Silencing of c-jun decreases cell migration, invasion, and EMT in radioresistant human nasopharyngeal carcinoma cell line CNE-2R

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Background: Previously, we found that c-jun was highly expressed in radiation-resistant human nasopharyngeal carcinoma cells (CNE-2R) compared with human nasopharyngeal carcinoma cells (CNE-2).

Materials and methods: In this study, we first used the scratch assays and transwell assays to detect the migration and invasion of CNE-2R and CNE-2 cells and tested the epithelial mesenchymal transformation (EMT)-related proteins E-cadherin and N-cadherin by Western blot analysis. Subsequently, c-jun was knocked down to establish the effect of c-jun on EMT, migration, and invasion of CNE-2R cells both in vitro and in vivo.

Results: A high EMT level, CNE-2R cells were more capable of migration and invasion than CNE-2 cells. Moreover, silencing of c-jun has upregulated the expression of E-cadherin and downregulated N-cadherin in CNE-2R cells, and subsequently the migration and invasion capacity of the cells was decreased. Consistent with in vitro results, in vivo studies indicated that the c-jun silencing reduced pulmonary migration of CNE-2R cells. Immunohistochemistry of lung metastatic tumor tissue showed that E-cadherin was upregulated, and N-cadherin was downregulated.

Conclusion: These findings suggest that silencing of c-jun in CNE-2R cells reduces cells migration, invasion, and EMT both in vitro and in vivo.

Keywords: nasopharyngeal carcinoma, c-jun, migration, invasion, EMT

Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common malignant tumors of the head and neck, which is highly prevalent among Chinese population, especially in Southern China.1,2 Radiotherapy and concurrent chemoradiotherapy are the recommended primary treatments for NPC in their early and advanced stages, respectively.3 Although early stages of NPC are highly radiocurable, local recurrence, metastasis, and radioresistance are the three significant factors that mainly restrict the efficacy and prognosis of radiotherapy.4 Therefore, in order to improve the prognosis of NPC, further exploration is required to delineate the underlying mechanisms of migration and invasion of radiologically resistant cells.

Transcription factors play an important role in the expression of genes. The activating protein-1 (AP-1) transcription factor is a dimeric complex comprising members of the jun and fos gene families. As the most potent transcriptional activator of the AP-1 family, c-jun is expressed in high levels in most of the multiple cancers,5–9 and plays an important role in the migration and invasion of transformed cells in malignant tumors such as liver cancer, soft tissue sarcoma, breast cancer, oral cancer, and ovarian cancer.10–14
Epithelial mesenchymal transformation (EMT) is a latent embryonic process which can be aberrantly reactivated during tumor progression. It is generally viewed as one of the main forces driving metastatic dissemination, by providing cells with invasive and motility capabilities. EMT is a biological process that allows a polarized epithelial cell, which normally interacts with basement membrane via its basal surface, to undergo multiple biochemical changes. These modifications enable EMT to assume a mesenchymal cell phenotype, which can enhance migratory capacity and invasiveness, elevate resistance to apoptosis, and greatly increase the production of extracellular matrix components. EMT is closely related to the development of tumor, and its metastasis and invasion at an early stage is highly reversible. This transformation process involves molecular recombination in the epithelial cells, and the loss and redistribution of the E-cadherin protein acting as an epithelial-specific marker, interstitial markers, vimentin and N-cadherin proteins. Previous studies have shown that c-jun plays a positive regulatory role in EMT of various tumor cells, such as lung cancer, breast cancer, and melanoma, and further inhibits the expression of c-jun or its associated pathways to reduce tumor cell EMT. But the effect of c-jun on EMT and cell migration of human NPC is rarely reported. In our previous work, CNE-2R cells were constructed by CNE-2 through intermittent high-dose γ-ray multiple irradiation, and we found that c-jun gene was highly expressed in CNE-2R cells compared with its expression in CNE-2 cells. Interestingly, silencing of c-jun by RNA interference has sensitized the CNE-2R cells to radiation and altered the cells proliferation. Considering that CNE-2R cells are radioresistant NPC cells, and c-jun is highly expressed in them, whether the c-jun influences migration, invasion, and EMT of CNE-2R cells was the subject of our interest.

In the present study, we first compared the migration and invasion ability and the level of EMT of CNE-2R and CNE-2 cells by scratch and transwell assays and Western blotting in vitro and found that the migration, invasion, and EMT of CNE-2R cells were higher than in CNE-2 cells. In order to verify and confirm the effect of c-jun silencing on migration and invasion of CNE-2R cells and its possible mechanism, we knocked down c-jun by RNA interference in CNE-2R cells and repeated these assays. Furthermore, by injection of tumor cells through the nude mice tail vein, we observed the invasion of CNE-2R cells in the lungs and confirmed the EMT-related protein expression in pulmonary metastasis by performing immunohistochemistry (IHC) assays. Both in vivo and in vitro experiments have demonstrated that c-jun gene silencing inhibited EMT and subsequent migration and invasion of CNE-2R cells. These observations demonstrated that c-jun gene silencing reduces the cells migration, invasion, and EMT in CNE-2R cells.

Materials and methods

Cell culture and transfection

A human NPC cell line CNE-2, constructed by the Cancer Hospital of Chinese Academy of Medical Sciences and Guangdong Medical University, was purchased from the Cancer Hospital of Shanghai Fudan University. CNE-2R, a radioresistant human NPC cell line, was previously constructed and maintained at the Cancer Laboratory of Guangxi Medical University. CNE-2 and CNE-2R cells were cultured in RPMI-1640 medium (HyClone, Logan, UT, USA) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA), penicillin (100 U/mL), and streptomycin (100 µg/mL), and were incubated in a humidified 5% CO₂ atmosphere at 37°C. The lentiviral infection was performed as described previously. The CNE-2R cells were cultured in six-well plates, and the c-jun-shRNA-expressing lentivirus (c-jun-shRNA, sequence: CAAAACCTCAGGACCTAATTCAACACTTCA) or the scrambled shRNA-expressing lentivirus (NC, sequence: TTCTCCGAACGTGTCACGT) was added with a multiplicity of infection of 20 in the CNE-2R cells and incubated for 72 h. The transduction efficiency was determined by inverted fluorescence microscopy (Olympus, Tokyo, Japan), quantitative real-time PCR (qRT-PCR), and Western blot.

qRT-PCR analysis

Total RNA was extracted from cells using Trizol reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions. For cDNA synthesis, 1 µg RNA was transcribed using the RT-PCR kit (Takara Biotechnology, Dalian, China). Primers for c-jun and β-actin were designed as follows: c-jun forward, 5′-AAAGGAAGCTGGAGAATCG-3′ and reverse, 5′-TGTTTAAGCTGTGCCACGT-3′; β-actin forward, 5′-ACCGAGCGCGCTACAGC-3′ and reverse, 5′-CTCATGGCCAATGTTGAT-3′. PCR amplification of cDNA was performed in a final volume of 20 µL. The experimental reaction was subjected to 40 cycles at 95°C for 5 s, 60°C for 30 s, followed by a final elongation at 95°C for 30 s, and 60°C for 1 min. β-actin was used as a control to normalize the amount of cDNA among samples. Expression of c-jun was normalized against β-actin, using the comparative threshold cycle 2ΔΔCt method. Results were expressed as the relative expression of mRNA levels compared with the controls. The experiment was performed in triplicate.
Western blot
The quantitated cell lysates were separated on 8%–12% sodium dodecyl sulfate polyacrylamide gels and electrophoretically transferred onto polyvinylidene fluoride membranes. After blocking for 90 min, the membranes were incubated sequentially with primary antibodies against c-jun (1:1,000 dilution; Cell Signaling Technology [CST], Boston, MA, USA, #9165), E-cadherin (1:1,000 dilution; CST, #3195), N-cadherin (1:1,000 dilution; CST, #13116), and β-actin (1:1,000 dilution; CST, #4970) at 4°C overnight. The membranes were washed and then incubated with a secondary antibody (1:15,000 dilution; Beyotime Biotechnology, Shanghai, China, #A0208) for 1 h at room temperature. Blotting images of membranes were obtained by the Bio-Rad infrared fluorescence imaging system Universal Hood III (Bio-Rad Laboratories Inc., Hercules, CA, USA). β-actin served as a control.

Scratch assay
The effects of CNE-2 and CNE-2R cells on migration capacities were assessed using scratch wound assay. A total of 5×10⁴ cells were seeded into six-well culture plates and cultured to complete confluence. Subsequently, three parallel, linear wounds were produced in each dish with a 200-μL plastic pipette tip. The cells were then cultured with serum-free medium. At different time points, three representative images of scratched areas from each dish were photographed to estimate the migration of cells using ImageJ. The scratch healing rate was calculated using the following formula to demonstrate the cell migration ability: Scratch healing rate=([0 h scratch width − 24 h scratch width]/0 h scratch width)×100%. Through the scratch wound assay, a comparison of cell healing capacity of the c-jun-shRNA group, NC group, and control group were estimated and analyzed. All experiments were repeated three times with identical findings.

Transwell cell migration and invasion assays
Invasion assays were performed using transwell chambers with 8-μm pore polycarbonate membrane inserts (Corning Inc., Corning, NY, USA). The cells were cultured with serum-free medium for 24 h, then 1×10⁵ cells/well were plated onto the upper chambers of Matrigel-coated inserts and the cells were incubated with serum-free medium for 24 h at 37°C. FBS and fibronectin were used in the lower chambers as chemoattractants. Noninvading cells were removed and the invaded cells in the membrane were fixed in methanol, washed and stained with 0.1% crystal violet. Inserts were washed, briefly air-dried, and mounted. Invading cells were quantified using an inverted microscope. The results in the bar diagram represent mean±SD of the number of migratory and invasive cells from five random 10× objective fields. All experiments were repeated three times with identical findings. For migration assays, the general transwell chamber was used instead of the matrix-coated transwell chamber.

In vivo metastasis analysis
For in vivo metastasis assay, 4–6-week-old nude mice (Shanghai Slake Experimental Animal Co. Ltd., Shanghai, China) were housed (five mice per cage) and fed with autoclaved food. PBS was used to reconstitute cells from c-jun-shRNA, NC, and control groups, and each nude mouse was injected with 5×10⁴ cells through the tail vein. At 6 weeks post-injection, the mice were sacrificed, and their lungs were harvested, fixed in Bouin’s solution, and photographed. The metastatic tumor nodules in the lungs were enumerated under a dissecting microscope. The representative lung tumors were removed, fixed, and embedded in paraffin. A 5-μm section was stained with H&E for histological analysis.

Immunohistochemistry evaluation
All sections were dewaxed in xylene and rehydrated in graded ethanol, followed by incubating in 3% hydrogen peroxide for 10 min to quench endogenous peroxides. Samples were heated in 0.01 mol/L citrate buffer for 1 min at 100°C, and then kept at room temperature for 30 min. After cooling, the samples were mixed with 2% normal goat serum in PBS for 30 min to block antigenic epitopes, and then incubated with primary antibody (1:500 dilution) at 4°C overnight. After that, the sections were washed with PBS three times and then incubated with system-labeled horseradish peroxidase anti-mouse secondary antibody at room temperature for 20 min. Next, the sections were incubated in diaminobenzidine color reagent and counterstained in Mayer’s hematoxylin, and dehydrated in alcohol and xylene. PBS was used as a negative control. Under the light microscope, the positive areas seem like brownish yellow granules. Staining intensity was categorized as follows: no staining, 0; weakly stained, 1; moderately stained, 2; strongly stained, 3. Percentage of stained cells was categorized as follows: no staining, 0; <30%, 1; 30%–60%, 2; >60%, 3. The overall staining score (0–6) for each tissue sample was calculated by adding the above scores. An overall staining score of ≤3 was considered to reflect low expression of the corresponding antigen, and a score of >3 reflected high expression.

Statistical analysis
Data are presented as mean±SD. Statistical analyses were performed using SPSS 17.0 (SPSS, Chicago, IL, USA) or
the GraphPad Prism 5.0 software. Statistical differences were determined by a two-tailed t-test. The analysis of variance (ANOVA) test was used to assess the significance of the differences among the experimental groups. All the cellular experiments were performed three or four times. A P-value <0.05 indicates a statistically significant difference and is indicated by *.

Ethics and legal approval statement
The use of the cells was approved by the Tumor Hospital of Guangxi Medical University. The study was performed in accordance with the guiding principles for the care and use of laboratory animals approved by the Ethics Committee of Tumor Hospital of Guangxi Medical University (Permit Numbers: LW2018002).

Results
Higher levels of EMT and c-jun expression augmented migration and invasion in CNE-2R cells
Through microscopic observation, we found that the morphology of CNE-2R cells showed significant changes compared with their parent cells, CNE-2. CNE-2 cells showed a paving stone structure with cell–cell adhesion, while CNE-2R cells were arranged in a loosely organized spindle cell pattern, resembling the epithelial type (Figure 1A). Western blot was

Figure 1 (Continued)
performed to detect the expression levels of EMT-related proteins. The results showed that with the high expression of c-jun in CNE-2R cells, the epithelial marker E-cadherin was downregulated, whereas mesenchymal protein N-cadherin was upregulated (Figure 1B). These findings indicated that the CNE-2R cells have significantly altered EMT compared with CNE-2 cells. Subsequently, to investigate the migration and invasion of CNE-2R cells and CNE-2 cells, scratch assay and transwell assay were performed. CNE-2R cells showed a statistically significant (*P<0.05) higher rate of migration and invasion (Figure 1C and D), confirming the high EMT level and migration and invasion capacity compared with CNE-2 cells.

Construction of c-jun gene silencing cell model, CNE-2R
As explained in a previous experimental strategy,22 the lentiviral shRNA was successfully constructed and transduced into the CNE-2R cells. The transduction rate was beyond 90% at 96 h (Figure 2A). RT-PCR and Western blot were used to detect the effect of c-jun gene silencing. Both the mRNA and protein expression levels of c-jun were significantly decreased in the c-jun gene shRNA-transduced group (c-jun-shRNA group) compared with the non-transfected (control group) and scrambled shRNA-transfected group (NC group; Figure 2B and C; **P<0.01). In addition, flow cytometry was used to detect the transfection rate of cells, and the results showed that the percentage of CNE-2R cells transfected was >90%.

Stable knockdown of c-jun expression suppressed migration and invasion of CNE-2R cells in vitro and in vivo
As CNE-2R cells with a high expression of c-jun gene had shown a strong ability for migration and invasion, the effects of c-jun silencing on migration and invasion of CNE-2R cells were detected both in vitro and in vivo. The migration and invasion of cells in vitro was detected using scratch and transwell assays. The results of the scratch test showed that the rate of scar healing of the c-jun-shRNA group cells was 15.266±1.855% after 24 h, which was significantly lower (**P<0.01) than that of the control group (28.851±2.914%) and NC group (26.526±3.02%) cells, while the comparison between control group and NC group was not statistically significant; P>0.05 (Figure 3A). In the transwell migration assays, the traversing number of c-jun-shRNA group cells was 172.25±29.93, which was significantly lower (**P<0.01) than that of the NC group (370.5±36.82) and the control group (350.75±30.96), and the comparison between...
control group and NC group was not statistically significant, P>0.05 (Figure 3B-above). In addition, in the transwell invasion assays, the traversing number of c-jun-shRNA group cells was 143±20.00, which was significantly lower than that of the NC group (234±17.33) and the control group (242±26.01); **P<0.01 (Figure 3B-below).

To test the effect of c-jun in vivo, we used nude mice to establish the animal models (Figure 3C-I), and formed three groups, namely c-jun-shRNA group, NC group, and control group. Later, these mice were injected with tumor cells in their tail vein, and metastatic tumor formation was detected in lungs. In vivo studies showed that the tumor metastasis of CNE-2R cells after the gene silencing in the lungs of the nude mice was significantly lower than before the gene silencing. Moreover, the metastatic lesions of the c-jun group were smaller and less in number than in the control group and NC group (Figure 3C-II). Subsequently, on H&E staining of lung tissue, it was observed that the pulmonary metastasis of the c-jun-shRNA group was significantly less than that of the control group and NC group, with a mild lesion formation and a smaller effect on the lung; **P<0.01 (Figure 3C-III). These results demonstrated that migration and invasion of
c-jun silencing decreases cell migration, invasion, and EMT in CNE-2R

Figure 3  c-jun silencing inhibited the migration and invasion of CNE-2R cells both in vitro and in vivo.

Notes: (A–C) Scratch assay and transwell assay to compare the cells migration and invasion in c-jun-shRNA group, NC group, and control group in vitro. (C) The establishment of the nude mice model and the demonstration of metastasis of CNE-2R cells in the lung. (I) Nude mice used in the experiment. (II) The formation of metastatic tumor in the lung. The differential formation of metastatic tumors in the lungs of the three groups after 6 weeks, the number of metastatic tumors in the lungs of c-jun-shRNA group was significantly lower than that of NC group and control group (*P<0.01). (III) Microscopic analysis of the lung metastatic tumors by H&E staining in the three cell groups. The level of pulmonary metastasis of c-jun-shRNA group was significantly lower than that of NC group and control group (**P<0.01). Control represents non-transfected group and NC represents scrambled shRNA transfected group. Magnification ×100.

Abbreviation: NC, negative control.
CNE-2R cells were suppressed by knockdown of c-jun gene expression both in vitro and in vivo.

**c-jun silencing altered EMT expression in CNE-2R cells both in vitro and in vivo**

Many studies have shown that EMT is a major driver of tumor cell invasion and metastasis, and our results have confirmed that c-jun silencing can reduce the migration and invasion ability of CNE-2R cells. To further investigate the possible role of underlying c-jun-mediated silencing and suppression of CNE-2R cells migration and invasion, the expression of the epithelial markers, E-cadherin and N-cadherin was determined by Western blot analysis and IHC in CNE-2R cells after c-jun knockdown. Firstly, we looked at the CNE-2R cells morphology by microscopy after c-jun silencing. Obviously, CNE-2R cells morphology has significantly changed after c-jun silencing, and the form of a loose spindle cell pattern has been altered to an oval-shaped cell form with more close alignment, and it appears that the CNE-2R cells epithelial type has been reversed (Figure 4A). Secondly, by performing the Western blot assays using the antibodies specific to EMT-related markers, the differential expression of proteins, such as E-cadherin and N-cadherin, in CNE-2R cells after c-jun knockdown was estimated. The results showed that the E-cadherin protein was upregulated, and the N-cadherin was downregulated, which indicates that the c-jun silencing had inhibited the occurrence of EMT in CNE-2R cells in vitro (Figure 4B). Finally, the lung metastatic tumor tissue of nude mice was subjected to immunohistochemical analysis to detect the expression levels of c-jun, E-cadherin, and N-cadherin proteins in c-jun-shRNA group, NC group, and control group. We found that in the lung metastatic tumor tissue, c-jun was weakly positive in c-jun-shRNA group (score of 1) but was strongly positive in both NC (scores of 3) and control groups (scores of 3). In addition, the expression of E-cadherin protein in the c-jun-shRNA group was strongly positive (scores of 5), while N-cadherin was weakly positive compared with control (scores of 2) and NC groups (scores of 2; Figure 4C). Collectively, these results indicated that the c-jun silencing has effectively inhibited the occurrence of EMT in CNE-2R cells in vitro and in vivo.

**Discussion**

Radiotherapy and concurrent chemoradiotherapy are recommended as primary treatments for NPC, local recurrence and distant metastasis are the main reasons for the failure of nasopharyngeal carcinoma treatment, especially in patients with radiation-resistant tumors. Therefore, it is of great significance to study the migration and invasion mechanism of radiation-resistant tumor cells. In our earlier studies, we successfully established the radioresistant human NPC cell line CNE-2R and showed that c-jun was highly expressed in CNE-2R cells. Moreover, the silencing of c-jun could lead to elevated radiosensitivity of CNE-2R cells and inhibited cell growth.\(^{21,22}\) In this study, we reported that CNE-2R cells with a high EMT level exhibited a strong ability of migration and invasion. Moreover, silencing of c-jun, which is highly expressed in CNE-2R, could significantly inhibit the EMT of CNE-2R cells and reduced the cells migration and invasion ability in vitro. Results also showed that migration, invasion, and EMT of CNE-2R cells were suppressed after the c-jun gene was silenced in vivo. Considering all the results of our experiments both in vivo and in vitro, we strongly believe that c-jun silencing could reduce the EMT, migration and invasion of CNE-2R cells.

AP-1 is formed as a composite c-jun/c-fos dimer, which takes part in the regulation of the downstream genes expression and plays an important role in the regulation of cell proliferation, apoptosis, and malignant transformation.\(^{23-27}\) Furthermore, AP-1 is shown to participate in the formation, invasion, and metastasis of the tumors,\(^{13,14,28,29}\) and c-jun plays a major interactive role in these processes. c-jun proto-oncogene is one of the members of the family of immediate early genes, belonging to either the nuclear transcription factors in the alkaline leucine zipper family, or intracellular c-jun and other members of jun family genes, or members of the family of fos homologous or heterologous dimers. As an important part of AP-1, c-jun is closely related to the transcriptional regulation of various cytokines and growth factors, which are highly expressed in many malignant tumors. In our previous experiment, the CNE-2R cells were constructed from the CNE-2 cells, and the expression of c-jun was significantly upregulated compared with that in CNE-2 cells.\(^{21}\) In the present study, we found that EMT as well as the cells ability of migration and invasion were more obvious in the CNE-2R cells than in CNE-2 cells. These findings indicated that the high expression of c-jun in CNE-2R cells could enhance the cells EMT and their ability to migrate. Sabichi had previously demonstrated that c-jun has a critical role in the migration and invasion characteristics of human breast cancer cells.\(^{30}\) Subsequently, we investigated to find out whether the expression of the c-jun gene would change the ability of cell migration and invasion. We showed that the ability of CNE-2R cells migration and invasion had decreased considerably, after c-jun expression was silenced.

EMT is believed to play an important role in tumor initiation. Studies have shown that c-jun plays a positive regulatory role in EMT of various tumor cells, such as in lung cancer,
c-jun silencing decreases cell migration, invasion, and EMT in CNE-2R

breast cancer, and melanoma, and inhibits the expression of c-jun or its associated pathways to reduce tumor cell EMT.17–20 Our results indicated that CNE-2R cells morphological characteristics altered from loose spindle cell structure to cell–cell adhesion of CNE-2 cells after c-jun silencing, and also this reversed the high expression of N-cadherin and low expression of E-cadherin in CNE-2R cells. These findings were consistent with a previous study by Ying et al, who demonstrated that c-jun knockdown in gastric cancer cells led to EMT reversion.31 These results demonstrated that the c-jun silencing has reversed the EMT of CNE-2R cells.

EMT is a process in which the epithelial cells lose epithelial characteristics and gain mesenchymal features, such as motility and invasiveness.32 It is well established that EMT is associated
with invasion and migration in various malignancies; regulating the level of EMT in tumor cells may affect the metastasis of tumor cells, such as in gallbladder cancer, malignant melanoma, lung cancer, and squamous cell carcinoma. In our experiment, the level of EMT of CNE-2R cells decreased significantly after silencing of c-jun expression, and the ability of cell migration and invasion decreased both in vitro and in vivo. These results strongly suggest that c-jun silencing suppressed the ability of cell migration, invasion, and EMT in CNE-2R cells. In conclusion, due to the high expression of c-jun gene, the CNE-2R cells EMT level and their ability to migrate have increased. Silencing of c-jun has inhibited the EMT, migration, and invasion of CNE-2R cells. However, c-jun promoting the internal mechanism of cell migration in the reflex resistance cells of NPC is complicated, and more experimental studies are needed to determine the relationship between EMT and migration in NPC cells. Additionally, delineating the complete mechanism of this interactive role played by c-jun expression in cell migration that facilitates metastasis, and further identifying the role of c-jun serving as a potential therapeutic candidate in the treatment of NPC, may require more exploration.

Conclusion

In this study, we found that CNE-2R cells were higher in EMT level and more capable of migration and invasion than CNE-2 cells. Furthermore, the level of EMT of CNE-2R cells decreased significantly after silencing of c-jun expression, and the ability of cell migration and invasion decreased both in vitro and in vivo. Based on these findings, we proposed that silencing of c-jun reduces cell migration, invasion, and EMT in CNE-2R cells.

Acknowledgments

The present work was supported by grants from the National Natural Science Foundation of China (81760544 and 81360343), the Guangxi Natural Science Foundation (2016GXNSFAA380127), and the Key Laboratory of High-Incidence-Tumor Prevention and Treatment, Independent Research Project (Contract number: GKZ201605).

Author contributions

XZ and GL conceived and designed the study. GL, BY, QL, and YS performed the experiments. ZL, KC, and LZ helped in analyzing the data and provided constructive discussions. GL and BY drafted the manuscript. LL and SQ critically reviewed and edited the manuscript. All authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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