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Original Article

c-Jun and Camk2a contribute to the drug resistance of induction docetaxel/cisplatin/5-fluorouracil in hypopharyngeal carcinoma

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Abstract: Hypopharyngeal carcinoma (HPC) is a subtype of head and neck squamous cell carcinoma, and prognosis has improved significantly over the past three decades. Induction docetaxel/cisplatin/5 fluorouracil (TPF) chemotherapy is regarded as the standard of treatment for locoregionally advanced HPC. However, patients who do not respond to cisplatin suffer, rather than benefit, from chemotherapy treatment. The goal of this study was to identify molecules involved in TPF resistance and to clarify their molecular mechanisms. Using the FaDu cell line as the cell model, the TPF IC50 was identified, and c-Jun, IL6, Camk2a, c-fos knockdown using siRNAs resulted in a significant declined TPF IC50. Retrospective analysis of the expression status of c-Jun, IL6, Camk2a, and c-fos by immunohistochemistry staining in sectioned HPC tissues from TPF-sensitive and TPF-insensitive patients shows that Camk2a and c-Jun were associated with the clinical pathogenesic features in HPC. The *in vitro* experiments also indicate that both Camk2a and c-Jun were responsive to TPF treatment. This study identified Camk2a and c-Jun as candidate genes that confer induction TPF resistance, which would help in the discovery of potential therapeutic markers and in developing a personalized and precise treatment approach for HPC patients.

Keywords: Hypopharyngeal cancer, induction TPF, drug resistance, c-Jun, Camk2a

Introduction

Hypopharyngeal carcinoma (HPC) is an uncommon cancer and accounts for approximately 3% to 5% of all head and neck squamous cell carcinomas (HNSCC) [1, 2]. Tissues of HNSCC incidence include the oral cavity, oropharynx, hypopharynx, and larynx [3]. Most HPC cases are locally or locoregionally advanced at presentation. Surgery and/or radiotherapy are commonly applied as definitive therapies, often in combination with chemotherapy [4]. Platinum-based concurrent chemoradiotherapy (CRT) has become a popular approach and the standard treatment, and induction chemotherapy (ICT) is also performed for locally advanced cancer when the goal is to preserve the larynx [5, 6].

Generally speaking, compared with radiotherapy alone, concurrent chemoradiotherapy significantly improves survival rates for patients with squamous cell carcinoma of the head and neck [7, 8]. A phase II-III trial shows that induction TPF followed by concomitant treatment versus concomitant treatment alone in locally advanced head and neck cancer improved the therapy outcome [9]. The most common ICT recipe is consisted of docetaxel/cisplatin/5 fluorouracil (TPF). Recently, multiple clinical trials of induction TPF have been carried out and several modified TPF recipes were developed, indicating the clinical potential of induction TPF. TPF is not well tolerated in clinical practice and hard to accomplish because of severe toxicity.The overall response rate after a new biweekly TPF recipe chemotherapy was significantly improved in a Phase II trial for advanced esophageal squamous cell carcinoma [10]. Induction chemotherapy with dose-modified TPF in Asian patients with borderline resectable or unresectable head and neck cancer is a suitable option [11]. A high response rate and good tolerability were obtained in the early response of esophageal cancer to neoadjuvant chemotherapy with TPF treatment [12]. TPF treatment may be applied both to adolescents and adults [13].

However, some patients still do not respond well to TPF-induced chemotherapy [14, 15]. In a phase II clinical trial, the combination therapy of ICT and CRT did not show any advantage compared to CRT alone, while the incidence of adverse events increased [5]. Thus, discrimination of TPF sensitivity is of high clinical significance. It may be a viable strategy to evaluate the chemotherapeutic effect of TPF in advance on the genetic level. Based on the advances of HPC molecular biology, several cisplatin-resistance genes including BST2, TIMELESS and COX-2 were identified [16-18]. Patients with insensitivity to induction TPF benefit little from the CRT followed chemotherapy and suffer from delayed treatment, therefore finding the right biomarkers for induction TPF is essential and critical for clinical practice. However, no related research has been reported on TPF-resistant genes. In the present study, we identified c-Jun and Camk2a as candidate TPF-resistant genes in HPC. These findings suggest that c-Jun and Camk2a likely mediate TPF resistance in HPC, offering guidance for personalized and precise treatment strategies for patients with HPC.

Materials and methods

Patients and samples

Surgical specimens were collected from 108 cases of hypopharyngeal squamous cell carcinoma (6 females and 102 males). The inclusion criteria were as follows: primary hypopharyngeal squamous cell carcinoma confirmed through histopathology, well-preserved specimens, complete clinical records and pathologic data, and no anti-tumor treatment before operation, including radiotherapy, chemotherapy, biotherapy, and so on.

This study was conducted in accordance with the Declaration of Helsinki. This study was conducted with approval from the Ethics Committee of the Capital Medical University. Written informed consent was obtained from all participants.

FaDu cell culture, transient transfection

FaDu (ATCC) cells were cultured in RMPI1640 supplemented with 10% FBS, 2 mM glutamine, penicillin G sodium (100 U/ml), and streptomycin sulfate (100 U/ml). The incubation criteria were set as saturated humidity, 5% CO_2, and thermostatic 37°C. siRNAs were synthesized by Oligio, BeiJin Inc and transfected using lipofectamine 2000.

MTT assay

Cells (1×10^5/well) were plated in 0.2 ml of medium/well in 96-well plates. For MTT assay, 10 μl of MTT (5 mg/ml) was added. The plates were incubated for 4 h in 5% CO_2 incubator for cytotoxicity. After incubation, the medium from the wells was removed carefully then 100 μl of DMSO was added to each well. 10 minutes later, presence of viable cells was visualized by the development of purple color due to formation of formazan crystals. The OD (optical density) values were read at 595 nm by using Microplate reader. DMSO serve as a blank. Measurements were performed, and the concentration required for a 50% inhibition of viability (IC50) was determined graphically Standard Graph was plotted by taking concentration of the drug on the X axis and relative cell viability on the Y axis.

Cell viability (%) = Mean OD/Control OD × 100%

RNA extraction, first strand cDNA synthesis and relative quantitative real-time PCR: Total RNA was extracted using Trizol reagent (Life Technologies, Carlsbad, USA) according to the manufacturer's protocol. RNA concentration was determined using a Nanodrop200C spectrophotometer (Thermo Scientific, Waltham, USA), and RNA integrity was detected by 1% agarose gel electrophoresis and staining with ethidium bromide (Biovision, San Francisco, USA).

One microgram of total RNA was incubated with DNase I (NEB, Ipswich, USA) to eliminate contaminant DNA at 37°C for 5 min and later supplemented with EDTA (5 mM) and heated at 75°C for 5 min. Random primers were added for first strand cDNA synthesis using M-MLV Reverse Transcriptase (Life Technology, Carlsbad, USA) according to the manufacturer's protocol, and human β-actin was used as an internal control. Primer sequences: Camk2a-F (5'-GAAGAGCGATGGTGTGAAGA-3'), Camk2a-R (5'-CGGGTGTTGGTGCTCTCTGA-3'), JUN-F (5'-GCGGACCTTATGGCTACAGT-3'), JUN-R (5'-GTGAGGAGGTCCGAGTTCTT-3').

Table 1. Knockdown using siRNA altered the TPF IC50

Groups	IC50 (ug/ml)		
	Cisplatin	Docetaxel	5-FU
CK	0.165	0.247	4.603
NC	0.148	0.223	4.156
JUN	0.111	0.167	3.115
IL6	0.0667	0.1	1.87
Camk2a	0.126	0.188	3.517
FOS	0.0924	0.139	2.586
PAK2	0.113	0.169	3.161
SFRP1	0.199	0.299	5.601

Relative gene expression was detected by relative quantitative real-time polymerase chain reaction (PCR) using Thunderbird SYBR Green qPCR Mix (TOYOBO, Osaka, Japan). The quantitative PCR was performed using the CFX-96 Touch™ Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, USA), following the manufacturer's instructions. The conditions for the reaction were as follows: initial denaturation at 94°C for 1 min, followed by 40 cycles starting with denaturation at 94°C for 10 s, annealing at 58°C for 10 s, and extension for 20 s. The fold change of gene expression was determined using the $2^{-\Delta\Delta CT}$ method.

Immunohistochemistry (IHC)

Paraffin wax-embedded sections were deparaffinized with 100% xylene and ethyl alcohol; endogenous enzyme was inactivated by treatment with 3% hydrogen peroxide for 5-10 min. After heat-induced antigen retrieval by heating to boiling point twice in 0.01 M citrate buffer solution (pH 6.0), sections were blocked using 5% rabbit serum for 20 min at room temperature. Immunohistochemical staining was performed using a streptavidin-biotin complex immunohistochemistry kit (Boshide Biotechnology, Wuhan, China). Briefly, sections were incubated overnight at 4°C with 1:50 diluted goat antihuman c-Jun or Camk2a polyclonal antibody (clone N-19, SC-6967; Santa Cruz Biotechnology, Santa Cruz, CA, USA), then washed three times in phosphate-buffered saline (PBS; pH 7.4) Sections were then treated with biotinylated rabbit antigoat immunoglobulin (Boshide Biotechnology) for 20 min at room temperature and washed three times in PBS, followed by incubation with streptavidin-biotin-peroxidase complex for 20 min at room temperature. Immunostaining was visualized using a DAB kit (Boshide Biotechnology). Sections were incubated with fresh 3,30-diaminobenzidine (DAB) for 5-15 min at room temperature, counterstained with hematoxylin and mounted in neutral gum. Specimens were viewed using a light microscope, the level of expression was assessed by using Image Pro-Plus (IPP) (Silver Springs, MD) [19].

Statistical analysis

Results were expressed as mean ± SD, and the significance was determined by Student's t-test or one-way ANOVA followed by Duncan's multiple range test. A *P*-value <0.05 was considered significant. All statistical analyses were performed using SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Knockdown key oncogenes using siRNA significantly altered the TPF IC50

Changes in gene expression patterns are closely related to tumor resistance to chemotherapy. However, which gene contributes to the efficacy of TPF chemotherapy is still unknown. In order to explore this issue, we test whether changing certain gene expression levels affects the lethality of TPF to FaDu cells, a human epithelial cell line isolated and established from a squamous cell carcinoma of the hypopharynx [20]. First, we determined the half maximal inhibitory concentration (IC50) of 5-FU, cisplatin and docetaxel to FaDu cells by using MTT assay. The TPF reagent containing 5-FU (4.156 μg/ml), cisplatin (0.148 μg/ml) and docetaxel (0.223 μg/ml) was used for the subsequent experiments.

By combining a literature review and previous research by our group [21], then we selected for c-Jun, IL6, Camk2a, c-FOS, PAK2, SFRP1 and tried to figure out that how knockdown of the selected oncogenes affected the TPF-sensitivity. As **Table 1** shows, knockdown of PAK2 (cisplatin (0.113 μg/ml), docetaxel (0.169 μg/ml), 5-FU (3.161 μg/ml)), SFRP1 (cisplatin (0.199 μg/ml), docetaxel (0.299 μg/ml), 5-FU (5.601 μg/ml)) didn't altered the TPF IC50. Meanwhile, the TPF IC50 declined significantly after c-Jun (cisplatin (0.111 μg/ml), docetaxel (0.167 μg/ml), 5-FU (3.115 μg/ml)), IL6 (cisplatin (0.0667 μg/ml), docetaxel (0.1 μg/ml), 5-FU (1.87 μg/ml)), Camk2a (cisplatin (0.126 μg/ml), docetaxel (0.188 μg/ml), 5-FU (3.517 μg/ml)),

Table 2. Relationship between the expression of Camk2a and c-Jun and clinicopathological manifestations in hypopharyngeal cancer

Parameter	N	Camk2a (mean ± SD)	*P* value	c-Jun (mean ± SD)	*P* value
Gender					
Male	102	0.000194115±0.000201977	0.441	0.000074246±0.000066154	0.510
Female	6	0.000129635±0.000105120		0.000092722±0.000074256	
Age (years old)					
<60	65	0.000185614±0.000187485	0.753	0.000080985±0.000067594	0.274
≥60	43	0.000197967±0.000215223		0.000066638±0.000064323	
Pathologic differentiation degree					
High	7	0.000120431±0.000228472	0.431	0.000081801±0.000073993	0.898
Moderate	89	0.000189068±0.000196564		0.000073889±0.000065722	
Low	12	0.000242289±0.000228472		0.000081727±0.000072357	
Primary site					
T2-3	63	0.000181266±0.000211802	0.568	0.000073601±0.000067162	0.758
T4	45	0.000203507±0.000178680		0.000077613±0.000065959	
Lymph nodes					
N0	34	0.000116878±0.000119326	0.008	0.000085338±0.000069612	0.288
N+	74	0.000224374±0.000217709		0.000070648±0.000064805	
Stage					
III	22	0.000110032±0.000183077	0.032	0.000070577±0.000068377	0.712
IV	86	0.000211126±0.000197519		0.000076474±0.000066219	

c-fos (cisplatin (0.0924 μg/ml), docetaxel (0.139 μg/ml), 5-FU (2.586 μg/ml)) knockdown, among which, IL6 and c-fos knockdown led to the most dramatic IC50 decline. These results indicate that these genes could play a role of conferring TPF-resistance and targeting these genes with a combination of TPF could make a better recipe for HPC therapy. Then we examined the expression status of those genes in clinical samples and screened the candidate TPF-resistance genes.

The expression status of c-Jun and Camk2a was associated with the TPF sensitivity

Then we tried to reason out the association between the expression status of key oncogenes and TPF-sensitivity. A retrospective analysis of the expression status of Camk2a and c-Jun was done by immunohistochemistry staining in sectioned HPC tissues from TPF-sensitive and TPF-insensitive patients. TPF-sensitivity and TPF-insensitivity were defined according to the prognosis results. As **Table 2** shows, the expression of c-Jun in hypopharyngeal carcinoma was not related to age, sex, degree of differentiation, depth of invasion, lymph node metastasis,orclinical stage (P>0.05). The expression of Camk2a was highly expressed in hypopharyngeal carcinoma with lymph node metastasis or clinical stage IV disease (P<0.05), but the expression is not related to age, sex, degree of differentiation, and depth of infiltration (P>0.05). As **Figure 1** shows, Camk2a was down-regulated in the TPF-insensitive group with $P = 0.185$, meanwhile c-Jun was up-regulated in the TPF-insensitive group with $P = 0.0008$. The up-regulation of c-Jun was quite dramatic with a more than fivefold increase, which indicates that c-Jun is a more sensitive candidate biomarker of TPF-resistance. On the contrary, the expression level of IL6, c-fos, PAK2, SFRP1 was slightly altered and no overall change was observed (data not shown). Thus, if a patient expressing less c-Jun or more Camk2a is suitable for treatment with TPF. On the basis of the above results, we chose Camk2a and c-Jun as the candidate TPF-resistant genes and checked whether the two genes were responsive to TPF treatment.

The expression level of c-Jun and Camk2a was upregulated when FaDu cells were treated with TPF

We also wondered if the expression of c-Jun and Camk2a in HPC cells would be regulated by TPF. Consequently, the mRNA levels of c-Jun and Camk2a were examined after the FaDu cells were treated with TPF. Results showed

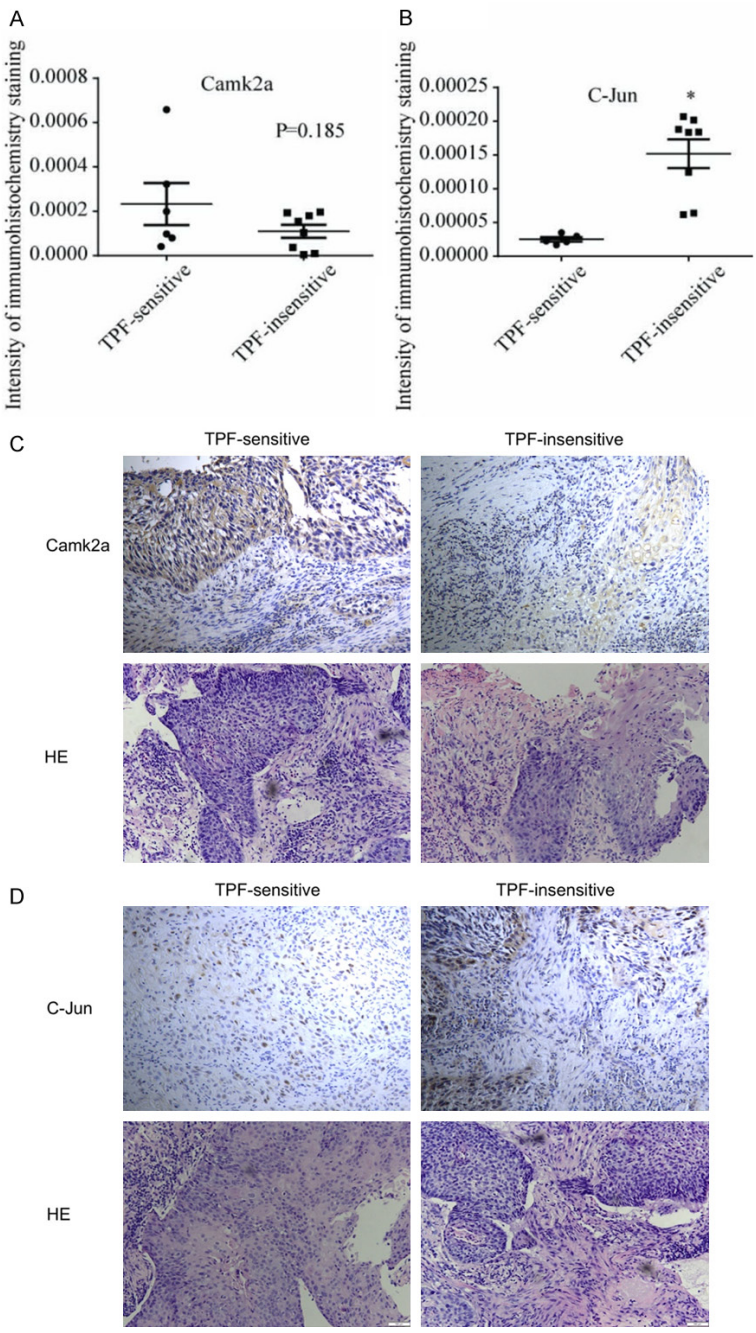

Figure 1. Retrospective analysis of the expression status of Camk2a and c-Jun by immunohistochemistry staining in sectioned HPC tissues from TPF-sensitive and TPF-insensitive patients. A, B. The statistical results of immunohistochemical staining for Camk2a and c-Jun in TPF-sensitive and TPF-insensitive HPC biopsies. C. Representative images of immunohistochemical staining for Camk2a and HE staining in TPF-sensitive and TPF-insensitive HPC biopsies. D. Representative images of immunohistochemical staining for c-Jun and HE staining in TPF-sensitive and TPF-insensitive HPC biopsies.

that TPF treatment of FaDu cells led to the up-regulation of both c-Jun and Camk2a on the mRNA level (**Figure 2**), indicating both Camk2a and c-Jun are both responsive to TPF treatment *in vitro*. Compared to c-Jun, the up-regulation of Camk2a was more remarkable and Camk2a was more sensitive to TPF treatment. Combined results suggest that the expression of c-Jun and Camk2a might confer the TPF-resistance of HPC.

Discussion

The primary endpoint of this study was to screen the TPF-resistance genes and discover potential therapeutic markers for HPC patients. In the present study, we demonstrated that c-Jun, IL6, Camk2a, and c-fos knockdown using siRNA resulted in a significant decline in TPF IC50 and sensitivity to TPF. The expression status of c-Jun, Camk2a by immunohistochemistry staining in sectioned HPC tissues from TPF-sensitive and TPF-insensitive patients was associated with the clinical pathogenesis features in HPC. Both Camk2a and c-Jun were responsive to TPF treatment. Thus, Camk2a and c-Jun might be considered as candidate therapeutic markers of TPF sensitivity in clinical practice.

Chemoinsensitization to cisplatin in HPC cells is mediated through several ways, including the inhibition of mitotic arrest [22-24], the resistance to apoptosis [23-26], increased tumor stemness [27], acquisition of epithelial-mesenchymal transition [28], metastasis [24], activation of DNA damage and repair pathways [29, 30]. In our study, knockdown of c-Jun and Camk2a resulted a decline in IC50 of TPF and significantly increased sensitivity to TPF (**Table**

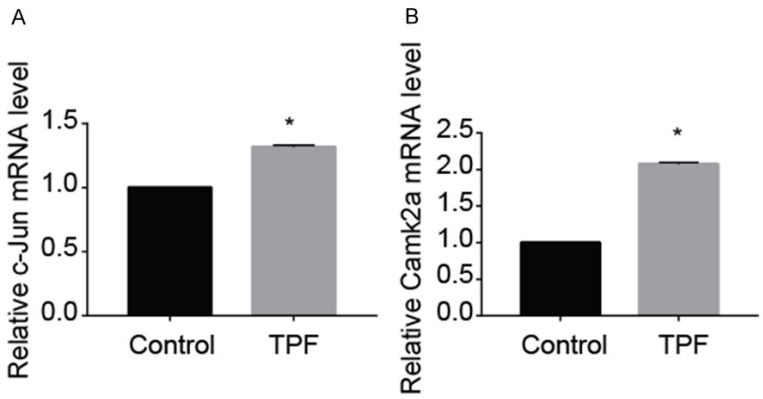

Figure 2. The expression level of c-Jun and Camk2a was upregulated when FaDu cells were treated with TPF. The FaDu cells were treated with TPF with an IC50 concentration, after 48 h, c-Jun (A) and Camk2a (B) expression level was analyzed by RT-qPCR.

1). Especially, we noticed that knockdown of IL6 and c-fos led to a dramatic TPF IC50 decline, which means that a modified and sensitive TPF therapy could be developed by targeting these genes. Reports show that the correlation between IL6 and the prognosis of HPC was established, and elevated IL6 levels were positively correlated with poorer 9-year overall survival (OS), disease-free survival (DFS), distant metastasis-free survival (DMFS), and lung metastasis-free survival (lung-MFS) [31], enhanced IL-6/IL-6R signaling promoted growth and malignant properties of HPC cancerous cells [32]. Knockdown of c-fos may enhance c-fos-associated growth arrest and apoptotic cell death in HPC cells [33], thus promoting the TPF IC50 sensitivity.

Ping *et al* report that MLN4924 suppressed c-Jun degradation in human HPC cells increased cell proliferation, cell apoptosis, and triggered the cell cycle [23]. This study confirmed c-Jun as the major effector molecule conferring TPF resistance. c-Jun is a well-known substrate of SAG-SCF E3 ligase, forming the AP-1 early response transcription factor with c-fos [34], and c-Jun might confer the TPF resistance by triggering cell cycle progression and facilitating anti-apoptotic activity. The variated expression confirmed the possible role of TPF-resistance in HPC (**Figure 1**). Surprisingly, TPF treatment led to an up-regulation of c-Jun in HPC cells (**Figure 2**), indicating that c-Jun was responsive to TPF treatment.

Unlike c-Jun, Camk2a is not well characterized in cisplatin-resistance. A research shows that in hypopharyngeal squamous cell carcinoma, the expression of Camk2a was altered in TPF chemotherapy-responsive tissues and [35]. Combined with the data of the clinical samples and the in vitro experiments, these findings suggested that TPF chemotherapy may induce an increase in c-Jun and Camk2a in TPF-sensitive patients, whose levels were otherwise altered before TPF therapy. Camk2a belongs to the Ca^{2+}/calmodulin-dependent protein kinase II subfamily and has been proven to be essential for normal learning and synaptic plasticity in mice, and human brain development [36]. So far, its role in tumorigenesis is not clear. Camk2a was discovered to affect treatment outcomes in patients with non-Hodgkin lymphoma or glioblastoma [37, 38], this study indicates that Camk2a might be involved in the chemoresistance of other type of tumors.

In this study, Camk2a and c-Jun are assumed and proved to the candidate TPF-resistance genes. Meanwhile, the limitations of this study should be taken into notice. We noticed an alteration of the expression status of Camk2a and c-Jun, but the expression level of IL6, c-fos, PAK2, and SFRP1 was slightly altered and no overall change was observed. Due to small numbers of clinical samples, effects on important clinical outcomes could not be adequately assessed. More clinical samples with a larger magnitude should utilized to examine the association between the expression status of Camk2a and c-Jun and the clinicopathologic features of HPC. We proved that Camk2a and c-Jun were responsive to TPF treatment *in vitro*, however, due to the TPF treatment procedure, we didnotexamine the expression status of Camk2a and c-Jun in the clinical samples after TPF treatment, thus the responsiveness of Camk2a and c-Jun in clinical practice was unknown. Therefore, the results and conclusions we gain from this research are an initial conclusion.

In conclusion, we identified c-Jun and Camk2a as candidate genes conferring the TPF-resistance in HPC. This study could provide an insight into the mechanism underlying TPF che-

motherapeutic response in HPC, and these candidate biomarkers maycontribute to HPC individualized treatment.

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Disclosure of conflict of interest

None.

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