Small interfering RNA-mediated knockdown of KRT80 suppresses colorectal cancer proliferation

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Abbreviations: DEGs, differentially expressed genes; KRT80, keratin 80; TCGA, The Cancer Genome Atlas; RT-qPCR, reverse transcription-quantitative PCR; CCK-8, Cell Counting Kit-8; siRNA, small interfering RNA; EdU, 5'ethynyl 2'-deoxyuridine; PPP1CA, protein phosphatase 1 catalytic subunit α; p21, cyclin-dependent kinase inhibitor 1A; p27, cyclin-dependent kinase inhibitor B

Key words: colorectal cancer, keratin 80, proliferation, cell cycle, DNA replication

Abstract. Colorectal cancer (CRC) is the third most common cancer in the world and its development is associated with oncogenic dysfunction. Therefore, the present study aimed to identify differentially expressed genes (DEGs) in CRC tissues and to determine the role of keratin 80 (KRT80) in CRC cell proliferation. DEGs were initially screened in 32 paired CRC tissues and matched adjacent normal tissues from RNA-Seq datasets in The Cancer Genome Atlas database using the limma package in R software. In total, 2,114 DEGs were identified, of which KRT80 was discovered to be the most upregulated in CRC tissues. Moreover, increased KRT80 expression levels were confirmed in tissues collected from 50 patients with CRC using reverse transcription-quantitative PCR, and its increased expression levels were significantly associated with increased lymph node and distant metastasis and a higher pathological stage. Furthermore, KRT80 knockdown using siRNA decreased the viability and proliferation of CRC cells. Finally, pathway analysis revealed that the proteins co-expressed with KRT80 in CRC were enriched in the cell cycle, DNA replication, immune system, metabolism of protein and RNA, signal transduction and other cellular processes. Among them, the cell cycle and DNA replication pathways contained the highest number of the proteins identified. In conclusion, the findings of the present study suggested that KRT80 may be overexpressed in CRC tissues. Furthermore, KRT80 may be involved in the proliferation of CRC cells, which is likely through its ability to regulate the cell cycle and DNA replication pathways, thus it may serve as a potential therapeutic target for patients with CRC.

Introduction

Colorectal cancer (CRC) is the most common malignancy of the digestive system and it is associated with high morbidity and mortality rates (1). According to the cancer statistics from 185 countries, ~800,977 new CRC cases were reported in 2018 and the mortality rate was as high as 47.8% (2). Although radical surgical resection combined with radiotherapy or chemotherapy is widely used for the treatment of CRC, its efficacy remains low; the 5-year survival rate of patients with CRC without metastasis is 40-90%, which drops to 10-15% in patients exhibiting metastasis (3). Thus, clinically effective therapeutic targets for CRC are still limited (4), and the identification of novel specific molecular targets is required to identify the mechanisms underlying CRC tumorigenesis and for the development of new drugs.

Gene expression profiling analysis based on large datasets serves an increasingly important role in determining potential molecular markers for different types of cancer (5,6). To investigate new CRC-related genes, the present study screened differentially expressed genes (DEGs) in 32 patients with CRC from RNA-Seq datasets in The Cancer Genome Atlas (TCGA) database and identified keratin 80 (KRT80) as the most upregulated gene. KRT80 is located on chromosome
12q13 and encodes a 452-amino-acid protein that is a type II keratin (7,8). Keratins are intermediate filament cytoskeletal proteins that maintain the structural integrity of epithelial cells and they have also been reported to be representative markers for epithelial cells (9,10). Keratin expression is tissue-specific and related to advanced tissue or cell differentiation (10). Previous studies have revealed that keratins were extensively expressed in human cancer and suggested that they may be used as molecular markers for the diagnosis of multiple types of tumor, such as basal cell carcinoma, oral squamous cell carcinoma, bladder cancer, breast cancer, hepatocellular carcinoma, cervical cancer and gastric adenocarcinoma (11). Furthermore, it has been reported that keratins served an important role in the regulation of cancer cell migration and invasion (12,13). In fact, the keratin family in humans consists of 28 type I keratins (KRT9-KRT40) and 26 type II keratins (KRT1-KRT8, KRT71-KRT86) (14,15). Among them, KRT7, KRT8 and KRT18-KRT20 were found to participate in the proliferation and differentiation of colon cells (16-18). However, to the best of our knowledge, there are few reports investigating the association between KRT80 expression levels and human cancer. Especially in CRC, only one previous study has demonstrated that KRT80 promoted the migration and invasion of CRC cells (19). Thus, the functional role of KRT80 in CRC cell proliferation remains relatively unclear.

In the present study, gene expression profiling analysis was performed to identify DEGs associated with CRC. Following the identification of KRT80, the study aimed to investigate the expression, function and underlying mechanisms of KRT80 in CRC cell proliferation. The findings supported the role of KRT80 in CRC development, and provided further experimental evidence and a theoretical basis for using KRT80 as a therapeutic target in patients with CRC.

**Materials and methods**

**Data mining and analysis.** The mRNA expression profiles of human CRC cases from RNA-Seq datasets in the TCGA database (TCGA_COADREAD_exp_HiSeq2013-05-24) were downloaded from the UCSC Xena Browser (https://xenabrowser.net/datapages/). The DEGs between 32 CRC tissues and adjacent normal tissues were identified using the limma package (v3.34.9) of R software (20). DEGs with the cutoff criteria of false discovery rate (FDR) <0.001, log₂(fold-change)>1 and average expression value ≥5 were considered to be statistically significant. The mRNA expression levels of KRT80 in multiple forms of cancer (including bladder, brain, breast, cervical, colorectal, esophageal, gastric, head and neck, kidney, ovarian, liver, lung, pancreatic and prostate cancer and lymphoma, melanoma, myeloma, leukemia and sarcoma) were further analyzed using ONCOMINE database (access date: January 9th 2019; www.oncomine.org) (21), with cut-off values of P<0.001 and a fold-change of 2.0. The proteins co-expressed with KRT80 in TCGA-CRC cases (false discovery rate values <0.05 and absolute Spearman's rank correlation coefficient ≥0.4) were identified using the eBioPortal database (accessed March 2019; http://www.ebiportal.org) (22,23). The co-expressed proteins were subjected to pathway analysis using the Reactome database (accessed March 2019; https://reactome.org/PathwayBrowser) (24) and P<0.05 as the cut-off value.

**Cell culture.** Human CRC cell lines (HCT116, RKO, LoVo, HT29, SW480 and SW620), the normal colorectal epithelial cell line FHC and normal colorectal fibroblasts CCD118CO were obtained from the American Type Culture Collection. HCT 116 and HT-29 cells were cultured in McCoy's 5A medium (Gibco; Thermo Fisher Scientific, Inc.); RKO and CCD18CO cells were cultured in Eagle's MEM medium (Gibco; Thermo Fisher Scientific, Inc.); LoVo cells were cultured in Ham's F-12K medium (Gibco; Thermo Fisher Scientific, Inc.); SW480 and SW620 cells were cultured in RPMI-1640 medium (HyClone; GE Healthcare Life Sciences); and FHC cells were cultured in DMEM/F12 medium (Gibco; Thermo Fisher Scientific, Inc.). All media were supplemented with 10% (v/v) FBS (Gibco; Thermo Fisher Scientific, Inc.), and maintained in a humidified incubator at 37°C with 5% CO₂.

**Clinical tissue samples.** The present study was conducted in accordance with the Declaration of Helsinki and the protocol was approved by the Ethics Committee of Shantou University Medical College (approval no. SUMC-2015-42). All patients provided their oral informed consent prior to participation in the study. The need for additional written consent was waived by the Ethics Committee of Shantou University, as the patients had authorized the use of their samples in additional studies in a written consent form they signed when donating their tissues for use in an earlier study. None of the patients had received preoperative treatment or been diagnosed with other types of primary tumors. In total, 50 pairs of CRC tissues and matched adjacent normal tissues were collected from patients (age range, 30-86 years; mean age, 60.7 years; 31 males and 19 females) at the First Affiliated Hospital of Shantou University Medical College between October 2015 and January 2017. The samples were obtained during surgery following the removal of tissue for routine pathology examination. Postoperatively, the tumors were histologically classified and staged based on the AJCC 7th edition tumor node metastasis (TNM) system (25), and the clinical characteristics of these patients with CRC are presented in Table I. All the tissue samples were immediately flash frozen in liquid nitrogen and subsequently stored at -80°C for reverse transcription-quantitative PCR (RT-qPCR) analysis.

**RT-qPCR.** Total RNA was extracted from CRC tissues and cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA using a PrimeScript RT-reagent kit with gDNA Eraser (Takara Bio, Inc.) according to the manufacturer's protocol. qPCR was subsequently performed using a QuantitalNova™ SYBR Green PCR mix kit (Roche Diagnostics) and an ABI Prism 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The following primer pairs were used for the qPCR: KRT80 forward, 5'-CTGCCTCTAATATG GCAAGGTG-3' and reverse, 5'-AGATGCCCCAGGTCTG AAGAT-3'; and β-actin forward, 5'-CTGGAGCGTGGTAGG TGACA-3' and reverse, 5'-AGGGACTTCCTGTAACATG CA-3'. The following thermocycling conditions were used for the qPCR: Initial denaturation at 95°C for 10 min; and 40 cycles of 95°C for 10 sec and 60°C for 30 sec. Expression levels of KRT80 were quantified using the 2^−ΔΔCq method for
Table I. Clinicopathological characteristics of the patients with colorectal cancer (N=50).

| Variable                        | Cases n (%) |
|---------------------------------|-------------|
| Age                             |             |
| ≤60 years                       | 25 (50)     |
| >60 years                       | 25 (50)     |
| Sex                             |             |
| Male                            | 31 (62)     |
| Female                          | 19 (38)     |
| Degree of differentiation       |             |
| Moderate to low                 | 9 (18)      |
| High                            | 38 (76)     |
| Unknown                         | 3 (6)       |
| Tumor invasion                  |             |
| T1-2                            | 6 (12)      |
| T3-4                            | 44 (88)     |
| Distant metastasis              |             |
| No                              | 40 (80)     |
| Yes                             | 10 (20)     |
| Lymph node metastasis           |             |
| No                              | 32 (64)     |
| Yes                             | 18 (36)     |

Cell experiments and the 2−ΔΔct method for tissue samples (26), and normalized to the loading control β-actin.

Cell transfection. RNA interference was used to knock down KRT80 expression levels in SW480 and SW620 cells. Briefly, small interfering RNA (siRNA) against KRT80 (siRNA-KRT80; 5'-CCCTGGATGTCAGTTGGA-3') and siRNA-negative control (NC: 5'-ACUGACACGUC GGAGAATT-3') were obtained from Guangzhou RiboBio Co., Ltd. A total of 2x10⁶ cells/well were seeded into six-well plates in RPMI-1640 medium, supplemented with 10% FBS. Following overnight attachment in a humidified incubator at 37˚C with 5% CO₂, SW480 and SW620 cells were transfected with 100 nM siRNA-KRT80 or siRNA-NC using Lipofectamine RNAiMAX reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol. A total of 5x10⁴ SW480 and SW620 cells/well were plated into 96-well plates in RPMI-1640 medium, containing 10% FBS. Following overnight attachment in a humidified incubator at 37˚C with 5% CO₂, SW480 and SW620 cells were transfected with siRNA-KRT80 or siRNA-NC for 48 h. Subsequently, the cells were incubated with 100 µl 50 µM EdU reagents at 37˚C. Following 2 h of incubation, the cells were fixed with 50 µl 4% paraformaldehyde for 30 min at 25˚C. Cells were washed three times in 100 µl PBS -0.1% Triton X-100 prior to incubation with 100 µl 1X Apollo solution (containing deionized water 93.8 µl, Apollo® reaction buffer 5 µl, Apollo® catalyst solution 1 µl, Apollo® fluorescent dye solution 0.3 µl and Apollo® buffer additive 0.9 mg) for 30 min at room temperature in the dark. The cells were subsequently stained with 100 µl 1X Hoechst 33342 nuclear dye for 30 min at room temperature. Stained cells were visualized using a DM IL LED fluorescence microscope (magnification, x200; Leica Microsystems GmbH), with an excitation wavelength of 567 nm. Image analysis was performed using ImageJ v1.8.0 software (National Institutes of Health).

Western blotting. SW480 cells were transfected with siRNA-KRT80 or siRNA-NC for 48 hand were lysed in radioimmunoprecipitation assay buffer (Sigma-Aldrich; Merck KGaA), supplemented with protease inhibitors (Roche Diagnostics). Total protein was quantified using a bichinonic acid assay kit (Pierce; Thermo Fisher Scientific, Inc.) and 20 µg protein/lane was separated via 12% SDS-PAGE. The separated proteins were subsequently transferred to PVDF membranes (EMD Millipore) and blocked with 5% skimmed milk at room temperature for 1 h. The membranes were incubated with the following primary antibodies overnight at 4˚C: Anti-KRT80 (1:800; cat. no. 16835-1-AP; ProteinTech Group, Inc.), anti-protein phosphatase 1 catalytic subunit α (PPP1CA; 1:2,000; cat. no. 67070-1-Ig; ProteinTech Group, Inc.), anti-cyclin-dependent kinase inhibitor 1A (p21; 1:1,000; cat. no. 2947; Cell Signaling Technology, Inc.), anti-cyclin-dependent kinase inhibitor 1B (p27; 1:1,000; cat. no. 3686; Cell Signaling Technology, Inc.) and anti-β-actin (1:2,500; cat. no. A5491; Sigma-Aldrich; Merck KGaA). Following the primary antibody incubation,
membranes were incubated with a horseradish peroxidase (HRP)-conjugated anti-mouse IgG (H+L) secondary antibody (1:5,000; cat. no. SA00001-1; ProteinTech Group, Inc.) or an HRP-conjugated anti-rabbit IgG (H+L) secondary antibody (1:5,000; cat. no. SA00001-2; ProteinTech Group, Inc.) at room temperature for 2 h. Protein bands were visualized using the SuperSignal™ West Dura Extended Duration Substrate reagent (Thermo Fisher Scientific, Inc.) and an Amersham Imager 600 (GE Healthcare). Expression levels were quantified using ImageJ v1.8.0 software (National Institutes of Health) and normalized to β-actin, the loading control.

Statistical analysis. Statistical analysis was performed using SPSS 23.0 software (IBM Corp.). Statistical differences between groups were either determined using a two-tailed unpaired Student's t-test, a paired Student's t-test or one-way ANOVA, followed by Dunnett's multiple comparison test. All data are presented as the mean ± SD. P<0.05 was considered to indicate a statistically significant difference.

Results

Screening of DEGs identifies KRT80 as the most upregulated gene in CRC. Among the CRC tissue samples from RNA-Seq datasets in the TCGA database, only 32 CRC specimens had matching adjacent normal tissues. Therefore, DEG analysis was only performed on these 32 pairs of tissue samples. A total of 2,114 DEGs were screened (consisting of 844 upregulated and 1,270 downregulated DEGs) between CRC and normal tissues, and KRT80 was discovered to be the most upregulated gene (Fig. 1A; Data S1). The ONCOMINE database was further used to compare the transcriptional levels of KRT80 in different types of cancer and the corresponding normal tissue; compared with the normal tissues, the mRNA expression levels of KRT80 were revealed to be significantly increased in 29 datasets (P<0.001), including CRC, gastric, lung and kidney cancer (Fig. 1B). Among these cancers, the CRC dataset accounted for 72.4% (21/29) of the cases (Fig. 1B).
To verify the above findings, KRT80 expression levels in CRC cell lines and cancerous tissues were further analyzed using RT-qPCR analysis. The expression levels of KRT80 mRNA were significantly increased in the CRC cell lines HCT116, RKO, LoVo, HT29, SW480 and SW620, compared with that noted in the normal colorectal cells FHC and CCD18CO (Fig. 2A). Consistent with the increased expression of KRT80 in CRC cell lines, increased expression levels of KRT80 was also observed in CRC tissues compared with the adjacent normal tissues (Fig. 2B). Furthermore, KRT80 expression was upregulated in 84% (42/50) of the patients with CRC (Fig. 2C). These findings are consistent with a previous study (19) and suggested that KRT80 expression levels may be increased in CRC tissues and cells.

**Increased KRT80 expression levels are associated with the pathological stage and metastasis of patients with CRC.** To investigate the association between KRT80 expression levels and clinicopathological characteristics of patients with CRC, the clinical data of patients with CRC in the TCGA database were analyzed. The expression levels of KRT80 mRNA were significantly increased in patients with a high pathological stage, and both distant and lymph node metastasis compared with patients with a low pathological stage and metastasis, respectively (Fig. 3A-C). Moreover, clinical tissue sample analysis from 50 patients with CRC validated the above findings; compared with patients without distant metastasis, KRT80 expression levels were significantly increased in patients demonstrating metastasis (Fig. 3D).

**Knockdown of KRT80 expression suppresses CRC cell proliferation.** To determine the effects of KRT80 on the viability of CRC cells, cell viability was analyzed following KRT80 knockdown with siRNA. As hypothesized, the genetic knockdown of KRT80 using siRNA for 48 h significantly decreased KRT80 expression levels to ~40 and 30% of the control levels in SW480 and SW620 cells, respectively (Fig. 4A and B). The viability of SW480 cells was significantly decreased following 48 h in siRNA-KTR80-transfected cells compared with the siRNA-NC-transfected cells, whereas the cell viability in
siRNA-KRT80-transfected cells SW620 cells was significantly decreased from 72 h compared with siRNA-NC-transfected cells (Fig. 4C and D). Moreover, the inhibitory effect of KRT80 knockdown on cell viability was more pronounced the longer the cells were cultured (Fig. 4C and D).

Furthermore, the effect of KRT80 on cell proliferation was determined using colony formation and EdU incorporation assays. The colony formation assay demonstrated that the genetic knockdown of KRT80 significantly decreased the number of colonies formed by ~50 and ~40% in SW480 and SW620 cells, respectively, compared with siRNA-NC-transfected cells (Fig. 5A). The EdU assay was conducted to assess the function of KRT80 with respect to cell proliferation. Compared with siRNA-NC-transfected cells, the number of EdU-positive cells was significantly reduced in both SW480 and SW620 cells transfected with the siRNA-KRT80 compared with the siRNA-NC-transfected cells (Fig. 5B). These data further supported the notion that KRT80 may be involved in cell proliferation in CRC.

Pathway analysis of KRT80 co-expressed proteins in CRC samples from TCGA. To predict the underlying mechanisms of KRT80 in CRC cell proliferation, data mining using the cBioPortal for TCGA was used to identify 354 proteins significantly co-expressed with KRT80 in CRC. In total, 112 co-expressed proteins with absolute Spearman’s r ≥ 0.4 were loaded into the Reactome database for pathway analysis (Data S2). The proteins were enriched in the following processes: Cell cycle, DNA replication, immune system, metabolism of RNA and proteins, transport of small molecules and signal transduction (Fig. 6). Among them, the pathways containing the most proteins were the cell cycle and DNA replication pathways.
Moreover, to verify the pathway analysis results, the expression levels of PPP1CA, which is co-expressed with KRT80 (Data S2) and involved in cell cycle and cell division (28), were analyzed in SW480 cells following the genetic knockdown of KRT80 expression. Western blotting discovered that the knockdown of KRT80 significantly decreased the protein expression levels of PPP1CA in SW480 cells compared with the siRNA-NC-transfected cells (Fig. 7). Additionally, KRT80 knockdown also significantly reduced the expression levels of cell cycle-related proteins, p21 and p27, compared with the siRNA-NC-transfected cells (Fig. 7).

Discussion

Keratin, as a molecular marker of epithelial cells (10), serves an important role in maintaining the stability and integrity of epithelial cells, and it is also involved in various intracellular signal transduction processes, such as cell stress, proliferation and metabolism (29,30). Numerous studies have reported that several proteins of the keratin family were closely related to the development of CRC; for example, keratins were discovered to regulate colonic epithelial cell differentiation through the Notch 1 signaling pathway (18); keratin 8 deletion-induced colitis caused a predisposition to murine CRC through the inflammasome and IL-22 pathway (17); and increased expression levels of KRT7 were observed in CRCs with lymph node metastasis, which was associated with a poor prognosis (31). However, although KRT80 has been reported to mediate migration and invasion in CRC through activating the AKT signaling pathway (19), the role of KRT80 in CRC proliferation remains unknown.

In the present study, the TCGA and ONCOMINE databases were used to identify KRT80 as the most upregulated gene in CRC compared with the normal tissues. Furthermore, it was confirmed that KRT80 expression levels in clinical CRC tissues and CRC cell lines were significantly increased compared with the normal tissues and cells, and increased KRT80 expression levels were associated with the pathological stage and metastasis in patients with CRC. These results were consistent with the study by Li et al (19), which demonstrated that KRT80 was highly upregulated in CRC compared with the normal tissues and associated with poor overall survival.
expressed in CRC and promoted the migration and invasion of CRC cells, suggesting that KRT80 may serve as an oncogene to promote the proliferation of CRC cells. To confirm this, KRT80 expression was knocked down and it was subsequently found that reduced KRT80 expression levels decreased the cell viability, reduced the number of colonies formed and suppressed cell proliferation.

Figure 5. Knockdown of KRT80 expression levels suppresses colorectal cancer cell proliferation. SW480 and SW620 cells were transfected with siRNA-KRT80 or siRNA-NC for 48 h. (A) Colony formation and (B) EdU incorporation assays were used to determine the cell proliferation rate (magnification, x200). ImageJ v1.8.0 software was used for cell counting. Data are presented as the mean ± SD from 3 independent experimental repeats. *P<0.05, **P<0.01 vs. siRNA-NC group. KRT80, keratin 80; siRNA, small interfering RNA; NC, negative control; EdU, 5-ethynyl 2'-deoxyuridine.
proliferation. Together with previous reports (19), these findings validated that KRT80 may be a novel potential oncogene for CRC.

In addition, the results of the present study revealed that proteins co-expressed with KRT80 were enriched in the cell cycle and DNA replication processes, and the genetic knockdown of KRT80 significantly reduced the expression levels of cell cycle-related proteins, including PPP1CA, p21 and p27. PPP1CA, which is co-expressed with KRT80 in CRC, is reported to be one of three catalytic subunits of protein phosphatase 1 (28); it has been demonstrated to be involved in prostate cancer and CRC tumorigenesis via the mitogen activated protein kinase signaling pathway (32,33). Although p21 and p27 mediate cell cycle arrest through binding to and inhibiting cyclin-dependent kinase/cyclin complexes, numerous studies over the past decade have revealed that p21 and p27 also serve an important role in carcinogenesis and tumor development (34-39). In fact, p21 and p27 have been reported to serve as oncogenic proteins or tumor suppressors, depending on their localization in the cytoplasm or the nucleus, respectively (40-43). In brief, cytoplasmic p21 and p27 have been found to favor antiapoptotic activities, whereas nuclear p21 and p27 have been associated with cell cycle arrest (36,39).
Therefore, the findings of the present study provided a valuable reference for further study to investigate the function and underlying mechanisms of KRT80 in CRC tumorigenesis. However, this study has some limitations. Firstly, only 50 pairs of tissue samples were used in this study, which is a small sample size. Additionally, some clinicopathological data related to CRC, such as patient survival, were not included. Finally, although pathway analysis indicated that proteins co-expressed with KRT80 were largely enriched in the cell cycle and DNA replication, the detailed underlying mechanisms have not been further explored. The above limitations should be resolved in future studies.

In conclusion, the findings of the present study suggested that KRT80 may be overexpressed in CRC tissues and it may function as an oncogene to promote the proliferation of CRC cells. KRT80 co-expressed proteins were discovered to be largely enriched in the cell cycle and DNA replication pathways, which are related to the process of tumorigenesis. Therefore, in-depth research into KRT80 to further reveal its functions and underlying mechanisms in CRC may provide novel insights for the early diagnosis and gene-targeted treatment of CRC.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the UCSC Xena Browser (https://xenabrowser.net/datapages/), ONCOMINE gene expression array datasets (www.oncomine.org), cBioPortal database (http://www.cbioportal.org) and the Reactome database (https://reactome.org/PathwayBrowser).

Authors' contributions

HY and XX designed the study; JL, XF and JC performed the experiments and analyzed the data; XX contributed to obtaining the patient tissue samples; and JL and HY wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All patients provided informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki and the study protocol was approved by the Ethics Committee of Shantou University Medical College (approval no. SUMC-2015-42).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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