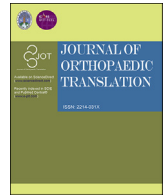


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Personalized medicine modality based on patient-derived xenografts from a malignant transformed GCTB harboring H3F3A G34W mutation

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ABSTRACT

Background: The function of H3F3A G34W mutation, which has been observed in almost all GCTB, remains poorly characterized. Breakthrough in malignant GCTB has been trapped by the lack of clinical available drugs, limited canonical patient samples and paucity of fidelity preclinical models.

Methods: Tumor samples obtained from a malignant GCTB was implanted in immunodeficient mice for the generation of PDX. Histological examination and short tandem repeat (STR) were used for inherited features analyses. An epigenetic/transcriptional targeted compound library was selected for drug screening. The in vivo effects of selected drug were validated in PDX model.

Results: We established the PDX model with recurrent malignant GCTB specimens, histological examination and STR analyses revealed that PDX and their corresponding parental patients shared the same STRs and histologic features, suggesting common origins. ITF-2357 was the most significant compound with an IC50 lower than 0.1 μM. The results of the drug screening and in vivo PDX validation demonstrated that ITF-2357 might be a promising drug targeted H3F3A G34W mutation MGCTBs.

Conclusion: Our study demonstrates that PDX model maintained the same histologic and genetic features as those in the original patient. targeting HDAC through ITF-2357 effectively overcomes malignant GCTB progression in vitro and in vivo.

Translational potential statement: As PDX retain the principal histologic and genetic characteristics of the primary tumors, mad it a valuable research tool in predictive clinical efficacy. In this study, we first established a malignant GCTB PDX model, which might further accelerate the progress of drug development in malignant GCTB.

1. Introduction

Giant cell tumor of bone (GCTB) is a benign but locally aggressive bone tumor, which is composed of mononuclear cells with large, osteoclast-like giant cells [1,2]. It is 1.8–7% of all GCTBs that are malignant GCTB. Malignant GCTB is a high-grade sarcoma diagnosed either at the primary site (primary malignant GCTB) or at the site of a recurrent lesion previously diagnosed, after surgery, radiotherapy or both (secondary malignant GCTB) [2,3]. Malignant GCTB are rare but now there is

no consensus on the treatment for them.

Previous finding demonstrating that there are about 90% of GCTBs harbored H3F3A G34W variants reminds the H3F3A G34W mutation plays an important role in the generation of GCTBs [4]. The classical clinical and radiological approaches can be no challenge to diagnose a GCTB, but the greatest challenges involve distinguishing malignant GCTB from conventional GCTBs [5]. Recently, mutation analysis and immunohistology of H3F3A G34W mutation has been a tool for the diagnosis and differential diagnosis of GCTBs [6,7], and it is proved that

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H3F3A G34W mutation is reliable marker to define benign GCTBs and malignant GCTB [5]. Our study hypothesizes H3F3A G34W mutation will be not only a diagnostic tool but also a drug target for malignant GCTB treatments.

For the reason that malignant GCTB are rare and lack of treatments, the personalized medicine applications for those patients are urgently needed. Recently, patient derived tumor xenografts (PDXs) as a more advanced pre-clinical model have been applied for drug development in bone tumors [8–10]. Compared with cell lines, PDX models are more faithful and stable for reserving the clinical tumor heterogeneity and molecular diversity of human cancers [11], and will be an approach for pre-clinical drug development.

In our study, we first established a PDX model of malignant GCTB from a frequent recurrent GCTB patient, carrying a H3F3A G34W mutation, and behaved similar heterogeneity and molecular features. Furthermore, based on the established PDX model, we carried out a drug screening targeting H3F3A G34W mutation for pre-clinical malignant GCTB drug development.

2. Material and methods

2.1. Relative materials

2.1.1. Patients information

The PDX model was established from a malignant GCTB patient, which was diagnosed as GCTB (Primary GCTB) in the first surgery, then experienced one recurrence (Recurrent GCTB), and finally became malignant GCTB in the third operation. Other 4 GCTB patients admitted to Shanghai General Hospital were included in our study. The clinical complaints and radiological features were collected. All of the patients were re-reviewed and confirmed by two pathological experts. The basic clinical-histological features of patients have also been collected (Supplementary Table S1). The present research was approved by the Institutional Research Ethics Committee of Shanghai General Hospital and informed consent was obtained from all patients.

2.1.2. Reagents and antibodies

CellTiter-Glo® Luminescent Cell Viability Assay reagent were purchased from Promega (Tokyo, Japan). Primary PDCs were maintained in Dulbecco modified Eagle medium (DMEM)/High Glucose medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS; Sigma–Aldrich, St Louis, MO), streptomycin (100 mg/mL), and penicillin (100 U/mL). Cultures were incubated at 37 °C with 5% CO₂ humidified incubator. Antibodies against PCNA, and c-caspase 3 were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against H3F3A G34W mutation were obtained from Active Motif.

2.2. Tissue sampling

Tumor samples were obtained from surgical specimens in sterile conditions. The available tumor samples were divided into three parts. The first part of the tissue, placed in the DMEM medium supplemented with 10% FBS and antibiotics, was implanted in immunodeficient mice for the generation of PDX. The second part was frozen in liquid nitrogen and stored at –80 °C for genetic analyses. The last remaining tissue was fixed in 10% formalin solution for histopathology and necessary immunohistochemistry.

2.3. Establishment of PDX models

Immunodeficient NOD Scidy (NSG) mice were bred in sterile condition in the animal facility. Fresh tumor specimens measuring approximately 4 mm³ for generating PDXs were implanted subcutaneously at the level of trans-scapular brown fat of 5 to 11-week-old NSG mice within an average of about 2 h after obtaining the tumor samples from surgery. Tumor growth was monitor at least twice weekly using calipers until it

reached a maximal volume of 1000 mm³, then the mouse was sacrificed by cervical dislocation and the tumor tissue was removed. The tumor was minced with scissor in sterile condition and tumor fragments were re-implanted in NSG mice. Some of the tumor fragments was used for in vitro cultures and the remaining fragments were immersed in 90% FBS+10% DMSO for viable storage in liquid nitrogen at –80 °C or used for histopathological and molecular analysis.

2.4. Establishment of primary PDX-derived cell lines (PDC)

Tissue samples obtained from the patient or from a PDX were minced in small pieces and placed in complete medium into 20 mm dishes (Falcon) incubated at 37 °C in a 5% CO₂ condition. When the cell cultures formed monolayer, the cells were sub-cultured after enzymatic removal with dissociation reagent (Sigma–Aldrich). The cell morphology were observed continuously during the passages.

2.5. Histopathology and immunohistochemistry

The tissue sections of 4 μm thickness were cut and placed on slides and were immersed in 4% paraformaldehyde for 4h, then transferred to 70% ethanol. The sections were placed in processing cassettes, dehydrated through a serial alcohol gradient, and embedded in paraffin wax blocks. Before staining, the sections were dewaxed in xylene, rehydrated through decreasing concentrations of ethanol, and washed in PBS, and then stained with hematoxylin and eosin (H&E). After staining, sections were dehydrated through increasing concentrations of ethanol and xylene. And for immunohistochemistry, tumor slides were subsequently deparaffinized with xylol. After washing the slides with 100% methanol, the endogenous peroxidase activity was blocked by incubating the slides for 20 min in 0.3% methanol/H₂O₂. Then the slides were rehydrated in 70% ethanol, 50% ethanol, and demi water. Antigen retrieval was performed by incubating the slides in boiling EDTA and followed by 2 h of cooling down in the same solution. Subsequently, the slides were blocked with PBS/1%BSA/5% milk for 30 min and incubated overnight with the first antibody in PBS/1%BSA/5% milk at 4 °C. Then we used the Anti-target Antibody. Slides were washed with PBS for 30 min. This was followed by a washing step with PBS for 10 min. After staining the nuclei with hematoxylin, the slides were dehydrated in demi water, 50% ethanol, 70% ethanol, and 0.3% methanol/H₂O, followed by washing with xylol and mounting.

2.6. Sanger sequencing analysis for H3F3A G34W mutation

For DNA isolation, paraffin-embedded tumor tissue cores were taken from the tumor samples and genomic DNA was isolated with the Chelex extraction method. The hotspot mutation containing H3F3A (128bp) was amplified by polymerase chain reaction (PCR). All PCR products were obtained on a Bio-Rad amplifier using the following program: 5 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 10 s at 60 °C, and 10 s at 72 °C, ending with a melt curve analysis. The PCR products were purified using MinElute 96 UF PCR Purification Plates (Cat. No. 28051, Qiagen) and used specific primers for sanger sequencing and analysis. The primers in this study were listed in Supplementary Table S2.

2.7. STR profiling

Genomic authentication of the primary GCTB, recurrent GCTB, malignant GCTB, PDX and PDC was conducted ensure donor identity. This analysis was performed by short tandem repeat (STR) profiling using Genetica PowerPlex 16HS Cell line PCR kit according to the manufacture.

2.8. Drug screening

For selection of PDCs sensitive-targeted drugs, we used an Epigenetic/Transcriptional Targeted Compound Library with 45 small-

molecule compounds (Supplementary Table S3). ITF-2357 used in animal study was purchased from MedChem Express™.

2.9. Cell viability assay

To determine the PDX cell viability, we used CellTiter-Glo® cell viability assay. The tumor cells were cultured with tested compounds added to experimental wells. After standing at room temperature (22–25 °C) for approximately 30 min, a volume of CellTiter-Glo® Reagent equal to the volume of cell culture medium was added in each well and then the contents should be mixed vigorously for 5 min to induce cell lysis. Incubating the plate at room temperature for an additional 25 min to stabilize the luminescent signal and record the luminescence.

2.10. In vivo drug efficiency validation

After successful tumor transplantation, tumor tissue samples were

passed through nude mice to prepare for follow-up studies. After 2 weeks, DMSO and ITF-2357 (10 mg/kg) were given intragastric administration. Mice injected with DMSO were used as control. Tumor volume was measured every 3 days. After 4 weeks of treatment, all mice were killed and tumor tissue was collected for subsequent histological testing.

3. Results

3.1. Establishment of the malignant GCTB PDX model

Currently, the lack of malignant GCTB experimental model restricted the pre-clinical drug development of malignant GCTB. We collected the patients samples and successfully established one model from four malignant GCTB tumor samples (25%). The PDX model was established from a malignant GCTB patient, which was diagnosed as GCTB (Primary GCTB) in the first surgery, then experienced one recurrence (Recurrent GCTB), and finally became malignant GCTB in the third operation with a

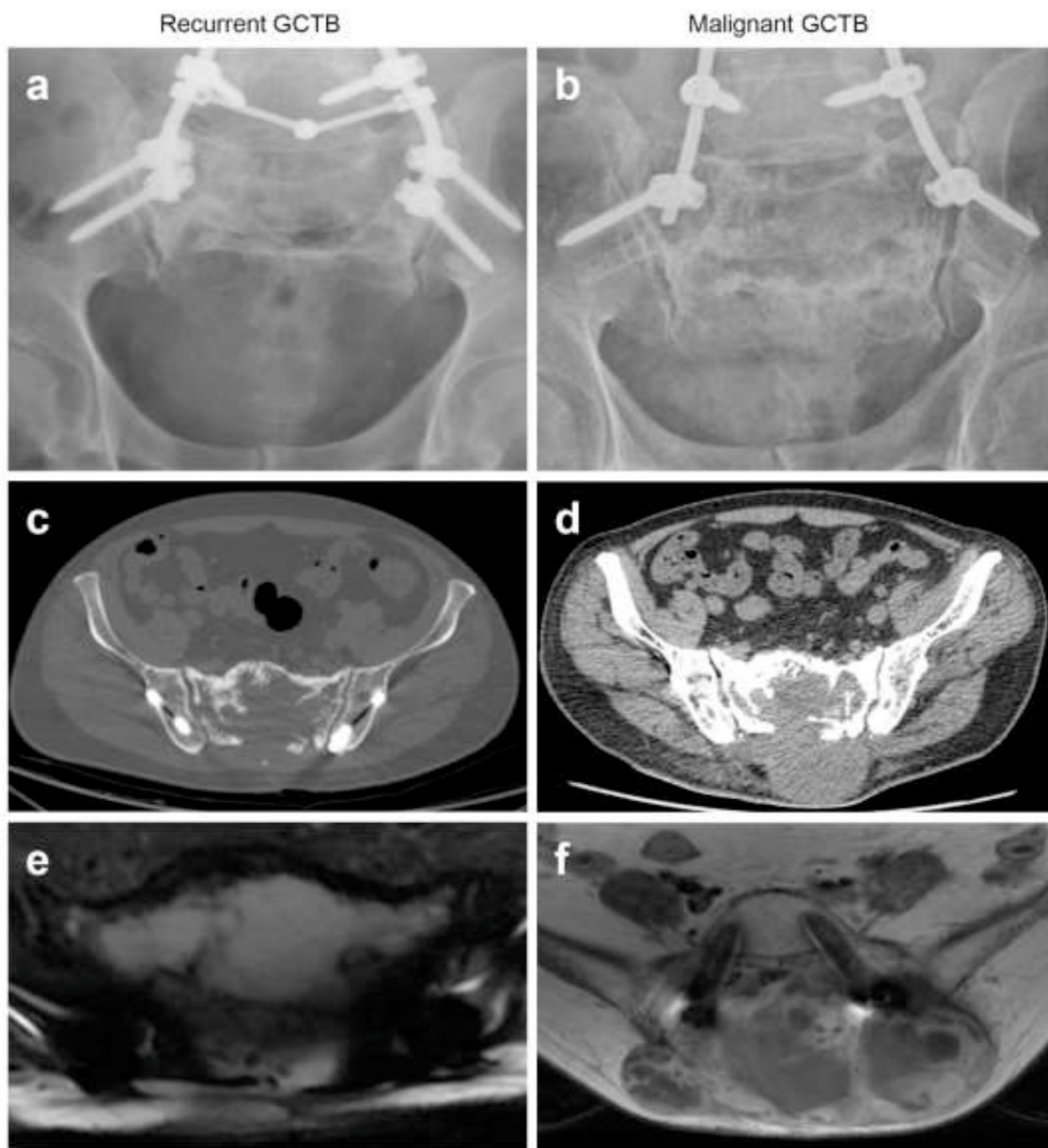


Figure 1. The radiography of the relevant patient of the successfully established Malignant GCTB PDX model. The patient was diagnosed as malignant GCTB repeatedly and at the last time of the recurrence. The X-ray feature of the recurrent GCTB (a) and malignant GCTB (b); CT feature of the recurrent GCTB (c) and the malignant GCTB (d); The MRI feature of the recurrent GCTB (e) and malignant GCTB (f).

high grade sarcoma tendency in the radiography (Fig. 1). The PDX model of GCTB was stable and growing fast at a 30-day interval for passaging. We collected the tissue of the malignant GCTB PDX model for the histopathology experiments. Compared with the relevant patient's tumor samples, the tissue of the PDX model inherited histopathological

features. The PDX model mirrored the mononuclear tumor cells with osteoblast-like giant cells from the patient tissue and the immunohistochemical series of PDX model revealed a similar histological features to the relevant patient tissue as well, with specific markers positive such as the stromal marker KP-1 and p63 (Fig. 2A). These results demonstrated a

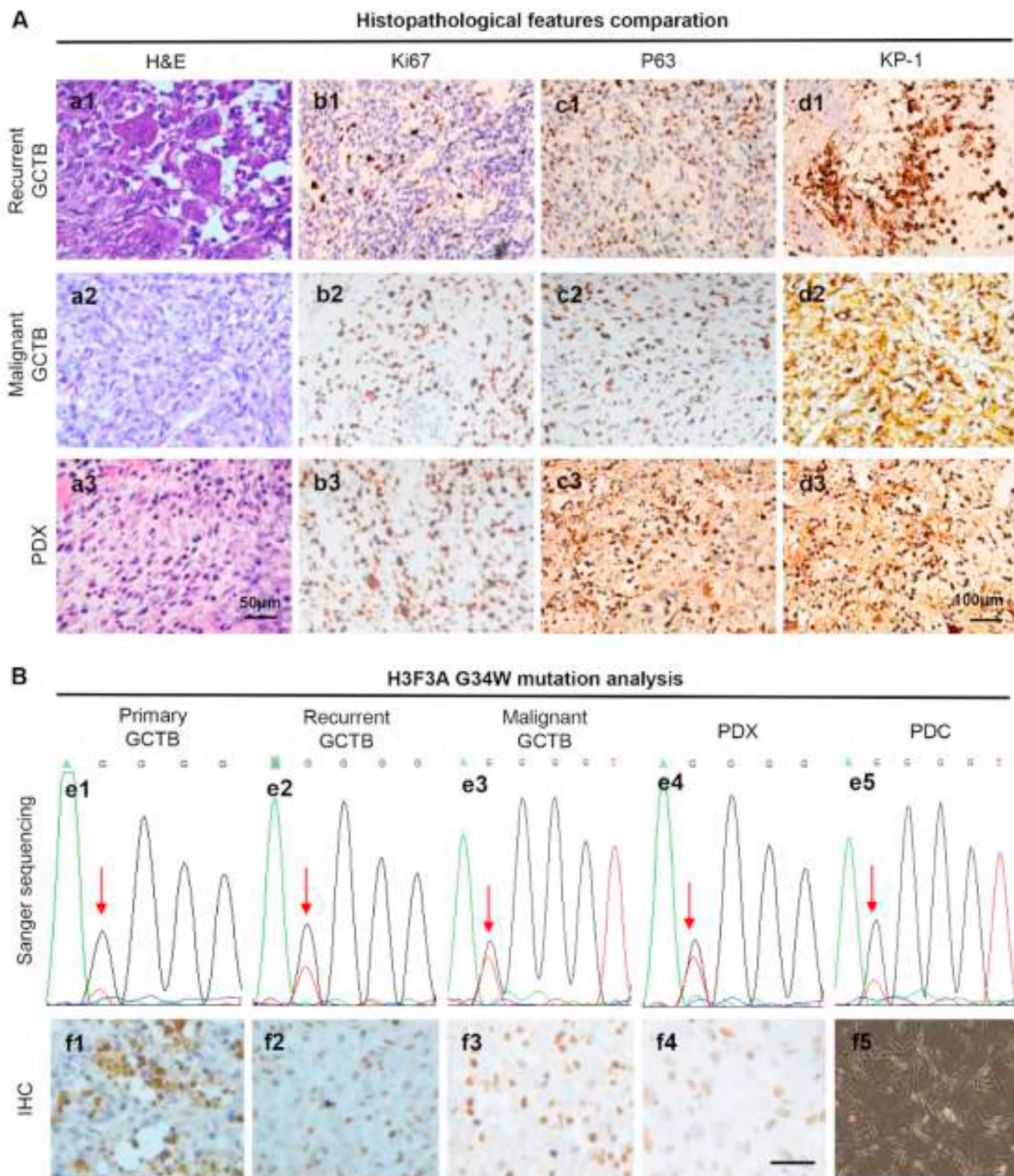


Figure 2. Comparison of the PDX & PDC model with the relevant patient. (A) (a1-a3) The PDX model inherited a similar H&E features of high-grade sarcoma cells with osteoblast-like giant cells from the relevant tumor tissue. (b1-b3) The Ki67 staining of relevant tumor tissue and the PDX model. (c1-c3) The P63 staining of relevant tumor tissue and the PDX model. (d1-d3) The KP-1 staining of relevant tumor tissue and the PDX model. (B) (e1-e5) The sanger sequencing analysis revealed an H3F3A G34W mutation. The abundance of H3F3A G34W mutation showed the primary site was the lowest which was diagnosed as primary GCTB but the recurrent GCTB and malignant GCTB tumor tissues with a high abundance of H3F3A G34W mutation. the PDX model inherited the H3F3A G34W mutation. (f1-f4) The immunohistochemical features confirmed the results of sanger sequencing analysis. (f5) The morphology of the PDC model.

malignant GCTB PDX model was successfully established with inherited features from relevant patient.

3.2. Stable inheritance of remarkable H3F3A G34W mutation

On account of the high prevalence of H3F3A G34W mutation in the generation of GCTB, a sanger sequencing analysis was performed to identify whether an H3F3A G34W mutation existed in order to confirm the specific feature of GCTB. As our expectation, the relevant tumor samples, both the primary site and the recurrence site from the patient, carried a H3F3A G34W mutation. The malignant GCTB PDX model also carried a H3F3A G34W mutation at a similar abundance. The inheritance of the H3F3A G34W mutation was confirmed by immunohistochemistry (Fig. 2B). These results demonstrated that the PDX model carried the specific malignant GCTB mutation. To proceed a pre-clinical drug screening on this malignant GCTB PDX model would acquire results with high reliability.

3.3. Establishment of the PDC from the PDX model

For further study, we used the tissue of successfully established malignant GCTB PDX model to isolates primary PDC. During the first passage of PDC, the cultured osteoclast-like giant cells were still observed, which would disappear at the second generation. A sanger sequencing was also performed to the PDC model at the 3rd generation and the results revealed the presence of H3F3A mutation as well (Fig. 2B). These results demonstrated that the PDC model, like the PDX model, carried an inherited specific malignant GCTB feature and could be used for cytological studies.

STR genotyping further investigated homology, the results of Primary GCTB, Recurrent GCTB, Malignant GCTB, PDX, and PDC with $\geq 90\%$ match, which were the same individual source, derived from a common

ancestry (Supplementary Fig. S1 and Table S4).

3.4. Drug screening of the PDC model

Unlike conventional benign GCTBs, malignant GCTB was insensitive to conventional therapy, which was in urgent need of a drug development. After successfully establishing an ideal PDC model, we conduct a pre-clinical drug screening based on an epigenetic/transcriptional targeted compound library, which including 45 small-molecule compounds and conventional chemotherapy (Supplementary Table S3). There were top five drugs (ITF-2357, SB939, (+)-JQ1, Panobinostat and CBL0137) that had an IC_{50} lower than 1 μM , and particularly, ITF-2357 was the most significant compound of all with an IC_{50} lower than 0.1 μM (Fig. 3A). The results of the drug screening demonstrated that ITF-2357 might be a promising drug targeted H3F3A G34W mutation malignant GCTBs.

PDC was used to further verify the inhibitory effects of ITF-2357 in vitro, and the results were consistent with that of the drug screening (Fig. 3B). Based on those, we tested the inhibitory effects of ITF-2357 with another pair of primary cells SGH-19 and SGH-20 isolated from a GCTB patient, in which SGH-19 was isolated from the primary tumor and SGH-20 was isolated from the recurrent surgical specimens (Supplementary Table S1). The results showed that the inhibitory effects of ITF-2357 on SGH-20 was stronger than that of SGH-19 (Fig. 3C). This suggests that ITF-2357 may have a stronger inhibitory effects on recurrent, more aggressive GCTB. To analyze the toxicity of ITF-2357 on normal cells, we further investigated the effects of ITF-2357 on human BMSCs (hBMSCs, without H3F3A G34W mutation, which was considered as the control), and the results indicated that ITF-2357 had no significant inhibitory effects on normal hBMSCs (Supplementary Fig. S2).

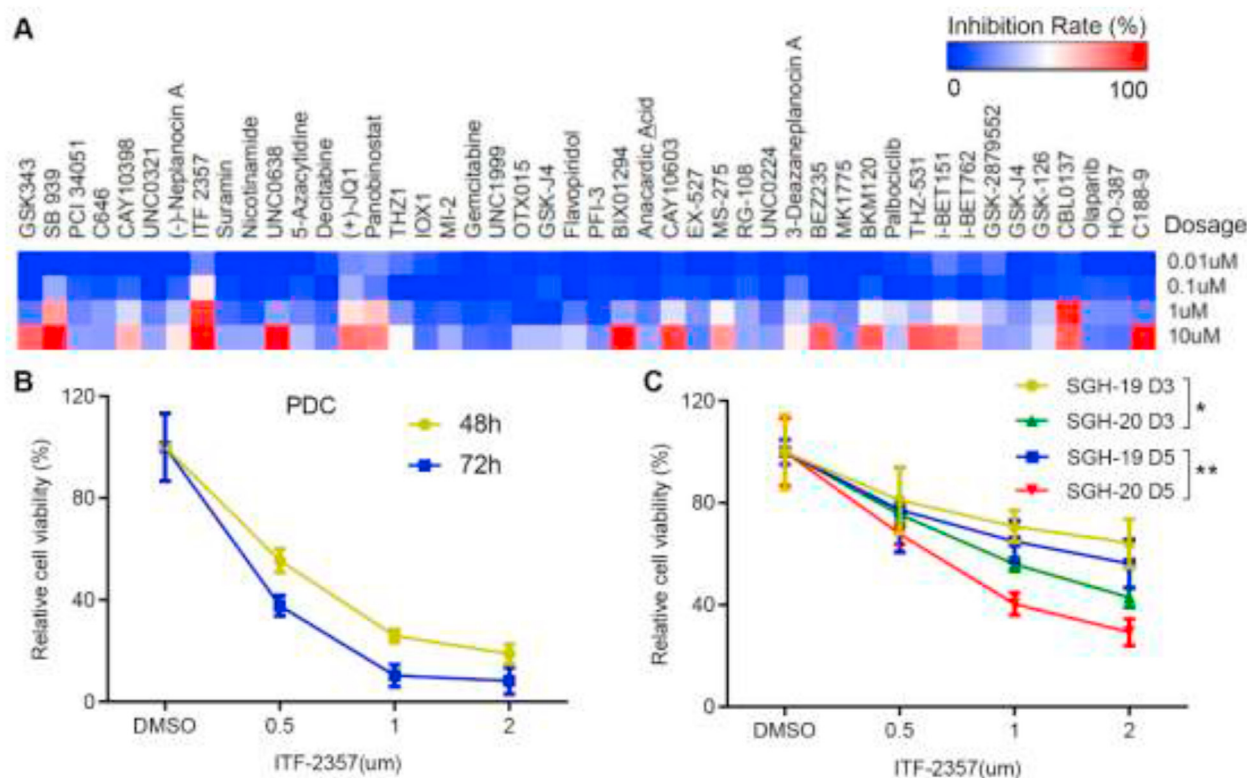


Figure 3. Drug screening of the PDC model in vitro for pre-clinical drug development. (A) The results of drug screening of Epigenetic/Transcriptional Targeted Compound Library. ITF-2357 had an IC_{50} lower than 0.1 μM . (B) The relative cell viability of ITF-2357 was performed in the malignant GCTB PDC model (48h & 72h). The results revealed a decrease of the relative cell viability. (C) A comparison between the malignant GCTB PDX model (SGH-20) and the benign conventional GCTB model (SGH-19) under the stimulation of ITF-2357 and the relative cell viability of SGH-20 decreased more obviously.

3.5. Specific drug validation on the malignant GCTB PDX models

ITF-2357 was then used for further experiments to confirm its efficiency in the malignant GCTB models *in vivo*. The tumor volume decreased at the 7th day after ITF-2357 administration and appeared a significant difference at the 28th day compared with DMSO group (Fig. 4A and B). Histological features of ITF-2357 stimulated PDX tumor tissue revealed a decrease of mononuclear cells as well as proliferation marker Ki67 compared with the control group, while frequently cell apoptosis was identified in ITF-2357 groups (Fig. 4C). Taken together, those results validated the promising efficiency of ITF-2357 in malignant GCTB model *in vivo*.

4. Discussion

GCTB was considered as an intermediate locally aggressive bone tumor featured with aggressive bone destruction [3]. Repeatedly recurrence, severe complications such as malignant transformation and lung metastasis often leads to poor clinical prognosis [12]. Even though the human monoclonal antibody denosumab shown the great potential in treating tumor-associated bone lysis through the RANKL pathway and has been used as neoadjuvant therapy for GCTB, detailed molecular

mechanism of malignant transformation of GCTB has not been clarified [13–15]. Efficient strategies in reducing tumor recurrence, malignant transformation has always been a major challenge in the field of basic and clinical translational research of GCTB [16].

In recent years, several studies have shown that mutations in histones themselves can also lead to cancer, so mutant histones also called “oncohistones” [17–20]. Histone residues carrying mutations cannot be modified by modifying enzymes, leading to the changes in genomic stability and gene expression patterns, and ultimately affecting the genesis and progression of tumors. Previous studies has shown that the 34th codon mutation of H3F3A was present in more than 90% of GCTB patients, and the mutation of histone H3F3A G34W enhancing tumor malignant and invasiveness, which was considered as the most significant characteristics of GCTB [4,7,21].

Malignant transformation of GCTB causing great damage to bone and even leading to the lung metastasis of tumor. The poor prognosis of malignant GCTB, which needs to be taken seriously [22]. In this study, the patient was primarily diagnosed with GCTB but recurred for many times after surgical resection of the tumor, and was diagnosed as malignant GCTB in the last surgery, making it a typical malignant GCTB case with great research value.

PDX was a human xenograft model based on the direct

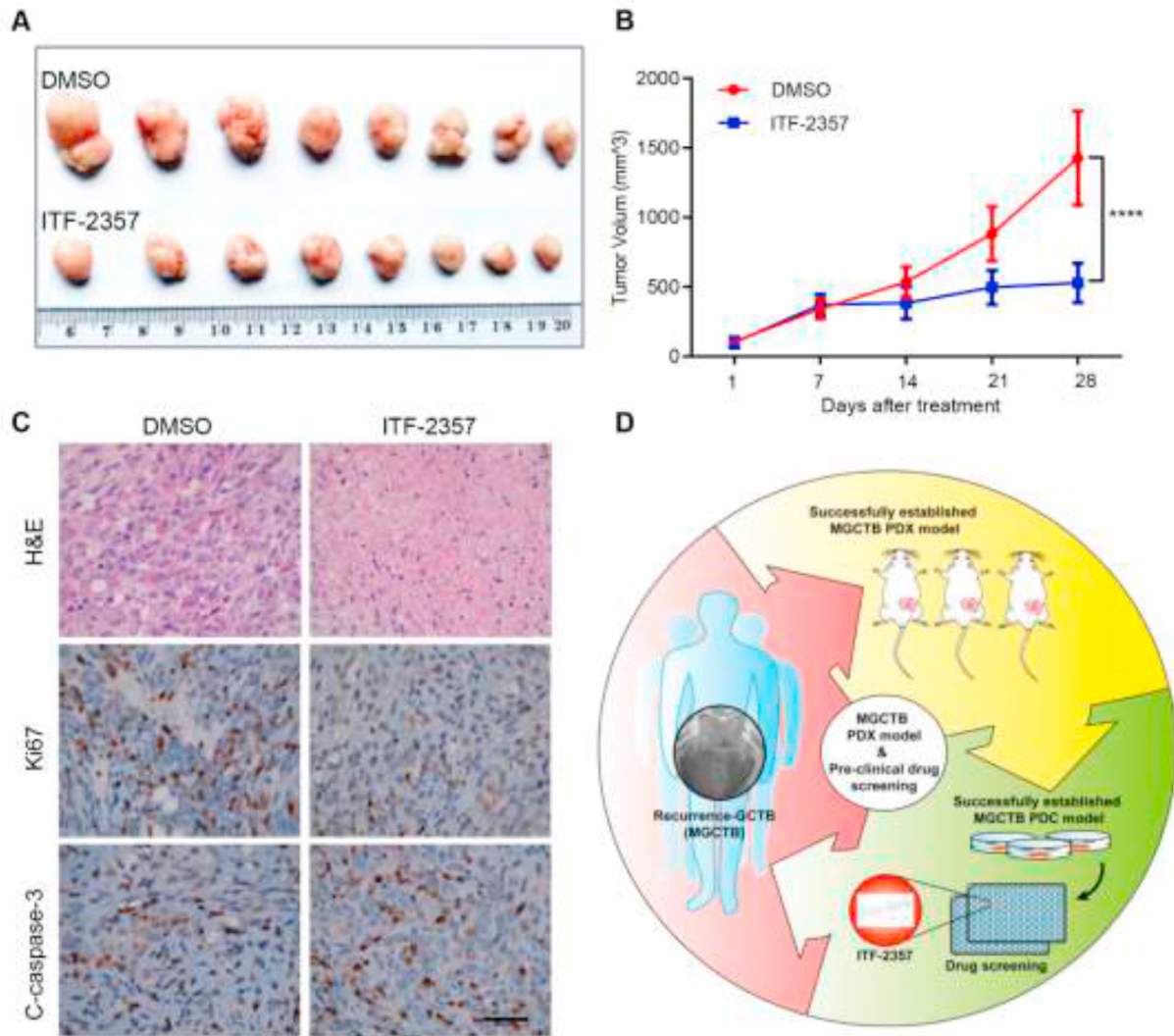


Figure 4. Verification of ITF-2357 *in vivo* and the graphical abstract of this study. The gross tumor specimens (A) and growth curves (B) of PDX model. (C) ITF-2357 inhibit the proliferation and induce cell apoptosis of malignant GCTB PDX model. (D) An graphical abstract to conclude our study which focused on the malignant GCTB pre-clinical drug development by using established the PDX and PDC models.

transplantation of the patient's tumor tissues into immunodeficient mice [23]. As PDX retain the principal histologic and genetic characteristics of the primary tumors, made it a valuable research tool in predictive clinical efficacy. In this study, PDX model was successfully constructed from the surgical specimens of a malignant GCTB, which retained the histological characteristics of the primary tumor. Recent studies have shown that H3F3A G34W mutation was mainly involved in the occurrence and malignant transformation of tumors through regulating histone modifications and gene expression of several key oncogenic factors involved in the tumor generation, the results further demonstrating the important role of epigenetic regulation in the occurrence and development of GCTB. On this basis, PDCs was isolated from the established PDX model, and the epigenetic-related small molecule drug library was used for drug screening, hoping for identified novel epigenetic regulation mechanism involved in malignant GCTB transformation. We demonstrated that the HDAC inhibitor ITF-2357 effectively inhibited the proliferation of the cell line. HDAC inhibitors has not previously been reported for the treatment of GCTB, but it shown to be effective in reverting the aberrant epigenetic states and reducing growth in osteosarcoma. In order to further verify the efficacy of ITF-2357 *in vivo*, we tested the efficacy of ITF-2357 in this malignant GCTB PDX model, and the results indicated that ITF-2357 effectively inhibits the growth of malignant GCTB tumor and promotes the apoptosis of tumor cells (Fig. 4D). Among previous studies, ITF-2357 exerts its anti-proliferative effects on down-regulates JAK/STAT signaling pathway or c-myc protein expression, which may be consistent with our studies. Together these data demonstrated that ITF-2357 has potential application prospects in malignant GCTB [24].

In summary, our study demonstrates that PDX model maintained the same histologic and genetic features as those in the original patient. targeting HDAC through ITF-2357 effectively overcomes malignant GCTB progression *in vitro* and *in vivo*. In addition, the compound library used in our drug screening was limited and a large-scale screening was required to systemically identify other crucial regulators and the corresponding drugs, which may provide novel theoretical basis for the malignant GCTB.

Declaration of competing interest

All authors declare that they have no conflicts of interest concerning this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jot.2021.04.004>.

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