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

Jia Cao (caojiamed@126.com)  
General Hospital of Ningxia Medical University  
https://orcid.org/0000-0002-3541-0689

Xiang Tao  
Ningxia Medical University

Bin Shi  
General Hospital of Ningxia Medical University

Jia Wang  
General Hospital of Ningxia Medical University

Rong Ma  
General Hospital of Ningxia Medical University

Jufen Zhao  
General Hospital of Ningxia Medical University

Jinhai Tian  
General Hospital of Ningxia Medical University

Qi Huang  
General Hospital of Ningxia Medical University

Jingjing Yu  
General Hospital of Ningxia Medical University

Libin Wang  
General Hospital of Ningxia Medical University

Research Article

Keywords: Colorectal cancer, NKD1, HHT, Cell cycle, Apoptosis

Posted Date: April 6th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2739531/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

**Background:** Colorectal cancer (CRC) is the most common primary malignancy. Homoharringtonine (HHT) has potential therapeutic effects on solid tumors. However, the regulatory target and mechanism of HHT in CRC progression remain elusive.

**Methods:** CCK-8, Edu staining, flow cytometry and Western blotting assay to detect the effect of HHT on proliferation and apoptosis of CRC cells. Transcriptome sequencing screened the drug delivery target of HHT. In vitro rescue experiment and in vivo tumorigenesis experiment detect the targeted interaction between HHT and NKD1. Quantitative proteomics combined with CO-IP and IF experiments to verify the targeted interaction between NKD1 and PCM1.

**Results:** HHT suppressed CRC cells proliferation by inducing cell cycle arrest and apoptosis in vitro and vivo. HHT inhibited NKD1 expression in a concentration and time dependent manner. NKD1 was overexpressed in CRC and its depletion enhanced the therapeutic sensitivity of HHT on CRC, which indicating that NKD1 plays an important role in the development of CRC as the drug delivery target of HHT. Furthermore, proteomic analysis revealed that PCM1 participated the process of NKD1-regulated cell proliferation and cell cycle. NKD1 interacted with PCM1 and promotes PCM1 degradation through the ubiquitin-proteasome pathway. The overexpression of PCM1 effectively reversed the inhibition of siNKD1 on cell cycle.

**Conclusions:** These results suggested that NKD1 targeting PCM1 to participate in regulating the therapeutic effects of HHT on CRC. Our findings provide evidence for clinical application of NKD1-targeted therapy in improving HHT sensitivity for CRC treatment

Introduction

Colorectal cancer (CRC) is a common malignancy, which is associated with a high risk of incidence and mortality worldwide [1]. Despite significant advancements in the diagnosis and treatment of CRC, the prognosis of patients with advanced stage CRC is still poor [2]. It has been reported that recurrence, metastasis and drug resistance are the leading causes of poor outcomes in CRC patients [3]. Due to the lack of understanding of the pathological mechanisms, it is difficult to predict the tumorigenesis of CRC. Therefore, the development of potential targeted drugs and therapeutic biomarkers is of great significance to improve the clinical outcomes of CRC patients.

Homoharringtonine (HHT) is a plant alkaloid, which is also known as a protein synthesis inhibitor and is used in the treatment of acute and chronic myeloid leukemia [4, 5]. Mechanistic studies have shown that HHT can prevent aminoacyl binding to the ribosomal subunit during the process of protein elongation, thereby disrupting the translation of oncogenes and ultimately leading to the apoptosis of leukemia cells [6, 7]. Recently, it was reported that HHT could exert anticancer effects on several solid malignancies. For example, Guo et al [8] reported that HHT could show anti-neoplastic activities in lung cancer by regulating TMEM16A activity. Liu et al [9] proved that HHT could induce PSMD11 protein...
synthesis by activating MEK1/ERK1/2 signaling pathway in pancreatic cancer. Zhu et al [10] proved that HHT could inhibit the proliferation and migration of hepatocellular carcinoma (HCC) cells by regulating the loss of EphB4-mediated β-catenin. Our previous study suggested that HHT could suppress the growth of breast cancer cells and promote apoptosis through the miR-18a-3p-AKT-mTOR signaling pathway [11]. Moreover, Shi et al [12] found that HHT could inhibit the growth of LoVo cells by inhibiting EphB4 and its downstream signaling. Qu et al [13] also demonstrated that HHT could inhibit cell proliferation and promote apoptosis in CRC by activating the PI3K/AKT/mTOR signaling pathway. Therefore, it is necessary to investigate the pharmacological targets of HHT as well as 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. It can affect the proliferation of cells by inhibiting the nuclear accumulation of β-catenin [14]. NKD1 is widely expressed in multiple tissues and plays a critical role in various tumors. The downregulation of NKD1 increased the invasive ability of non-small-cell lung cancer and correlates with a poor prognosis [15]. The NKD1 knockdown was associated with poor prognosis in invasive breast ductal carcinoma [16]. In HCC tissues, the low of NKD1 was associated with extrahepatic metastasis and histological differentiation [17]. Meanwhile, Wang et al [18] showed that NKD1 might be an important CRC biomarker and act as a curative target for the treatment of CRC. However, the specific function and mechanism of NKD1 in the progression of CRC remain poorly understood, and whether there is a link between HHT and NKD1 needs further investigation. This study aimed to elucidate the anti-CRC effects and molecular mechanisms of HHT.

This study aimed to elucidate the anti-CRC effects and molecular mechanisms of HHT. Our results demonstrated that HHT targeting NKD1 inhibits the proliferation of CRC cells by inducing cell cycle arrest and apoptosis. Silencing the NKD1 significantly improved the therapeutic sensitivity of HHT to CRC. Further mechanism revealed that NKD1 could interact with PCM1 and promote its degradation through the ubiquitin-proteasome pathway. The overexpression of PCM1 could effectively reverse the effects of NKD1 on promoting cell cycle arrest and apoptosis. These results suggested that HHT blocks CRC progression by inhibiting NKD1/PCM1 axis. Our study provided preliminary guidance for the treatment of CRC with HHT, and highlighting the inhibition of NKD1/PCM1 signals can improve the therapeutic effect of HHT, which will represent a promising therapeutic strategy for the diagnosis and treatment of CRC.

Materials And Methods

Cell culture and treatment

Human CRC cell lines, including HT29 and SW620, were purchased from the Shanghai Institute of Cell Biology at the Chinese Academy of Sciences. The cell lines were 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 at 37 °C containing 5% CO2 concentration. HHT was obtained from Minsheng
Pharmaceutical Co. Ltd (Zhejiang, China). The cells were treated with different concentrations of HHT (1.5 μM, 3 μM and 6 μM) to detect the cell cytotoxicity. The SW620 cells were transfected with the PCM1 plasmid using Lipofectamine 3000 transfection reagent (Invitrogen, USA), following the manufacturer’s instructions.

Cell viability and colony formation assays

For the CCK8 assay, the HT29 and SW620 cells (2×10^3 cells/well) were seeded into 96-well plates and incubated overnight at 37°C. The cells were then treated with HHT at the 6 μM for another 24 h, 48 h and 72 h. Then, a 10 μL CCK8 solution (KeyGEN BioTECH, China) was added to each well and further incubated for an additional 2 h at 37°C according to the manufacturer’s instruction. The cell viability was assessed by measuring the absorbance at 450 nm using a microplate reader. For the colony formation assay, the 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, followed by fixation with 100% methanol and staining with 0.5% crystal violet solution for 10 min. The number of colonies was counted using ImageJ software from the representative areas. All the experiments were performed in triplicates.

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

Cell proliferation ability and DNA synthesis were determined using the EdU staining assay. Briefly, the cells were treated with different concentrations of HHT for 48 h. Then the cells in the logarithmic growth phase were seeded on the coverslips (NEST, USA), and added with 10 μM EdU solution (Abcam, USA) to each well for incubation 2 h 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 washing the cells with cold PBS three times, each well was supplemented with 0.5% Triton X-100 (Solarbio, China) and blocked with 10% goat serum (Solarbio, China) for 1 h. For the visualization of nuclei, the cells were stained with DAPI reaction solution (Sigma, USA) for 20 min in dark and then fixed with a fluorescence quenching agent (Solarbio, China). The EdU-positive cells were finally photographed and counted using a fluorescence microscope (Olympus, Japan) under different fields.

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

Total RNA was extracted from tissues and cultured cells using TRIzol reagent (Invitrogen, USA) following the manufacturer’s instructions. RT-PCR assay was carried out as described in our previous study [19]. The following primer sequences were used for RT-PCR: NKD1: F: ACCATTGCGTAGATGAGAACAT, R: CCAAATTGGGACGTGTAGTTTT. GAPDH: F: TGTTGCCATCAATGACCCCTT, R: CTCCACGACGTACTCAGCG.

RNA interference vectors and cell transfection
The lentivirus vectors, which contained the small interfering RNAs (siRNAs), specifically targeting the NKD1 mRNA were designed and synthesized by Shanghai Genechem Co, Ltd (Shanghai, China). For lentiviral transduction, the SW620 cells were seeded into 24-well plates and allowed to grow to a cell confluence to 50%, which were then transfected with three siRNAs vectors or negative control vectors following the manufacturer's instructions. After culturing the cells in an incubator at 37°C for 8-12 h, the serum-free medium was replaced with a complete medium. The stably transfected siRNA-NKD1 cells were screened using 1 μg/mL puromycin reagent (Carlsbad, USA). Cell transfection efficiency was observed under a fluorescence microscope and examined using qRT-PCR and Western blot analyses. The NKD1 siRNA sequences are listed in Supplementary Table S1.

**Cell cycle and apoptosis analyses using flow cytometry**

For the cell cycle analysis, the HT29 and SW620 cells were seeded into a six-well plate and treated with 6 μM HHT for 48 h. The cells were then digested with trypsin without EDTA to prepare a single-cell suspension. After centrifugation, the supernatant was removed and the cells were resuspended in a cold PBS buffer. The cells were fixed by adding precooled 70% ethanol overnight at -20°C. The cells were then resuspended in 200 μL binding buffer and stained with PI staining solution for 30 min at room temperature. The apoptosis rate of the HT29 and SW620 cells was identified using AnnexinV-APC/PI double staining apoptosis detection kit (MULTI SCIENCE, China). The binding buffer containing 10 μL Annexin V-APC, was added to the cells and incubated for 15 min at room temperature in dark conditions, and then added 5 μL PI solution in dark for 10 min. The quantification of cell cycle distribution and cell apoptosis rate were examined using a flow cytometer system.

**Protein extraction and Western blot analysis**

In brief, the cells were collected, and the proteins were 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 and 4°C for 20 min, and the concentration of total extracted protein was measured using a BCA detection kit (Thermofisher Scientific, Inc). Equal amounts of proteins were electrophoresed on a 10% SDS-PAGE and then 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 at 4°C overnight. Subsequently, the membrane was incubated with the HRP-conjugated goat anti-rabbit or anti-mouse IgG respective secondary antibodies (ab205718; 1:5000, Abcam) at room temperature for 1 h. The protein bands were visualized using BiolImaging Systems (BIO-RAD, USA). Anti-GAPDH antibody was used as an internal control to the protein levels. Specific antibodies were listed in Supplementary Table S2.

**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 antibodies overnight at 4 °C. Protein A/G-agarose (Abcam) was washed with cold PBS, and then incubated with an antibody for 4 h. Then, the immunoprecipitated complex was washed three times with ice-cold PBS and the supernatant was removed. Then, 5X loading buffer was added to elute the proteins. The eluted proteins were separated using SDS-PAGE. The bound proteins were analyzed by Western blotting with an anti-PCM1 antibody (Santa cruz).

**Immunofluorescence (IF) staining**

The HT29 and SW620 cells were seeded on the coverslips in a 12-well plate (NEST, USA) for 48 h. After washing with cold PBS, the 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, the cells were incubated at 4°C with primary antibodies against C-PARP (Abcam, 1:200, USA) overnight, and followed by incubation with fluorophore-conjugated respective secondary antibody (1:200, Invitrogen, USA) for 2 h. The nuclei of cells were visualized by staining 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 the integrated fluorescence density was measured by the ImageJ software.

**Immunohistochemistry (IHC) staining**

The cells and tissue samples fixed in 10% formaldehyde were paraffinized and sliced into 5-μm-thick sections. The microarray sections were deparaffinized with xylene, rehydrated using graded alcohol solutions, and then treated with 0.3% hydrogen peroxide for 30 min. After repairing and blocking, the slides were incubated with primary antibodies, 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) at 4 °C overnight. The slides were then incubated with corresponding HRP-labeled secondary goat anti-rabbit antibodies at room temperature for 1 h. After the diaminobenzidine (DAB) reaction in dark, the slides were counterstained with hematoxylin. Typical images were captured under a microscope, and three equal-area non-repetitive fields were used for each slice to calculate the number.

**Tumor xenograft model**

BALB/C female nude mice (6-8 weeks old) were purchased from the Beijing Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). The siNKD1- transfected cells were subcutaneously injected into the mice to establish the CRC xenograft model. The mice in the two HHT groups were intraperitoneally injected with 0.5 mg/kg HHT for 15 days to observe its inhibitory effects on tumor growth. The length and width of tumor regions were measured using electronic calipers. Subsequently, the mice were euthanized and tumor tissues were isolated and frozen in liquid nitrogen or fixed in formalin to perform
IHC analysis. All the animal experiments were performed in accordance with the principles and guidelines approved by the Ethics Committee of Animal Research.

**Statistical analyses**

All the statistical analyses were performed using SPSS 18.0, GraphPad Prism version 8.0 and Illustrator 2017 software. The data were presented as the mean ± SD, and statistical differences among the groups were determined by a two-tailed Student t test or one-way analysis of variance (ANOVA). A $P$-value of <0.05 was considered statistically significant.

**Results**

**HHT potently inhibited the viability and proliferation of CRC cells**

The inhibitory effects of HHT on the viability of HT29 and SW620 cells were investigated by observing the cell morphology and CCK8 assay. The structures of both the cell lines became shattered and deformed with the increase in HHT concentration (0, 1.5 μM, 3 μM and 6 μM) (Fig. 1A), The HHT treatment significantly reduced cell viability in a concentration and time-dependent manner in comparison with the untreated cells (Fig. 1B). Meanwhile, the colony formation and Edu staining assays were performed to elucidate the cells proliferation abilities. As shown in Fig. 1C, the number of colonies decreased significantly with the increase in HHT concentration. Edu staining assay also showed that a significant decrease in the number of positively stained cells with the continuous increase in the HHT concentration (Fig. 1D, E). Taken together, these results suggested that HHT exhibited strong cytotoxic and cytostatic effects on CRC cells.

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

The effects of HHT on the distribution of cell cycle were analyzed by flow cytometry. As shown in Fig. 2A, the cell divisions of the cell lines were arrested at G0/G1 phase, the HHT treatment increased the proportion of cells in a dose dependent manner. On the contrary, the proportion of the cells in S phase or G2/M phase decreased as compared to those in the control group. This suggested that HHT could cause the arrest of CRC cells in the G0/G1 phase. Additionally, Western blot assay was used to detect the cell cycle-related proteins and corresponding kinases. The results showed that the expression levels of CyclinE, CyclinD1, CDK2, CDK4 and CDK6 proteins decreased with the increase in the HHT concentration (Fig. 2B, C). HHT also decreased expression levels of above proteins in a time-dependent manner (Fig. 2D, E). Collectively, these data indicated that HHT could inhibit the growth and proliferation of CRC cell by inducing cell cycle arrest at G0/G1 phase.
HHT induced apoptosis of CRC cells

To determine the apoptotic effects of HHT, the HT29 and SW620 cells were exposed to various concentrations of HHT for 48 h 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 the cell lines in a dose-dependent manner as compared to that in the untreated group. Besides, the immunofluorescence staining assay demonstrated that HHT induced the nuclear accumulation of cleaved-PARP (Fig. 3B, C). Then, Western blot analysis was used to further investigate the levels of apoptosis-related proteins in CRC cells. The results indicated that HHT treatment increased the protein levels of cleaved-PARP and Bax, and decreased those of the Caspase3, Caspase9 and Bcl-2 expression (Fig. 3D, E). Taken together, these results strongly proved that HHT could induce the expression of pro-apoptotic proteins to activate the cascade reaction, which ultimately facilitated the apoptosis of CRC cells.

NKD1 expression was correlated with response to HHT

In order to illustrate the underlying molecular mechanism of HHT’s anti-cancer effects, transcriptomic sequencing analysis was performed on the HHT-treated SW620 cells. The results showed a total of 3201 upregulated genes and 3495 downregulated genes in HHT treatment groups as compared to the control group (fold change>2 and p<0.05). Cluster and volcano map analyses revealed that HHT treatment downregulated the NKD1 expression (Fig. 4A, B). Gene Ontology (GO) analysis revealed that downregulated genes were significantly enriched in GO terms including with cell cycle, DNA replication and apoptotic process (Fig. 4C). Reactome pathway database showed significant enrichment of the downregulated genes in cell cycle, G1/S transition, and apoptosis signaling pathway (Figure S1), this was largely consistent with our detection of the function of HHT in vitro. In order to verify the results of bioinformatics analysis, we completed the RT-PCR and Western blot experiment, and found that HHT reduced NKD1 mRNA and protein expression both in a concentration and time-dependent manner (Fig.4D, E). In addition, in order to prove the correlation between HHT and NKD1, xenograft tumorigenic experiments suggested that the tumor formation was reduced in the HHT group as compared to that in the control group (Fig. 4F), and the tumor weight and volume were also significantly reduced synchronously (Fig. 4G, H). As shown in Fig. 4I, HHT treatment decreased the mRNA and protein expression of NKD1 in nude mice tissues. The apoptosis-related protein Bcl-2 expression decreased, while that of Bax increased in HHT treated group (Fig. 4J). These results suggested that NKD1 might act as a drug delivery target of HHT and plays an important role in inhibiting CRC cell proliferation by HHT.

Expression of NKD1 in the CRC tissues and cell lines

In order to investigate the specific role of NKD1 in HHT, the Pan-cancer expression level of NKD1 was first analyzed using the TCGA database. The results demonstrated that NKD1 was highly overexpressed in
COAD and READ (Fig. 5A). And NKD1 expression was significantly upregulated in 288 CRC tissues matching with 41 normal samples (Fig. 5B). However, there was no difference in the early stage (G1+G2) and late stage (G3+G4) patients (p=0.051) (Fig. 5C). RT-PCR and IHC analyses also confirmed that NKD1 was highly expressed in CRC (Fig. 5D, E). Furthermore, the NKD1 expression was also detected in CRC cell lines. As shown in Fig. 5F, G, NKD1 mRNA and protein were highly expressed in CRC cells as compared to normal cells (NCM460). Meanwhile, NKD1 siRNAs were designed and tested by RT-PCR and Western blot analyses. The results indicated the mRNA and protein levels of NKD1 siRNA1 decreased (Fig. 5H and 5I). The above results demonstrated that NKD1 acts as an oncogene to play an important role in regulating the development of CRC.

Sensitivity of CRC cells to HHT was associated with NKD1

SW620 cells were transfected with siNKD1 and exposed to the specified concentration of HHT to elucidated the correlations between NKD1 and HHT. 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 assays showed that the knockdown of NKD1 increased the inhibitory effects of HHT on the growth and proliferation of cells (Fig. 6B, C). Besides, as shown in Fig. 6D, E, the knockdown of NKD1 dramatically increased the sensitivity of SW620 cells to HHT, which was manifested in the increase of apoptosis rate and number of cells in the G0/G1 phase. For further validation, Western blot analysis was performed to detect the expression levels of cell cycle and apoptosis-related proteins. The results indicated that in the NKD1-knockdown cells, the HHT treatment significantly reduced the expression levels of CDK2, CDK4, CDK6, CyclinE and anti-apoptotic protein Bcl-2 expression and increased those of pro-apoptotic protein Bax and C-caspase9 (Fig. 6F, G). These results suggested that HHT induced the cell growth inhibition, cell cycle arrest and cell apoptosis largely attributed to the inhibitory effects of NKD1.

NKD1 deletion promoted CRC cells sensitivity to HHT in vivo

The xenograft nude mouse model was established to observe the inhibitory effects of HHT in combination with NKD1 depletion on tumor growth in vivo. The tumor cells were inoculated subcutaneously into the nude mice, and intraperitoneal injection 0.5 mg/kg HHT. Consistent with the in vitro results, the HHT treatment decreased the average tumor volume and weight as compared to those in the control group, and knockdown of NKD1 enhanced the suppressive effects of HHT against tumor growth (Fig. 7A-7C). Moreover, Western blot analysis demonstrated that the expression levels of NKD1, CDK2, CDK4, Cyclind1 and Bcl-2 decreased, while that of Bax increased in the NKD1 knockdown mice treated with HHT (Fig. 7D, E). IHC results also showed that the NKD1-knockdown mice treated with HHT decreased the protein levels of NKD1, Ki67 and CDK4 (Fig. 7F). These findings further highlighted the crucial roles of NKD1 deletion in impairing the CRC tumorigenesis and improving the HHT sensitivity.
**PCM1 as a potential regulatory partner of NKD1**

To reveal the potential mechanism of NKD1-mediated responses to HHT sensitivity, quantitative proteomics analysis was used (Fig. 8A). The number of differentially expressed (DE) upregulated and downregulated proteins in the three groups were identified (P value <0.05 and FC >1.2; Supplementary Fig. S2a). The downregulated proteins were analyzed and visualized using a heat map and Venn diagram, showing a total of 58 common intersected proteins (Fig. 8B, C). They were mainly associated with viral life cycle, viral transcription and DNA metabolic process by the ontology (GO) terms (Fig. S2b and S2c). The KEGG pathway enrichment analysis showed that the 58 common intersected proteins were mainly enriched cell cycle and apoptosis signaling pathways, these results were consistent with the previous experimental results (Fig. S2d and Fig. 8D). Next, we cluster four biological functions, including cell proliferation, viral process, cell cycle and apoptosis-related proteins, and found that PCM1, DHFR and PCNA were significantly enriched and decreased in the HHT combined with siNKD1 knockdown group (Fig. 8E). We confirmed with Western blot experiment that the expression of PCM1 was the lowest in both the isolated and combined groups (Fig. 8F). Taken together, these results demonstrated that PCM1 might be a target protein related to NKD1 and potential regulatory partner of NKD1.

**NKD1-mediated PCM1 regulated the inhibitory effects of HHT on CRC**

Further experiments were conducted to verify whether PCM1 was involved in the regulatory effects of NKD1 on the sensitivity of CRC cells to HHT. The intracellular localization of NKD1 and PCM1 was analyzed by immunofluorescent staining, and found that NKD1 and PCM1 were colocalized in the nucleus (Fig. 9A). Endogenous immune-precipitation showed that NKD1 interacted with PCM1 in SW620 cells (Fig. 9B, C). Meanwhile, depletion of NKD1 decreased the protein expression levels of NKD1 and PCM1, however, the overexpression of PCM1 could not affect the expression of NKD1 (Fig. 9D). Further examination showed that the NKD1 depletion decreased PCM1 protein levels in SW620 cells, and this effect was rescued by the treatment of the proteasome inhibitor MG132 (Fig. 9E). The results also indicated that NKD1 knockdown reduced the stability of PCM1 by acting through protein synthesis inhibitor CHX (Fig. 9F). Meanwhile, CCK8 and colony formation assays indicated that the overexpression of PCM1 could reverse the NKD1 knockdown-induced inhibition of cell growth (Fig. 9G, H). Western blot results also indicated that the knockdown of NKD1 could cause cell cycle arrest and reduce the expression levels of anti-apoptotic proteins. Conversely, these results were partially rescued by the overexpression of PCM1 (Fig. 9I, J). These data indicated that NKD1 targeting PCM1 regulates the therapeutic effects of HHT on CRC.

**Discussion**

CRC is a common malignancy with high recurrence and drug resistance. Due to the lack of effective treatment strategies, this disease has become a heavy burden on human health. Seeking effective
therapeutic drugs and action targets is the main direction and prerequisite for the treatment of CRC [20, 21]. Previous studies have reported HHT as a highly effective chemotherapeutic drug for the treatment of acute myeloid leukemia [22, 23]. Recent studies illuminated that HHT might also have therapeutic potential against solid tumors [24, 25]. The current study evaluated the effects of HHT and its potential underlying mechanism in CRC. Based on the experiments, this study demonstrated that HHT treatment could inhibit the proliferation of CRC cells in vitro as well as tumor growth in vivo. Further analysis suggested that the inhibitory effects might be attributed to HHT-induced cell cycle arrest and apoptosis. The results implied that HHT might be a potential therapeutic agent for the treatment of CRC.

Cell cycle arrest and apoptosis are the types of cell death with a series of regulated signaling cascades, which are essential for the initiation and progression of cancer [26, 27]. Understanding the mechanisms of how HHT induced CRC apoptosis is important for its possible applications in cancer treatment. Transcriptome sequencing was performed to analyze HHT targets and enrichment signaling pathways. The results showed that the concentration and time dependent expression of NKD1 was significantly decreased in HHT treatment group. Cell cycle and apoptosis signaling pathways were enriched after HHT treatment. NKD1 is a negative regulator of the canonical Wnt signaling pathway [28] and plays a key role in numerous tumors [29, 30]. However, the studies on the correlations between NKD1 and CRC progression are limited and require further investigation. The present study systematically analyzed the relationship between HHT, NKD1 and CRC. Based on the experimental results, it was suggested that NKD1 was highly expressed in CRC tissues and cell lines, the inhibition of NKD1 enhanced the sensitivity of cells to HHT exposure and promoted the HHT-induced cell cycle arrest and apoptosis of CRC cells. Similarly, the knockdown of NKD1 robustly suppressed tumor growth in xenograft tumor mouse models. These findings confirmed that NKD1 promoted CRC progression and served as a novel HHT drug delivery target for CRC patients.

In order to further explore the molecular mechanism by which HHT targeting NKD1 regulates the therapeutic efficiency of CRC, subcellular proteomic and bioinformatic analysis were performed to identify the candidate proteins which participated in the pathways affected by HHT treatment in combination with NKD1 knockdown. The result showed that PCM1 was related to HHT treatment and NKD1 expression. It is well known that PCM1 has a dynamic role in cell cycle distribution and is responsible for protein recruitment and cell division [31]. PCM1 also has been recognized as a key regulator of multiple malignancies. For example, PCM1 was shown to be involved in chromosomal mutations and was associated with glioblastoma and multiple hematological malignancies [32, 33]. A complex formed by the combination of PCM1 and canonical autophagy protein GABAPAP was involved in regulating apoptosis and autophagy [34]. Based on these observations, this study proposed that the targeting of NKD1 by HHT was mediated by the modulation of PCM1, and the NKD1-PCM1 axis might be a driver event in CRC. Mechanistic studies revealed a novel function of NKD1 based on its interaction with PCM1 in the nucleus, which led to the promotion of PCM1 degradation through the ubiquitin-proteasome pathway. Furthermore, the results indicated that the overexpression of PCM1 could effectively reverse the NKD1 knockdown-induced cell cycle arrest and apoptosis in CRC cells, thereby establishing a feedback loop between NKD1 and PCM1 to regulate the sensitivity of CRC to HHT. These findings provided an
insight into potential role of NKD1 as a key factor in the regulation of the occurrence and development of CRC, and supported the possibility of HHT-targeting NKD1 for CRC treatment.

**Conclusions**

In summary, this study proved that HHT could inhibit CRC progression via an NKD1/PCM1 dependent mechanism. This study provided preliminary guidance on developing a potential drug using HHT as the lead compound for CRC treatment, and highlighting the inhibition of NKD1/PCM1 oncogenic signaling to improve HHT sensitivity, which represented a promising therapeutic strategy for the diagnosis and prognosis of CRC.

**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

All animal experiments have complied with the ARRIVE guidelines and have been carried out in accordance with the U.K. Animals Act. Animal study was conducted after approval from the Ethics Committee of Ningxia Medical University.

Conflict of interest

The authors have declared that no competing interest exists.

Funding

This study was funded by First-Class Discipline ConstructionFounded Project of Ningxia Medical University and the School of Clinical Medicine (No. NXYLXK3020071). Ningxia Natural Science Foundation (No. 2021AAC03350). Special Talent IntroductionProject of Ningxia Autonomous Region Key R&D Programs (No. 2021BEB04046). The sixth group of Ningxia Autonomous Region Young Scientific and Technological Talent Lifting Project (No. NXXJTJ2021119). Ningxia high level science and technology innovation leading talent project (Grant No. KJT2019003). The Scientific Research Platform Open Project of the General Hospital of Ningxia Medical University (Grant No.2020-146).

Author contributions

Jia Cao contributed to the experimental work, paper writing and editing, performed the data evaluation and material management. Xiang Tao and Rong Ma 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. (Magnification: 100X, Scale bar: 100μm) 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. (Magnification: 200X, Scale bar: 50μm)) (*P<0.05, **P<0.01, ***P<0.001).

**Figure 2**

HHT accelerated cell cycle arrest of CRC cells at the G0/G1 phase. Different concentrations of HHT acted on HT29 and SW620 cells. A Cell cycle distribution was detected by flow cytometry analysis. B, C Protein expression levels of cell cycle markers were detected in HT29 and SW620 cells using Western blotting.
after HHT treatment at different concentrations. D, E Protein expression levels of cell cycle markers were detected by Western blotting after HHT treatments at different times. (*P<0.05, **P<0.01).

**Figure 3**

HHT induced apoptosis of CRC cells. A Total apoptotic rate of HT29 and SW620 cells was detected by flow cytometry analysis. B, C Apoptotic ratio of cells were detected by immunofluorescence staining.
against C-PARP. (Magnification: 400X, Scale bar: 20μm) D, E Protein expression levels of apoptotic markers were detected in HT29 and SW620 cells by Western blotting. (*P<0.05, **P<0.01).

Figure 4

NKD1 was a delivery target of HHT. 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. C GO analysis showing the biological functions of top 30 differentially downregulated genes. D, E Relative mRNA and protein expression levels of NKD1 were verified using RT-PCR and Western blot. F Representative photographs obtained from nude mice in control group and HHT groups are shown. Tumor weight (G) and tumor volume (H) were measured every 2-3 days in each group. I NKD1 expression after HHT treatment was detected using RT-PCR. J Protein levels of NKD1, Bax and Bcl-2 in each group were measured using 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 was verified by RT-PCR. E NKD1 localized expression was determined by IHC staining. (Upper picture magnification: 100X, Scale bar: 100μm; lower picture magnification: 400X, Scale bar: 20μm) 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 promoted cell cycle arrest and apoptosis. A Western blot analysis was performed to detect the protein expression levels of NKD1. B Cell growth curve of each group was evaluated by CCK8 assay. C Colony formation assay of SW620 cells with the NKD1 depletion or HHT treatment. D Cell cycle analysis was performed to assess the effects of NKD1 silencing or HHT treatment on SW620 cells. E Effect of HHT in combination with siNKD1 on apoptosis were analyzed by flow cytometry. F, G Protein expression levels of cell cycle and apoptosis markers were detected using Western blot. (*P<0.05, **P<0.01, ***P<0.001).
Figure 7

HHT combined with NKD1 knockdown inhibited CRC tumor growth in vivo. A Representative photographs of tumor were shown in each group. Tumor weight (B) and tumor volume (C) were measured in each group of xenograft mouse models. D Protein levels of cell cycle and apoptosis markers in each group were measured by immunoblotting. E Relative density of the protein band was measured using ImageJ.
software. Tumor sections underwent IHC staining using antibodies against NKD1, Ki67, CDK4 and Bax in the xenograft tumor. (Magnification: 400X, Scale bar: 20μm). (*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 proteins in each 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. E Enriched Gene Ontology terms for the candidate target proteins. F The expression of predicted proteins was assessed by Western blotting in different treatment groups.

Figure 9

PCM1 was involved in the inhibitory effect of siNKD1 on CRC A Analysis of the intracellular localization of NKD1 and PCM1 using IF staining. (Magnification: 400X, Scale bar: 20μm). B, C The Co-IP assay
revealed an association between endogenous NKD1 and PCM1 in SW620 cells. NKD1 depletion decreased the PCM1 protein level, and this effect was reversed by the PCM1 overexpression. Inhibition of siNKD1 on PCM1 by MG132 (E) and CHX (F). CCK8 (G) and clonal formation (H) assay were performed to detect the effect of siNKD1 combined with PCM1 overexpression on cell proliferation. I, J Immunoblotting analysis of cell cycle and apoptosis markers in SW620 cells. (*P<0.05, **P<0.01, ***P<0.001).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterials.docx