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Effects of HIF-1α on ERRα/γ protein expression in mouse skeletal muscle

Ying Zhang, Weixiu Ji, Lianfeng Zhang, Sixue Liu, Gang Liu, Jianxiong Wang

ABSTRACT

Aims: HIF-1α plays an important role in the adaptive responses to hypoxia. The ERRα and γ are crucial regulators of energy metabolism in skeletal muscle. The aim of the present study was to generate the inducible HIF-1α transgenic mice and examine the effects of different HIF-1α protein expression levels on ERRα/γ in mouse skeletal muscle. Methods: We generated the HIF-1α high-expression transgenic mice (HT) and HIF-1α low-expression transgenic mice (LT), and then compared the expressions of ERRα/γ and its target genes in skeletal muscles of three kinds of mice: HT, LT, and non-transgenic mice (NT).Results: The results showed that (1) the double positive mice from the founder of 3# and 68# showed an obvious expression of HIF-1α induced by tamoxifen and both of them were maintained to the further research as HT and LT mice, respectively; and (2) the nucleoprotein expressions of ERRα/γ and the mRNA levels of the ERRα/γ target genes: MCAD, PPARα, NRF1 and PDK4 were higher in the LT mice than the values in the NT, but only the mRNA levels of MCAD and PPARα were significantly higher. The HT mice showed significantly lower ERRα protein content than that of the NT mice. Conclusion: Our study was the first to report the generation of the inducible HIF-1α transgenic mice and effects of HIF-1α on ERRα/γ protein expression in mouse skeletal muscle in vivo. These data demonstrate that the low HIF-1α protein expression may associate with an up-regulation of ERRα/γ and their target genes in skeletal muscles, while the aggravated HIF-1α protein expression would reduce the effects.

Keywords: ERRα/γ, HIF-1α, Skeletal muscle, Target genes, Transgenic mouse,

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INTRODUCTION

The hypoxia inducible factor (HIF)-1 plays a key role to mediate the cellular responses to hypoxia. Its target genes control many cellular signal transductions, such as erythropoietin which induces red blood cell

production, vascular endothelial growth factor (VEGF) which promotes angiogenesis, and glucose transporter 1 (GLUT1) which increases the efficiency of the glucose uptake [1, 2]. Previous studies have examined the role of HIF-1α in skeletal muscle. A research group found that the HIF-1 protein and mRNA levels are varied in different fiber types of rodent skeletal muscles [3]. Another study demonstrated that acute exercise can elevate the HIF-1 protein level in human muscle [4]. Deletion of HIF-1 in mouse skeletal muscle might lead to impaired glycolytic flux and decreased exercise endurance [5]. These studies focused on the effects of HIF-1 on the tissue or organ level. Skeletal muscle function and metabolism are controlled by many signaling pathways and molecules. At the molecular level, there are few studies about the relationship between HIF-1α and other signaling pathways or molecules which control energy metabolism in skeletal muscle.

Estrogen-related receptors (ERRs) are crucial regulators of energy metabolism [6]. There are three members in the ERR family: ERRα, ERRβ and ERRγ. They are active nuclear receptors that contain high levels of the DNA sequence identified to that of estrogen receptors [7]. ERRα and γ isoforms are ubiquitously expressed at high levels in the heart, skeletal muscle and brown adipose tissue; while ERRβ expression is restricted to the brain, kidney and heart. ERRs control many downstream target genes which are important to glycolytic metabolism, fatty acid oxidation, mitochondrial oxidative phosphorylation, and mitochondrial biosynthesis. The main downstream target genes include pyruvate dehydrogenase kinase 4 (PDK4) [8], medium-chain acyl coenzyme A dehydrogenase (MCAD) [9], peroxisome proliferator-activated receptor α (PPARα) [10], and nuclear respiratory factors (NRFs) [11].

There are a few reports about the relationship between HIF-1 and ERRs in cell lines in literature. A previous study has shown that ERRγ mRNA and protein levels were increased by a hypoxic treatment in the hepatoma cell lines and this change in ERRγ was associated with the increased HIF-1α and β expression [12]. The knockdown of endogenous HIF-1α reduced the hypoxia-mediated induction of ERRγ. In addition, hypoxia also increased the promoter activity and mRNA level of PDK4 in the HepG2 cells [12]. Another study suggested that ERRs physically interact with HIF and stimulate HIF-induced transcription. Transcriptional activation of hypoxic genes in cells cultured under hypoxia was largely blocked by suppression of ERRs through the treatment with diethylstilbestrol, an ERR inhibitor [13]. These results have suggested a possible relationship between HIF-1α and ERRs. However, these observations are restricted to the cell culture studies. There are few researches of the relationship between HIF1α and ERRα/γ in vivo. The purpose of this study was to generate the inducible HIF-1α transgenic mice and evaluate the effects of HIF-1α expression on the ERRα/γ protein and the mRNA expression levels of the ERRα/γ's target genes in skeletal muscle of the mouse with low- or high-expression in HIF-α protein.

MATERIALS AND METHODS

The generation of the inducible HIF-1α transgenic mice

The eGFP gene was labeled with two LoxP sites in the 5' and 3' end and fused with a full length mouse HIF-1α cDNA (Genbank accession No.15251) (Figure 1). The transgenic plasmid (pBactin2(+)-GFP-Loxp-mHIF-1α) was constructed by inserted the DNA fragment containing the eGFP and HIF-1α cDNA under the chicken β-actin promoter, that the chicken β-actin promoter enhanced the eGFP expression directly and could enhance the HIF-1α expression after the eGFP to be removed by Cre. The inducible expression of HIF-1α mRNA was tested in the 293T cells (Figure 2). The 293T cells were transfected with pcDNA3.1(-)-CRE, pBactin2(+)-GFP-Loxp-mHIF-1α, and pcDNA3.1(-)-CRE + pBactin2(+)-GFP-Loxp-mHIF-1α, respectively. The total RNA was isolated and mRNA levels of the HIF-1α were detected by RT-PCR. The construct was microinjected into male pronuclei of fertilized mouse oocytes and implanted into pseudo-pregnant females to generate the transgenic mouse lines [14]. The Genotyping of GFP-Loxp-mHIF-1α transgenic mice was verified by PCR using primers 5'TGGCAATGTCTCCTTTACCTTC and 5'GCAGTGGTCGTTTCTTGAGG to amplify the desired 654bp fragment of the mHIF-1α (Figure 3). Totally, 12 founder mice were found and they were crossed with a pUbc-Cre-ert2 mice (from JAX, No.008085), which could expressed the Cre-ert2 systematically in an inducible way with tamoxifen (Figure 4). The double positive mice were generated from 2#, 3#, 7# and 68# founders and

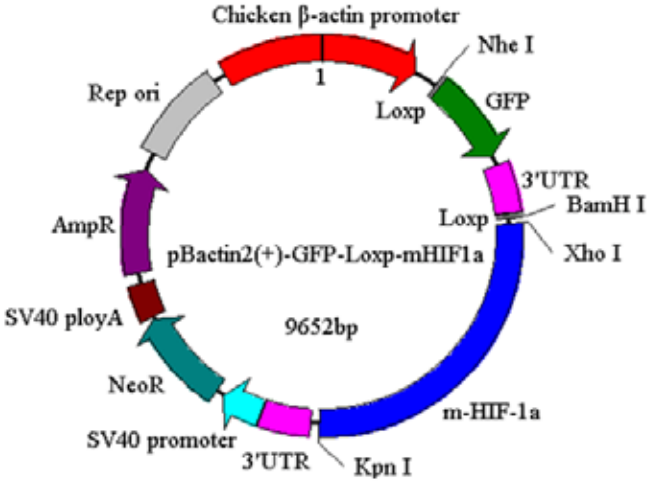

Figure 1: A LoxP labeled eGFP between the chicken β-action promoter and the HIF-1α cDNA.

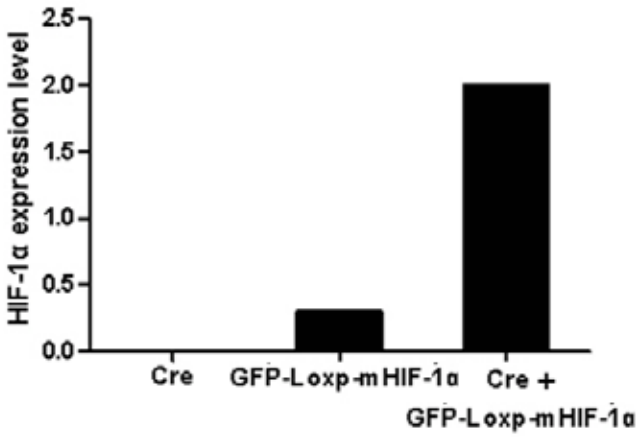

Figure 2: The HIF-1α expression in 293T cells.

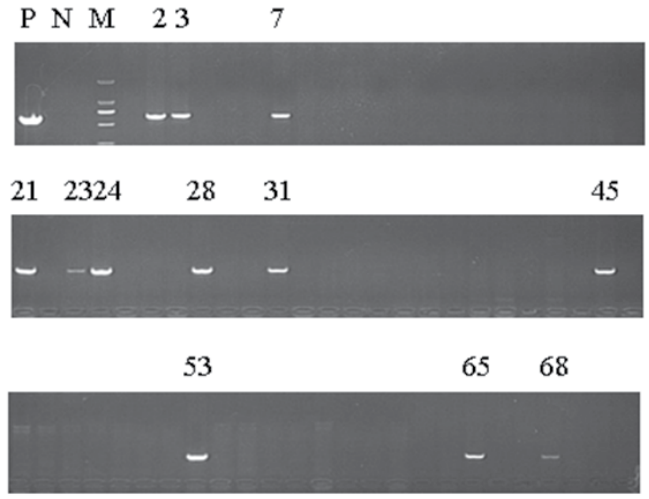

Figure 3: The Genotyping of GFP-Loxp-mHIF-1α transgenic mice was verified by PCR.

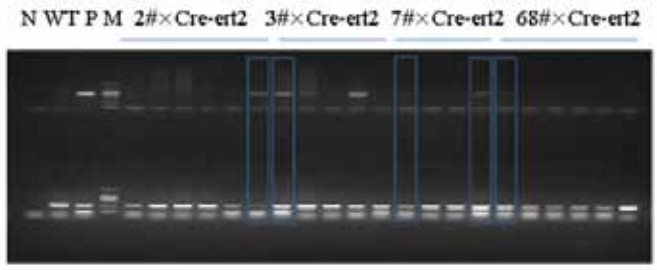

Figure 4: There were four double positive F1 mice from the 12 founder crossed with the pUBC-Cre-ert2 mice respectively.

these mice were induced with tamoxifen in a dose of 100 μg/d by oral administration for 10 days to get HIF-1α low-expression (LT) and HIF-1α high-expression (HT) transgenic mice. The double positive mice were maintained on a C57BL/6J genetic background and all the mice were bred in an AAALAC-accredited facility.

The present study protocol was approved by the Animal Care and Use Committee of Beijing Sport University. Non-transgenic mice (NT) (n=10), LT mice (n=10), and HT mice (n=10) were used to evaluate the effects of HIF-1α expression on the ERRα/γ protein and the mRNA expression levels of the ERRα/γ's target genes in skeletal muscle of the mouse. All mice with a mean body weight of 20±2 g were housed with controlled room temperature (20–25°C), 12:12-h light-dark cycle, and free access to food and water. After allowing acclimatization to their housing for three days, all mice were euthanized and their skeletal muscles from the legs were excised, cleaned of blood and connective tissue, quick-frozen with aluminum tongs, pre-cooled in liquid nitrogen, and stored at -80°C.

Western blot analyses

For Western blot analyses of HIF-1α, ERRα and ERRγ protein levels, homogenates were made from the skeletal muscle samples. Nucleoproteins were isolated from 100 mg of muscle using NE-PER nuclear extraction reagents (87792, Pierce, Rockford, IL, USA). Protein concentration was measured using the BCA protein assay kit (23225, Pierce, Rockford, IL, USA). Nucleoproteins (20 μg) were separated on NuPAGE®Novex®Bis-Tris Gels (separating gel 4–12%) (Life Technologies, USA) by electrophoresis, and the fractionated proteins were transferred to a nitrocellulose membrane by iBlot Gel Transfer System (Invitrogen, USA). The blots were probed with HIF-1α antibody (Cell signaling, 3434), ERRα antibody (abcam, 76228), ERRγ antibody (abcam, 128930), and the same blots were stripped and reprobed with GAPDH (Santa Cruz. sc-48166)/β-actin (Santa Cruz. sc-47778) or histone-1 (H1) antibody (Santa Cruz. sc-8030). The individual values were originally expressed as a ratio of a standard (GAPDH/β-actin or H1 content) and then expressed as a fold change of the NT group value.

Real-time PCR

Total RNA was isolated from 50 mg of crushed muscle tissue using the TRI reagents according to the manufacturer's instructions. Reverse transcription (RT) of total RNA to cDNA was performed using a Reverse Transcription Kit (Toyobo, Japan). RT-PCR was performed in a ABI 7500 Real-Time PCR System (USA) using 10 ul of SYBR Green Real time PCR Master Mix (Toyobo, Japan), 2 ul of cDNA template, 6 ul of ddH_2O and 2ul of respective Qiagen primer sets (Germany) for PDK4 (QIAGEN, QT00157248), MCAD (QIAGEN, QT00111244), PPARα (QIAGEN, QT00137984), NRF1 (QIAGEN, QT01051820) and HIF-1α (QIAGEN, QT01039542). Copy numbers of cDNA targets were quantified using Ct values, and the mRNA expression levels for all samples were normalized to the level of the housekeeping gene 18S rRNA (QIAGEN, QT010036875). The difference in expression between control and experimental was calculated as $2^{-\Delta\Delta CT}$, as described previously [15].

Statistical analysis

All data were presented as means ± SE. Statistical analysis was conducted via one-way ANOVA, and a Tukey post hoc test was used when significance was found. Differences were considered statistically significant at $p < 0.05$.

RESULTS

The generation of the inducible HIF-1α transgenic mice

The generation of the inducible HIF-1α transgenic mice was shown in Figure 5. The double positive mice from the founder of 3# and 68# showed an obvious expression of HIF-1α induced by tamoxifen and both of them were maintained to the further research as LT and HT transgenic mice, respectively.

HIF-1α and nucleoprotein ERRα expressions in skeletal muscle

Skeletal muscles of NT, LT and HT mice were harvested and their expression of HIF-1α and ERRα were analyzed by Western blot. LT and HT mice showed higher HIF-1α signals than that of the NT mice and HT mice showed higher HIF-1α signal than that of the LT mice when using the HIF-1α antibody in muscle (Figure 6A). The nucleoprotein expression of ERRα in LT mouse skeletal muscle was increased, but did not reach significant level; while the nucleoprotein expression of ERRα in HT mouse skeletal muscle was decreased significantly, compared with that of the NT muscles (Figure 6B).

The nucleoprotein expression of ERRγ in skeletal muscle

There was a trend that the nucleoprotein expression of ERRγ was higher in the LT group and was lower in the HT group, compared to that of the NT group, but the differences were not significant between the groups (Figure 7). The ERRγ nucleoprotein level in LT and HT groups had a similar change pattern of the ERRα in Figure 6.

The mRNA expression of MCAD, NRF1, PPARα and PDK4 in skeletal muscle

The levels of MCAD, NRF1, PPARα and PDK4 mRNA expressions were measured using RT-PCR and normalized with 18S rRNA in the three groups (Figure 4). The mRNA expressions of MCAD and PPARα in the LT mice were significantly increased, compared with the value in the NT. The mRNA expressions of NRF1 and PDK4 showed increases but the trends were not statistically significant. The HT mice did not show significant difference in the genes' mRNA expressions to those of the LT and NT mice (Figure 8).

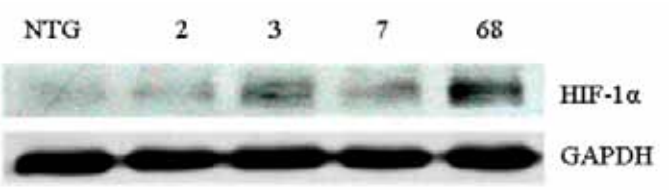

Figure 5: The inducible expression of HIF-1α in tissue of muscle was compared with Western blot.

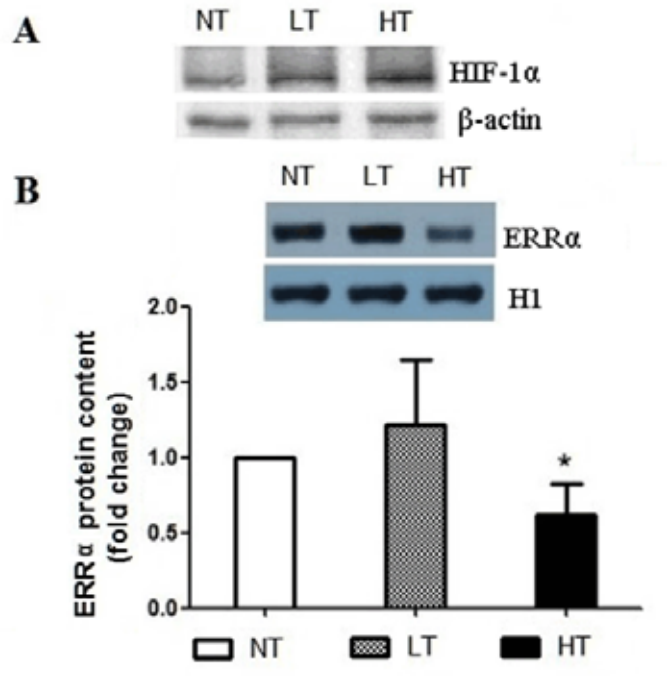

Figure 6: HIF-1α protein content (A) and ERR α nucleoprotein content, (B) in NT, LT and HT mice muscles.
*significantly different from the corresponding NT mice values at $p < 0.05$.

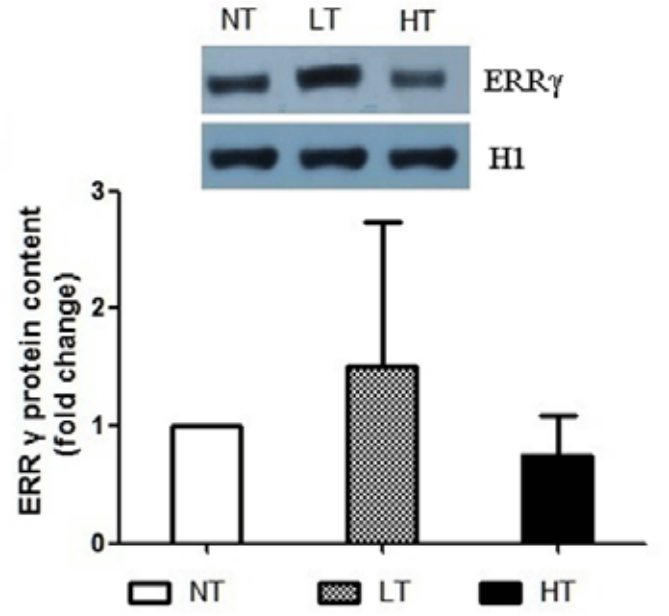

Figure 7: Western blot analysis for ERRγ in mouse skeletal muscle.

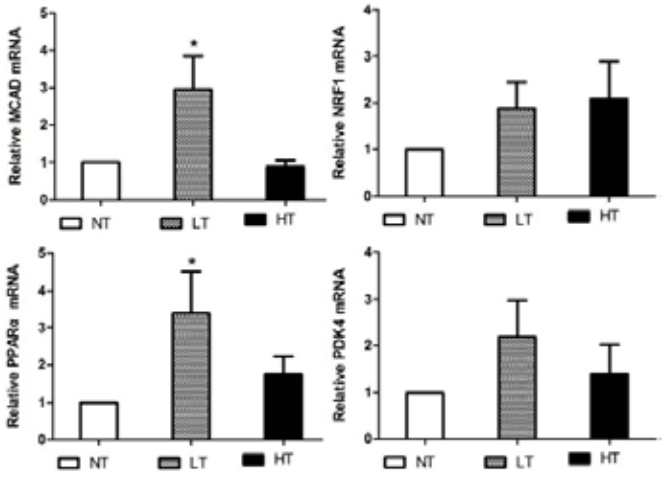

Figure 8: The levels of MCAD, NRF1, PPARα and PDK4 mRNA. * significantly different from the corresponding NT mice values at $p < 0.05$.

DISCUSSION

The importance of HIF-1 signaling in metabolic regulation has spurred much interest in recent years [12, 16]. Previous studies have been mainly focused on the metabolic role of HIF in

(i) stimulating the expression of genes that encodes glucose transporters and glycolytic enzymes to accelerate glucose uptake and the anaerobic glycolytic ATP production [17],
(ii) down-regulating mitochondrial oxygen consumption by directly or indirectly inducing pyruvate dehydrogenase (PDK), which inhibits the mitochondrial pyruvate dehydrogenase complex (PDC) from converting pyruvate into acetyl-CoA [18,19], and
(iii) regulating the differential expression of cytochrome c oxidase subunit 4 isoforms to optimize the efficiency of respiration at different O2 tensions [20].

There are few studies referred to the relationship of HIF and ERR. Studying the effects of HIF-1α on ERRα/γ would improve our understanding about the metabolic regulation under hypoxic condition in skeletal muscle. Therefore, in the present study for the first time, we generated the inducible HIF-1α transgenic mice and reported the impact of HIF-1α on ERRα/γ protein expression in mouse skeletal-muscle through the new generated HIF-1α transgenic mice.

There are no conclusive results of the effects of HIF-1 on ERRs in hypoxia. Some studies have reported that hypoxia stimulates the expression of ERRα through peroxisome proliferator-activated receptorγcoactivator-1α (PGC-1α), but not HIF-1. [21, 22]. While another study showed that HIF-1α had an effect on ERRs in hypoxic cell culture in vitro [12]. This statement has been supported by the finding of two putative binding sites of HIF-1α on the ERRγ promoter in a cell culture experiment. The promoter activity in response to hypoxia was abolished in these site-mutated constructs [12]. Furthermore, the evidence of ERRγ regulation of HIF-1α under hypoxic condition was confirmed by the ChIP assays in the hypoxia treated HepG2 [12]. In the present study, we observed that the nucleoprotein expressions of ERRα/γ and the mRNA levels of the ERRα/γ target genes: MCAD, PPARα, NRF1 and PDK4 were higher in the LT mice compared to the values in the NT mice, although only the mRNA levels of MCAD and PPARα were increased significantly. The results suggest that HIF and ERR may act in a common pathway and form a regulatory complex. Interestingly, we also found that the ERRα/γ protein contents were decreased in the HT mice and the reduced ERRα was significantly different from the corresponding NT value. HIF-1α is able to induce apoptosis during severe or prolonged (>24 hours) hypoxia, although it has been generally accepted as a pro-survival factor [13, 23]. Thus, the molecular mechanism causing different ERRα/γ protein levels, transcriptional regulation or output of ERRα/γ for downstream targets requires further clarification.

There are limitations in our study, we had no single transgenic mice of GFP-Loxp-mHIF-1α and Ubc-Cre-ert2 as controls. More controls would provide more clear results to the research. In addition, we did not measure the transcriptional activity of the ERRα/γ genes which are regulated by HIF-1α, as well as the ERRα/γ mRNA level and the transcriptional activity of PDK4, MCAD, PPARα and NRF1 genes which are regulated by ERRα/γ. Though the present results have shown the effects of HIF-1α on the expressions of ERRα/γ and their target gene, future studies should measure these directly. Moreover, in the present study, we only investigated the gene and protein expressions but did not do any histomorphological and functional measurements. These histomorphological and functional measurements would be desirable and add more comprehensive insights into the scope of the study.

CONCLUSION

Our study was the first to report the generation of the inducible HIF-1α transgenic mice and effects of HIF-1α on ERRα/γ protein expression in mouse skeletal muscle in vivo. We found that the nucleoprotein expressions of ERRα/γ and the mRNA levels of the ERRα/γ target genes were higher in the LT mice; while the ERRα/γ protein contents were decreased in HT mice, compared to the values in the NT mice respectively. These data reveal a relationship that HIF-1α affects ERRα/γ and their target genes in mouse skeletal muscle.

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Author Contributions

Ying Zhang – Substantial contributions to conception and design, Acquisition of data, Drafting the article, Revising it critically for important intellectual content, Final approval of the version to be published
Weixiu Ji – Substantial contributions to conception and design, Acquisition of data, Drafting the article, Revising it critically for important intellectual content, Final approval of the version to be published
Lianfeng Zhang – Substantial contributions to conception and design, Acquisition of data, Drafting the article, Final approval of the version to be published
Sixue Liu – Substantial contributions to conception and design, Acquisition of data, Drafting the article, Revising it critically for important intellectual content, Final approval of the version to be published
Gang Liu – Substantial contributions to conception and design, Acquisition of data, Drafting the article, Final approval of the version to be published
Jianxiong Wang – Substantial contributions to conception and design, Drafting the article, Revising it critically for important intellectual content, Final approval of the version to be published

Guarantor

The corresponding author is the guarantor of submission.

Conflict of Interest

Authors declare no conflict of interest.

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