THZ1 targeting CDK7 suppresses c-KIT transcriptional activity in gastrointestinal stromal tumours

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Abstract

Gastrointestinal stromal tumour (GIST) is the most common mesenchymal tumour of the gastrointestinal tract and is characterized by activating mutations of the *KIT* or *PDGFRa* receptor tyrosine kinases (RTKs). Despite the clinical success of tyrosine kinase inhibitors (TKIs), more than half of GIST patients develop resistance due to a second mutation. Cyclin-dependent kinase 7 (CDK7) is the catalytic subunit of CDK-activating kinase (CAK), and it plays an important role in the regulation of cell cycle transitions and gene transcription. THZ1, a CDK7 inhibitor, exhibits a dose-dependent inhibitory effect in various cancers. Here, we demonstrated that CDK7 was relatively overexpressed in high-risk GIST and predicted a poor outcome. A low concentration of THZ1 exhibited a pronounced antineoplastic effect in GIST cells in vivo and in vitro. Moreover, THZ1 exerted synergistic anticancer effects with imatinib. THZ1 treatment resulted in transcriptional modulation by inhibiting the phosphorylation of Ser2, Ser5, and Ser7 within RNA polymerase II (RNAPII). c-KIT, an oncogene driver of GIST, was transcriptionally repressed by THZ1 treatment or CDK7 knockdown. Transcriptome sequencing analysis showed that OSR1 acted as a downstream target of CDK7 and was a novel oncogene driven by c-KIT. Taken together, our results highlight elevated CDK7 expression as a predictor of poor outcome in GIST and the combination of CDK7 and RTK inhibitors as a potent therapeutic strategy to improve the efficacy of GIST treatment.

Introduction

Gastrointestinal stromal tumours (GISTs) originate from interstitial cells of Cajal (ICCs) and are the most common malignant mesenchymal neoplasms of the gastrointestinal tract[1, 2]. The majority of GISTs harbour activating mutations in *KIT* (75–80%) or platelet-derived growth factor receptor alpha (*PDGFRA*) (10–15%)[3–5]. The constitutive activation of these receptor tyrosine kinases (RTKs) in turn drives oncogenesis by activating downstream signalling pathways[6]. By inhibiting RTK signalling, tyrosine kinase inhibitors (TKIs) have been widely used as adjuvant therapy for GIST and they significantly prolong survival[7–9]. However, despite initial responses, resistance to TKIs limits their long-term benefit due to acquired secondary mutations[10]. Nearly half of patients with advanced GIST develop tumour progression within the first two years of imatinib treatment, and the estimated 10-year progression-free survival and overall survival rates are 7%-9% and 19.4%-23%, respectively[11, 12].

Several core transcription factors have been revealed to play essential roles in driving GIST cell proliferation and metastases by binding to enhancers of GIST-associated genes and facilitating *KIT* gene expression[13–15]. *FOXF1* is highly expressed in GIST and colocalizes with *ETV1* at enhancers to directly control the transcription of two major oncogenes, *ETV1* and *KIT*[13]. Therefore, characterization of transcription factor deregulation in GIST may provide innovative insights into its pathogenesis mechanisms and offer new therapeutic approaches.

Cyclin-dependent kinases (CDKs) catalyse the phosphorylation of cyclins and control cell cycle transitions. CDK7 is the catalytic subunit of CDK-activating kinase (CAK), which can stimulate cell cycle progression and activate multiple other CDKs through T-loop phosphorylation, such as CDK1, CDK2,

CDK4, and CDK6[16–18]. In addition, CDK7 is a component of the transcription factor TFIIH, which phosphorylates the C-terminal domain (CTD) of RNA polymerase II (RNAP II) and therefore activates transcription initiation and elongation[19, 20]. CDK7 was found to be overexpressed and promote tumorigenesis in various cancers, such as breast cancer and osteosarcoma[21, 22]. THZ1, a selective CDK7 inhibitor, covalently binds to CDK7 and suppresses its kinase activity based on modification of a unique cysteine residue[23]. THZ1 potently represses the transcription of several the super-enhancer-associated oncogenes and elicits a tumour inhibition effect in various cancers[21, 24–28].

However, the role of CDK7 in the progression of GIST has not yet been elucidated. In this study, we found that CDK7 expression was elevated in high-risk GISTs and related to a poor prognosis. CDK7 knockdown or THZ1 treatment inhibits GIST cell proliferation and leads to inhibition of transcriptional activity and protein expression of *c-KIT.* Whole-transcriptome sequencing analysis was performed to decipher the mechanisms of CDK7 inhibition in GIST. THZ1 treatment led to inhibition of RNAPII phosphorylation, an indication of transcriptional inhibition, suggesting that targeting CDK7 may provide a mechanism to block transcriptional activation of *c-KIT* in GISTs. Taken together, our findings revealed that targeting CDK7 may be a potent therapeutic strategy for GIST patients.

Methods

Cell line and reagents

The GIST-T1 cell line was purchased from Cosmo Bio Co. Ltd. (Tokyo, Japan). The GIST-882 cell line was kindly provided by Dr. Fletcher from Harvard Medical School. GIST-T1 and GIST-882 cells were cultured at 37°C with 5% CO_2 in RPMI-1640 medium (Gibco, USA) supplemented with 10% foetal bovine serum and 1% penicillin–streptomycin (Gibco, USA). Antibodies against CDK7 (#2916), Rpb1 CTD (#2629), phospho-Rpb1 CTD (Ser2) (#13499), phospho-Rpb1 CTD (Ser5) (#13523), phospho-Rpb1 CTD (Ser7) (#13780), cyclin D1 (#55506), CDK4 (#12790), γ(p-)H2AX (#9718), cleaved PARP (#5625), c-kit (#3074), phospho-c-kit (#3073), ERK1/2 (#4695), phospho-ERK1/2 (#4370), AKT (#9272), phospho-AKT (#4060) and Alexa Fluor conjugated anti-rabbit IgG (H + L) (#8889) were purchased from Cell Signaling Technology (CST, MA, USA). Antibody against Ki67 (ab16667) purchased from Abcam (Cambridge, MA, USA). Antibody against OSR1 (sc-376545) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against GAPDH (60004–1-Ig) and Caspase-3 (19677-1-AP) and HRP-conjugated secondary antibodies (SA00001-1 and SA00001-2) were purchased from Proteintech (Wuhan, China). Imatinib was kindly provided by Novartis Pharmaceuticals (Basel, Switzerland). THZ1 cells (HY-80013A) were purchased from MedChemExpress (MCE, Monmouth Junction, NJ, USA). Imatinib was dissolved in phosphate buffer solution (PBS), and THZ1 was dissolved in dimethyl sulfoxide (DMSO) for the in vitro cell culture studies.

Tissue microarray and immunohistochemistry

For immunohistochemistry (IHC), we employed a constructed tissue microarray (TMA) containing 223 paraffin-embedded primary GIST surgical samples resected at Zhongshan Hospital between 2009 and 2012 with Institutional Review Board approval. IHC staining was performed with anti-CDK7 antibody

(#2916, CST). Two researchers separately evaluated the staining intensity and divided the samples into low expression and high expression groups.

Cell transfection

Small interfering (si)RNAs targeting CDK7 and OSR1 and control siRNA were synthesized by Obio Technology (Shanghai, China). Plasmids and vector plasmids as controls were synthesized by Shanghai GeneChem Co., Ltd. siRNA and plasmid transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After transfection for 48 h, the knockdown efficiency was tested by western blotting.

Cell viability assay

Cell proliferation was examined using Cell Counting Kit-8 (Yeasen, China) according to the instructions. GIST-T1 or GIST-882 cells were plated in 96-well plates at 1.5×10^3 cells in 100 μl of medium per well. After incubation overnight, the medium was replaced with 100 μl medium with 10 μl CCK-8 and incubated for 2 h. The absorbance value was detected with a microplate reader at 450 nm. Similarly, the OD values were assessed at the indicated time points: 24, 48, and 72 h.

For the drug inhibition assay, 5×10^3 GIST-T1 or GIST-882 cells/well were seeded and incubated at 37°C overnight. Cells were treated with THZ1 and imatinib at various doses, and the mean inhibitory concentration (IC50) was calculated using nonlinear regression analysis in GraphPad Prism 8.0. The synergistic effect of the combination treatment was measured using CompuSyn software (ComboSyn, Inc. Paramus, NJ, USA)[29]. The combination index (CI) was generated by CompuSyn software, and $CI < 1$, $= 1$, and > 1 indicate synergic, additive or antagonistic effects, respectively.

Cell cycle and apoptosis analysis

Approximately 1×10^6 cells were collected and fixed with 75% ethanol at 4°C overnight. After centrifugation and resuspension in PBS, the cells were incubated with 500 μL propidium iodide (PI)/RNase staining solution (Absin, China) at 37°C for 30 minutes. Cell apoptosis was detected with an Annexin V-fluorescein isothiocyanate (FITC) apoptosis assay kit (Absin, China). Briefly, cells in 6-well plates were harvested by EDTA-free trypsinization, washed twice with cold PBS buffer and then resuspended in binding buffer with 5 μl of Annexin V and 5 μl of PI for 15 min at RT in the dark. The distribution of the cell cycle and apoptosis was determined in a BD Accuri C6 plus flow cytometer (BD Biosciences). The results were analysed by ModFit 3.0 software (Verity software house, Topsham, ME, USA) and FlowJo software (FlowJo, LLC, Ashland, OR, USA).

Colony formation assay

The cells were seeded in 6-well plates at a density of 500 cells/well and cultured for 2 weeks. The medium was replaced every five days. To examine the colony formation, the cells were fixed with 4% paraformaldehyde for 20 min. Next, crystal violet (0.5%) was used to stain the cells for 20 min. The colonies were photographed and then counted using ImageJ software.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using TRIzol Reagent (Yeasen Biotechnology Shanghai, China) and reverse transcribed into cDNA using cDNA Synthesis SuperMix (Yeasen) according to the manufacturer's instructions. Real-time PCR was performed using SYBR Green Master Mix (Yeasen) on a StepOne Real-Time PCR System (Applied Biosystems). The qRT-PCRs were run in triplicate. GAPDH was used as an endogenous control.

Western blot

Total protein was extracted from the cells with radioimmunoprecipitation assay (RIPA) buffer (Beyotime Biotechnology Shanghai, China) supplemented with protease inhibitors (Beyotime) and phosphatase inhibitors (Beyotime). The membranes were then blocked with 5% skim milk for 90 minutes at room temperature and incubated with the primary antibodies overnight at 4°C. The blots were then washed 3 times for 10 minutes with TBST (TBS with 0.1% Tween 20) and incubated with HRP-conjugated goat anti-rabbit or mouse IgG secondary antibodies (1:5000) for 1 hour at room temperature. The bands were visualized by incubating with enhanced chemiluminescence (ECL) solution and imaged by a Bio-Rad Imaging system detector.

Immunofluorescence staining

Cells were treated with 100 nml/L THZ1 for 24 hours. Then, the cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.2% Triton X-100 (Beyotime) for 15 min, blocked with 3% BSA for 60 min, and incubated with anti-c-KIT antibody (1:400) overnight at 4°C. The cells were incubated with Alexa Fluor conjugate-anti-rabbit IgG (H + L) secondary antibody (1:1000) for 60 min in the dark and then with DAPI for 5 min.

Mouse xenograft tumor assay

The mice experiments were approved by the Institutional Review Boards of Zhongshan Hospital. Female BALB/c nude mice (4-weeks old) were obtained from Shanghai Jiesijie Laboratory Animal Co., Ltd. GIST-T1 cells were mixed with Matrigel (BD Biosciences) at a ratio of 1:1 and were subcutaneously injected into BALB/c nude mice at 3×10^6 cells per mouse. When the tumors of all mice grow to be visible, mice were divided into two groups randomly (n = 5). Mice were treated with THZ1 (10 mg/kg) or PBS intratumoral injection for every three day. Tumor volume was measured every three days by using the formula: V = 1/2* length (mm) * width (mm)2. The mice were sacrificed when tumor volume in control group reached approximately 500 mm^3. The tumors were harvested, weight and used for IHC.

Library preparation and sequencing

The RNA sequencing service was supplied by Applied Protein Technology (Shanghai, China). In brief, GIST-T1 and GIST-882 cells were treated with DMSO or THZ1 (100 nmol/L) for 6 h. Then, RNA was extracted in triplicate by using TRIzol Reagent (Yeasen). Paired-end libraries were prepared using an ABclonal mRNA-seq Lib Prep Kit (ABclonal, China) following the manufacturer's instructions. Sequencing

was performed with an Illumina NovaSeq 6000/MGISEQ-T7 instrument. The ClusterProfiler R software package was used for Gene Ontology (GO) analysis[30]. When $p < 0.05$, it was considered that the GO function was significantly enriched.

Statistical analysis

All statistical analyses were performed with Prism 8.0 (GraphPad Software). Continuous variables are presented as the mean ± standard deviation (SD). A two-tailed Student's t-test was used to analyse significant differences between the two groups, and one-way ANOVA was used for multiple groups. Statically significant differences were considered when $P < 0.05$.

Results

CDK7 mRNA and protein were elevated in high-risk GISTs and associated with poor clinical outcomes

To analyse the differential CDK expression in GISTs, we obtained publicly available transcriptomic data of GSE136755 from the Gene Expression Omnibus (GEO), which includes gene microarray data and clinicopathological information for 59 primary GIST tumour samples without preoperative imatinib treatment[31]. CDK1-CDK10 had significantly higher mRNA levels of CDK4, CDK7 and CDK9 than the other CDKs (Fig. 1A). Next, we measured the mRNA levels of CDK4, CDK7 and CDK9 in different GIST risk groups. CDK7 was significantly elevated in the high-risk group compared with the very low-, low- and intermediate-risk groups ($P < 0.05$ Fig. 1B), while the mRNA levels of CDK4 and CDK9 did not increase with the risk category (supplemental material).

To further validate our findings at the protein level in GIST samples, we examined CDK7 protein expression in tissue microarrays (TMAs) of 223 GIST samples by immunohistochemistry (IHC). Two researchers separately evaluated the staining level and divided the patients into CDK7 high and low expression groups (Fig. 1C). We focused on the relationships between the expression level of CDK7 and the clinicopathological characteristics, including sex, age, tumour size, mitotic index and risk category (Table 1). A significant correlation was found between elevated CDK7 expression, a higher mitotic index and a higher risk of GIST ($P < 0.05$), while no significant relationship was found between CDK7 expression and sex, age or tumour size. Kaplan–Meier survival analysis demonstrated that high CDK7 expression was strongly correlated with a reduced RFS ($P = 0.032$, HR = 2.024, CI = 1.046–3.917, Fig. 1D).

Table 1
Relationship between CDK7 expression and clinicopathological characteristics of GIST patients

Factor	CDK7 expression		*P* value
	Low (134)	High (89)	
Age			
< 60	68	54	0.145
≥ 60	66	35	
Sex			
Male	61	43	0.682
Female	73	46	
Tumour site			
Stomach	86	53	0.470
Intestine, colorectum	43	34	
Others	5	2	
Tumour size (cm)			
0–5.0	74	44	0.676
5.1–10.0	44	32	
> 10	15	13	
Mitotic index (per 50 HPFs)			
0–5	90	43	0.005
> 5	42	44	
Modified NIH criteria			
Very low/low	56	24	0.014
Intermediate	23	11	
High	55	55	

In summary, CDK7 was highly expressed in high-risk GISTs and it may participate in GIST progression.

CDK7 knockdown attenuated GIST cell growth and induced cell cycle arrest

To investigate the role of CDK7 in GIST cells, two independent siRNAs were transfected into GIST-T1 and GIST-882 cells. The transfection efficacy was detected by western blotting (Fig. 2A). As shown in Fig. 2B and Fig. 2C, CDK7 knockdown significantly suppressed cell proliferation and colony formation in both GIST T1 and GIST-882 cells. We next assessed the effect of CDK7 knockdown on GIST cell cycle progression. We found that CDK7 knockdown resulted in marked cell cycle arrest at the G1/S stage (Fig. 2D). These results indicate that CDK7 may play an oncogenic role in GIST cells. We also found that the cell cycle-related protein cyclin D1, a regulator of the G0–G1 to S-phase transition, was significantly suppressed. Additionally, γH2AX was significantly upregulated, suggesting increased DNA damage after CDK7 knockdown (Fig. 2E). Taken together, these data demonstrated that CDK7 might be a promising treatment target for GISTs.

THZ1 exerted dose-dependent tumour inhibition and induced apoptosis in GIST cells

The CDK7 inhibitor THZ1 exerts antitumorigenic effects in various cancers. To evaluate the antitumour effects of THZ1 in GIST, GIST-T1 and GIST-882 cells were treated with THZ1 for 72 h. As shown in Fig. 3A and Fig. 3B, THZ1 significantly suppressed the viability of GIST cells in a dose-dependent manner. The IC50 values were 41 nmol/L and 79 nmol/L for GIST-T1 and GIST-882 cells, respectively. At concentrations as low as 25, 50, or 100 nmol/L, THZ1 potently reduced cell viability of GIST-T1 and GIST-882 in a time-dependent manner (Fig. 3C).

The extent of apoptosis was assessed after THZ1 treatment with flow cytometry analysis. Annexin V/PI staining illustrated that the percentage of apoptotic cells significantly increased in a THZ1 dose-dependent manner (Fig. 3D,3E). The apoptotic related proteins were also detected. As shown in Fig. 3F, the expression of cleaved PARP, cleaved caspase-3 and γH2AX was markedly increased in GIST-T1 and GIST-882 cells after THZ1 treatment.

Collectively, these results revealed that THZ1 could inhibit GIST cell proliferation and induce apoptosis.

THZ1 shows anti-neoplastic properties in vivo

The tumour-inhibition effect of THZ1 in vivo was assessed in subcutaneous xenograft model of GIST- T1. As expected, the results showed that THZ1 treatment led to profound reduction of tumour volume and weight, corroborating the tumour inhibition effect in vitro (Fig. 4A,4B). No significant loss of body weight was observed (Fig. 4C). IHC assay of tumour samples showed that THZ1 treatment dramatically inhibited cell proliferation and promoted cell apoptosis (Fig. 4D). Collectively, these results revealed that THZ1 possesses potent antitumor effect of GIST cells in vivo.

THZ1 exhibited synergistic inhibition with imatinib in GIST cells

Furthermore, we assessed the potential tumour inhibition efficacy of THZ1 in combination with imatinib in GIST-T1 and GIST-882 cells. Cells were treated with a series of different drug concentrations. The

synergistic antitumour effect was analysed by using CompuSyn software, and combination index (CI) values were calculated based on the drug combination principles proposed by Chou-Talalay[29]. Our data revealed that single treatment with THZ1 or imatinib significantly suppressed GIST-T1 and GIST-882 cell viability (Fig. 5A). THZ1 had strong synergistic effects with imatinib in both GIST-T1 and GIST-882 cells, and there was an advantage of combination treatment (Fig. 5A-5C). Immunoblotting analyses showed that combination treatment of THZ1 and imatinib lead to more upregulation of cleaved caspase 3 and PARP as compared with single agent treatment (Fig. 5D). In summary, combination treatment with THZ1 and imatinib significantly improved the viability-suppressing capability of GISTs.

THZ1 treatment causes selective transcription suppression in GIST

We next probed the mechanisms underlying the antitumour effect of THZ1 in GISTs. Considering the prominent role of CDK7 in regulating the cell cycle and RNAPII-mediated transcription, we next assessed THZ1-induced transcription alterations in the gene expression profiles of GIST cells by whole-transcriptome sequencing (RNA-sequencing, RNA-seq) analyses. Treatment with 50 nmol/L THZ1 for 6 hours resulted in a dramatic decrease in global messenger RNA levels (Fig. 6A). We performed gene ontology (GO) analysis to explore the functional enrichment of most differentially expressed genes. We found that THZ1-sensitive genes were associated with pathways involved in the regulation of transcription regulation by RNAPII (Fig. 6B). Because CDK7 can preferentially downregulate RNAPII CTD phosphorylation, we detected the related proteins by western blot. THZ1 treatment reduced the phosphorylation of Ser2, Ser5 and Ser7 on Pol II CTD in GIST-T1 and GIST-882 cell lines in a dose-dependent manner (Fig. 6C). Although our results showed that CDK7 knockdown led to cell cycle arrest in GIST cells, CDK7 preferentially regulated transcription instead of directly regulating the cell cycle in GIST.

CDK7 knockdown and THZ1 treatment inhibited *c-kit* transcription

Previous research revealed that THZ1 treatment induces EGFR and PDGFRa expression and multiple downstream oncogenic signalling pathways in glioma[32]. CDK7 inhibition suppresses GIST cell proliferation and survival via inhibition of transcription. Therefore, we analysed the c-kit expression changes after CDK7 knockdown or THZ1 treatment. It was of great interest to observe that *c-kit* mRNA and protein levels were significantly inhibited after CDK7 knockdown (Fig. 7A,7B) and THZ1 treatment (Fig. 7C-7E). The phosphorylated forms of AKT and ERK were all prominently downregulated by CDK7 or THZ1 knockdown (Fig. 7B,7E). Similarly, compared with monotherapies of imatinib or THZ1, the combination more effectively inactivated the *c-kit* and downstream AKT and ERK signalling cascades (Fig. 7F).

CDK7 inhibited *c-kit* transcription via OSR1 in GIST cells

Previous studies have indicated that THZ1 impairs the transcriptional activity of the super-enhancer (SE) of oncogenic genes, and to explore the mechanism underlying c-kit transcription inhibition induced by CDK7 knockdown, we investigated SEs. Previous research has identified a cluster of SEs of GISTs, including MEIS1, OSR1, and FOXF1. Interestingly, we found that odd-skipped related transciption factor 1 (OSR1) was the most significantly downregulated gene in GIST-T1 cells after THZ1 treatment (Fig. 8A, Table S1). Immunoblotting analyses verified that OSR1 expression was significantly inhibited by THZ1 treatment with CDK7 knockdown (Fig. 8B,8C). Next, we searched the gene expression profile in the Oncomine database and MediSapiens IST Online transcriptome database. As showed in Fig. 8D,8E, OSR1 was significantly upregulated in GIST compared with normal gastric tissue and was highly expressed in GIST and prostate cancer compared with other human cancer. To prove the result, we detected the OSR1 expression in clinical samples of GIST, leiomyoma and schwannoma. As expected, OSR1 was significantly high expressed in GIST (Fig. 8F). Furthermore, we performed correlation analysis of OSR1 and c-kit expression, and the results showed that OSR1 levels were positively associated with c-kit levels ($r = 0.407$, $P < 0.001$, Fig. 8G). The qRT-PCR and immunoblotting analyses showed that OSR1 knockdown significantly inhibited c-kit transcript and protein expression in GIST-T1 and GIST-882 cells (Fig. 8H,8I). In contrast, overexpressed OSR1 increased c-KIT expression (Fig. 8J). When CDK7 siRNA and OSR1 plasmid were co-transfected in GIST-T1 and GIST-882 cells, the inhibition of c-KIT expression was reversed (Fig. 8K). Collectively, these results revealed that CDK7 mediate c-KIT expression through OSR1.

Discussion

Despite advances in genetic alteration diagnoses and tyrosine kinase inhibitor treatment, the prognosis of patients with GIST remains unsatisfactory, especially for high-risk GISTs. The effectiveness of standard targeted therapy is hampered by secondary resistance following initial responses due to acquired secondary mutations[10].

CDKs play a significant role in regulating the cell cycle and gene transcription. CDK7 is one of the subunits of the multiprotein transcription factor complex TFIIH and it is critical for facilitating transcription initiation and elongation via phosphorylation of the CTD of RNAPII[19, 20]. CDK7 has been reported to be a potential therapeutic target in transcription-dependent cancers and is associated with a poor prognosis[21, 24–28]. However, the role of CDK7 in GIST tumorigenesis remains unknown.

In our study, we conducted an analysis of the transcriptomic data of 65 GIST patients from the GEO public database. The CDK4, CDK7 and CDK9 mRNA levels were significantly higher than those of other CDKs, and further, we found that the CDK7 mRNA level was significantly elevated in high-risk GISTs, while there was no significant difference in CDK4 and CDK9. Previously, Yu Liu analysed quantitative proteome profiling of GIST and adjacent normal tissue and found that several kinases were significantly upregulated in GIST, including KIT and CDK7[33]. Consistent with this, we validated with tissue microarrays (TMAs) that CDK7 overexpression in GISTs was correlated with tumour progression and an unfavourable prognosis. Inhibition of CDK7 or CDK7 inhibitor treatment significantly inhibited GIST cell proliferation. These results revealed that CDK7 might play an oncogenic role in GIST progression.

GISTs exhibit a homogeneous repertoire of transcription factors, which supports its associated gene expression program throughout all stages of the disease. Several core transcription factors have been revealed to play essential roles in driving GIST cell proliferation and metastasis by binding to enhancers of GIST-associated genes and facilitating *KIT* gene expression[13–15]. Moreover, a previous study revealed that the KIT-regulated enhancer domain in GISTs could be targeted by BRD4, a key activator of RNAPII transcription at active chromatin marks, and the BET bromodomain inhibitor (BBI) can downregulate *KIT* transcription[34, 35]. Therefore, characterization of transcription factor deregulation in GIST may provide innovative insights into the pathogenesis mechanisms and offer new therapeutic approaches.

In our study, RNA-seq analysis was used to detect alterations in total transcripts in GIST cells after treatment with THZ1. We observed that a cluster of genes was particularly sensitive to THZ1 treatment and was mainly enriched in biological processes of transcription regulation mediated by RNAPII. Considering the original genetic alteration of GIST, we investigated the transcriptional activity and protein expression of *c-kit* after CDK7 knockdown or THZ1 treatment. Interestingly, we found that *c-kit* transcription and protein expression were significantly inhibited after CDK7 knockdown or THZ1 treatment in both GIST T1 and 882 cells. This result indicated that CDK7 might be a key driver of *c-kit* expression in GIST and that it is a possible therapeutic target.

CDK inhibitors are of great interest to explore as novel therapeutic agents against cancer and several CDK inhibitors have been applied in the clinic. CDK4/6 inhibitors have gained FDA approval for the treatment of hormone receptor-positive breast cancer, and inhibitors targeting other cell cycle CDKs are currently in clinical trials for non-small cell lung cancer and other solid tumours[36]. THZ1, as a CDK7 inhibitor, exerts synergistic anticancer effects when combined with TKIs against neuroblastoma, glioma and non-small-cell lung cancer[32, 37–39]. Therefore, targeting CDK7 may provide an alternative therapeutic option to block the reactivation of receptor tyrosine kinase pathways in RTK-driven neoplasms, especially for TKI-resistant cancer. Our research revealed that a combination of THZ1 and imatinib exerts synergistic antitumour effects in GIST cells. Both THZ1 and imatinib treatment led to *c-kit* expression inhibition, and the combination treatment enhanced the inhibition of *c-kit* expression and the downstream AKT and ERK signalling pathways. Taken together, our results indicate that THZ1 has effective antitumour activity against GISTs and may provide an additional therapeutic strategy for GIST patients with a poor response to imatinib.

Super-enhancer (SE) is a large cluster of genomic regulatory elements typically exhibiting an enrichment of histone H3 lysine 27 (H3K27ac) and densely bound by transcription factors and cofactors, playing critical roles in defining cell fate and identity[40]. Interestingly, superenhancers frequently drive the expression of prominent oncogenes in cancer cells[41]. Previous research has used H3K27ac chromatin immunoprecipitation with sequencing (ChIP-seq) of GIST tumour samples and cell lines and identified the SE clusters that drive c-KIT gene expression and are unique to GISTs [15]. Subsequently, studies indicated that the SE domain was essential for c-KIT gene expression and tumorigenesis, including FOXF1, HAND1 and BARX1[13, 14]. Disruption of the SE domain represents a therapeutic vulnerability in GIST[34]. Among

the genes screened out by ChIP-seq of GIST tumour samples and cell lines, OSR1 was hypothesized to bind to the c-KIT locus by ATAC sequencing[15]. In our research, we found that CDK7 knockdown significantly inhibited the expression of c-KIT, and we identified the genes downregulated after THZ1 treatment by RNA-seq. Interestingly, we found that OSR1 was the predominantly downregulated gene. Therefore, we hypothesized that CDK7 knockdown inhibited the expression of c-KIT via OSR1. Subsequently, we proved that OSR1 expression was inhibited by THZ1 treatment in a dose-dependent manner and that OSR1 knockdown also significantly inhibited c-KIT expression. Moreover, OSR1 overexpression reversed the inhibition of c-KIT expression induced by CDK7 knockdown. In summary, our research may have revealed the role of OSR1 in c-KIT expression attenuated by CDK7 inhibition.

In summary, our results uncovered the positive correlations between CDK7 and the malignant potential of GISTs and indicated that targeting CDK7 with the selective inhibitor THZ1 may be a promising treatment for GIST patients.

Declarations

Disclosure of Potential Conflicts of Interest

The authors declare no conflicts of interest.

Authors' contributions

Jianyi Sun and Qiang Zhang: conceptualization, formal analysis, methodology, writing review and editing. Xiangfei Sun and Anwei Xue: methodology and investigation. Xiaodong Gao and Kuntang Shen: conceptualization, research administration, supervision and funding acquisition. All authors read and approved the final manuscript.

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Availability of data and materials

All data supporting the conclusions are included within the article and the additional file. More supporting data is available under reasonable request.

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Figures

Figure 1

High CDK7 protein expression is associated with poor prognosis of GIST.

A. Based on data from the GSE136755 dataset, mRNA level of CDK4, CDK7 and CDK9 was relatively higher than other CDKs in CDK1-10. The mRNA level was calculated via GEO2R. B. CDK7 expression was significantly elevated in high risk of GIST based on the data from GSE136755. C. Representative scanned images of GIST samples with low or high CDK7 protein expression, as determined by IHC. D. Kaplan–Meier survival curves showing the high CDK7 protein expression significantly positively related with poor recurrence-free survival in GIST (*P*=0.032).

Figure 2

Knockdown of CDK7 diminishes cell viability, proliferation and induced cell cycle arrested. A. Immunoblotting analysis of CDK7 expression after targeting siRNA-mediated CDK7 knockdown in GIST-T1 and GIST-882 cells. A nontargeting siRNA and two independent siRNA (siRNA1 and siRNA2) are represented by siNC, siCDK7-1, and siCDK7-2. B. CCK8 cell viability assay after CDK7 knockdown in GIST-T1 and GIST-882 cells. C. Colony formation assays of GIST-T1 and GIST-882 cells after CDK7 knockdown. D. Flow cytometry analysis was used to detect and analyze cell-cycle distribution of cell cycle after CDK7 knockdown. E. Immunoblotting analysis of cyclin-D1, CDK4 and γH2AX expression after CDK7 knockdown.

Figure 3

THZ1 impedes the proliferation and induces apoptosis of GIST in vitro. A. Dose-response curves of GIST-T1 and GIST-882 cells after treatment with THZ2 for 72 hours. Cell viability was assessed with the CCK8 assay. B. Colony formation assays of GIST-T1 and GIST-882 cells with THZ1 treatment. C. Time-response curves of GIST-T1 and GIST-882 cells upon treatment with THZ1 at concentrations as low as 25, 50, and 100 nmol/L.D. THZ1 induces the apoptosis of GIST cells. The GIST-T1 and GIST-882 cells were treated with THZ1 or vehicle. E. Immunoblotting analysis of cleaved-caspase3, cleaved-PARP protein expression after THZ1 treatment for 24 hours.

Figure 4

THZ1 shows anti-neoplastic properties in vivo. A. Images of subcutaneous tumours from vehicle and THZ1 treatment groups (n=5 each). B,C. Tumour volume and weight of two groups. THZ1 treatment lead to significant reduction of volume and weight. C. Immunohistochemistry staining of Ki67 and cleaved-caspase3 of tissue sections from vehicle or THZ1 treated subcutaneous tumour.

Figure 5

Combination treatment of THZ1 and imatinib for GIST. A. CCK8 viability assay following treatment for 72 hours with escalating concentrations of THZ1 and imatinib in GIST-T1 and GIST-882 cells. B. Synergy was calculated using CompuSyn and a combination index value of under 1.0 is considered synergy. C. Immunoblotting analysis of cleaved-caspase3, cleaved-PARP protein expression after combination treatment of THZ1 and imatinib in GIST-T1 and GIST-882 cells for 24 hours.

Figure 6

CDK7 inhibition inhibitied of RNA transcription in GIST cells. A. Heatmap showing the change of global active transcripts in GIST-T1 and GIST-882 cells following treatment with 50nmol and 100 nmol/L THZ1 for 6 hours. B. Enriched GO functional categories of transcripts were reduced over two-fold in GIST-T1 and GIST-882 cells following treatment with 50 nmol/L and 100 nmol/L THZ1 for 6 hours. C. Immunoblotting analyses of RNAPII, RNAPII CTD phosphorylation (S2, S5, and S7), and CDK7 in GIST-T1 and GIST-882 cells treated either with THZ1 or DMSO at the indicated concentrations for 24 hours.

Figure 7

THZ1 treatment inhibited c-KIT transcription activity and protein expression in GIST cells

A.B. qRT-PCR and immunoblotting analysis of c-KIT expression after siRNA-mediated CDK7 knockdown in GIST-T1 and GIST-882 cells.

C.D. qRT-PCR and immunofluorescence assay showed c-KIT expression change THZ1 treatment in GIST-T1 and GIST-882 cells.

E. Immunoblotting analysis of c-KIT expression and downstream ERK and AKT signaling pathway was inhibited following indicated concentration of THZ1 treatment in GIST-T1 and GIST-882 cells for 24 hours.

F. Immunoblotting analysis indicated that combination treatment of imatinib and THZ1 enhanced the c-KIT expression inhibition in GIST-T1 and GIST-882 cells for 24 hours.

Figure 8

CDK7 inhibited c-KIT expression through OSR1 in GIST.

A. Volcano plot of RNA-seq data displaying the distribution of differential gene expression between THZ1 treatment and DMSO treatment. The up-regulated and down-regulated genes were highlighted in red and blue. The result illustrated that OSR1 was the top down-regulated gene with lowest *P* value.

B,C. qRT-PCR and immunoblotting analyses showed that OSR1 expression was inhibited by CDK7 knockdown or THZ1 treatment in dose manner. OSR1 expression was inhibited by CDK7 knockdown in GIST-T1 and GIST-882 cells.

D. Gene expression profile in the Oncomine database. The result showed that OSR1 was significantly upregulated in GIST compared with normal gastric tissue.

E. Gene expression profile in the MediSapiens IST Online transcriptome database. The result showed that OSR1 was uniquely overexpressed in GIST and prostate cancer compared with other cancer types.

F. Immunoblotting analyses of OSR1 expression in clinical sample tissue of GIST, liomyoma and schwannoma. Expression of OSR1 was significantly higher in GIST than liomyoma and schwannoma.

G. Data from MediSapiens database showed that c-KIT and OSR1 expression was positively related (n=77, r=0.407, $P<0.001$).

H,I qRT-PCR and immunoblotting analyses showed that OSR1 knockdown significantly inhibited c-KIT mRNA and protein expression in GIST-T1 and GIST-882 cells.

J. Immunoblotting analyses showed that over-expressed OSR1 promote c-KIT expression.

K. The inhibition effect of c-KIT expression was reversed when CDK7 siRNA and OSR1 plasmid were co-transfected.

Supplementary Files

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

- Supplementarymaterial.docx
- TableS1.xls