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Regular paper

JNK promotes the progression of castration-resistant prostate cancer

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Background: Prostate cancer is one of the most common cancers in men worldwide. This study aims to elucidate the roles of c-Jun N-terminal kinase (JNK) in the progression of castration-resistant prostate cancer (CRPC). Methods: JNK overexpressing and knockdown cell lines were established on the PC-3 prostate cell line. qPCR and Western blotting were performed to determine the mRNA and protein levels of target genes in prostate tissues and cell lines. MTT and Matrigel invasion assays were conducted to evaluate the cell viability and invasive ability, respectively. The Kaplan-Meier estimator was performed to estimate the overall survival rate and second progression-free survival rate. Pearson's correlation coefficient was used to evaluate the relationship between JNK and prostate-specific antigen (PSA). Results: Relative JNK expression was correlated with Gleason score and PSA value in patients with CRPC. Kaplan-Meier analysis revealed that patients with low JNK expression exhibited high overall survival and second progression-free survival rate. *In vitro* assays demonstrated that JNK overexpression promoted cell viability and invasion as well as the protein expressions of extracellular signal-regulated kinase (ERK) and matrix metalloproteinase 1 (MMP1) in PC-3 cell lines. Conclusions: JNK overexpression promotes the development of CRPC via the regulation of ERK and MMP1.

Keywords: Prostate cancer, PC3, prostate-specific antigen, castration, JNK

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Abbreviations: AR, androgen receptor; CRPC, castration-resistant prostate cancer; ECOG, Eastern Cooperative Oncology Group; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MMP1, matrix metalloproteinase 1; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI3K, phosphoinositide 3-kinase; PSA, prostate-specific antigen; SD, standard deviation

INTRODUCTION

Prostate cancer is ranked as the second most common cancer among men and the fourth most common cancer among all types of cancers worldwide (Siegel *et al.*, 2021; Sung *et al.*, 2022). The incidence of prostate cancer is gradually increasing over the past decades (Schatten, 2018; Siegel *et al.*, 2022). For instance, the incidence rates of prostate cancer in the advanced stage have increased by 4%~6% annually from 2014 to 2018 (Schatten, 2018). In 2020, there are an estimated 1.4 million people who are diagnosed with prostate cancer in the world (Sung *et al.*, 2021). Depending on the stage of prostate cancer and its metastasis status, therapeutic options for prostate cancer include surgery, chemotherapy, radiation, androgen deprivation therapy (ADT), and combination therapy (Keyes *et al.*, 2013; Sowery *et al.*, 2007). Androgens are known as a key factor for stimulating prostate cancer cell growth, by lowering or stopping androgens are able to suppress proliferation and invasion of cancer cells (Harris *et al.*, 2009; Sharifi *et al.*, 2005). As one of the standard therapy options for prostate cancer, ADT is commonly used for patients with advanced prostate cancer and is shown to effectively control tumor growth. However, one of the major challenges is castration resistance, as supported by ADT therapy became less effective towards inhibit prostate cancer growth, and most of the patients who had ADT therapy developed castration-resistant prostate cancer (CRPC) (Chandrasekar *et al.*, 2015; Harris *et al.*, 2009; Sharifi *et al.*, 2005). Hence, developing new strategies that can overcome castration resistance is urgently required.

Several molecular pathways, such as the androgen receptor (AR) signaling pathway, c-Jun-N2-terminal kinase (JNK) signaling pathway, phosphoinositide 3-kinase (PI3K) signaling pathway, etc., are known to be involved in the prostate cancer (Mazaris & Tsiotras, 2013; Shtivelman *et al.*, 2014). The major underlying molecular mechanisms inducing CRPC are closely linked with the dysregulation of those signaling pathways (Dutt & Gao, 2009). For instance, the activation of PI3K leads to the phosphorylation of Akt and downstream cellular events, which is known as one of the major drivers of CRPC (Bitting & Armstrong, 2013). The aberrant modifications of AR increase the post-transcriptions of AR-related genes, leading to castration resistance (Yuan *et al.*, 2014). Interestingly, *in vitro* and *in vivo* data supported that targeting PI3K and AR signaling pathways by a combination of AR inhibitor (BEZ235) and mTOR inhibitor (EPI-002) effectively inhibited tumor growth as well as reduced prostate cancer resistance to ADT (Kato *et al.*, 2016). In addition, JNK is known to play a crucial role in the invasive prostate cancer (Xu & Hu, 2020). In 2020, Li and colleagues reported that a combination of JNK inhibitor (AS602801) and AR inhibitor (Enzalutamide) effectively suppressed proliferation and invasion of androgen-resistant prostate cancer cells *in vitro* and reduced tumor growth *in vivo* (Li *et al.*, 2020). These results suggest that targeting JNK might be an effective strategy for ameliorating prostate cancer resistance to ADT.

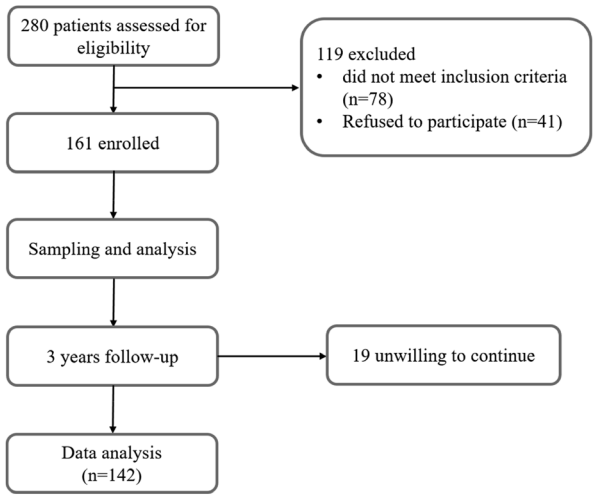

Figure 1. Schematic diagram of study profile.

MATERIAL AND METHODS

Enrollment of participants

As demonstrated in Fig. 1, a total of 280 patients were enrolled to assess eligibility. All patients have read and signed the informed consent. The patients that were aged less than 18 years old were excluded. Eastern Cooperative Oncology Group (ECOG) performance was assessed for all patients. Patients with ECOG performance status scored at 0 or/and 1 were included. In addition, only CRPC patients CRPC without any metastatic diseases were included. Finally, 161 patients who meet eligibility criteria participated in this study. The study was approved by the ethics committee of LONGHUA Hospital Shanghai University of Traditional Chinese Medicine, and the patients signed written consent.

Construction of cell lines

The PC3 cell line was obtained from ATCC (Manassas, VA) and cultured in the complete RMPI medium containing 10% fetal bovine serum and Penicillin-Streptomycin antibiotics. The construction of JNK overexpressing PC3 cell line (JNK OE) and JNK knockdown cell line (sh-JNK) were performed as previously reported. The cells were seeded into the 6-well microplates and incubated overnight to reach 70~80 % confluency. After that, the cells were transfected with plasmid containing JNK sequence or the plasmid containing JNK sequence plus shJNK by using Lipofectamine Transfection Reagent (ThermoFisher Scientific, Waltham, MA).

Cell viability and cell invasion assays

To evaluate the cell viability and cell invasion, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and Matrigel invasion assay were determined, respectively. For the MTT assay, cells including PC3 cells, JNK overexpressing PC3 cells, and PC3 cells that were transfected with shJNK were seeded into the 96-well microplates at 1000 cells per well. Abiraterone at concentrations of 5, 10, and 20 μM was added and incubated for another 3 days. Next, MTT working solution (5 mg/mL) was added to each well and incubated for 4 hours in the dark. Dimethyl sulfoxide solution was then added to dissolve the purple crystals. The plate was read by using a microplate reader at wavelengths of 590 nm and 690 nm (reference wavelength).

For the Matrigel invasion assay, the invasion chambers were coated with Matrigel (0.5 mg/mL). Next, the cells were seeded into the upper chamber with serum-free medium and the lower chamber was filled with complete RPMI medium. Next, the cells were incubated for 48 hours and the non-invasive cells in the upper chamber were removed, and invasive cells were then fixed and stained with crystal violet staining solution. The cells were observed under a microscope and the number of invasive cells was counted.

qPCR

The prostate cancer tissue samples were collected from the participants. Total RNAs were isolated from the prostate cancer tissues by using the RNA extraction reagent (Invitrogen, Waltham, MA). The cDNA library was then constructed by using a reverse transcription kit (Invitrogen). The primers of JNK and internal control *GADPH* were synthesized by Descript. After that, the PCR reaction was performed and the mRNA levels of JNK were normalized to *GAPDH*. The Melt curves were applied to ensure the accuracy of the PCR reaction.

Western blotting

Protein was isolated from the prostate cancer tissues as previously reported (Yang *et al*., 2017). In brief, a cold radio-immunoprecipitation assay reagent (Bio-Rad, Hercules, CA) was added to the shredded tumor tissues. A homogenizer was used to create the uniformed protein mixture buffer and centrifuge was used to remove the tissue debris. Next, the bicinchoninic acid assay reagent was used to qualify the protein concentration. Protein was loaded into the gel followed by the incubation with the primary antibody solution including anti-JNK (1:2000, Abcam, Cambridge, MA), anti-extracellular signal-regulated kinase (ERK, 1:2000, Abcam), anti-matrix metalloproteinase 1 (MMP1, 1:2500, Abcam), or GADPH (1:3000, Sigma, St. Louis). Next, the membrane was blocked with blocking buffer (5% bovine serum albumin) followed by the incubation of the secondary antibody solution. Chemiluminescence blot imaging was used to detect the proteins. The protein expression was normalized to GAPDH.

Statistical analysis

The Kaplan–Meier estimator was performed to estimate the overall survival percentage and second progression-free survival in patients with low JNK and high JNK. Pearson's correlation coefficient was used to analyze the correlation between JNK expression and PSA levels in patients with CRPC. Prism Software was used for data analysis. Data were expressed as the means ± standard deviation (S.D.). One-way ANOVA with Tukey's multiple-comparisons test was applied for multiple groups. Any p-values that were less than 0.05 were considered as statistical difference.

RESULTS

Trial patients and their clinical characteristics

As displayed in Fig. 1, initially 280 patients were assessed for eligibility, and we finally recruited 161 eligible patients for this study. After sampling and analysis,

Table 1. Patient Characteristics.

	Low JNK level (n=71)	High JNK level (n=71)	*p*-value
Age (years)	63.2 (7.0)	62.8 (6.7)	0.729
ECOG performance status			
0	58 (81.7)	55 (77.5)	0.678
1	13 (18.3)	16 (22.5)	
PSA (ng/ml)	7.2 (2.7)	8.8 (3.2)	0.002
Gleason score			
≤7	48 (67.6)	30 (42.3)	0.004
>7	23 (32.4)	41 (57.7)	
Metastasis during follow-up			
Bone metastases	21 (29.6)	29 (40.8)	0.219
Lymph node metastases	24 (33.8)	38 (53.5)	0.028
Visceral metastases	13 (18.3)	17 (23.9)	0.538
Therapies			
Abiraterone	63 (88.7)	61 (85.9)	0.802
Docetaxel	52 (73.2)	55 (77.5)	0.697
Cabazitaxel	12 (16.9)	14 (19.7)	0.829

Data are n (%) or mean (S.D.). ECOG, Eastern Cooperative Oncology Group. PSA, prostate-specific antigen.

we conducted a 3-year follow-up study. After excluding the participants who were unwilling to continue, we analyzed the data from the 142 patients. Two cohorts including patients with low JNK levels and patients with high JNK levels were included (Table 1). We noticed that the levels of PSA in patients with low JNK levels were significantly decreased as compared to patients with high JNK levels (p=0.002). In addition, we observed less lymph node metastases in patients with low JNK levels as compared to patients with high JNK levels (24 *vs* 38). These results demonstrated that the levels of JNK were associated with the levels of PSA and lymph node metastases in patients with CRPC.

Relative JNK expression was correlated with Gleason score and PSA value in patients with CRPC

Next, we determined the relative JNK expression in CRPC patients with different Gleason scores. Interestingly, we found that the relative JNK expression in CRPC patients with low Gleason score (Gleason score ≤7) was significantly decreased as compared to the CRPC patients with high Gleason score (Gleason score>7), indicating the correlation between JNK and Gleason score (Fig. 2a). Besides, we also observed a positive correlation of JNK and PSA in patients with CRPC (Fig. 2b).

Patients with low JNK expression exhibited high overall survival and second progression-free survival rate

To explore the relationship between JNK expression and survival rate, we conducted a 3-year follow-up study. We found that patients with low JNK expression showed a higher overall survival rate as compared with those patients with high JNK expression (p=0.033, Fig. 3a). Consistently, we observed that patients with low JNK expression showed high second progression-free survival rate as compared with those patients with a high JNK expression (p=0.005, Fig. 3b). These results supported that patient with low JNK expression exhibited higher overall survival and second progression-free survival rate as compared to patients with high expression.

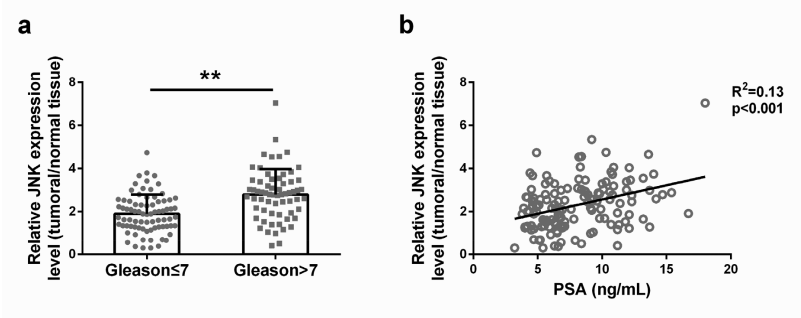

Figure 2. Relative JNK expression was correlated with Gleason score and PSA value in patients with castration-resistant prostate cancer (CRPC).
(**a**) The relative JNK expression in patients with different Gleason scores. (**b**) Pearson correlation showed the correlation of relative JNK expression with PSA levels in patients with CRPC. Data were expressed as the means ± S.D. **p<0.01.

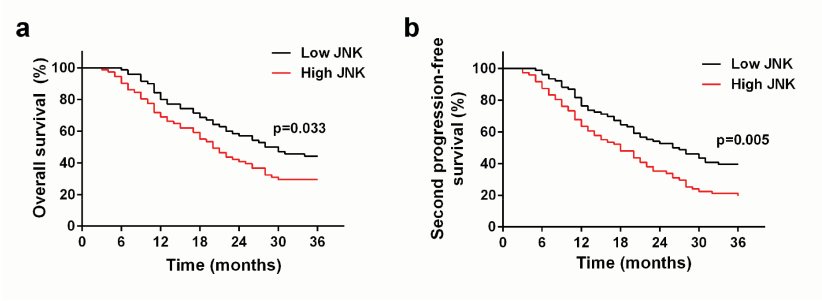

Figure 3. Kaplan-Meier analysis estimated the overall survival percentage (a) and second progression-free survival (b) in patients with low JNK and high JNK.

JNK overexpressing promoted cell viability and invasion of PC3 cells

To confirm the roles of JNK expression in the CRPC, we tested cell viability and invasion in the abiraterone-treated prostate cancer cell line. We found that the cell survival rate of PC3 cells was significantly decreased in the treatment of abiraterone. However, cells with JNK overexpression displayed a higher survival rate as compared with those cells transfected with shJNK (Fig. 4a). Consistently, cell invasion assay also showed that JNK overexpressing cell lines have a higher number of invasive cells, whereas cells that were transfected with shJNK have a smaller number of invasive cells (Fig. 4b). Taken together, these results suggested that JNK overexpression promoted cell viability and invasion of prostate cancer cells.

JNK overexpression enhanced the protein levels of ERK and MMP1 in PC3 cells

Finally, we explored the changes of other cytoplasm proteins in the presence of JNK overexpression in PC3 cells. Cytoplasm proteins including ERK and MMP1 were determined by using Western blotting. The protein expressions of JNK were significantly increased in the JNK overexpressing cells, indicating that the JNK overexpressing cells were successfully constructed (Fig. 5a and 5b). In addition to JNK, we found that protein expressions of ERK and MMP1 were also increased in the JNK overexpressing cells as compared to the PC3 cells and JNK overexpressing cells that were transfected with shJNK (Fig. 5a, 5c, and 5d). These results suggested that JNK overexpression enhanced the protein levels of ERK and MMP1 in PC3 cells.

DISCUSSION

In this study, we found that JNK expression was positively correlated to Gleason score and PSA value in patients with CRPC. Interestingly, high JNK expression in prostate tumor tissues is associated with low overall survival and second progression-free survival rate as compared with those patients with low JNK expression. In vitro data suggested that JNK overexpression promotes cell proliferation, invasion, and sensitivity to abiraterone treatment, whereas inhibiting JNK reversed these cellular events, suggesting the relationship between JNK and CRPC. Moreover, targeting JNK also resulted in the changes in ERK and MMP1, indicating the roles of JNK on prostate cancer associated with those cytoplasm proteins.

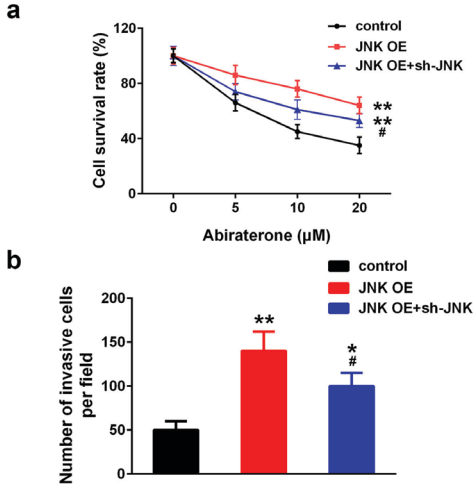

Figure 4. Effects of JNK on cell viability and invasion of PC3 cells. PC3 cells with JNK overexpressing or knockdown were constructed.
(**a**) JNK overexpressing enhanced abiraterone resistance, whereas the presence of sh-JNK decreased cell viability in PC3 cells. Besides, (**b**) Cell invasion of PC3 cells was evaluated by using the Transwell assay. Data were expressed as the means ± S.D. *p<0.05, **p<0.01 as compared with the control group. #p<0.05 as compared with the JNK OE group.

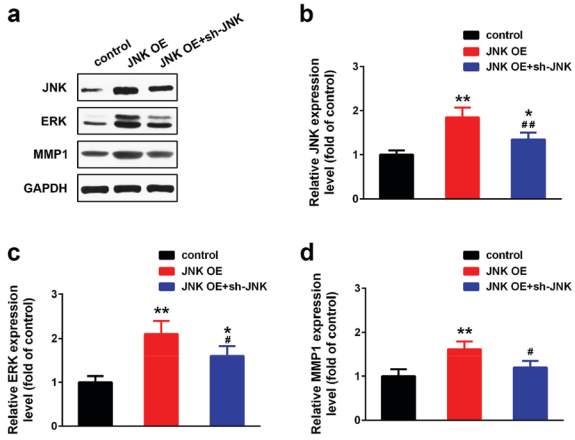

Figure 5. Effect of JNK on the protein levels of ERK and MMP1 in PC3 cells.
(**a**) Protein expressions of JNK (**b**), ERK (**c**), and MMP1 (**d**) were determined by using Western blotting. The relative expressions of those biomarkers were expressed as the fold of control. Data were expressed as the means ± S.D. *p<0.05, **p<0.01 as compared with the control group. #p<0.05, ##p<0.01 as compared with the JNK OE group.

ADT is commonly recommended for patients with advanced prostate cancer. However, CRPC is still one of the major challenges (Gunner *et al*., 2016). Previous studies have demonstrated that JNK exerts a variety of functions in the regulation of cell differentiation, proliferation, invasion, and apoptosis in prostate cancer cells (Bode & Dong, 2007; Xu & Hu, 2020). Clinical studies revealed that JNK is highly expressed by the prostate cancer tissues (Bode & Dong, 2007). Interestingly, our study revealed less lymph node metastases in patients with low JNK levels as compared to those with high JNK levels. Consistently, JNK is also positively correlated to the levels of PSA and Gleason score, indicating that high JNK was more frequently observed in patients with advanced prostate cancer. In 2017, Zhang and colleagues reported the positive expression rate of the JNK in prostate patients with survival >5 years (60%) and survival ≤5 years (45%) in a cohort of forty samples (Zhang *et al*., 2017). Interestingly, they found that the phosphorylation of JNK was higher in patients with survival >5 years than with survival ≤5 years (Zhang *et al*., 2017). Our study revealed that patients with low JNK expression in their prostate cancer tissues showed a higher overall survival rate and second progression-free survival rate as compared with those patients with high JNK expression.

The link between JNK and AR signaling pathway has been well established by a series of studies (Li *et al*., 2020; Xu & Hu, 2020). For instance, interactions between AR and JNK substrates regulate the therapeutic effects of the chemotherapy agent, taxane, against CRPC (Tinzl *et al*., 2013). A reduction in the phosphorylation of JNK is associated with the repression of AR in prostate cancer cells (Shah & Bradbury, 2015). In this study, to confirm the roles of JNK in the CRPC, we established a series of cell lines including JNK overexpressing cells and JNK overexpressing cells that were transfected with shJNK. Interestingly, we observed that JNK overexpression promoted cell viability and invasion of PC3 cells that were treated with abiraterone. However, the presence of shJNK suppressed cell viability and invasion of PC3 cells. These results indicated that the levels of JNK are critical in the CRPC.

Targeting JNK exhibited broad effects in the treatment of prostate cancer (Xu & Hu, 2020). For instance, Kim and colleagues found that targeting ERK/JNK/AKT pathway with Oleanolic acid inhibits tumor growth in the DU145 animal model (Kim *et al*., 2018). Another study initiated by Ma and colleagues reported that Corosolic acid regulates cell apoptosis in CRPC in part by the regulation of Inositol-requiring enzyme 1 and the JNK signaling pathway (Ma *et al*., 2018). In our study, we found that targeting JNK regulated cell proliferation and invasion in the abiraterone-treated PC3 cells. However, when the JNK was overexpressed, the PC3 cells became less sensitive to the abiraterone treatment. These results confirmed that targeting JNK might be an effective strategy for regulating castration resistance of prostate cancer cells.

The Extracellular signal-regulated kinase (ERK) is another important kinase in CRPC, which is also known as a therapeutic target for prostate cancer (Gan *et al*., 2010). The phosphorylation of ERK is a feature of CRPC and is associated with the incidence of prostate cancer recurrence (Nickols *et al*., 2019). The activation of ERK leads to the overexpression of matrix metalloproteinase-1 (MMP-1) in the tumor tissues, which promotes tumor growth and metastasis (Nickols *et al*., 2019; Quintero-Fabián *et al*., 2019; Yang *et al*., 2015). In this study, we further explored the underlying mechanism of JNK on the regulation of CRPC. Interestingly, we observed a positive correlation of JNK with ERK and MMP1, as supported by the protein expressions of ERK and MMP1 were increased in the JNK overexpressing cells. However, the presence of shJNK inhibited the protein levels of ERK and MMP1 in PC3 cells. These results suggested that the effects of JNK on the regulation of cell viability and invasion in prostate cancer cells were associated with ERK and MMP1.

CONCLUSION

JNK expression is positively correlated with Gleason score and PSA value in patients with CRPC. Low JNK expression is also associated with high overall survival and second progression-free survival rate. Interestingly, JNK overexpression promotes cell viability and invasion, which is in part by the regulation of ERK and MMP1. These results suggest that JNK may be considered as an effective target for the CRPC therapy.

Declarations

Statement of Ethics. The study was approved by the ethics committee of LONGHUA Hospital Shanghai University of Traditional Chinese Medicine, and the patients signed written consent. This study followed the Declaration of Helsinki, Ethical Principles for Medical Research Involving Human Subjects.

Consent for publication. Current study is available from the corresponding author on reasonable request.

Author contribution. Concept or design: Yigeng Feng, Hongwen Cao, Dan Wang, Lei Chen, Renjie Gao, Peng Sun. Acquisition of data: Yigeng Feng, Renjie Gao, Peng Sun. Analysis or interpretation of data: Yigeng Feng, Lei Chen, Renjie Gao, Peng Sun. Drafting of the manuscript: Yigeng Feng, Hongwen Cao, Dan Wang, Lei Chen, Renjie Gao and Peng Sun. Critical revision of the manuscript for important intellectual content: All authors. All authors had full access to the data, contributed to the study, approved the final version for publication, and take responsibility for its accuracy and integrity.

Conflict of Interest Statement. None declared.

Data Availability Statement. The data that support the findings of this study are available from the corresponding author, Lei Chen, upon reasonable request.

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