microscope (Olympus, Japan) with different field.

RNA extraction and quantitative real-time PCR (qRT–PCR)

Total RNA was extracted from tissues and cultured cells using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA concentration was measured on Nanodrop Spectrophotometer (Thermo Fisher Scientific, USA) and reverse transcription kit (Takara, Japan) was utilized to perform cDNA reverse transcription. RT-PCR amplification was performed with specific primers and carried out using the SYBR-Green PCR system (Takara, Japan). GAPDH served as the internal control throughout the experiment. The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The sequences of the primers used for qPCR were as follows: NKD1: F: ACCATTGCGTAGATGAGAACAT, R: CCAAATTGGGACGTGTAGTTTT. GAPDH: F: TGTTGCCATCAATGACCCCTT, R: CTCCACGACGTACTCAGCG.

RNA interference vectors and cell transfection

The lentivirus vector containing the small interfering RNAs (siRNAs) that specifically target NKD1 were designed and synthesized from Shanghai Genechem Co, Ltd (Shanghai, China). For lentiviral transduction, SW620 cells were seeded into 24-well plates to facilitate the cell confluence to 50%, and transfected with three siRNAs vector or negative control vector according to the manufacturer's protocol. After culture in an incubator at 37°C for 8–12 h, the serum free medium was replaced with complete medium. Screening of stably transfected siRNA-NKD1 cells with 1 µg/ml puromycin reagent (Carlsbad, USA). Cell transfection efficiency was observed under fluorescence microscope and examined using qRT-PCR and Western blot assays. The NKD1 siRNA sequences were shown in Table 1.
Table 1
The target sequences of NKD1 siRNA

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Cell cycle and apoptosis analysis by flow cytometry

For the cell cycle analysis, HT29 and SW620 cells were plated into a six-well plate and treated with indicated concentration of HHT for 48h. Cells were digested with trypsin without EDTA to prepare a single-cell suspension. After centrifugation, the supernatant was removed and the cells were resuspended in cold PBS buffer. Precooled 70% ethanol was added to fix the cells overnight at -20°C. Cells were resuspended in 200ul binding buffer and stained with PI staining solution for 30min at room temperature. Cell cycle distribution was measured and quantified by the flow cytometry System (BD Biosciences, USA).

For the apoptosis assay, AnnexinV-APC/PI double staining apoptosis detection kit (MULTI SCIENCE, China) was employed to detect the apoptosis rate of the HT29 and SW620 cells. The binding buffer containing 10µL Annexin V-APC was added to the cells and incubated for 15 min at room temperature in dark conditions, and then added 5µL PI in dark conditions for 10min. The cell apoptosis rate was examined by flow cytometer and the data was analyzed with BD Cell-Quest and FlowJo software.

Protein extraction and Western blot analysis

In brief, the cells were collected and extracted using a whole protein extraction kit (KeyGEN BioTHCH, China) containing lysis buffer, Protease Inhibitor Cocktail and PMSF for 45 min on ice. Then the lysates were centrifuged at 13,000g at 4°C for 20 min and the total protein concentration was measured by BCA detection kit (Thermofisher Scientific, Inc). Equal amounts of proteins were electrophoresed on 10% SDS-PAGE and transferred to PVDF membranes (Millipore, USA). The membranes were blocked with 5% defatted milk for 1 h at room temperature and incubated with specific primary antibodies 4°C overnight. Subsequently, the membrane was further incubated with HRP-conjugated secondary antibodies against goat anti-rabbit or anti-mouse IgG (ab205718; 1:5000, Abcam) at room temperature for 1 h. The protein bands were visualized using BioImaging Systems (BIO-RAD, USA). Anti-GAPDH antibody was used as an internal control to normalize protein levels. Specific antibodies were shown in Table 2.
### Table 2
The antibodies used in the western blotting analysis

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### Co-immunoprecipitation assay

The cells were harvested and lysed in cold lysis buffer. Equal amounts of cell lysates were incubated with normal IgG or special primal anti-NKD1 (CST) overnight at 4°C. Protein A/G-agarose (Abcam) were washed with cold PBS, and then incubated in antibody for 4h. After that, the immunoprecipitated complex was washed three times with ice-cold PBS and remove the supernatant. Then, adding 5x loading buffer into the precipitation to boil protein. The eluted proteins were separated by SDS-PAGE. Bound proteins were analyzed by western blotting with anti-PCM1 antibody (Santa cruz).

### Immunofluorescence (IF) staining

HT29 and SW620 cells were seeded into 12-well plate on coverslips (NEST, USA) for 48 h. After washing with cold PBS, cells were fixed in 4% paraformaldehyde for 20 min. Subsequently, the coverslips were permeabilized with 0.5% Triton X-100 for 10 min and blocked with 5% normal goat serum for 1 h at room temperature. Then, incubated with primary antibodies against C-PARP (Abcam, 1:200, USA) overnight at 4°C, and followed by incubation with fluorophore-conjugated secondary antibody (1:200, Invitrogen, USA).
for 2 h. The nucleus was visualized with DAPI solution (Sigma, 1:500, USA) for 15 min in the dark. Finally, the slides were observed under a fluorescence microscope (Olympus, Japan), and integrated fluorescence density was measured by ImageJ software.

**Immunohistochemistry (IHC) staining**

The 10% formaldehyde fixed samples and tissue were embedded in paraffin and sliced into 5-µm thick sections. The microarray sections were deparaffinized by xylene and rehydrated through graded concentrations of alcohol solution, and then treated with 0.3% hydrogen peroxide for 30 min to inactivate endogenous peroxidase. After being repaired and blocked, the slides were incubated with primary antibodies at 4°C overnight, including anti-NKD1 (1:200, Abcam, USA), anti-Ki67 (1:200, Abcam, USA), anti-CDK4 (1:200, Abcam, USA), anti-Bax (1:100, CST, USA). Subsequently, the corresponding HRP-labeled secondary goat anti-rabbit antibody was incubated at room temperature for 1h. After the diaminobenzidine (DAB) reaction was developed in dark conditions, the slides were counterstained with hematoxylin. The typical images were analyzed under microscope, and three equal-area non-repetitive fields were selected for each slice to calculate the number.

**Tumor xenograft Model**

BALB/C nude mice (6–8 weeks old) were purchased from the Beijing Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). siNKD1 transfected cells were subcutaneously injected to establish the colorectal cancer xenograft model. Mice were randomly assigned into five groups (Control group, NC group, siNKD1 group, HHT group and siNKD1 + HHT group, n = 7). 0.5mg/kg HHT were intraperitoneally injected for 15 days to observe the inhibitory effect on tumor growth. The length and width of tumors were measured with electronic calipers and tumor volume was determined using the formula: volume = (Length × Width²)/2. Subsequently, the mice were euthanized and tumor tissues were isolated and frozen in liquid nitrogen or fixed in formalin to perform IHC assay. All animal experiments were performed in accordance with principles and guidelines approved by the Ethics Committee of Animal Research.

**Statistical analysis**

The statistical analysis was performed using SPSS 18.0, GraphPad Prism version 8.0 and Illustrator 2017 software. All assays were presented as the mean ± SD and the repeated individual experiments were performed for each group. Statistical differences among groups were determined by two-tailed Student t test or one-way analysis of variance (one-way ANOVA). Values were considered to be significant when P < 0.05.

**Results**

HHT potently inhibited the viability and proliferation of CRC cells
The inhibitory effect of HHT on viability of HT29 and SW620 cells was investigated by cell morphology and CCK8 assay. Both of cells were shattered and deformed with the increase of HHT concentration (0, 1.5uM, 3uM, 6uM) (Fig. 1A). The cell viability significantly reduced towards HHT treatment in concentration and time dependent manner compared to the untreated cells (Fig. 1B). Meanwhile, the colony formation and Edu staining assay were performed to elucidate the cells proliferation. As shown in Fig. 1C, the number of colonies decreased significantly with the increase of HHT concentration. Edu staining experiment also showed that the number of positive staining cells decreased significantly when the HHT concentration increased continuously (Fig. 1D, E). Taken together, these results suggested that HHT had strong cytotoxic and cytostatic effects on CRC cells.

**HHT accelerated cell cycle arrest of CRC cells at G0/G1 phase**

The effect of HHT on cell cycle distribution in HT29 and SW620 cells was analyzed by flow cytometry. The results were shown in Fig. 2A, two cells were all arrested at G0/G1 phase and the proportion were increased in a dose dependent manner after incubation with HHT. On the contrary, the ratio of the S phase or G2/M phase were decreased compared with the control group. The results reflected HHT have function of blocking the CRC cells in G0/G1 phase. Additionally, western blot detected the cell cycle related proteins and corresponding kinase, and the results showed that the expression of CyclinE, Cyclind1, CDK2, CDK4 and CDK6 proteins were repressed with the increase of HHT concentration (Fig. 2B, C). HHT also decreased the above proteins expression in a time-dependent manner (Fig. 2D, E). Collectively, these data indicated that HHT inhibited CRC cell growth and proliferation by inducing cell cycle arrest at G0/G1 phase.

**HHT induced apoptosis of CRC cells**

To determine whether HHT could induce the cells apoptosis, HT29 and SW620 cells were exposed to various concentrations of HHT for 48h and the apoptotic rates were evaluated by AnnexinV-FITC/PI staining assay. As shown in Fig. 3A, HHT remarkably increased the total proportion of apoptosis in both cells in a dose-dependent manner compared to the untreated group. Besides, immunofluorescence staining assay demonstrated that HHT could induce cleaved-PARP condensed bright in nuclear (Fig. 3B, C). We next implemented western blot analysis to further investigate CRC cells apoptosis related protein expression. The results indicated that HHT treatment significantly increased the protein level of cleaved-PARP and Bax, whereas decreased the Caspase3, Caspase9 and Bcl-2 expression (Fig. 3D, E). Taken together, these results strongly proved that HHT induced the expression of pro-apoptotic proteins to activate the cascade reaction and ultimately facilitate the apoptosis of CRC cells.

**NKD1 expression correlates with response to HHT**

To illustrate the molecular mechanism underlying the anti-cancer effect of HHT, transcriptome sequencing analysis were performed on HHT-treated SW620 cells. A total of 3201 up-regulated genes and 3495 down-regulated genes were found in HHT treatment compared to the control group (fold change > 2 and p < 0.05). Cluster map and volcano map analysis revealed that the NKD1 expression was
downregulated following HHT treatment (Fig. 4A, B). Gene Ontology (GO) analysis revealed that downregulated genes were significantly enriched for GO terms associated with cell cycle, DNA replication, apoptotic process (Fig. 4C). Reactome database analysis showed a significant enrichment in Cell cycle, G1/S transition, and Apoptosis signaling pathway (Figure S1), which largely consistent with our verification of HHT function. RT-PCR and Western blot confirmed that HHT reduced NKD1 mRNA and protein expression both in a concentration and time-dependent manner (Fig. 4D,E). In addition, we completed the xenograft tumorigenic experiments in nude mice to further prove the targeting relationship between HHT and NKD1. We found that compared with the control group, the tumor formation was reduced in the intraperitoneal injection HHT group (Fig. 4F), and the tumor weight and volume were also significantly reduced synchronously (Fig. 4G,H). Meanwhile, the Fig. 4I results showed that the NKD1 mRNA expression was decreased in nude mice tissues after HHT treatment. The apoptosis related protein Bcl-2 expression was decreased whereas Bax was increased in HHT treated group (Fig. 4J). These results suggested that NKD1 may act as a drug delivery target of HHT and play an important role in HHT inhibiting CRC cell proliferation.

**Expression of NKD1 in the colon carcinoma specimen and cancer cells**

In order to investigate the expression of NKD1 in CRC development, we first analyzed the Pan-cancer expression overview of NKD1 through the TCGA database (https://portal.gdc.cancer.gov/), and found that NKD1 was highly overexpressed in COAD and READ (Fig. 5A). Then we detected 288 CRC tumors and 41 matched normal samples, and found the NKD1 expression significantly upregulated in the tumor specimens (Fig. 5B). But there was no difference in the early stage (G1 + G2) and late stage (G3 + G4) patients (p = 0.051) (Fig. 5C). In addition, we used RT-PCR and IHC experiments to verify the relative expression of NKD1 in 36 pairs of cancer and adjacent tissues, and found that NKD1 was significantly elevated in CRC patients compared to adjacent normal tissues (Fig. 5D,E). We also detected the NKD1 expression in CRC cell lines. As shown in Fig. 5F,G, NKD1 expression was highly expressed in CRC cells than that of normal cell (NCM460), with the greatest expression in SW620 cells. The above results demonstrated that NKD1 may play an important role in regulating the development of CRC. To further explore the function of NKD1, NKD1 siRNAs were designed and transfected into the SW620 cells. The interference efficiency was tested by RT-PCR and Western blot, and the results indicated that the mRNA expression of NKD1 siRNA1, siRNA2 and siRNA3 all decreased (Fig. 5H), but the protein expression of NKD1 decreased only in siRNA1 group (Fig. 5I). Therefore, interference sequence 1 (NKD1 siRNA1) was selected for the following study.

**The sensitivity of CRC cells to HHT is associated with NKD1**

To further elucidated the relationship between NKD1 and HHT sensitivity, SW620 cells were transfected with siNKD1 and exposed to the specified concentration of HHT. Western blot result showed that the protein expression of NKD1 was lowest in NKD1 knockdown cells upon HHT treatment than in the control group (Fig. 6A). CCK8 and clone formation assay showed that NKD1 silencing increased the inhibitory effect of HHT on cell growth and proliferation (Fig. 6B, C). Besides, as shown in Fig. 6D, E, knockdown of
NKD1 dramatically increased the sensitivity of SW620 cells to HHT and manifested in the increase of proportion of G0/G1 phase and apoptosis. For further validation, western blot was conducted to detect cell cycle and apoptosis-related protein expression. The results indicated that CDK2, CDK4, CDK6, CyclinE and anti-apoptotic protein Bcl-2 expression was significant reduction in HHT combined NKD1 deletion treatment group, while pro-apoptotic protein Bax and C-caspase9 expression were increased, suggesting that HHT induced cell growth inhibition, cell cycle arrest and cell apoptosis largely attributed to the inhibition of NKD1 (Fig. 6F, G). Taken together, these results indicated that NKD1 acts as a critical target of HHT to mediate the therapeutic effect of HHT on CRC.

**NKD1 deletion promotes CRC cells sensitivity to HHT in vivo**

In order to detect the NKD1 as a target for HHT to inhibit the progression of CRC, we established xenograft nude mouse model with prepared NKD1 NC or NKD1 siRNA to verify the inhibitory effect of HHT combined with NKD1 depletion on tumor growth in vivo. The tumor cells were inoculated subcutaneously and then sacrificed 20 days after intraperitoneal injection of HHT (0.5 mg/kg). In accordance with in vitro experimental results, the average tumor volume and weight were decreased with HHT treatment than that in the control group, and the addition of NKD1 knockdown enhanced the suppressive effect of HHT on tumor growth (Fig. 7A-7C). Moreover, western blot demonstrated that the expression of NKD1, CDK2, CDK4, CyclinD1 and Bcl-2 were all decreased, whereas Bax expression was increased in the tissues from the NKD1-depleted combined with HHT-treated group (Fig. 7D, E). IHC results also showed that the group treated with HHT and siNKD1 lentivirus contributed to decrease the level of NKD1, Ki67 and CDK4 in xenograft tumor tissues compare with control group (Fig. 7F). These findings further highlighted the crucial roles of NKD1 in impairing CRC tumorigenesis and improving HHT sensitivity.

**PCM1 as a potential regulatory partner of NKD1**

To reveal the potential mechanism of NKD1-mediated responses to HHT sensitivity, LC-MS/MS quantitative proteomics being applied to figure out the target protein (Fig. 8A). The number of differentially expressed up- and down-regulated proteins in three groups, including Control vs HHT; Control vs siNKD1; Control vs siNKD1 + HHT, were identified (P value < 0.05 and FC > 1.2; Fig. S2a). The down-regulated differentially expressed (DE) proteins in the three groups were analyzed and displayed in the form of heat map and Venny map, and a total of 58 common intersection proteins were found (Fig. 8B, C). With the KEGG pathway analysis, the 58 clustered proteins were mainly enriched to Cell cycle and Apoptosis signaling pathways, which was consistent with our previous experimental results (Fig. 8D and Fig. S2d). The ontology (GO) terms including biological process, cellular component, molecular function, were allocated to viral life cycle, viral transcription and DNA metabolic process (Fig. S2b and S2c). According to the four functions of the GO enrichment, the co-intersection proteins were clustered as apoptotic, cell cycle, cell proliferation and viral process (Fig. 8E). Among them, PCM1, DHFR and PCNA were selected and confirmed by western blot assay. The results showed that PCM1 protein was the most reduced in HHT and siNKD1 treatment alone or in combination group (Fig. 8F). Taken together, these
results confirmed that PCM1 as the target protein related to NKD1 and to be potential regulatory partner of NKD1.

**NKD1-mediated PCM1 regulates the inhibitory effect of HHT on CRC**

Further experiments were conducted to verify whether PCM1 was involved in the NKD1 regulation CRC cells sensitivity to HHT. The intracellular localization of NKD1 and PCM1 was analyzed by immunofluorescence staining. The yellow areas displayed in the cells illustrated that NKD1 co-localized with PCM1 in the nucleus (Fig. 9A). Endogenous immune-precipitation showed that NKD1 interacted with PCM1 in SW620 cells (Fig. 9B, C). In addition, depletion of NKD1 decreased the protein expression of NKD1 and PCM1, but overexpression of PCM1 could not effect the expression of NKD1 (Fig. 9D). We further examined NKD1 depletion in SW620 cells and found that NKD1 silencing could decrease PCM1 protein, and this effect was rescued by the proteasome inhibitor MG132 treatment (Fig. 9E). We also found that NKD1 deletion will reduce the stability of PCM1 through the action of the protein synthesis inhibitor CHX (Fig. 9F). Meanwhile, CCK8 and colony formation assay indicated that overexpression of PCM1 could reverse the cell growth inhibition induced by NKD1 depletion (Fig. 9G, H). Western blot results indicated that knockdown of NKD1 could reduce cell cycle and anti-apoptotic related protein expression, but increase the pro-apoptotic proteins expression. Conversely, the above results were partially rescued by PCM1 overexpression (Fig. 9I, J). Taken together, these data indicated that NKD1 modulates the therapeutic effect of HHT on CRC depends on PCM1.

**Discussion**

Colorectal cancer is a common malignant tumor with high recurrence and drug resistance. Due to the lack of effective treatment strategies, this disease has brought a heavy burden to human health. Seeking effective therapeutic drugs and action targets is the main direction and prerequisite for CRC [19, 20]. Previous studies have reported that HHT is a highly effective chemotherapeutic drug that has been confirmed in the treatment of acute myeloid leukemia [21, 22]. Currently, emerging studies illuminated that HHT may have therapeutic potential against solid tumors [23, 24]. In this study, the influence of HHT and its potential underlying mechanism in CRC were evaluated. Based on the experiments, we demonstrated that HHT treatment caused potent inhibition of CRC cells proliferation in vitro and substantial suppression of tumor growth in vivo, which inhibitory effects are likely attributed to HHT-induced cell cycle arrest and apoptosis. The results implied that HHT may be a potential therapeutic agent for CRC treatment.

To understand the mechanisms of how HHT induced CRC apoptosis is important for its possible application to cancer treatment. We employed transcriptome sequencing analysis to find the HHT action enriched signaling pathways and biological functions related to cell cycle and apoptosis. We employed transcriptome sequencing analysis to find the biological functions and signaling pathways enriched by HHT action were closely related to cell cycle and apoptosis. After validated the candidate gene, we
selected NKD1 as the target gene. NKD1 was high expression in CRC tissues and cell lines, and the expression of NKD1 was significantly decreased when treated with HHT, indicating that NKD1 may serve as a target of HHT. NKD1 is a negative regulator of the canonical Wnt signaling pathway [25] and plays a key role in many malignant tumors [26, 27]. However, few research have been carried out with CRC, and the relationship between NKD1 and CRC progression remains to be further addressed. For the first time, we systematically analyzed the expression and function of NKD1 in CRC. Based on the experiment results, we found that inhibition of NKD1 enhanced cell sensitivity to HHT exposure and promoted HHT-induced cell cycle arrest and apoptosis of CRC cells. Similarly, the knockout of NKD1 robustly suppressed tumor growth in xenograft tumor models. These findings confirmed that NKD1 promotes CRC progression and serve as a novel HHT drug delivery target for CRC patients.

Cell cycle arrest and apoptosis are one type of cell death with a series of regulated signal cascades, which are essential process for cancer initiation and progression [28, 29]. In this study, we performed subcellular proteomic and bioinformatic analysis to identify the candidate proteins which participated in HHT treatment combined with NKD1 knockdown. We found that PCM1 as a pivotal protein that was positively related to HHT treatment and NKD1 expression. It is well known that PCM1 has a dynamic cell cycle distribution and responsible for protein recruitment and cell division [30]. PCM1 also has been recognized as a key regulator of multiple malignancies. For example, PCM1 involved in chromosomal mutations and associated with glioblastoma and multiple hematological malignancies [31, 32]. PCM1 and canonical autophagy protein GABAPAP combined to form a complex and involved the regulation of apoptosis and autophagy [33]. Based on these observations, we propose that HHT targeting NKD1 is mediated by the modulation of PCM1, and NKD1-PCM1 axis could be one of the driver events of CRC. Mechanistic studies uncovered a novel function of NKD1 based on its interaction with PCM1 in the nucleus and promoted PCM1 degradation through the ubiquitin-proteasome pathway. Furthermore, the results indicated that overexpression of PCM1 could effectively reverse NKD1 knockdown-induced cell cycle arrest and apoptosis in CRC cells, establishing a feedback loop between NKD1 and PCM1 in regulating sensitivity of CRC to HHT. Our finding provides the potential role of NKD1 as a key factor regulating CRC development and progression, which supports the possibility of HHT targeting NKD1 for CRC treatment.

**Conclusion**

In summary, this study proved that HHT inhibits CRC progression via the inhibition of NKD1/PCM1 dependent mechanism. Our study provides preliminary guidance on developing a potential drug with HHT as the lead compound for CRC treatment, highlighting the inhibition of NKD1/PCM1 oncogenic signaling in improving HHT sensitivity, which represents a promising therapeutic strategy for CRC diagnosis and prognosis.

**Declarations**

Data availability statement
The corresponding author will provide the original data used to support the findings of this study upon reasonable request.

Ethics statement

Animal study was conducted after approval from the Ethics Committee of Ningxia Medical University.

Consent for publication

All authors have agreed to the publication of this manuscript.

Availability of data and materials

Not applicable.

Conflict of interest

The authors have declared that no competing interest exists.

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Authors’ contributions

Jia Cao contributed to the experimental work, paper writing and editing, performed the data evaluation and material management. Rong Ma and Xiang Tao were mainly responsible for completing some cell experiments. Jia Wang and Jufen Zhao contributed to the molecular experiments. Bin Shi collected and reviewed clinical data. Jinhai Tian was mainly responsible for biological information analysis. Jingjing Yu and Qi Huang contributed to the data evaluation and data sorting. Libin Wang contributed to the funding acquisition, data evaluation, paper editing and project administration.

References


Figures
Figure 1

Different concentrations of HHT effectively inhibit cell proliferation in vitro. A Representative images of HT29 and SW620 cells morphology after HHT treatment are shown. Scale bar, 100 μm. B CCK8 assay detected the effect of HHT on cell proliferation at different times. C Proliferated cells were measured by clone formation assay. D, E The number of HT29 and SW620 cell proliferation was calculated using EdU staining Scale bar, 50 μm. (*P<0.05, **P<0.01, ***P<0.001).
Figure 2

HHT accelerated cell cycle arrest of CRC cells at G0/G1 phase. Different concentrations of HHT acted on HT29 and SW620 cells. A The cell cycle distribution was detected by flow cytometry analysis. B, C The protein expression of cell cycle markers (CDK2, CDK4, CDK6, Cyclin1, CyclinE) was detected in HT29 and SW620 cells by Western blotting after HHT at different concentrations. D, E The protein expression of cell cycle markers was detected in both of cells by Western blotting after HHT at different times. ("P<0.05, **P<0.01).
Figure 3

HHT induced apoptosis of CRC cells. A The total apoptotic rate of HT29 and SW620 cells was detected by flow cytometry analysis. B, C HHT action was used to detect the apoptotic ratio of cells by immunofluorescence staining against C-PARP. D, E The protein expression of apoptotic markers (Bax, Bcl-2, Caspase3, Caspase9, PARP) was detected in HT29 and SW620 cells by Western blotting. (†P<0.05, **P<0.01).
NKD1 is a delivery target of HHT. A A heat map was constructed based on the differentially expressed genes (DEGs) between the control and HHT treatment cells. B An enhanced volcano plot of DEGs from RNA sequencing is shown. The red and blue dots represent upregulated and downregulated genes, respectively. C GO analysis of the biological functions of the enrichment of the top 30 differentially downregulated genes. D, E The relative expression of NKD1 mRNA and protein was verified by RT-PCR.
and Western blot. F Representative photographs formed by nude mice in control group and HHT group are shown. G The tumor weight and H tumor volume were measured every 2-3 days in each group (n=7 per group). I NKD1 expression after HHT treatment was detected in 7 mice by RT-PCR. J The protein levels of NKD1, Bax and Bcl-2 in each group were measured by immunoblotting. (*P<0.05, **P<0.01).

**Figure 5**

NKD1 overexpression in CRC tissues and cell lines. A TCGA database revealed NKD1 pan-cancer expression. B TCGA cohorts database detected NKD1 transcripts in 288 cases of CRC tissues (“Tumor”) and 41 cases of normal tissues (“Normal”). C The subgroup analyses of NKD1 mRNA expression of CRC patients in TCGA were shown. D The expression of NKD1 in 36 pairs of CRC tissues and normal tissues were compared.
was verified by RT-PCR. E, NKD1 localized expression was determined by IHC staining. F, G The expression of NKD1 in normal colorectal cells (NCM460) and different tumor cell lines was validated by RT-PCR and Western blotting. H, I Construction of NKD1 interference lentiviral vector and verification of the transfection efficiency by RT-PCR and Western blot. (*P<0.05, **P<0.01, ***P<0.001).

Figure 6
HHT combined with NKD1 knockdown promotes cell cycle arrest and apoptosis. The experiment was divided into five groups, including Control group, NC group, siNKD1 group, HHT group, and siNKD1 plus HHT group. A Western blot was used to detect the protein expression of NKD1. B The cell growth curve of each group was evaluated by CCK8 assay. C Colony formation assay of SW620 cells with NKD1 depletion or HHT treatment. D Cell cycle analysis was performed to assess the effect of NKD1 silencing or HHT treatment on SW620 cells. E The effect of HHT combined with siNKD1 on apoptosis was analyzed by flow cytometry. F, G The protein expression of cell cycle and apoptosis markers was detected by Western blot. (\(^*P<0.05, \^{**}P<0.01, \^{***}P<0.001\)).
Figure 7

HHT combined with NKD1 knockdown inhibits CRC tumor growth in vivo. A Representative photographs of tumors from SW620 cells in control group, NC group, siNKD1 group, HHT group and combined group are shown. The xenograft tumor weight B and tumor volume C were measured every three days in each group (n=7 per group). D The protein levels of cell cycle and apoptosis markers in each group were measured by immunoblotting. E The relative density of the protein band was measured by ImageJ.
software. F Tumor sections underwent IHC staining using antibodies against NKD1, Ki67, CDK4 and Bax in the xenograft tumor. Scale bar, 20 μm. (*P<0.05, **P<0.01, ***P<0.001).

Figure 8

PCM1 is a potential regulatory partner of NKD1. A Schematic diagram of study design. B Heatmap and hierarchical clustering analysis of differentially expressed (DE) proteins in the control group, HHT group,
siNKD1 group and combined group (two-tailed unpaired t test, fold change>1.2, p < 0.05). C Venn diagrams show the related proteins overlapping among groups. D The top 18 signaling pathways involved in DE proteins were annotated by the KEGG database and visualized by the cluster Profiler R software package. E Enriched Gene Ontology terms for the candidate target proteins. F The expression of predicted proteins (PCM1, DHFR and PCNA) was assessed by Western blotting in different treatment groups.

**Figure 9**
PCM1 overexpression rescued the NKD1 knockdown-inhibited proliferation in CRC cells. A Analysis of the intracellular localization of NKD1 (red) and PCM1 (green) by immunofluorescence staining. scale bar, 20 μm. B, C The Co-IP assay revealed an association between endogenous NKD1 and PCM1 in SW620 cells. D NKD1 depletion decreased the PCM1 protein level, and this effect was reversed by PCM1 overexpression. E In the presence of the proteasome inhibitor MG132, the effect of NKD1 on PCM1 did not result in a further increase in the PCM1 protein level. F In the presence of the protein synthesis inhibitor CHX, NKD1 depletion shortened the half-life of PCM1. G, H Cell proliferation ability was tested in SW620 cells of NKD1 knockdown group transfected with PCM1 plasmid. I, J Immunoblotting analysis of cell cycle and apoptosis markers in SW620 cells of NKD1 knockdown group transfected with PCM1 overexpression plasmid. (*P<0.05, **P<0.01, ***P<0.001).

**Figure 10**

Legend not included with this version.

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