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Original Research Article

Mechanism of hesperidin-induced apoptosis in human gastric cancer AGS cells

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Abstract

Purpose: *To investigate the effect of hesperidin on apoptosis of gastric cancer (GC) AGS cells, and the underlying mechanism of action.*
Methods: *The inhibitory effect of hesperidin was determined in AGS cells by MTT assay. Annexin V-FITC/PI double staining and flow cytometry (FC) were used to evaluate the apoptotic activity of hesperidin, reactive oxygen species (ROS), and changes in cell apoptosis after treatment of NAC cells with NAC. Expressions of B cell lymphoma/leukemia-2 (Bcl-2) and mitogen-activated protein kinase (MAPK) signaling pathway were determined by Western blotting.*
Results: *MTT assay data showed that hesperidin inhibited AGS and MKN-28 cells. Treatment of AGS cells with hesperidin resulted in apoptosis which manifested as nuclear condensation and cell shrinkage. Staining and FC demonstrated that hesperidin induced mitochondrial apoptosis in AGS cells and increased intracellular ROS. Pre-treatment of AGS cells with NAC inhibited hesperidin-induced apoptosis. Results from western blotting showed that the increased expressions of p-JNK, p-p38, Bad, Caspase-3 and PARP, and downregulation of anti-apoptotic proteins p-ERK and Bcl-2, indicating that hesperidin activated MAPK signaling pathway and mitochondria-dependent apoptosis in AGS cells.*
Conclusion: *Hesperidin promotes apoptosis in human GC AGS cells. This finding provides an insight into the development of new anti-GC drugs.*

Keywords: *Hesperidin, Human gastric cancer cells, Cell Apoptosis, MAPK signal pathway*

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INTRODUCTION

Gastric cancer (GC) is a life-threatening tumor that occurs in the stomach. According to a global survey conducted by the World Health Organization, there were over 1,000,000 new cases of GC worldwide in 2018, with 800, 000 people dead from the disease. The incidence of GC ranks fifth, while the mortality ranks third among the most malignant tumors. Gastric cancer (GC) in China accounts for more than 40 % of all new cases of GC in the world [1, 2]. At present, the treatment of GC in the clinic involve chemotherapy, surgical treatment and radiation therapy, but these treatments are associated with adverse side effects and poor prognosis [3]. Most of the extant anticancer drugs approved for clinical use are unmodified natural products or

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semi-synthetic derivatives. Natural extracts are more potent but have more toxic side effects than existing chemotherapeutic drugs. However, natural extracts provide a promising research direction for development of future chemotherapy drugs [4].

Hesperidin is a natural flavonoid derived from plants belonging to different genus such as *Leguminosae*, *Labiatae*, *Duccinaceae*, *Rutaceae* and *Citrus*. The molecular formula of hesperidin is $C_{28}H_{34}O_{15}$ [5]. Recent studies demonstrated hesperidin possesses many pharmacological effects such as anti-aging, anti-inflammation, anti-coagulant, immunity-boosting, cardio-protective, neuro-protective and anticancer effects [6]. It has been reported that hesperidin activated the expressions of Caspase-9, Caspase-8 and Caspase3 in HepG2 cells by regulating Bcl2/Bax protein, and also induced apoptosis in HepG2 cells [7]. In addition, hesperidin regulated the transcriptional level of breast cancer MCF-7 cells through regulation of the binding of c-JNK N-terminal kinase and extracellular regulated protein kinase (ERK) signaling pathway between AP-1 and C/EBP [8]. Other studies have shown that hesperidin induced apoptosis and inhibited the proliferation of lung cancer cells, but exhibited no cytotoxicity towards normal pulmonary fibroblasts [9].

Although hesperidin effectively inhibits the proliferation of a series of cancer cells and induces apoptosis by regulating multiple signaling pathways and proteins, its efficacy, and the pharmacological mechanisms involved in its effect on GC have not been reported. This study investigated the inhibitory effect of hesperidin on the proliferation and apoptosis of human GC AGS cells, as well as the underlying molecular mechanisms. The results obtained provide new ideas for GC treatment, and may form a basis for development of new anti-GC drugs which may also be useful against other cancers.

EXPERIMENTAL

Cells

Human GC cell lines AGS, NCI-H23 and MKN-45, human normal gastric epithelial cell line GES-1, human normal liver L-02 cell line, and human normal kidney 293T cell line were bought from Shanghai Huiying Biotechnology Company.

Reagents and drugs

Hesperidin was purchased from Chengdu Ruifens Biotechnology Co. Ltd; 5-fluorouracil (5-FU), dimethyl sulfoxide (DMSO), RPMI-1640 medium and fetal bovine serum (FBS) were products of Gibco Company, USA. Green-streptomycin chain double antibody (PS), trypsin (TE) and phosphate buffer (PBS) were purchased from Hyclone Company, USA. Thiazolyl blue (MTT) was supplied by Dinghua Company, China, while JC-1 Fluorescent Probe kits and AnnexinV/PI kits were purchased from Shanghai Beyotime Biotechnology. Antibodies for Bad, α-tubulin, Bcl-2, Caspase-3, PARP, ERK, p-ERK, JNK, p-JNK, p38 and p-p38, as well as HRP-labeled goat anti-rabbit IgG, HRP-labeled goat anti-mouse IgG and sheep anti-mouse IgG were obtained from American Santa Company. 2, 7-Dichloro fluorescent yellow diacetate (DCFH-DA) and ECL chemiluminescence reagents were purchased from China Seymour Technology Co. Ltd.

Methyl thiazolyl diphenyl-tetrazolium (MTT) assay

Cell suspensions of human GC AGS cells in logarithmic growth phase, normal liver L- 02 cells and normal kidney 293T cells were collected (1×10^5 cell/mL). They were inoculated into 96-well plates, each at a volume of 100uL, and incubated in a 5 % CO_2 incubator at 37 ℃. After 24h, the cells were starved for 2 - 4 h in the culture solution with 1 % FBS, and a positive control group and a negative control group were simultaneously set up. In the experimental groups, 1 μL of different concentrations of hesperidin (1, 3, 10, 30 and 100 μM) was added to each well. The control group was treated with the same concentration of 5-FU which was usually used as chemotherapeutic drug.

In the negative control group, 1 μL of DMSO was added to each well (in place of hesperidin). Eight replicate wells were set up in each group. After 24 h of incubation, 15 μL 0.5 % MTT solution was added to each well, followed by incubation for 2 h. Thereafter, the supernatant was discarded. Then, 100 μL DMSO was added to each well to solubilize the formazan crystals formed, and after oscillation for 10 min, the absorbance of each well was measured at a wavelength of 490 nm.

Cell survival and IC_{50} values were calculated. Each experiment was repeated 3 times. The effect of hesperidin on the proliferation of AGS cells, expressed as cell viability (C), was calculated as in Eq 1.

C (%) = {(Ae – Ab)/(Ac – Ab)100 ………. (1)

where Ae, Ab and Ac are the absorbance of experimental, blank and control group samples,

respectively.

Annexin V/PI double staining

The GC AGS cells in logarithmic growth phase and in good growth condition were collected and seeded into 6-well plate at a density of 1 × 10^5 cells/well. The cells were cultured in 5 % CO_2 and 37 ℃ incubator for 24 h. They were then treated with different concentrations of hesperidin (1, 3, 10, 30 and 100 μM) and resuspended in 1 mL of cell staining solution. Then, Annexin V staining solution and PI staining solution (5 μL) were added to each group, and after incubation for 30 min in an ice bath, the morphological changes in AGS cells were observed under an inverted microscope and analyzed.

Flow cytometry

The GC AGS cells were collected and inoculated into 6-well plates at a density of 10^5 cells/well. The cells were cultured for 24 h in a 5 % CO_2 incubator at 37 ^{o}C, after which they were treated with different concentrations of hesperidin (1, 3, 10, 30 and 100 μM) in 1 mL cell staining buffer. Then, Annexin V staining solution and PI staining solution (5 μL each) were added to each group. After incubating in ice bath for 30 min, the morphologies of the AGS cells were examined under an inverted microscope and analyzed.

Evaluation of the effect of hesperidin on ROS levels

The AGS cells were treated with 20 μM hesperidin for 3, 6, 12 and 24 h. Then, the culture medium was discarded, and the cells were rinsed once with PBS and incubated with 10 μM DCFH-DA for 30 min at 37 ℃ in a constant temperature water bath. Thereafter, the cells were rinsed once with PBS, and re-suspended in 500 μL PBS. Finally, the level of ROS in the cells was determined using flow cytometric analysis.

Assessment of te effect of NAC on apoptosis of AGS cells

The AGS cells were pretreated with 5 mM NAC for 30 min, and then treated with hesperidin at a concentration corresponding to its IC_{50} for 24 h. The cells were resuspended in 195 μL Annexin V-FITC solution after washing with PBS. Annexin V-FITC and PI (3 μL each) were added, followed by incubation for 15 min in the dark. Then, the cells were resuspended in 300 μL PBS, and apoptosis was determined using flow cytometry.

Western blotting

Gastric cancer AGS cells with good growth status were inoculated in 6-well plates (2×10^5 cells/well) and treated with hesperidin at a final concentration corresponding to its IC_{50} for different time periods (3, 6, 12 and 24 h). Total protein was extracted from the cells, and the protein concentration of the cell lysate was determined using Coomassie brilliant blue method. Equal amounts of protein were subjected 12% SDS–PAGE, and the bands were transferred to nitrocellulose membrane. The membrane was blocked with 5 % skim milk for 2 h, and washed with TBST solution prior to overnight incubation with primary antibodies for Bcl-2, Bad, cleaved-caspase-3, cleaved-PARP-1, ERK, p-ERK, JNK, p-JNK, p38, p-p38 and α-tubulin at 4 ℃. Thereafter, the membrane was washed with TBST solution and incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 2 h, followed by washing with TBST solution. The resultant bands were quantitatively analyzed using Image J image analysis software.

Statistical analysis

Data are expressed as mean ± standard deviation ($\bar{x} \pm s$). Statistical analysis was done using SPSS version 19.0. Comparison between two groups was done using *t*-test, and values of $p > 0.05$ were considered statistically significant.

RESULTS

Inhibitory effect of hesperidin on the proliferation of human GC cells

The results of MTT test are shown in Figure 1. Hesperidin decreased the viabilities of the three GC cells gradually and in a concentration-dependent manner, indicating that hesperidin had a good inhibitory effect on the proliferation of GC cells. Comparison of the effects of hesperidin and 5-FU (positive control) on proliferation (at the same concentration) showed that there was significant difference between the two groups. Furthermore, the same concentration gradient experiments were carried out on human normal gastric epithelial cells GES-1, human normal liver LV02 and human normal kidney 293T cells. The results showed that hesperidin treatment produced less toxic side effects than 5-FU positive control group (Figure 1). Furthermore, the IC_{50} values of hesperidin for the three GC cells calculated using SigmaPlot software were 21.85 ± 2.57, 34.58 ± 3.69 and 26.45 ± 2.38 μM. The IC_{50} value of hesperidin for AGS cells was low, indicating that AGS cells were the most

sensitive to hesperidin. Thus, AGS cells were used in the subsequent experiments.

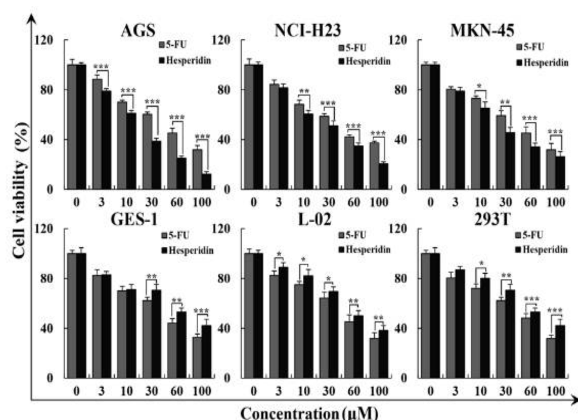

Figure 1: Inhibitory effect of hesperidin on the proliferation of three kinds of GC cells and three kinds of normal cells; **p* < 0.05; ***p* < 0.01; ****p* < 0.001, compared with control group

Effect of hesperidin on apoptosis of human GC AGS cells

In order to investigate whether hesperidin inhibited the proliferation of AGS cells by inducing apoptosis, Annexin V/PI double staining and FC were performed. The results showed that with increase in duration of exposure to hesperidin, the observed green fluorescence intensity gradually increased, indicating that the degree of apoptosis in AGS cells increased gradually, and peaked after 24 h. Under the open field, morphological changes in apoptosis, such as cell shrinkage and decrease of cell density were observed. It can be seen from Figure 3A that with continuous increase in duration of hesperidin treatment, the number of apoptotic cells increased gradually, and the apoptosis reached the highest level when the treatment duration was 24 h. The percentage of cells in the late stage of necrosis and apoptosis in the 3, 6, 12 and 24h groups were 19.37 ± 2.14, 22.34 ± 3.24, 25.63 ± 2.87 and 40.29 ± 4.54, respectively. Changes in mitochondrial membrane potential were determined using JC-1 kit.

With increase in treatment time, the number of cells in the right upper quadrant gradually decreased, while the number of cells in the right lower quadrant increased gradually (Figure 3 A), indicating that mitochondrial membrane potential decreased gradually. Cell apoptosis appeared to be mitochondrial-dependent (Figure 3 B). The expressions of apoptosis-promoting proteins Bad, cleaved-caspase-3 and cleaved-PARP increased with the prolongation of hesperidin treatment, while the expression of Bcl-2 decreased (Figure 3 A). These experimental data demonstrate hesperidin induces apoptosis in human GC AGS cells.

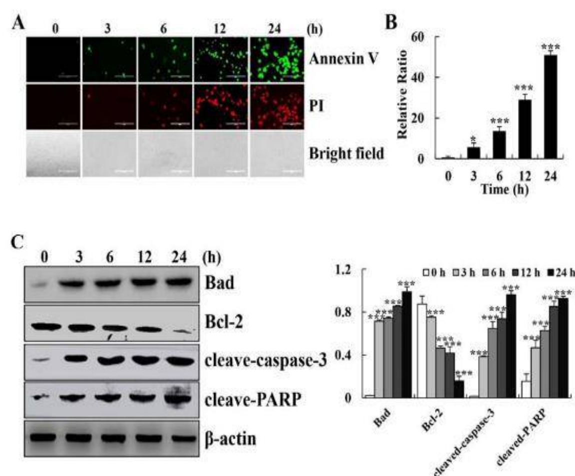

Figure 2: Induction of apoptosis in AGS cells by hesperidin. A: Annexin V-FITC/PI double staining (200×); B: quantitative analysis of A; C: expression levels of apoptosis-related proteins; F: quantitative analysis of C; **p* < 0.05; ****p* < 0.001, compared with control group)

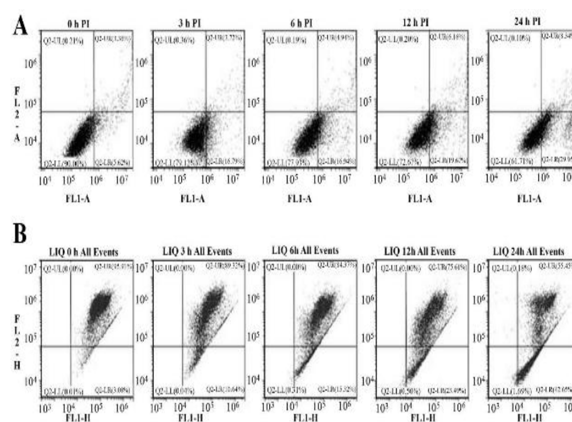

Figure 3: Induction of apoptosis in AGS cells by hesperidin. A: apoptotic ratios as determined using flow cytometry; B: changes in mitochondrial membrane potential, determined using JC-1

Effect of hesperidin on the expressions of signal pathway-related proteins

As shown in Figure 4, the expressions of pro-apoptotic proteins p-JNK and p-p38 increased with increase in treatment time of hesperidin, while the expression of anti-apoptotic protein p-ERK decreased. These results show that hesperidin induced apoptosis AGS cells by regulating the MAPK signaling pathway. Furthermore, it exerted a good cytotoxic effect on AGS cells (Figure 4).

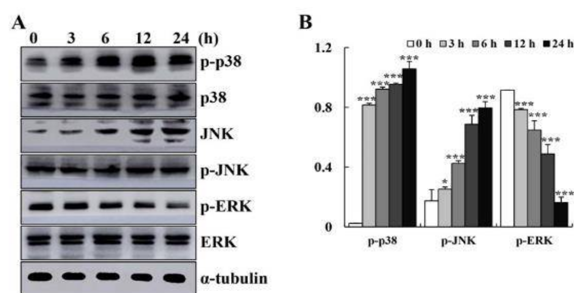

Figure 4: The expression levels of upstream-related proteins in AGS cells treated with hesperidin. A: Expression levels of upstream-related proteins, as determined using western blotting. B: Quantitative analysis of A; * $p < 0.05$; ***, $p < 0.001$, compared with control group

Hesperidin induced apoptosis by regulating ROS levels in AGS cells

Hesperidin treatment led to gradual and time-dependent increases in ROS (Figure 5 A). Moreover, apoptosis was significantly decreased in the group treated with hesperidin and NAC, when compared with the group treated with hesperidin alone (Figure 6 A). The expressions of p-p38, p-JNK and cleaved-caspase-3 protein in hesperidin group were significantly higher than the corresponding expressions in the control group, while the expressions of p-ERK and p-STAT3 protein were significantly lower in the hesperidin group than in the control group. However, the expressions of p-p38, p-JNK and cleaved-caspase-3 protein in hesperidin-and-NAC group were significantly lower than those in hesperidin group, while the protein expression of p-ERK was significantly increased. The results in Figure 6 suggest that hesperidin activated the MAPK signaling pathway by regulating the levels of ROS in GC AGS cells, thereby inducing apoptosis in the cells.

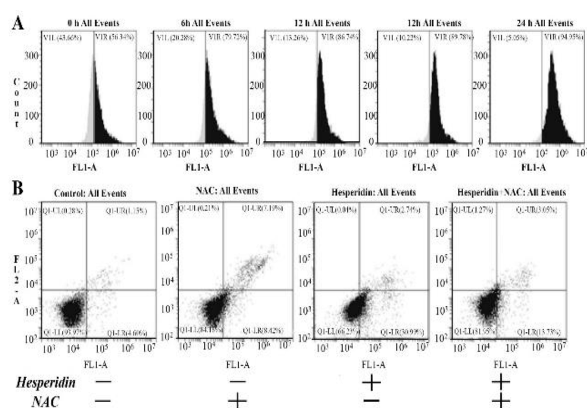

Figure 5: Effect of hesperidin on the expression levels of ROS and cell apoptosis. A: Intracellular ROS levels as determined using flow cytometry. C: Apoptosis in AGS cells treated with NAC, hesperidin and CT+NAC

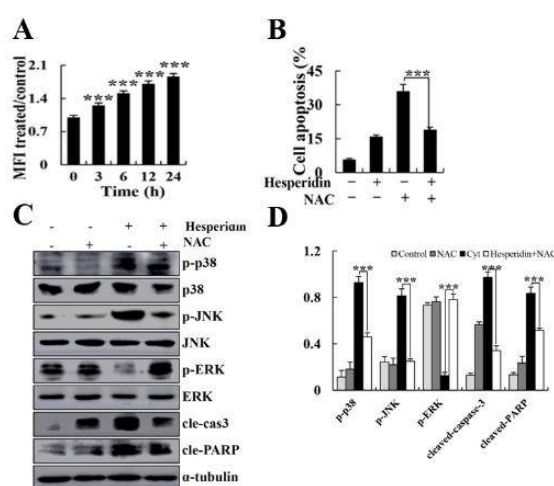

Figure 6: Effect of hesperidin on the expression levels of ROS and cell apoptosis. A: Quantitative analysis of Figure 5A.B: Quantitative analysis of Figure 5B. C: Expression levels of upstream-related proteins. ***$p < 0.001$, compared with control group

DISCUSSION

The induction of apoptosis in cancer cells is one of the promising strategies for cancer treatment [10,11]. Reactive oxygen species (ROS) are normal products of metabolism which are important in the regulation of cell signal pathway and homeostasis *in vivo*. Many studies have shown that apoptosis is regulated by intracellular ROS levels and their related signal pathways. Therefore, regulation of intracellular ROS levels lead to changes in cell homeostasis, and then to cell apoptosis [12]. Hesperidin is a dihydroflavone derivative with pharmacological properties. It induces apoptosis in various tumor cells such as liver cancer cells, breast cancer cells and lung cancer cells. However, not much is known about its effect on GC cells.

It is well known that cell apoptosis occurs through two routes: the mitochondria-dependent pathway and mitochondria-independent pathway, the former of which is regulated by Bcl-2 protein family. The balance between Bad and Bcl-2 proteins affects the activation of Caspase-3 protein. Activated Caspase-3 splits the downstream poly ADP-ribose polymerase (PARP) protein, causing PARP to lose its enzyme activity and eventually leading to apoptosis in the mitochondrial pathway [13,14]. A decrease in cell membrane potential can be easily detected by monitoring changes in fluorescence color of JC-1, which is also a marker of early apoptosis [15,16]. In this study, JC-1 fluorescence probe was used to mark the cells treated with hesperidin. With increase in time of exposure to hesperidin, the green/red fluorescence ratio increased, mitochondrial

membrane potential decreased, and the cells underwent mitochondrial-dependent apoptosis. Subsequently, changes in the expressions of related apoptotic proteins were determined. It was found that hesperidin upregulated the protein expressions of Bad, cleaved-caspase-3 and cleaved-PAPR, and down-regulated the expression of Bcl-2 protein. These results suggest that hesperidin induces mitochondrial dependent apoptosis in human GC AGS cells.

The MAPK family of protein kinases includes three sub-families (ERK, JNK and p38) which regulate many life processes such as cell growth, differentiation and apoptosis [17]. Some studies have shown that the phosphorylation of P38 and JNK upregulates the expressions of cleaved-caspase-3 and cleaved-PARP. Induction of apoptosis in GC cells involves the regulation of several members of the Bcl-2 protein family through the ERK signaling pathway, leading to activation of mitochondria-dependent cell apoptosis [18, 19]. This suggests that MAPK is involved in development of tumors. In order to determine whether hesperidin induced apoptosis in GC AGS cells by regulating the MAPK signaling pathway, the expressions of upstream-related proteins were determined. It was found that hesperidin upregulated the expressions of p-JNK and p-p38 proteins, but downregulated the protein expression of p-ERK. These results suggest that hesperidin induced apoptosis of human GC AGS cells by regulating the MAPK signaling pathway.

The level of ROS is directly related to homeostasis of the intracellular environment. Increases in the level of ROS increase the permeability of mitochondria through matrix swelling and rupture of the outer membrane, leading to the release of apoptosis signal molecules. In addition, ROS regulates a number of intracellular signaling pathways such as NF-kappa B, STAT3 and MAPK, thereby affecting biological processes such as cell growth, differentiation, apoptosis and invasion [20,21].

In this study, the level of intracellular ROS in AGS cells increased with duration of hesperidin treatment. Co-treatment with hesperidin and NAC led to significant reduction in the degree of apoptosis, when compared with the hesperidin treatment group, and the ability of hesperidin to induce apoptosis was inhibited. Thus, hesperidin induced apoptosis by upregulating intracellular ROS in human GC AGS cells. In order to investigate whether the activation of MAPK signaling pathway was regulated by ROS, the expressions of MAPK-related proteins and apoptosis-related proteins were determined at the molecular level. The results revealed that the ROS-scavenging agent NAC significantly reversed the activation of MAPK signal pathway, and also reversed changes in levels of apoptotic proteins. These findings clearly indicate that hesperidin regulates AGS cell apoptosis by regulating ROS levels.

CONCLUSION

The findings of this study demonstrate the inhibitory effect of hesperidin on human GC AGS cells. The results also show that hesperidin induces mitochondria-dependent apoptosis by increasing ROS levels and regulating MAPK signaling pathway in AGS cells. Thus, these findings provide a new strategy for the development of new drugs for the treatment of GC.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication. Wangmei Yu and Zheke Yu conceived and designed the study. Xiaolong Xie, Qingzheng Jin and Huizhong Wu collected and analyzed the data, while Wangmei Yu wrote the manuscript which was approved by all authors.

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