

lncRNA DGCR5 inhibits the proliferation of colorectal cancer cells by downregulating miR-21

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Abstract. Long non-coding RNA (lncRNA) DiGeorge syndrome critical region gene 5 (DGCR5) serves roles as a tumor suppressor or oncogene in different types of cancer. The current study aimed to explore the role of DGCR5 in colorectal cancer (CRC). It was revealed that the expression of DGCR5 was downregulated, while microRNA (miR)-21 was upregulated in CRC. The expression level of DGCR5 in tumor tissue decreased, while expression levels of miR-21 increased, with advancing stages of the disease. The expression levels of DGCR5 and miR-21 were inversely associated in tumor tissues. In CRC cells *in vitro*, miR-21 overexpression failed to significantly affect DGCR5, while DGCR5 overexpression resulted in reduced expression levels of miR-21. DGCR5 overexpression showed no significant effects on cancer cell migration and invasion, but suppressed cancer cell proliferation *in vitro*. miR-21 overexpression increased cancer cell proliferation and attenuated the effects of DGCR5 overexpression. Therefore, lncRNA DGCR5 may inhibit the proliferation of CRC cells by downregulating miR-21.

Introduction

Incidence of colorectal cancer (CRC) was the third most common type of cancer in certain regions of the world in 2018, such as the USA (1). CRC is a fatal disease, resulting in >500,000 mortalities every year worldwide (2). Patients with early stages of CRC are treated surgically, followed by adjuvant therapy to inhibit cancer cell proliferation and metastasis (3). However, a considerable number of patients with CRC are diagnosed with metastasis to the lymph nodes and

major organs, such as the lungs and liver, leading to poor treatment outcomes and a low overall 5-year survival rate (4,5). Therefore, novel therapeutic targets are required to improve the survival of patients with advanced CRC.

Genome-wide analysis of genetic alterations has revealed that certain somatic and germline mutations are critical determinants of tumorigenesis in CRC (6,7). Long non-coding RNAs (lncRNAs) are RNA transcripts >200 nucleotides in length with non-protein-coding abilities, and have been identified as critical players in cancer biology through their regulatory roles in gene expression at posttranscriptional or translational levels and through epigenetic pathways (8). In effect, certain lncRNAs are potential therapeutic targets for the treatment of many types of cancers, such as lung cancer, liver cancer and CRC (9,10). However, the application of most lncRNAs in cancer therapies is limited by their unknown functions. lncRNA DiGeorge syndrome critical region gene 5 (DGCR5) has been implicated in many types of cancer, and serves a cancer-specific role as a tumor suppressor in papillary thyroid carcinoma or as an oncogene lung adenocarcinoma (11,12). The majority of studies investigating the role of DGCR5 in cancer biology have revealed its interactions with microRNAs (miRNAs/miRs), a group of small non-coding RNA molecules ~22 nucleotides in length which serve important roles in gene expression regulation through the cleavage of transcripts (11,12). miR-21 is a well-characterized oncogenic miRNA in several types of cancer, including CRC (13). miR-21 participates in CRC by regulating various cancer cell behaviors through the interaction with multiple downstream tumor suppressive and oncogenic pathways, such as the phosphatase and tensin homolog (PTEN) and signal transducer and activator of transcription 3 pathways, respectively (13). Preliminary data has revealed that DGCR5 and miR-21 were inversely correlated in human CRC tissues (data not shown). The present study investigated the role of DGCR5 in CRC as well as the possible interaction between DGCR5 and miR-21.

Materials and methods

Patients. The present study included 65 patients with CRC enrolled at The Second Affiliated Hospital of Guangxi University of Science and Technology between March 2016 and

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March 2018. The patients included 35 males and 30 females and their mean age was 51.3 ± 5.7 (range, 34–66 years). The diagnosis of the patients was confirmed by histopathological examinations. The inclusion criteria for participation in the current study were as follows: i) patients diagnosed with CRC for the first time; ii) patients had not been treated prior to admission; and iii) patients with complete medical records. The exclusion criteria were as follows: i) patients with previous history of malignancy; ii) patients who received treatment within 3 months prior to admission; iii) patients who were transferred from other hospitals. Based on the American Joint Committee on Cancer staging criteria (AJCC) used (14), there were 14, 20, 18 and 13 patients at stage I, II, III and IV, respectively. The Ethics Committee of The Second Affiliated Hospital of Guangxi University of Science and Technology approved the current study and all patients signed informed consent.

Local blast. A local blast analysis was performed using NCBI-blast software (version 2.2.30; National Center for Biotechnology Information). In this analysis, human miRNAs sequences (<http://www.mirbase.org>) were used as queries, and DGCR5 was used as a targeted database.

Specimens and cell lines. A biopsy was performed on each patient to obtain cancer and adjacent healthy tissues (2 cm distance from the tumor site) from each patient. The weight of the tissues ranged between 0.07 and 0.11 g. Tissues were stored at -70°C until use. All tissue samples were confirmed by histopathological examination. RKO (American Type Culture Collection) and CR4 (Sigma-Aldrich; Merck KGaA) human CRC cell lines were used to perform *in vitro* cell experiments. Cells were cultured according to the manufacturer's protocol using Eagle's Minimum Essential Medium (Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA) at 5% CO_2 and 37°C .

Total RNA extraction and reverse-transcription quantitative polymerase chain reaction (RT-qPCR). For the analysis of DGCR5 expression levels, RNAzol[®] RT reagent (Sigma-Aldrich; Merck KGaA) was used to extract total the RNA from CRC and adjacent healthy tissues as well as the RKO and CR4 cell lines. RNA samples were reverse transcribed into cDNA using the AMV Reverse Transcriptase kit (Promega Corporation) at the following thermal conditions: 15 min at 55°C and 10 min at 80°C . qPCR was subsequently performed using the SYBR[®]-Green master mix (Bio-Rad Laboratories, Inc.) using 18S rRNA as an endogenous control. The following primer sequences were used: DGCR5 forward, 5'-CCAAGCCTGTCTGTGTGTTC-3' and reverse, 5'-GGGAGACACAGACCACAAGA-3'; and 18S forward, 5'-GCTTAATTTGACTCAACACGGGA-3' and reverse, 5'-AGCTATCAATCTGTCAATCCTGTC-3'. Thermal conditions were: 1 min at 95°C , followed by 40 cycles of 10 sec at 95°C and 40 sec at 95°C . For the analysis of miR-21 expression levels, extraction of miRNAs from CRC and adjacent healthy tissues as well as the RKO and CR4 cell lines was performed using a microRNA purification kit (cat. no. 21300; Norgen Biotek Corp.) according to manufacturer's protocol. TaqMan microRNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.) was used to perform reverse transcription according to manufacturer's protocol. TaqMan[™] Fast Advanced Master mix

(Thermo Fisher Scientific, Inc.) was used to prepare all qPCR reaction mixtures using U6 as the endogenous control. The following primer was used for the reaction: miR-21 forward, 5'-GCCCGCTAGCTTATCAGACTGATG-3'. The miR-21 reverse primer and U6 forward and reverse primers were included in the TaqMan[™] Fast Advanced Master mix kit. The thermal conditions were: 1 min at 95°C , followed by 40 cycles of 10 sec at 95°C and 20 sec at 95°C . All RT-qPCR reactions were performed in triplicate.

All data were analyzed using the $2^{-\Delta\Delta\text{C}_q}$ method (15) and each experiment was performed in triplicate.

Transient transfection. Sequences for the negative control (5'-CGCGCUUCGGUUUAACUAGC-3') miRNA and miR-21 mimic (5'-CAACACCAGUCGAUGGGCUGU-3') were obtained from Sigma-Aldrich; Merck KGaA. The DGCR5-expression pcDNA3.1 and empty pcDNA3.1 vectors were obtained from by Sangon Biotech Co., Ltd. A total of 10^5 CRC or RKO cells were transfected with 40 nM miRNA or 10 nM vector using Lipofectamine[®] 2000 (Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. Two types of control cells, including cells without transfection (C) and cells transfected with empty vectors or negative control miRNAs (NC) were included. Subsequent experiments were performed 24 h following transfection.

Cell proliferation assay. CRC and RKO cells were harvested 24 h following transfection and single cell suspensions were prepared using Eagle's Minimum Essential Medium supplemented with 10% FBS to a final cell density of 4×10^4 cell/ml. Cells suspensions were cultured at 37°C and 5% CO_2 in a 96-well plate containing 0.1 ml per well. A total of 10 μl Cell Counting Kit-8 solution (Sigma-Aldrich; Merck KGaA) was added into each well every 24 h for 96 h. The optical density was measured at a wavelength of 450 nm to represent cell proliferation.

Statistical analysis. Three biological replicates were included in each experiment. Differences between CRC and healthy adjacent tissues were analyzed by the paired t-test. Differences among different clinical stages and cell transfection groups were analyzed by the one-way ANOVA and Tukey test. Linear regression was performed to analyze the association between the expression levels of DGCR5 and miR-21 in CRC and healthy adjacent tissues. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

DGCR5 and miR-21 are dysregulated in CRC tissues. The expression levels of DGCR5 and miR-21 in CRC and healthy adjacent tissues were quantified by RT-qPCR and the expression data were analyzed using the paired t-test. It was observed that DGCR5 was significantly downregulated (Fig. 1A), while miR-21 was significantly upregulated (Fig. 1B), in CRC tissues compared with healthy adjacent tissues in patients with CRC ($P < 0.05$), indicating the involvement of DGCR5 and miR-21 in CRC.

Expression levels of DGCR5 and miR-21 in CRC tissues are affected by clinical stages. The expression levels of DGCR5

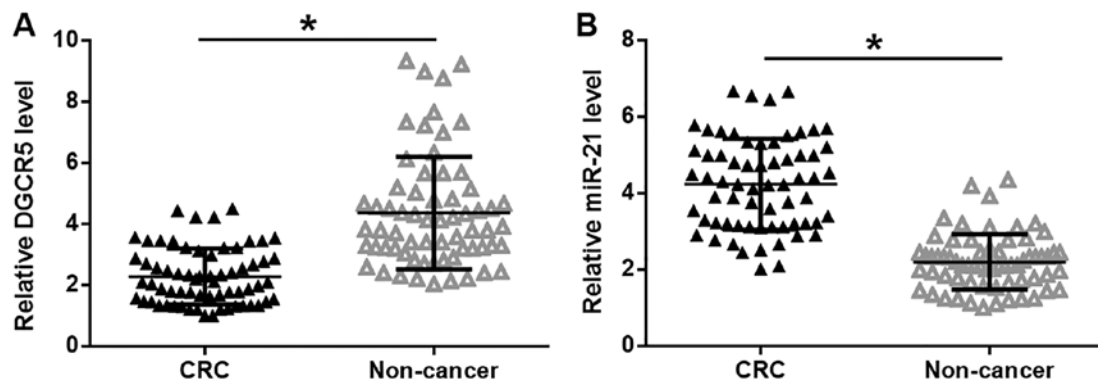


Figure 1. DGCR5 and miR-21 were dysregulated in CRC tissues. Analysis of reverse-transcription quantitative polymerase chain reaction data using the paired t-test revealed that (A) DGCR5 was significantly downregulated while (B) miR-21 was significantly upregulated in CRC tissue compared with adjacent non-cancer tissue in patients with CRC. * $P < 0.05$, as indicated. DGCR5, DiGeorge syndrome critical region gene 5; miR, microRNA; CRC, colorectal cancer.

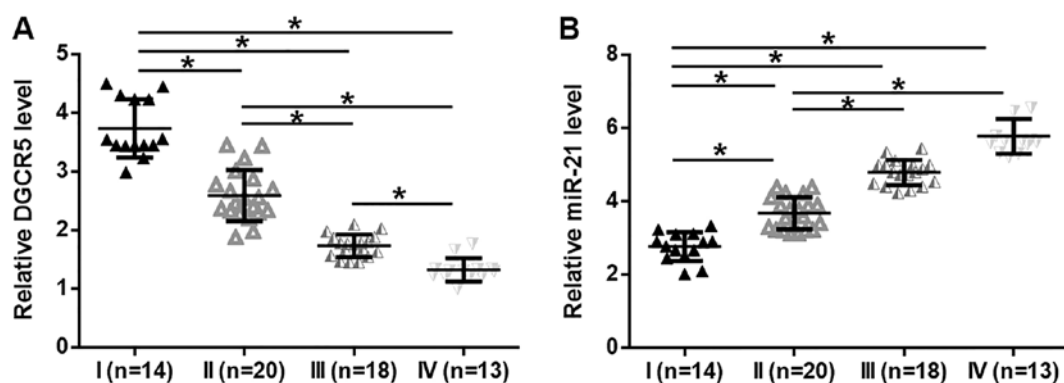


Figure 2. Expression levels of DGCR5 and miR-21 in CRC tissues were affected by clinical stages. Differences in expression levels of DGCR5 and miR-21 among patients with different clinical stages were analyzed using the one-way analysis of variance followed by the Tukey post hoc test. (A) DGCR5 expression levels decreased while (B) miR-21 expression levels increased with advanced clinical stages. * $P < 0.05$, as indicated. DGCR5, DiGeorge syndrome critical region gene 5; miR, microRNA; CRC, colorectal cancer.

and miR-21 in patients with different clinical stages of CRC were analyzed using the one-way ANOVA and Tukey test. The expression levels of DGCR5 in CRC tissues were significantly decreased (Fig. 2A), while expression levels of miR-21 were significantly increased (Fig. 2B), with advancing clinical stages ($P < 0.05$).

DGCR5 and miR-21 expression levels are inversely associated in CRC tissues. Linear regression was performed to analyze the association between the expression levels of DGCR5 and miR-21. It was observed that DGCR5 and miR-21 were inversely associated in CRC tissues (Fig. 3A). However, the association between DGCR5 and miR-21 was not statistically significant in healthy adjacent tissues (Fig. 3B). It suggested that DGCR5 and miR-21 may interact with each other in CRC.

DGCR5 overexpression causes the downregulation of miR-21 in CRC cells. In order to explore the interaction between DGCR5 and miR-21 in CRC, a DGCR5 expression vector and miR-21 mimic were transfected into CRC and RKO cell lines. Compared with control (C) and negative control (NC) groups, expression levels of DGCR5 and miR-21 were upregulated by more than 2 fold at 24 h after transfection (Fig. 4A; $P < 0.05$), indicating the successful transfections. In addition, miR-21

overexpression failed to significantly affect DGCR5 (Fig. 4B), while DGCR5 overexpression led to reduced expression levels of miR-21 (Fig. 4C; $P < 0.05$).

DGCR5 regulates cancer cell proliferation but not migration and invasion through miR-21. Comparing with the C and NC groups, DGCR5 overexpression showed no significant effects on RKO and CR4 cell migration and invasion (data not shown; revealed by Transwell migration and invasion assay). By contrast, DGCR5 overexpression resulted in inhibited RKO and CR4 cell proliferation compared with the C and NC groups (Fig. 5; $P < 0.05$). miR-21 overexpression resulted in increased cancer cell proliferation and attenuated the effects of DGCR5 overexpression (Fig. 5; $P < 0.05$).

Discussion

lncRNA DGCR5 plays a role as tumor suppressor in certain types of cancer, including lung cancer, but serves as an oncogene in other types of cancer, including papillary thyroid carcinoma and liver cancer (11,12,16). The present study revealed the tumor suppressive role of DGCR5 in CRC and demonstrated that DGCR5 may inhibit the proliferation of CRC cells by downregulating miR-21, an oncogenic miRNA in CRC (13).

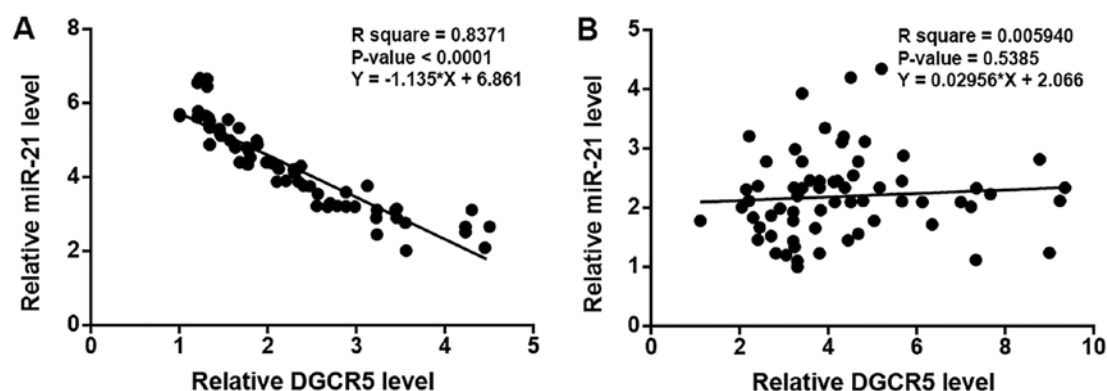


Figure 3. DGCR5 and miR-21 were inversely associated in CRC tissues. Linear regression analysis showed that DGCR5 and miR-21 were (A) inversely associated in CRC tissues but not in (B) adjacent non-cancer tissues. DGCR5, DiGeorge syndrome critical region gene 5; miR, microRNA; CRC, colorectal cancer.

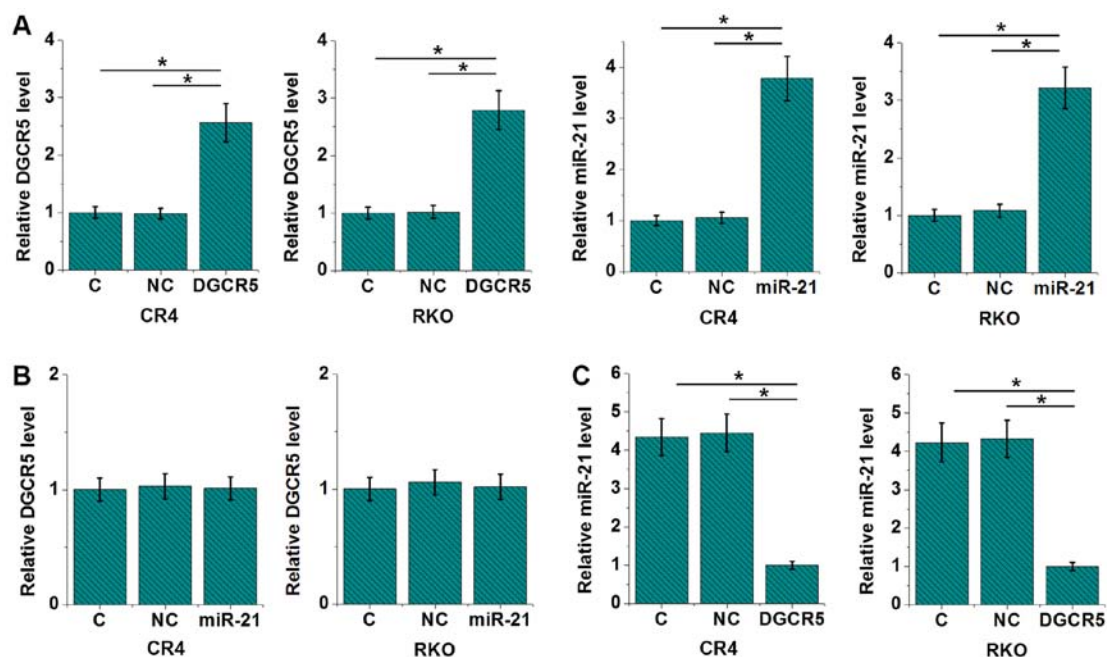


Figure 4. DGCR5 overexpression downregulated miR-21 expression in CRC cell lines. (A) Compared with the C and NC groups, expression levels of DGCR5 and miR-21 were upregulated >2-fold 24 h following transfection. (B) miR-21 overexpression failed to significantly affect DGCR5 expression and (C) DGCR5 overexpression reduced expression levels of miR-21. * $P < 0.05$, as indicated. DGCR5, DiGeorge syndrome critical region gene 5; miR, microRNA; CRC, colorectal cancer; C, control; NC, negative control.

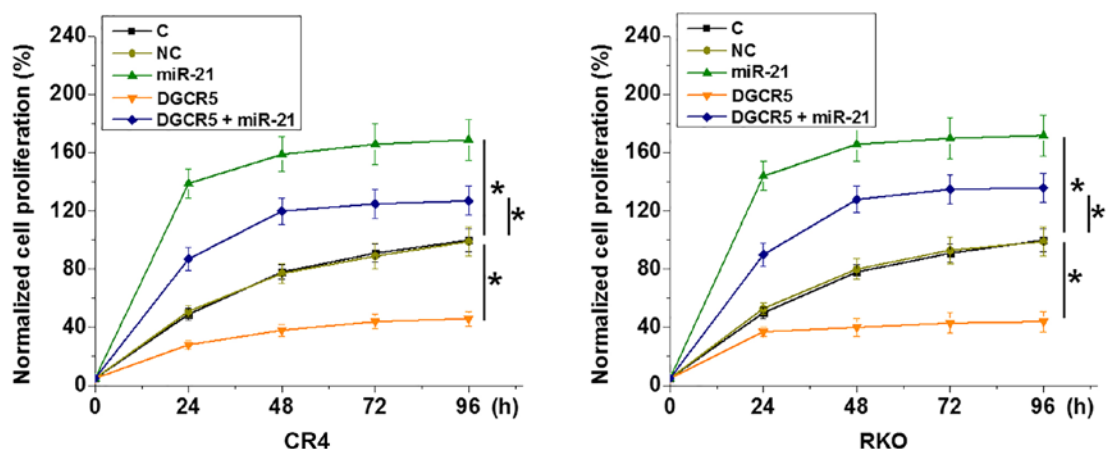


Figure 5. DGCR5 regulated cancer cell proliferation through miR-21. DGCR5 overexpression inhibited cancer cell proliferation. miR-21 overexpression promoted cancer cell proliferation and attenuated the effects of DGCR5 overexpression. * $P < 0.05$, as indicated. DGCR5, DiGeorge syndrome critical region gene 5; miR, microRNA; C, control; NC, negative control.

DGCR5 has opposite functions in different types of cancer. DGCR5 was upregulated during the development of lung cancer, and DGCR5 overexpression inhibited hsa-mir-22-3p to promote cancer development (11). By contrast, DGCR5 inhibits miR-2861 to serve a tumor suppressive role in papillary thyroid carcinoma (12). The present study revealed that DGCR5 was downregulated in CRC tissues compared with healthy adjacent non-cancer in patients with CRC patients. Additionally, overexpression of DGCR5 decreased the proliferation of CRC cells *in vitro*. Therefore, the results obtained in the present study suggested that DGCR5 serves a tumor suppressive role in CRC.

To the best of our knowledge, the present study was the first to report the inhibitory role of DGCR5 on miR-21, which as a well-characterized oncogenic miRNA in several types of cancer (17). miR-21 expression in cancer development may be regulated by certain lncRNAs (18,19). One possible mechanism of the interaction between lncRNAs and miRNAs is that lncRNAs may sponge miRNAs and inhibit their function (20). However, a BLAST (NCBI) search did not reveal potential binding sites for miR-21 on DGCR5, suggesting that other molecular mechanisms may mediate interactions between lncRNAs and miRNAs. Consistently, preliminary luciferase assay data (data not shown) suggested an indirect interaction between miR-21 and DGCR5. The relative luciferase signals of miR-21 and negative control were 100 ± 5.6 vs. 99.7 ± 4.9 . It has been shown that both DGCR5 and miR-21 interact with PTEN (21,22). In addition, PTEN is downregulated in CRC (23), and PTEN can mediate the interaction between certain lncRNAs and miRNAs (24). Therefore, PTEN may mediate the interaction between DGCR5 and miR-21; however, future studies are required to elucidate the molecular mechanism involved.

DGCR5 overexpression resulted in downregulated miR-21 but failed to significantly affect CRC cell migration and invasion (data not shown). miR-21 may regulate *in vitro* CRC cell invasion and migration (16). Therefore, DGCR5 may interact with multiple effectors to regulate CRC cell behavior. Future studies are required to identify the effectors involved.

In conclusion, DGCR5 was downregulated in CRC tissues compared to healthy adjacent samples in patients with CRC and overexpression of DGCR5 *in vitro* inhibited CRC cell proliferation by downregulating miR-21.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

HH and XY designed the experiments. HH, XY, JC and JF performed the experiments. CC, JW and QM collected and

analyzed the data. XY drafted the manuscript. All authors have read and approved this manuscript.

Ethics approval and consent to participate

The Ethics Committee of The Second Affiliated Hospital of Guangxi University of Science and Technology approved the current study and all patients signed informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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