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Full paper

EphB4 inhibitor overcome the acquired resistance to cisplatin in melanomas xenograft model

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ABSTRACT

The purpose of this paper is to investigate the possible mechanisms of resistance to chemotherapy in melanoma from the perspective of molecular biology and to discuss the strategies to overcome them. Cisplatin, a DNA-damaging compound that triggers apoptotic cell death, is commonly used in the treatment of malignant melanoma. However, most patients develop mechanisms of acquired resistance and about 25% of them do not achieve tumor regression at all, due to intrinsic resistance to therapy. In the current study, we reported the tumor xenografts of the human A375 melanoma, after 40-weeks' consecutive therapy with cisplatin that developed resistance as a result of *EphB4* overexpression. Moreover, the expression of phospho-AKT and phospho-ERK were significantly increased in cisplatin-resistant tumors. In addition, combined of cisplatin with EphB4 selective inhibitor could abrogate this acquired mechanism of drug resistance due to an enhanced apoptotic effect in cisplatin-resistant xenografts. In summary, these results help to understand the mechanisms of acquired resistance to chemotherapy and provide important information for clinical treatment strategies.

1. Introduction

Cutaneous melanoma (primary site) is the most aggressive form of skin cancer, which is highly curable when localized to the primary site. However, when melanoma spreads to the regional lymph nodes, the 5-year survival rate is only 29% and once major organs are disseminated with disease the rate would fall to 7% (1). Alkylating agent dacarbazine usually was used as a standard treatment to treat metastatic melanoma, which frequently leads to poor outcomes, while combinations of chemotherapeutics have shown only marginally higher response rates, paying the price of systemic toxicity (2,3). In addition, most patients with metastatic melanoma are incurable because of melanoma cells are generally insensitive to chemotherapy-induced cell death (3).

Cisplatin is a neutral inorganic, square planar complex, which is one of the most potent anti-tumor agents. It exert cytotoxic effect by interaction with DNA to form DNA adducts, which culminate in either repair of the DNA damage and cell survival or activation of DNA damage-mediated apoptotic program. The clinical benefits of cisplatin as an anti-tumor agent have been recognized for over 30 years, which is also commonly used in the treatment of malignant melanoma (4). However, melanoma is relatively resistant to cisplatin even if it is highly effective in the treatment of many types of cancer (5). Despite some mechanisms of tumor resistance to cisplatin in other tumor types have been proposed in pre-clinical studies, the mechanisms of chemoresistance are still unknown in melanoma. Possible explanations include disrupted accumulation of agents caused by drug pumps, up-regulated DNA repair, defective apoptosis signaling, and survival factor activation (6).

In the current study, we developed a melanoma A375 xenografts which sensitive to cisplatin firstly and then acquired tumor resistant by continuous dosing cisplatin. To our knowledge, this is the first time to investigate the possible mechanisms of resistance to chemotherapy in melanoma from the perspective of molecular biology and to discuss the strategies to overcome them.

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2. Materials and methods

2.1. Reagents and cell line

Cisplatin were purchased from Sigma (St. Louis, MO, USA). For *in vivo* studies, Cisplatin was dissolved in normal saline delivered by intraperitoneal (IP, 2 mg/kg) injection twice weekly. EphB4 inhibitor NVP-BHG712 purchased from Selleck Chemicals (Houston, TX, USA). Phospho-AKT (Ser473, p-AKT), phospho-ERK1/2 (Thr202/Tyr204, p-ERK1/2), EphB4, AKT, and ERK1/2 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Tissue lysis buffer and phosphatase inhibitor cocktails were purchased from Sigma (St. Louis, MO, USA). Human melanoma cell line A375 was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in DMEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), penicillin and streptomycin (ICN Biomedicals, Inc., Costa Mesa, CA, USA), and sodium pyruvate and maintained at 37 °C with 5% CO_2 in a humidified atmosphere.

2.2. Cisplatin-resistant A375 xenograft model establishment and efficacy study in vivo

Female athymic BALB/c nude mice (6–8 weeks) were purchased from Vital River (Beijing, China). Mice were maintained under super pathogen-free conditions and housed in barrier facilities on a 12-h light / dark cycle, with food and water ad libitum. Tumor volume was calculated using the following formula: tumor volume = (length × width^2)/2, where length and width are the longest and shortest dimensions of the tumor, respectively. Mice were injected subcutaneous (s.c.) with 4×10^6 A375 cells, which had been resuspended in 100 μL of PBS. When tumor volume reached 150–200 mm^3, 10 mice in each group were treated with 2 mg/kg cisplatin twice weekly by IP administration. Tumors were monitored for the development of cisplatin resistance, defined as marked tumor growth in the presence of continued cisplatin therapy (7). Approximately 40 weeks following the injection, the tumors (1.5 cm in diameter) was aseptically resected from cisplatin-resistance group and minced into small pieces (3 mm in diameter). Then a piece of tumor was implanted into the left flank of host mice. When tumor volume reached 150–200 mm^3, mice were randomly divided into four groups (n = 10 for each group) to receive of vehicle (IP, daily), 2 mg/kg cisplatin (IP, twice weekly), 10 mg/kg NVP-BHG712 (Oral, once daily), or a combination treatment for 21 days. In the end of studies, we isolated tumor tissues from A375 xenografts and transfer tumor tissues to fresh sterile DMEM medium. Next we transferred tumor tissues to a new dish and dissected off necrotic areas, fatty tissues, blood clots, and connective tissues with forceps and scalpels. Finally, the tumor tissues were washed with PBS and stored in liquid nitrogen. All animal experiments were performed in accordance with protocols approved by the Experimental Animal Center of the Third Military Medical University Animal Care and Use Committee.

2.3. RNA sequencing (RNA-seq)

Gene expression analysis of both cisplatin-sensitive or resistant A375 tumor tissues was carried out on RNA extracted from tumors using Qiagen RNA easy kits (QIAGEN Inc., Valencia, CA, USA). RNA quality was verified by running samples on an Agilent Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA, USA), and preparation of complementary RNA, array hybridizations, scanning, and subsequent array image data analysis were done using the manufacturer's specified protocol. The sequencing library was constructed according to Illumina's TruSeq RNA Sample Preparation Protocol (8). After normalization, the DNA sample libraries were pooled into 4 libraries, and the pooled libraries were sequenced on an Illumina HiSeq 2000 sequencing machine (Illumina Inc., San Diego, CA, USA).

2.4. Western blot analysis

The mice were sacrificed with CO_2, and then tumors were resected from A375 xenograft after last treatments for 4 h in the last day of efficacy study. Protein extracts were prepared from the tumor tissues using lysis buffer containing 50 mM Tris–HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxy-cholate, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin (Sigma, St. Louis, MO, USA). The protein concentrations were determined using the Bradford assay. The protein lysate (50 μg) was separated on a 10% SDS-PAGE gel and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) for western blot analysis detection. The blot was blocked with 5% non-fat dry milk in a buffer containing 10 mM Tris (pH 7.5), 100 mM NaCl, and 0.1% Tween-20 (Sigma). The blot was washed and incubated with primary antibodies (1:1000 dilution) for 1 h and then incubated for 30 min with secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (1:3000 dilution). Immunoreactive protein signals were visualized by an enhanced chemiluminescence kit (Thermo Scientific, Amersham, USA).

2.5. Apoptosis assays

The mice were sacrificed with CO_2, and then tumors were resected from A375 xenograft after last treatments for 4 h in the last day of efficacy study. Apoptosis in tumors were determined by TUNEL, using an in-situ cell-death detection kit (Boehringer Mannheim, Indianapolis, IN, USA) according to the manufacturer's protocol. Tumor tissues were fixed with freshly prepared paraformaldehyde [4% in PBS (pH 7.4)], rinsed with PBS, and incubated in permeabilization solution. After cross-reaction with TUNEL reaction mixture for 60 min at 37 °C and cross-reaction with converter-alkaline phosphatase solution for 30 min at 37 °C in a humidified chamber, the slides were reacted with alkaline phosphatase substrate solution for 5–10 min (Vector Laboratories, Burlington, MA, USA), rinsed and mounted under a coverslip for analysis with a light microscope. The number of TUNEL-positive cells was counted in five different fields under a light microscope at ×40 magnification, and representative fields were photographed. The percentages of apoptotic cells were calculated from the ratio of apoptotic cells to total cells counted.

2.6. ELISA analysis for p-EphB4

Phospho-EphB4 expression in tumor tissues level was detected by Phospho-EphB4 Duoset IC ELISA analysis (R&D, Minneapolis, MN, USA). According to the kit instruction procedures, 96 well ELISA high binding plates were incubated overnight at room temperature with 100 μl/well of the specific capture antibody diluted in sterile PBS to the proper working concentrations. In the second day wells were washed and blocked for 1 h at room temperature. After that, wells were washed and 100 μl/well of lysates were added at room temperature for 2 h, then wells were washed and incubated with Detection Antibody at room temperature for 2 h. Finally the biotinylation antibody working liquid was added and the OD450 value was measured to obtain the p-EphB4 expression level.

2.7. Statistical analysis of the data

All results and data were confirmed in at least three separate experiments. Data are expressed as means ± SD, and were analyzed by student's t-test using the GraphPAD InStat software, version 1.14 (GraphPAD Inc., San Diego, CA, USA). *In vivo* experiments Differences were considered to be statistically significant when the *P*-value was <0.05.

3. Results

3.1. Cisplatin-resistant A375 xenograft model establishment

In order to establish cisplatin-resistant A375 xenograft model *in vivo*. The tumor bearing mice were twice weekly IP injection with 2 mg/kg cisplatin for up to 40 weeks. As shown in Fig. 1A, B, treatments with 2 mg/kg cisplatin significantly inhibit tumor growth A375 xenografts at the first stage. However, #3 and #6 xenografts acquired resistance to cisplatin after 40-weeks' consecutive treatment. Any treatment was well tolerated by mice, with no weight loss or other signs of acute or delayed toxicity were observed in the cisplatin treated xenografts (Fig. 1C).

3.2. EphB4 was overexpressed in the cisplatin-resistant A375 xenografts

In order to study possible mechanisms of acquired resistance to cisplatin, we exposed A375 xenografts to cisplatin for a long time period. And then genome-wide gene profile between cisplatin-sensitive and cisplatin-resistant tumors were compared. The results showed that gene *EphB4* was overexpressed, whereas in the cisplatin-sensitive A375 xenografts, the *EphB4* was expressed at a normal level (Fig. 2). According to the results we deduced that *EphB4* may be contributed to the acquired resistance to the cisplatin. In addition, no overexpression were found in the signal transducers that are more frequently aberrantly activated in human tumors such as EGFR, KRAS, EphA2, B-RAF, and PIK3CA (Fig. 2). The result of Western blot also indicated that cisplatin-resistant A375 xenografts harboring EphB4 overexpression (Fig. 4B).

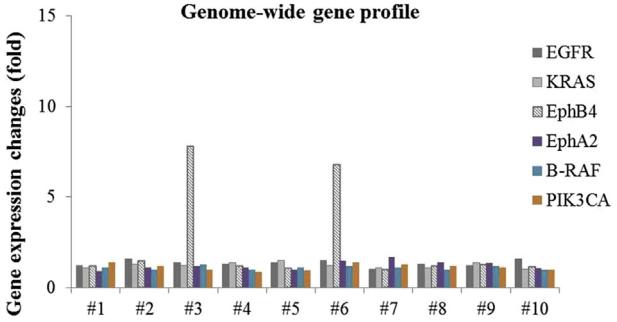

Fig. 2. Differences in gene expression between cisplatin-sensitive and resistant A375 xenografts by RNA-Seq analysis. The mice were sacrificed with CO_2, then Cisplatin-sensitive and resistant tumors were resected from A375 xenografts respectively. The gene expressions of tumors were analyzed by RNA-Seq.

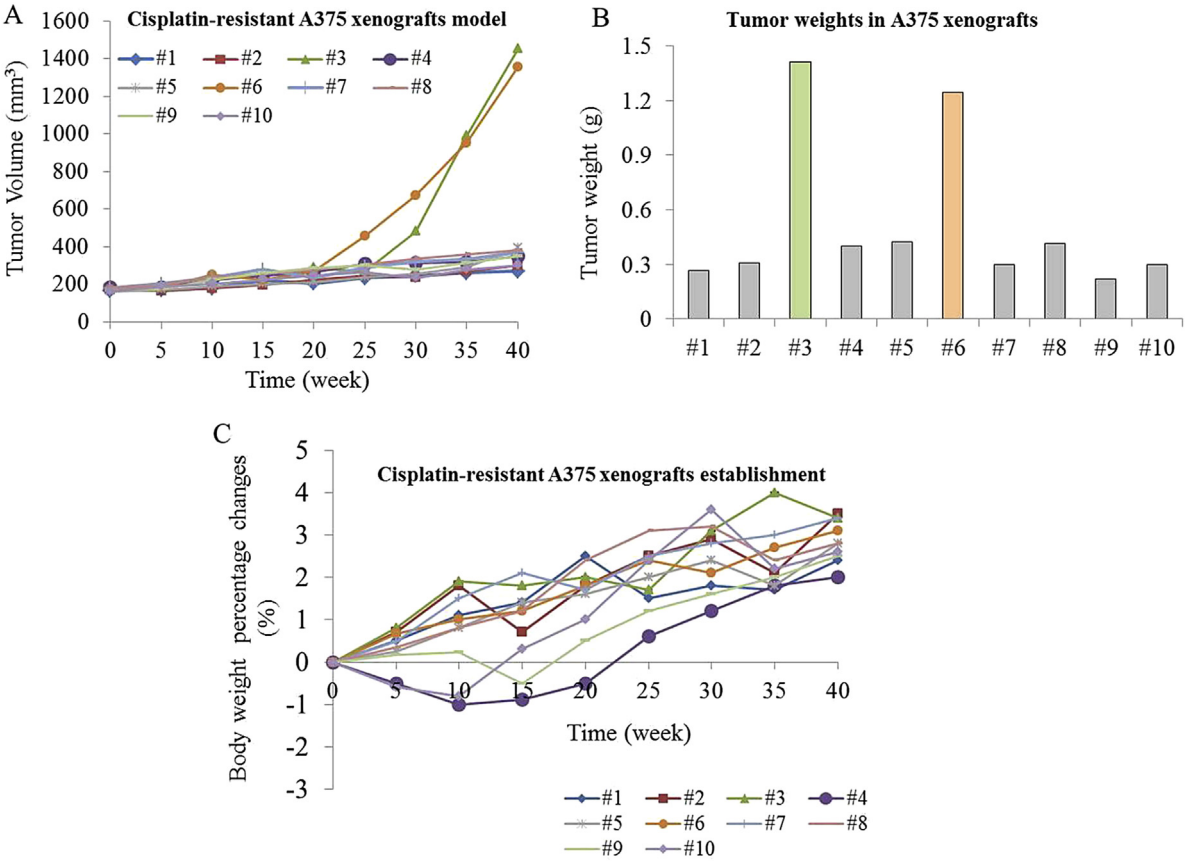

Fig. 1. Cisplatin-resistant A375 xenograft model establishment. (A) Nude mice-bearing A375 tumors were once-daily dosed with 2 mg/kg cisplatin twice weekly by IP administration for up to 40 weeks. Tumor volume was measured using Vernier calipers on the indicated days with the median tumor volume. (B) Tumors were resected from nude mice after 40 weeks' treatment. (C) Body weight was monitored on the indicated days.

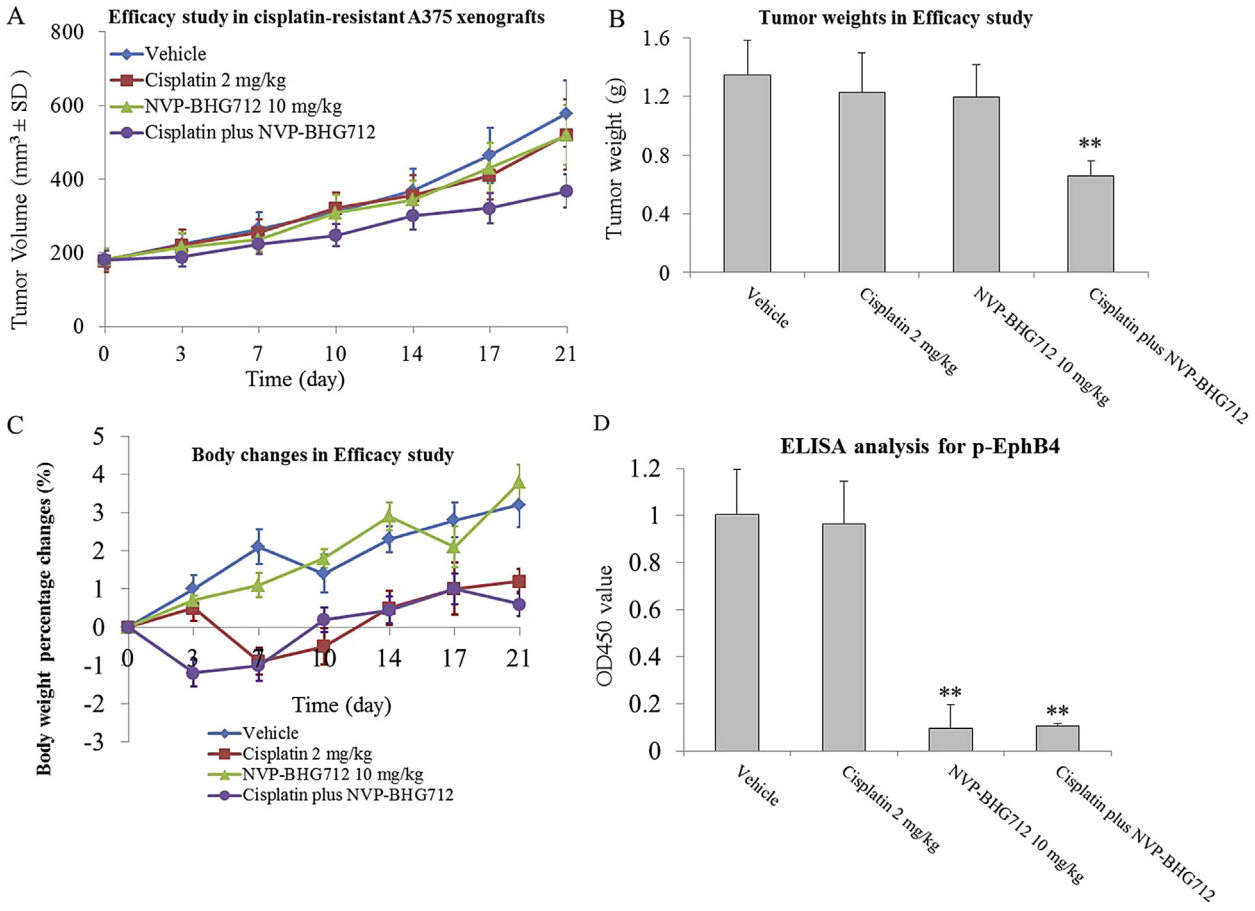

Fig. 3. Combination of cisplatin with EphB4 inhibitor had a synergistic anti-tumor effect on cisplatin-resistant A375 xenografts. (A) Cisplatin-resistant A375 xenografts were treated with 2 mg/kg cisplatin (IP, twice weekly), 10 mg/kg NVP-BHG712 (Oral, once daily), or a combination treatment for up to 21 days respectively. (B) The mice were sacrificed with CO_2, and then tumors were resected from mice and weighted in the end of efficacy study. (C) Body weight was monitored on the indicated days. (D) The tumors were also subjected to Phospho-EphB4 Duoset IC ELISA kit for detecting phosphor-EphB4 expression level. Mean ± SD, n = 10. **, $P < 0.01$ *vs* vehicle group.

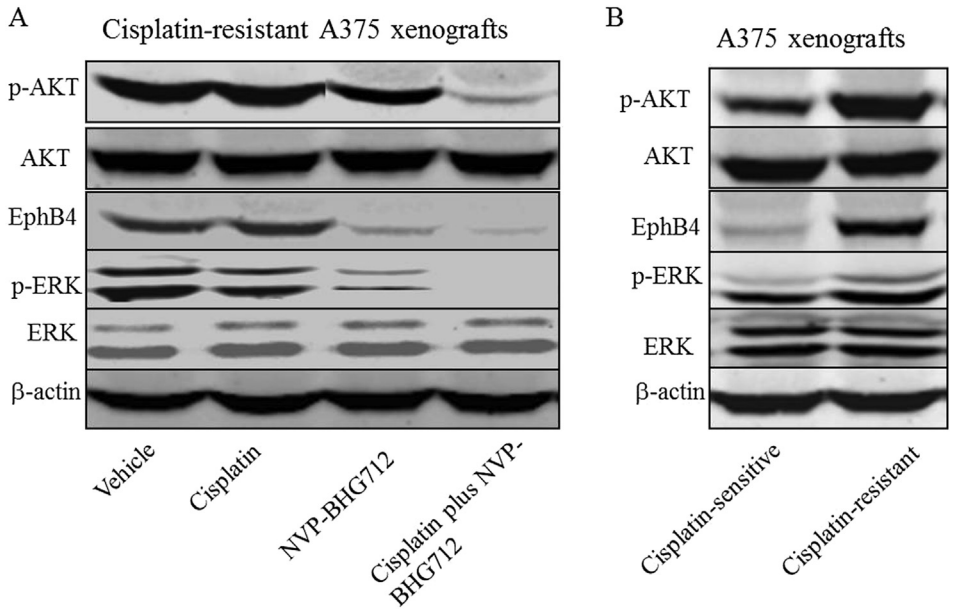

Fig. 4. Effects of Cisplatin or/and NVP-BHG712 on PI3K and MAPK pathways in cisplatin-resistant A375 xenografts. (A) The cisplatin-sensitive or resistant A375 tumor tissues were collected and subjected to western blot analysis for detection of EphB4 expressions. (B) The mice were sacrificed with CO_2, and then tumors were resected from cisplatin-resistant A375 xenografts and subjected to western blot analysis for detection of PI3K/MAPK signaling transduction in the end of efficacy study.

3.3. Combination of cisplatin with EphB4 inhibitor is synergistic in cisplatin-resistant A375 xenografts

To further validate our observations, a combination treatment efficacy study was performed. We combined of cisplatin with an EphB4 inhibitor NVP-BHG712 to treat cisplatin-resistant A375 xenografts. The results indicated that neither 2 mg/kg cisplatin nor 10 mg/kg NVP-BHG712 treatment could inhibit tumor growth in cisplatin-resistant A375 xenografts (Fig. 3A, B). As we expected, the combined treatments significantly inhibited tumor growth after 3 weeks of therapy. It is worth noting that no obvious weight loss or other signs of acute or delayed toxicity were observed in all treatment groups (Fig. 3C). Next, we turn to phospho-EphB4 Duoset IC ELISA kit for detecting phosphor-EphB4 expression level. The results showed that both NVP-BHG712 and the combination treatment would significantly inhibit phosphor-EphB4 expression in A375 xenografts (Fig. 3D).

3.4. Effects of cisplatin or/and NVP-BHG712 on PI3K and MAPK pathways in cisplatin-resistant A375 xenografts

To discriminate whether the observed overexpression was due to selection of a preexisting subpopulation or if it was acquired during the treatment, Western blot analysis was used to assess the impact of on cisplatin or/and NVP-BHG712 downstream molecules of the PI3K and ERK pathways in cisplatin-resistant A375 xenografts. The results indicated that the expression of p-AKT and p-ERK were not affected by cisplatin single treatment in cisplatin resistant A375 xenografts, whereas the expression of p-ERK was significantly inhibited by NVP-BHG712 alone treatment. In addition, EphB4 appeared to be significantly inhibited by NVP-BHG712 single treatment. Moreover, the protein levels of p-AKT as well as p-ERK were significantly inhibited by the combined treatment in cisplatin-resistant A375 xenografts (Fig. 4A). Comparing to cisplatin-sensitive A375 xenografts, the cisplatin-resistant ones showed more high expression levels of

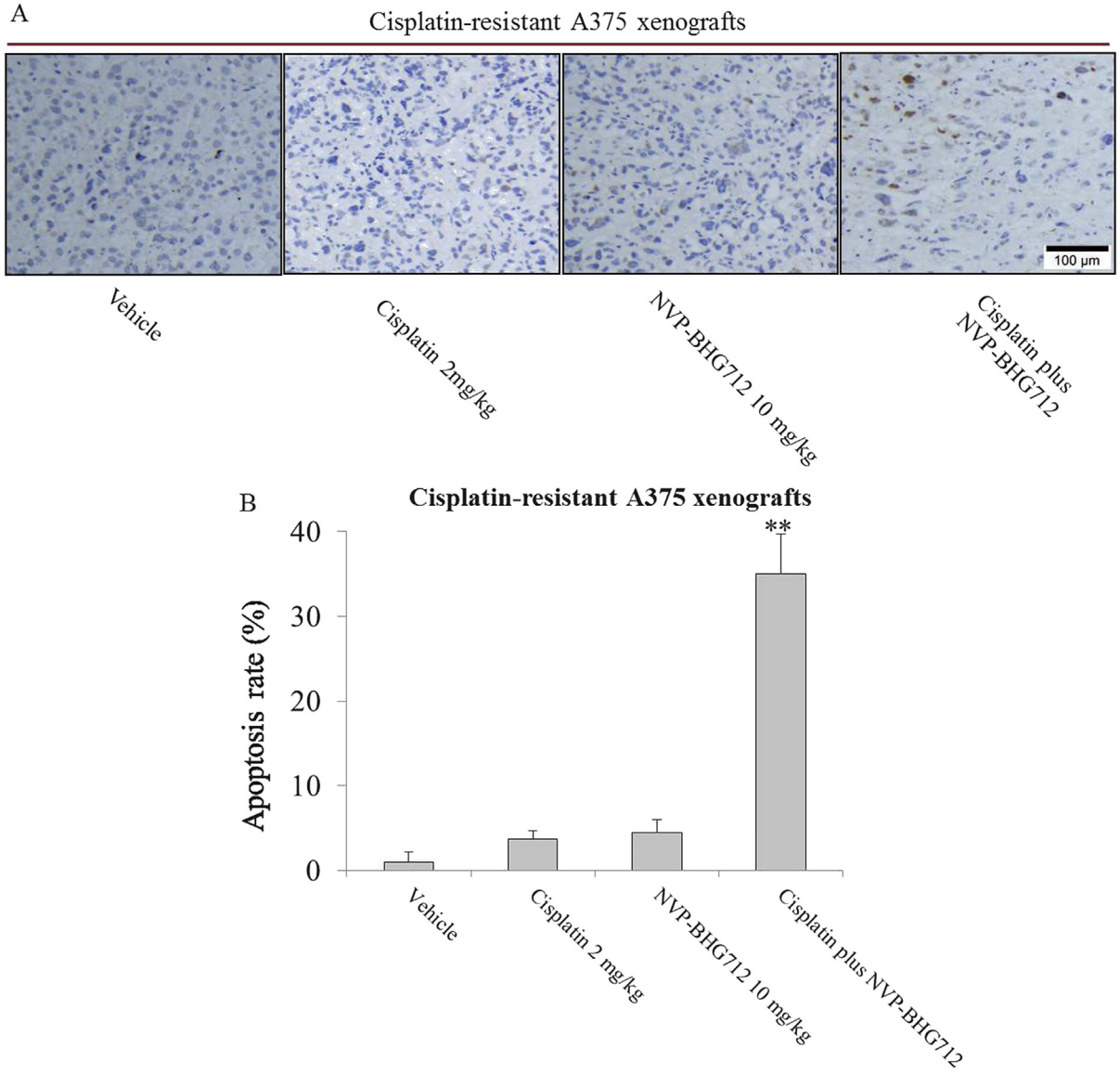

Fig. 5. Combination of cisplatin with EphB4 inhibitor induced apoptosis in cisplatin-resistant A375 xenografts. (A) The mice were sacrificed with CO_2, and then tumors were resected from A375 xenografts after last treatments for 4 h in the last day of efficacy study. Tumor tissues were then subjected to TUNEL alkaline phosphatase assay. (B) The number of TUNEL-positive cells was counted in five different fields under a light microscope at ×40 magnification. The percentages of apoptotic cells were calculated from the ratio of apoptotic cells to total cells counted. Mean ± SD, n = 10. **, $P < 0.01$ *vs* vehicle group.

p-AKT as well as p-ERK. In addition, consistent with the result of RNA-Seq, cisplatin-resistant A375 xenografts harboring EphB4 overexpression (Fig. 4B). This finding reveals that the observed genomic alterations were acquired during the selection.

3.5. Apoptosis in cisplatin-resistant A375 xenografts was induced by the combined of cisplatin with EphB4 inhibitor

A TUNEL alkaline phosphatase assay demonstrated that cisplatin-resistant A375 xenografts underwent apoptosis after the cisplatin in combination with NVP-BHG712 treatment. However, alone treatment with cisplatin or NVP-BHG712 could not induce apoptosis in cisplatin-resistant A375 xenografts (Fig. 5A, B). These results thus indicate that the EphB4 pathway inhibition is required for cisplatin-resistant A375 xenografts undergoing apoptosis.

3.6. Acquired resistance in cisplatin-resistant A375 xenografts was irreversible

We then established the second and third passages cisplatin-resistant A375 xenografts, in order to evaluate if resistant ones have acquired a permanent resistant phenotype. The results showed that both second and third passages xenografts still had a resistance to cisplatin, meanwhile the tumor growth speed of second and third passage xenografts were faster comparing with first passage ones (Fig. 6A, B and C). Moreover, the results of Western blot indicated cisplatin-resistant A375 xenografts including second and third passages also showed similar expression levels of EphB4, p-AKT and p-ERK (Fig. 6D).

4. Discussion

Cisplatin, as one member of a class of platinum-containing anticancer drugs, which displays a great deal of clinical activity on a wide variety of solid tumors. In addition, cisplatin often used in combination with other chemotherapy drugs to treat patients with advanced melanoma (9,10). As we know, cisplatin exert their function mainly by inducing DNA damage, and then cell apoptosis. However, melanoma cells are generally insensitive to chemotherapy or developing a resistance in a short time period (11,12). The mechanisms for drug resistance in melanoma is most likely dysregulation of apoptosis, accumulation of agents caused by drug pumps, DNA repair up-regulation, cell signaling dysfunction (13–16). Except for these pharmacologic based mechanisms, PI3K/AKT signaling activation, overexpression of HER2, p53 dysfunction could be the factors contribution to cisplatin resistance at the molecular level (17–19). In the current study, we developed a melanoma xenograft model, which resistant to cisplatin. The results of gene expression microarray showed that *EphB4* overexpression but not amplification by comparing the genome-wide gene profile of the sensitive and resistant xenografts. The results of Western blot also indicated EphB4 was highly expressed in cisplatin-resistant A375 xenografts. This founding may help us to understand acquired resistance in melanoma from a new angle.

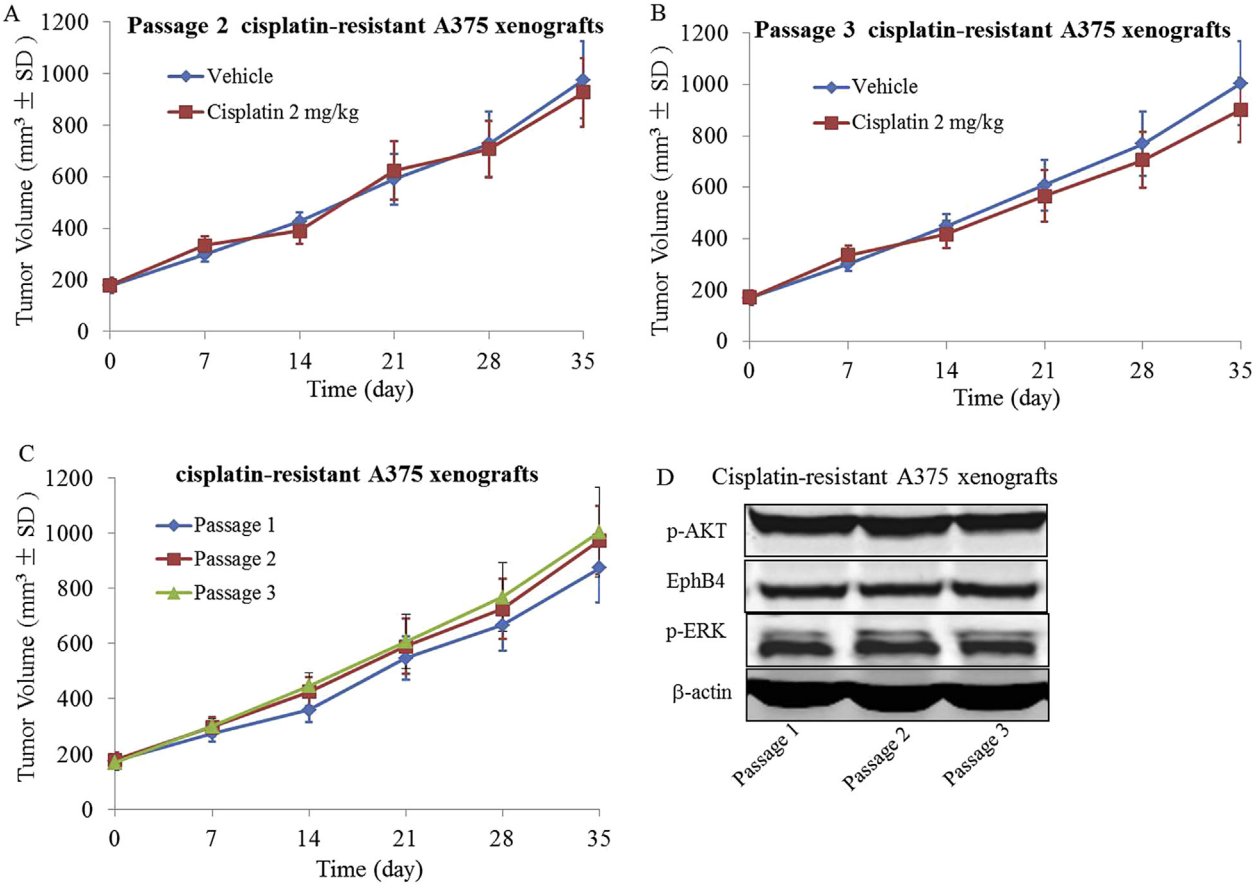

Fig. 6. Acquired resistance in cisplatin-resistant A375 xenografts was irreversible. (A and B) The established second and third passages cisplatin-resistant A375 xenografts were dosed with 2 mg/kg cisplatin (IP, twice weekly) for 21 days. (C) The tumor growth speed of first, second and third passage xenografts were monitored. (D) The mice were sacrificed with CO_2, and then tumors were resected from second and third passages cisplatin-resistant A375 xenografts. EphB4, p-AKT and p-ERK expression levels in the tumor tissues were detected with western blot analysis.

The Eph (erythropoietin producing hepatocellular) family of receptors make up the largest subgroup of the receptor tyrosine kinase, which could be divided into the A- or B-type based on their interactions with Ephrin ligands (20,21). Durg et al. reported that EphA2 is an important oncogene and potentially a common source of 'addiction' for many melanoma cells (22). Benchun et al. also mentioned that EphA2 was an important factor for vemurafenib resistance (23). However, we did not found high *EphA2* gene expression in the current resistant model. Since EphA2 and EphB4 have no cross-binding to Ephrins of the opposing subclass and NVP-BHG712 was well designed and further optimization led to a potent and selective inhibitor of the EphB4 receptor tyrosine kinase, we proposed *EphB4* overexpression contributed to the cisplatin resistance in the current model. Nevertheless, the relationship between EphB4 and apoptosis pathway is still unclear and needed further investigation.

It seems that melanoma would be a coreless disease with so many participants involved in cell survival. Therefore, it is possible to find the way cancer cells are using to escape treatment, and find the core of the intricate pathways in tumors with specific genetic background. In the current studies we focus on combined therapeutic strategies using chemotherapy and molecular inhibitors, and try to find pathway-specific novel therapeutics for sensitization to existing therapies with lower toxicity, which represent a significant progress made in understanding the biology of therapy resistance and in the management of malignant melanoma.

In conclusion, this study indicated overexpression of EphB4 in A375 xenografts treated with cisplatin for a long time period contributed to the tumor resistance. However, there are still some problems needed to be address in order to understand fully the delicate interplay between molecular factors that promote cell survival. If we are going to design new strategies to circumvent multifactorial mechanism of cisplatin resistance more effectively, the additional knowledge is needed.

Conflicts of interest

The authors declare that they have no competing interests.

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