

The circRNA_102911/miR-129-5p/SOX6 axis is involved with T lymphocyte immune function in elderly patients with laparoscopic left hepatectomy for hepatolithiasis

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Abstract. The aim of the present study was to investigate the impact of laparoscopic left hepatectomy (LLH) for hepatolithiasis on the T lymphocyte immune changes of elderly patients and to analyze underlying mechanisms of action behind these changes. A total of 164 patients who underwent LLH due to left-sided hepatolithiasis were recruited. In terms of T lymphocyte immune changes, it was found that firstly, the basic quantity of peripheral lymphocytes in the elderly group was significantly lower than that in a younger preoperative group. Secondly, after surgical trauma, the immune function of T lymphocytes had a significant decline and lasted longer when compared with younger patients, which was reflected by the perioperative changes in the T lymphocyte proliferative ability, levels of IL-2 secreted by T lymphocytes and the percentage of CD3⁺/CD4⁺ T lymphocytes in the peripheral blood. Circular RNA (circRNA) 102911 (102911) was upregulated and microRNA (miR)-129-5p was downregulated in CD3⁺/CD4⁺ T lymphocytes from elderly patients with LLH for hepatolithiasis. Furthermore, the overexpression of 102911 inhibited the proliferation of CD3⁺/CD4⁺ T lymphocytes as well as promoting cell apoptosis, with the opposite effects being observed on knockdown of 102911. miR-129-5p is involved in the proliferation and apoptosis of CD3⁺/CD4⁺ T lymphocytes and may be a promising target of 102911. Moreover, SOX6 is a downstream molecule of miR-129-5p. Immune function and number of T lymphocytes decreased significantly after surgical trauma compared to younger patients, and this decline lasted

longer in older patients treated with LLH for hepatolithiasis. The 102911/miR-129-5p/SOX6 axis was found to be involved in T lymphocytes immune function, which provided a novel insight for the treatment of elderly patients with hepatolithiasis.

Introduction

As the development of laparoscopic technology and the update of laparoscopic instruments, laparoscopic hepatectomy is considered to be a more favorable option for the treatment of hepatolithiasis compared with open hepatectomy (1). Compared to conventional open approaches, the laparoscopic approach has been shown to cause reduced intraoperative blood loss, fewer post-operative complications, a shorter hospital stay and a shorter intestinal function recovery time, even in elderly patients (2). However, for surgeons, a high infective complication rate in elderly patients is still a substantial challenge, even when using minimally invasive techniques such as laparoscopic hepatectomy, which results in mild trauma to patients (3).

The immune system is activated by surgical stress and trauma and plays important roles in resisting injury and facilitating wound healing (4). However, in the case of immune dysfunction, the temporary post-traumatic or post-operative immune suppression predisposes the patient for septic complications, especially for elderly patients as a result of immunosenescence (5). Studies have shown that circular RNA (circRNA) plays an important role in immune regulation (6,7). circRNAs show tissue-specific expression profiles and are abnormally expressed in various diseases. They also participate in post-transcriptional cutting, editing, translocation, translation and degradation (8). Studies have shown that circRNAs are involved in the development of various disease, including liver cancer, cholangiocarcinoma, leukemia, acute kidney transplantation, Alzheimer's disease and rheumatoid arthritis (6,7). However, the role of (circRNA)_102911 (102911) in T lymphocytes immune function in elderly patients with laparoscopic left hepatectomy (LLH) for hepatolithiasis has not been elucidated. MicroRNAs (miRNAs), a subgroup

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of non-coding RNA molecules which are ~17-24 nucleotides in length, can negatively regulate gene expression by base pairing with the 3'-untranslated regions (3'-UTRs) of their target mRNAs, resulting in transcript inhibition and/or mRNA degradation (9). Currently, 474 miRNAs have been verified in the human genome and they are estimated to regulate about ~30% of protein-coding genes (10). The proposed competing endogenous RNA theory, which suggests that circRNAs may work as miRNA sponges, preventing their binding to mRNAs, has received increasing attention (11). circRNAs exhibit a network-like regulatory pattern in age-related diseases and various circRNAs play important roles in immune regulation (12). Abnormally expressed miRNAs and circRNAs may prove to be a significant target for the prevention and effective management of post-operative complications for patients with post-operative atrial fibrillation (13). In the present study, it was confirmed that 102911 targets miR-129-5p and that miR-129-5p can target SOX6, which together play important roles in elderly-related diseases. In the present report, the difference of T lymphocyte immune changes between elderly and young patients after LLH for hepatolithiasis and the essential roles of 102911 on the proliferation and apoptosis of CD3⁺/CD4⁺ T lymphocytes were evaluated, which may provide a novel insight for the treatment of elderly patients with hepatolithiasis.

Patients and methods

Patients. The present study was approved by the ethics committee of the Eighth Medical Center of the Chinese PLA General Hospital (Beijing, China). All experiments were performed in accordance with the guidelines set by the medical laboratory management committee of the Eighth Medical Center of the Chinese PLA General Hospital. Informed consent was obtained from all participants. All of the operations were performed by the same surgical team. The inclusion criteria were as follows: i) Left-sided hepatolithiasis with irreversible diseases, such as biliary strictures, severe parenchymal fibrosis or atrophy; and ii) left-sided hepatolithiasis with or without common bile duct stones. The exclusion criteria included: i) Patients with immunological diseases or those who were on steroids or other immunosuppressive drugs; ii) Child-Pugh class C liver function, cardiopulmonary or hepato-renal impairment and associated tumors; iii) previous upper abdominal surgery, but not for cholecystectomy; and iv) necessity of bilio-enteric anastomosis or left caudate lobectomy. A total of 164 patients who underwent LLH for hepatolithiasis from Jan 2010-Dec 2017 were enrolled in the present study. Among these prospectively enrolled patients, 88 were elderly patients, >65 years (elderly group) and 76 were young patients (<65 years old, younger group). The age range of all patients was 18-85 years (37 males and 127 females). All patients received follow-up (5.2±2.9 months) after the operation. Any post-operative complication was diagnosed using either physical examination or standard diagnostic testing and treated based on surgeon discretion using accepted standard methods of treatments.

Operative procedures. LLH was performed under general anesthesia with patients in the supine position. After set of

carbon dioxide pneumoperitoneum at 12-15 mmHg accordingly, four ports were established: One 10 mm port below the umbilicus, one 12 mm port under the left costal margin, and two 5 mm ports in the right midabdomen. Ligaments around the left liver lobe were firstly divided, then the hepatoduodenal ligament was dissected bluntly, the left hepatic artery and the left branch of the portal vein were freed and clamped. The liver parenchyma was transected using laparoscopic hepatectomy by curettage and aspiration as previously described (11). Laparoscopic common bile duct exploration and T-tube drainage were performed routinely in patients with stones in the dilated common bile ducts (>8 mm) and the stones were removed using forceps or by choledochoscopy, according to their location. Endoscopic sphincterotomy was performed to remove stones in the nondilated common bile ducts.

Data collection. The patients' demographic data, operation duration and post-operative stay were recorded. Complications such as wound infection, pneumonia and peritonitis were assessed at follow-up (mean 3 months, by telephone). T lymphocyte proliferative abilities, levels of IL-2 in peripheral blood T lymphocytes, % CD3⁺/CD4⁺ T lymphocytes and the apoptotic rate of CD3⁺/CD4⁺ T lymphocytes in peripheral blood were compared between elderly group and the middle-aged group during the preoperative period and post-operative day 1, 3, 7 and 14.

Blood samples. Blood samples were collected from peripheral veins of all patients at five time points, namely prior to the operation and on post-operative day 1, 3, 7 and 14. A non-pyrogenic test tube containing Dipotassium EDTA was used to collect 3 ml blood samples and mixed with the same volume of aseptic isotonic saline. After slowly placing 6 ml blood solution on the surface of sterile, pyrogen-free Ficoll-Hypaque Solution (Inno-Train Diagnostik GmbH), the liquid was centrifuged at 18-20°C and 1,500 × g for 20 min to get the mononuclear cells in the second layer. The property of monocytes and polymorphonuclear leukocytes adhering to plastic and capillary glass tubes were used to further separate and purify lymphocytes (14). Trypan blue was used to detect cell activity >97%. The cells were counted, and the concentration was adjusted to 5×10⁶ cells/ml.

Isolation and detection of CD3⁺/CD4⁺ T lymphocytes. The cell concentration of lymphocytes was adjusted to 1×10⁶ cells/ml using PBS (Beijing Solarbio Science & Technology Co., Ltd.) and 100 µl suspension was added to a flow tube. After incubating with CD3-FITC (BD Pharmingen; BD Biosciences) 20 µl and CD4-allophycocyanin (APC; BD Pharmingen; BD Biosciences) 20 µl for 15 min in the dark, 400 µl PBS was added. The % of CD3⁺/CD4⁺ T lymphocytes was isolated using a flow cytometer (BD Pharmingen; BD Biosciences) and then analyzed using FlowJo version 7.6 software (FlowJo LLC).

Cell culture and transfection. Cells were maintained in RPMI-1640 medium (HyClone; GE Healthcare) supplemented with 10% FBS (HyClone; GE Healthcare), 100 U/ml penicillin and 100 µg/ml streptomycin (both from HyClone; GE Healthcare) at 37°C in a humidified incubator containing 5% CO₂. Small interfering RNA (siRNA) sequences targeting

102911 (si-102911, 5'-CUUCUAUUAAGUAAUUGU GUA-3'), negative control (si-NC, 5'-GAGCAAGAAGUAGAU GCCU-3'), miR-129-5p mimic (5'-CUUUUUGCGGUCUGG GCUUGC-3'), miR-129-5p inhibitor (the sequence was 5'-GCA AGCCCAGACCGCAAAAAG-3') and the corresponding negative controls (miR-NC; miR mimic control, 5'-ACA UACUUCUUUAUGCCCCAU-3' or miR inhibitor control, 5'-UUCUCCGAACGUGUCACGUTT-3') were obtained from Shanghai Genepharm Co., Ltd. Wild-type (WT) 102911 (LV-102911), SOX6 (LV-SOX6) or mutant (LV-NC; 5'-TGCTTC TATTAAGTAATTGTG-3') fragments were amplified using PCR from 293T cDNA using Taq polymerase (Thermo Fisher Scientific, Inc.) and subsequently inserted into a pcDNA3.1 vector (Shanghai Genepharm Co., Ltd.). The primer sequences used were as follows: 102911 forward, 5'-GCCTCCATAGGT GTGGAAGAT-3' and reverse 5'-TTCGCCTCCATAGGT GTGGAA-3'; SOX6 forward, 5'-CTTCGCCTCCATAGGTGT GGA-3' and reverse 5'-TCGCCTCCATAGGTGTGGAAG-3'. The following thermocycling conditions were used: 95°C for 30 sec, followed by 30 cycles at 95°C for 15 sec, 60°C for 20 sec, 68°C for 10 sec and a final extension at 68°C for 5 min. All the transfections were performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 24 h according to the manufacturer's protocols. All transfections and further western blotting at 8 h following transfection were performed on lymphocytes isolated from elderly patients.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA of CD3⁺/CD4⁺ T lymphocyte were extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Reverse transcription was performed using PrimeScript™ RT Reagent kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocols. PCR primers were designed and synthesized by Shanghai Genepharm Co., Ltd. and gene expressions were detected using SYBR Premix Ex Taq II (Takara Biotechnology Co., Ltd.). The expression levels of mRNAs and miRNA were normalized to β-actin and U6, respectively. The sequences of forward and reverse primers were as follows: 102911 forward 5'-ATGCCACAACGCAGATTGAT-3' and reverse 5'-CGA GAAACGCACAAGAAGG-3'; miR-129-5p forward 5'-GCG ACTGACGTCTTTGCGGTCTGG-3' and reverse 5'-CAG AACAGTGTGCGTACAGTGACGAT-3'; SOX6 forward, 5'-CCCCTCTGAACATGGTGGTGGC-3' and reverse, 5'-TGA GACTGCCCTGCCGAGT-3'; β-actin forward, 5'-GCACCA CACCTTCTACAAG-3' and reverse, 5'-TGCTGCTGATCC ACATCTG-3'; and U6 forward 5'-GCTTCGGCAGCACAT ATACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTG CGTGTCA-3'. The following thermocycling conditions were used for the qPCR: Initial denaturation at 95°C for 5 min, followed by 45 cycles of 95°C for 15 sec, 60°C for 20 sec and 72°C for 10 sec. Relative expression was calculated using the $2^{-\Delta\Delta C_q}$ method (15).

Western blotting. Total protein from cells was isolated using protein extraction reagent RIPA buffer (Beyotime Institute of Biotechnology). The protein concentration was measured using the BCA protein assay kit (Beyotime Institute of Biotechnology). A total of 40 µg protein was added to 10% SDS-PAGE and separated proteins were transferred

onto polyvinylidene fluoride membranes, which were then blocked with 5% non-fat milk in TBS at room temperature for 1 h. Subsequently, these membranes were washed with TBS-Tween-20 (1X TBS, 0.1% Tween-20 and 5% nonfat milk powder; Wuhan Boster Biological Technology, Ltd.) three times and incubated with antibodies targeting caspase-3 (1:500; cat. no. ab49822; Abcam), SOX6 (1:1,000; cat. no. ab64946; Abcam) or β-actin (1:5,000; cat. no. ab179467; Abcam) overnight at 4°C. Subsequently, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody targeting anti-rabbit IgG (1:5,000; cat. no. ab6721; Abcam) for 1 h. The protein signals were determined using an ECL detection kit (Thermo Fisher Scientific, Inc.).

Luciferase assay. WT fragments of the 3'UTR of 102911/SOX6 with potential binding sites of miR-129-5p were synthesized into pmiR-GLO (Promega Corporation) according to the manufacturer's protocols. 102911/SOX6-3'UTR-mutant reporter containing the mutant binding sites of miR-129-5p was established using the QuikChange Multi Site-Directed Mutagenesis kit (Stratagene; Agilent Technologies GmbH). The constructed vectors were then co-transfected with miR-NC or miR-129-5p mimics into DH5α competent cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Luciferase activity was determined 48 h post-transfection using the Promega luciferase assay (Promega Corporation) according to the manufacturer's instructions. Firefly luciferase activity was normalized to that of *Renilla* luciferase.

T cell proliferation assay. Cells (2x10⁶ cells/ml) were inoculated into 96-well plates and incubated at 37°C in 5% CO₂ for 4 h. Subsequently, cells were treated with 5 µg/ml concanavalin A (Sigma-Aldrich; Merck KGaA) at 37°C for 24 h. A total of 20 µl Thiazolyl blue (MTT; Sigma-Aldrich; Merck KGaA) was then added to each well. After culturing for 4 h, 100 µl Triton-ISOP (10% Triton X-100, 50% isopropanol and 0.01 M HCl) solution was added to dissolve the MTT crystals. When all the crystals had been dissolved through repeated blowing with a pipette, the optical density was measured using a microplate reader (Spectra MR, Dynex) at a wavelength of 540 nm.

Enzyme-linked immunosorbent assay. IL-2 and IFN-γ levels in culture supernatant were measured using commercially available ELISA kits for human proteins (human IL-2 Quantikine ELISA kit; cat. no. PD2050 and human IFN-γ Quantikine ELISA kit; cat. no. PDIF50C; R&D Systems, Inc.). ELISAs were performed strictly following the protocols provided by the manufacturer. Plates were read using a microplate reader (Spectra MR, Dynex).

Detection of the apoptotic rate of CD3⁺/CD4⁺ T lymphocytes. Using PBS, the concentration of isolated peripheral blood mononuclear cells was adjusted to 1x10⁶ cells/ml. The supernatant was discarded by centrifugation and the cell pellet was suspended in flow with 0.1 ml of PBS. A total of 20 µl of CD3-FITC, 20 µl of CD4-APC, 5 µl of Annexin V and 5 µl of 7-amino actinomycin D were then added and incubated for 15 min in the dark. Finally, 0.4 ml of binding buffer was added, and the cells were examined by a flow cytometer

(BD Pharmingen; BD Biosciences) within 1 h and analyzed using FlowJo version 7.6 software (FlowJo LLC).

Statistical analysis. SPSS 15.0 (SPSS Inc.) for Windows was used for data analysis and the normality assumption of parametric tests was checked. The χ^2 test and Student's t-tests were used to compare categorical, parametric and nonparametric data between two groups. A paired t-test was performed when analyzing the preoperative and post-operative changes within the groups. Statistical significance was analyzed using one-way ANOVAs with Tukey's post hoc tests among multiple groups. Two-way ANOVAs, followed by Bonferroni's corrections were used to analyze the main effect of time, main effect of age and the interaction between age and time. Pearson's correlation analysis was performed to determine the correlations between RNA expression levels. A P-value <0.05 was considered statistically significant for all tests.

Results

Patient demographics and clinical outcomes. The patient demographic data are presented in Table I. A total of 164 patients were included in the study. There were no significant differences in the ratio of male to female subjects, body mass index (BMI), Child-Pugh class or preoperative white blood count. However, the basic quantity of peripheral lymphocyte in the elderly group was significantly lower than that in the younger group, preoperatively. Table II shows the clinical outcomes of LLH categorized by the patients' age grouping. These 2 groups had similar operation duration, blood loss and types of operative procedures. The elder patients required a longer time to resume to a normal diet and a longer stay in hospital. In terms of infective complications, no noteworthy differences were found in wound infection rates, but the elder patients group had higher rates of pneumonia and peritonitis. The probability of peritonitis in elderly patients may be closely related to bile leakage.

T lymphocyte proliferative ability and IL-2 levels. Data from the preoperative period showed that compared with the younger group, the proliferative ability of peripheral T lymphocytes in elderly patients was significantly attenuated (1.17 ± 0.18 vs. 0.98 ± 0.22 , respectively). After operation, T lymphocyte proliferative ability of both groups was significantly inhibited; however, in the elderly patient group, this trend lasted for a longer period (Table III). IL-2 expression levels in the elderly patient group were significantly lower than that in middle-age group in both the pre- and post-operative period (Table IV). After operation, T lymphocytes secreted reduced IL-2 in both groups. In the elderly patient group, expression levels of IL-2 at POD14 were still significantly lower than the preoperative levels, but in the younger group, T lymphocyte secreted a normal level of IL-2 (Table IV).

Apoptotic rate of CD3⁺/CD4⁺T lymphocytes in the peripheral blood. As described in Table I, the basic quantity of peripheral lymphocytes in the elderly group was significantly lower than that in the younger group pre-operatively (2.64 ± 0.65 vs. 1.81 ± 0.78). However, data from Table V showed that there was no difference in the percentage of CD3⁺/CD4⁺T lymphocytes in the

Table I. Demographic data.

Parameter	Age ≤65 years	Age ≥65 years	P-value
Patients numbers	76	88	0.437
Sex ratio, male/female	17/59	20/68	0.353
BMI (kg/m ²),	23.1±2.6	22.7±3.1	0.447
Child-Pugh class			
A	70	79	-
B	6	9	
Preoperative WBC ($\times 10^9/l$)	6.47±1.73	7.34±1.8	0.254
Preoperative LYM ($\times 10^9/l$)	2.64±0.65	1.81±0.78	0.005

Data are presented as the count or the mean ± SD. BMI, body mass index; LYM, lymphocyte; WBC, white blood count.

peripheral blood from these two groups pre-operatively. After operation, the % of CD3⁺/CD4⁺T lymphocytes in the elderly patient group levels rapidly fell by POD1, which remained low during the entire monitoring period. For the younger-age group, the % CD3⁺/CD4⁺T lymphocytes dropped in POD3 and POD7, but in POD14 the percentage of CD3⁺/CD4⁺T lymphocytes in the peripheral blood raised to levels similar to the pre-operative levels (Table V). After the operation, the apoptotic rate of CD3⁺/CD4⁺T lymphocytes in the elderly patients group rose significantly by POD1 and still significantly increased until POD14. For the younger-age group, the apoptotic rate of CD3⁺/CD4⁺T increased in POD3 and POD7, but in POD14, the apoptotic rate of CD3⁺/CD4⁺T in the peripheral blood declined to levels similar to the pre-operative levels (Table VI).

102911 is involved in the proliferation and apoptosis of CD3⁺/CD4⁺T lymphocytes. The expression levels of 102911 were significantly elevated in the CD3⁺CD4⁺T lymphocytes obtained from the elderly patients (Fig. 1A). However, there was no significant difference in the pre-operative and post-operative (POD1-14) expression levels of 102911 in the CD3⁺CD4⁺T lymphocytes of the two groups of patients, and the expression levels of 102911 were significantly increased in the CD3⁺CD4⁺T lymphocytes obtained from the elderly patients in the pre-operative and post-operative groups (POD1-14) (Table VII). After CD3⁺CD4⁺T lymphocytes were transfected with LV-102911, the expression levels of 102911 were significantly increased (Fig. 1B). The expression of 102911 was significantly decreased following transfection of CD3⁺CD4⁺T lymphocytes with si-102911 (Fig. 1C). Overexpression of 102911 significantly inhibited CD3⁺CD4⁺T lymphocytes proliferation (Fig. 1D), promoted CD3⁺CD4⁺T lymphocytes apoptosis (Fig. 1F and G) and decreased the expression levels of IL-2 and IFN (Fig. 1J). Similarly, reduced expression of 102911 significantly promoted T cell proliferation (Fig. 1E), inhibited T cell apoptosis (Fig. 1H and I) and increased IL-2 and IFN expression levels (Fig. 1K). These results suggested that 102911 may be involved in the regulation of CD3⁺CD4⁺T lymphocytes in elderly patients.

Table II. LLH outcome according to age.

Parameter	Age ≤65 years	Age >65 years	P-value
Operation duration, min	231±75	229±59	0.762
Blood loss, ml	337±184	329±172	0.583
Operative procedures, n			-
LLH	32	37	
LLH with CBDE	25	28	
LLH with intraoperative endoscopic sphincterotomy	3	5	
LLH with preoperative endoscopic sphincterotomy	16	18	
Resumption to diet, days	1.9±0.7	2.8±1.1	0.056
Hospital stays	4.28±1.2	5.07±2.2	0.006
Infective complications			
Wound infection	2	5	0.212
Pneumonia	2	9	0.000
Peritonitis	1	7	0.000

Data are presented as the count or the mean ± SD. CBDE, common bile duct exploration; LLH, left hepatectomy.

Table III. Comparison of the T lymphocyte proliferative ability in the two age groups.

Group	Time-points for detection				
	Pre-operation	POD1	POD3	POD7	POD14
>65 years	0.98±0.22	0.68±0.15 ^a	0.53±0.14 ^a	0.59±0.25 ^a	0.63±0.21 ^a
≤65 years	1.17±0.18 ^b	0.93±0.27 ^{a,b}	0.63±0.17 ^{a,c}	0.81±0.27 ^{a,b}	1.02±0.17 ^{a,b}

Data are presented as the count or the mean ± SD. ^aP<0.001 vs. pre-operation; ^bP<0.001 vs. the >65 years group; ^cP<0.01 vs. the >65 years group. POD, post-operative day.

Table IV. Comparison of levels of IL-2 in peripheral blood T lymphocytes (pg/ml).

Group	Time-points for detection				
	Pre-operation	POD1	POD3	POD7	POD14
>65 years	21.7±7.8	11.3±4.8 ^a	10.8±3.3 ^a	11.1±3.6 ^a	13.1±4.5 ^a
≤65 years	27.8±5.9 ^b	22.7±6.7 ^{a,b}	15.8±5.6 ^{a,b}	17.3±4.8 ^{a,b}	25.7±3.6 ^b

Data are presented as the count or the mean ± SD. ^aP<0.001 vs. pre-operation; ^bP<0.001 vs. the >65 years group. POD, post-operative day.

102911 directly binds with miR-129-5p in CD3⁺CD4⁺ T lymphocytes. The target binding site of 102911 and miR-129-5p is shown in Fig. 2A. Dual luciferase reporter assays showed that the activity of double fluorescein was significantly decreased after transfection with miR-129-5p mimics in 102911-WT (Fig. 2B). Additionally, RT-qPCR was used to detect the expression level of miR-129-5p in the CD3⁺CD4⁺ T lymphocytes of the two groups. The results showed that the expression levels of miR-129-5p in CD3⁺CD4⁺ T lymphocytes of the elderly patients was significantly decreased (Fig. 2C). There was a significant negative correlation between the expression levels of 102911 and miR-129-5p in CD3⁺CD4⁺ T lymphocytes of elderly patients (Fig. 2D). Furthermore, the

Table V. Comparison of the % CD3⁺/CD4⁺T lymphocytes in the peripheral blood.

Group	Time-points for detection				
	Pre-operation	POD1	POD3	POD7	POD14
>65 years	42±7	25±6 ^a	23±7 ^a	27±5 ^a	26±5 ^a
≤65 years	44±9	40±5 ^{b,c}	30±7 ^{a,c}	38±5 ^{a,c}	42±7 ^c

Data are presented as the count or the mean ± SD. ^aP<0.001 vs. pre-operation; ^bP<0.01 vs. pre-operation; ^cP<0.001 vs. the >65 years group. POD, post-operative day.

Table VI. Comparison of the % apoptotic rate of CD3⁺/CD4⁺T lymphocytes in peripheral blood.

Group	Time-points for detection				
	Pre-operation	POD1	POD3	POD7	POD14
>65 years	12.5±4.9	17.5±5.4 ^a	19.3±6.8 ^a	18.4±7.3 ^a	16.3±6.7 ^a
≤65 years	10.7±5.8	12.5±3.1 ^b	18.9±5.7 ^a	15.3±4.7 ^{a,c}	11.7±4.5 ^b

Data are presented as the count or the mean ± SD. ^aP<0.001 vs. pre-operation; ^bP<0.001 vs. the >65 years group; ^cP<0.01 vs. the >65 years group. POD, post-operative day.

Table VII. Comparison of pre-operative and post-operative mRNA expression of 102911 in CD3⁺/CD4⁺T lymphocytes using RT-qPCR.

Group	Time-points for detection				
	Pre-operation	POD1	POD3	POD7	POD14
>65 years	2.7±0.5	2.8±0.7	2.9±0.6	2.7±0.8	2.6±0.5
≤65 years	1.4±0.5 ^a	1.4±0.4 ^a	1.5±0.7 ^a	1.6±0.6 ^a	1.5±0.6 ^a

Data are presented as the count or the mean ± SD. ^aP<0.001 vs. the >65 years group. POD, post-operative day.

expression levels of miR-129-5p were significantly reduced following the overexpression of 102911 (Fig. 2E) and the expression levels of miR-129-5p were significantly increased following the reduction of 102911 expression (Fig. 2F). After CD3⁺CD4⁺ T lymphocytes were transfected with miR-129-5p mimics, the expression levels of miR-129-5p were significantly increased (Fig. 2G). The expression levels of miR-129-5p were significantly decreased following transfection of CD3⁺CD4⁺ T lymphocytes with miR-129-5p inhibitors (Fig. 2H), demonstrating the successful transfection of these agents. These results suggested that 102911 may play a role in CD3⁺CD4⁺ T lymphocytes through targeting miR-129-5p.

miR-129-5p is involved in the proliferation and apoptosis of CD3⁺CD4⁺ T lymphocytes. To further explore the role of miR-129-5p in CD3⁺CD4⁺ T lymphocytes, miR-129-5p mimics and miR-129-5p inhibitors were transfected into CD3⁺CD4⁺ T lymphocytes. Overexpression of miR-129-5p significantly promoted T cell proliferation (Fig. 3A), inhibited T cell apoptosis (Fig. 3C) and increased IL-2 and IFN expression levels (Fig. 3E). In contrast, low expression of miR-129-5p inhibited CD3⁺CD4⁺ T lymphocyte proliferation (Fig. 3B), promoted CD3⁺CD4⁺ T lymphocyte apoptosis (Fig. 3D) and decreased IL-2 and IFN expression levels (Fig. 3F). These results showed that miR-129-5p was also involved in the proliferation and apoptosis of CD3⁺CD4⁺ T lymphocytes.

miR-129-5p directly binds with SOX6 in CD3⁺CD4⁺ T lymphocytes. To further study the downstream targets of miR-129-5p, the target binding site between miR-129-5p and SOX6 was found (Fig. 4A). Dual luciferase reporter assays showed that the activity of double fluorescein was significantly decreased after

transfection with miR-129-5p mimics in SOX6-WT (Fig. 4B). Furthermore, RT-qPCR was used to detect the expression level of SOX6 in CD3⁺CD4⁺ T lymphocytes of the two groups. The results showed that the mRNA expression levels of SOX6 in CD3⁺CD4⁺ T lymphocytes of elderly patients was significantly increased (Fig. 4C). Meanwhile, there was a significant negative correlation between the expression levels of miR-129-5p and SOX6 in the CD3⁺CD4⁺ T lymphocytes of the elderly patients (Fig. 4D) and a positive correlation between expression levels of 102911 and SOX6 (Fig. 4E). Furthermore, the expression level of SOX6 were significantly reduced after CD3⁺CD4⁺ T lymphocytes were transfected with miR-129-5p mimics (Fig. 4F) and the expression levels of SOX6 were significantly raised after the reduction of miR-129-5p expression levels (Fig. 4G). In order to investigate the effects of SOX6 on the biological behavior of CD3⁺CD4⁺ T lymphocytes, the cells were transfected with LV-NC or LV-SOX6, and the transfection efficiency were determined using RT-qPCR, with the results showing that a successful transfection, leading to an increased expression level of SOX6 (Fig. 4H). These results showed that miR-129-5p may play a role in CD3⁺CD4⁺ T lymphocytes through targeting SOX6.

The 102911/miR-129-5p/SOX6 axis is involved in the proliferation and apoptosis of CD3⁺CD4⁺ T lymphocytes. In order to further study the regulatory roles that 102911 has on CD3⁺CD4⁺ T lymphocytes through regulating the miR-129-5p/SOX6 axis, CD3⁺CD4⁺ T lymphocytes were co-transfected with LV-102911 and miR-129-5p mimics, or miR-129-5p and LV-SOX6 respectively. The effects of LV-102911 on the proliferation and apoptosis of CD3⁺CD4⁺ T lymphocytes were abolished following co-transfection with miR-129-5p mimics (Fig. 5A, C and E). Meanwhile, the effects of miR-129-5p mimics on the proliferation and apoptosis of CD3⁺CD4⁺

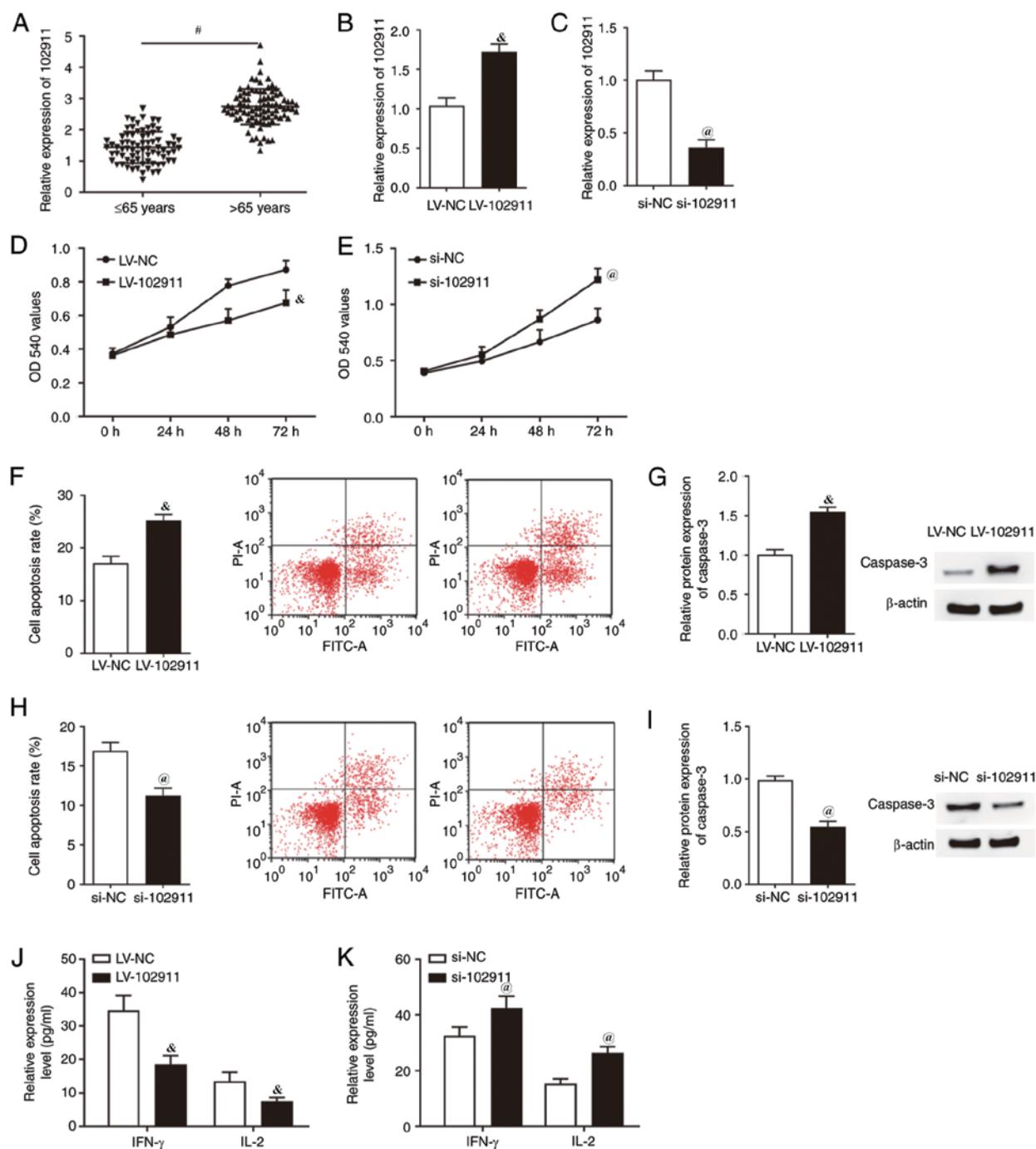


Figure 1. 102911 is involved in the proliferation and apoptosis of CD3+/CD4+ T lymphocytes. (A) The expression levels of 102911 were determined in CD3+/CD4+ T lymphocytes from the two age groups using RT-qPCR (Student's t-test). 102911 expression levels in CD3+/CD4+ T lymphocytes transfected with (B) LV-102911 or (C) si-102911 were determined using RT-qPCR (Student's t-test). The proliferative rates of CD3+/CD4+ T lymphocytes transfected with (D) LV-102911 or (E) si-102911 was determined using MTT assays (two-way ANOVAs). (F) The apoptotic rate of CD3+/CD4+ T lymphocytes transfected with LV-102911 was determined using flow cytometry (Student's t-test). (G) The protein expression levels of caspase-3 in CD3+/CD4+ T lymphocytes transfected with LV-102911 were determined using western blotting (Student's t-test). (H) The apoptotic rate of CD3+/CD4+ T lymphocytes transfected with si-102911 was determined using flow cytometry (Student's t-test). (I) The protein expression levels of caspase-3 in CD3+/CD4+ T lymphocytes transfected with si-102911 were determined using western blotting (Student's t-test). IL-2 and IFN-γ expression levels in CD3+/CD4+ T lymphocytes transfected with (J) LV-102911 or (K) si-102911 were detected using ELISAs (Student's t-test). #P<0.05 vs. <65 years; &P<0.05 vs. LV-NC; @P<0.05 vs. si-NC. All assays were repeated three times in duplicates. 102911, circular RNA_102911; NC, negative control; FITC-A, anti-fluorescein isothiocyanate; OD, optical density; RT-qPCR, reverse transcription-quantitative PCR; si, small interfering RNA.

T lymphocytes were abolished following co-transfection with SOX6 (Fig. 5B, D and F). Taken together, these data show that the 102911/miR-129-5p/SOX6 axis was involved in T lymphocytes immune function, which provided a novel insight for the treatment of elderly patients with hepatolithiasis.

Discussion

Increases in the average age of populations is a worldwide phenomenon (16). In recent years, as life expectancies increase, the aged population is expanding dramatically in China (17).

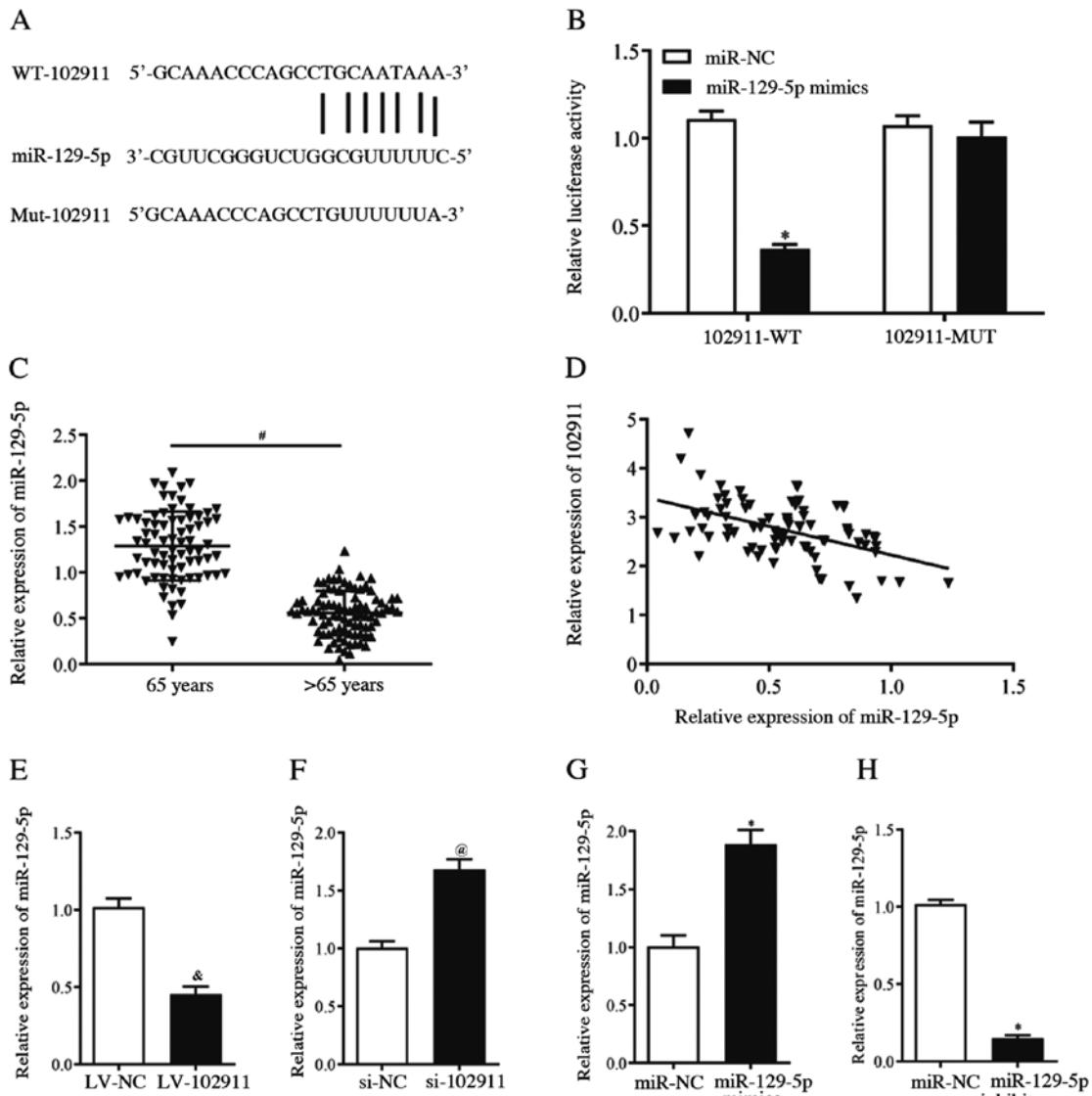


Figure 2. 102911 directly binds with miR-129-5p in CD3⁺/CD4⁺ T lymphocytes. (A) The putative binding sites between the transcripts of 102911 and miR-129-5p. (B) Luciferase gene reporter assays demonstrated that 102911 directly bound with miR-129-5p (Student's t-test). (C) The expression levels of miR-129-5p in CD3⁺/CD4⁺ T lymphocytes of two groups were determined using RT-qPCR (Student's t-test). (D) Spearman's correlation analysis indicated a negative correlation between 102911 and miR-129-5p expression levels in CD3⁺/CD4⁺ T lymphocytes of elderly patients. miR-129-5p expression levels of CD3⁺/CD4⁺ T lymphocytes transfected with (E) LV-102911, (F) si-102911, (G) miR-129-5p mimics or (H) miR-129-5p inhibitors, were determined using RT-qPCR (Student's t-tests). #P<0.05 vs. <65 years; *P<0.05 vs. miR-NC; &P<0.05 vs. LV-NC; @P<0.05 vs. si-NC. All assays were repeated three times in duplicates. 102911, circular RNA_102911; miR, microRNA; Mut, mutant; NC, negative control; RT-qPCR, reverse transcription-quantitative PCR; si, small interfering RNA; WT, wild-type.

Due to the increasing aged population, there is increased demand on surgical services to treat diseases and improve patient quality of life (18). Hepatolithiasis is a common disease in China and a risk factor for cholangiocarcinoma (19). Usually, open partial hepatectomy is used as an effective treatment for hepatolithiasis (20). The most frequent location of intrahepatic ductal stones in hepatolithiasis is in segments I-IV of Couinaud (21). With the development of minimally invasive concepts, technologies and anatomical features, laparoscopic partial left LLH has become widely used and established as a more effective treatment for hepatolithiasis (22).

The improved treatment protocol has become especially significant for aged patients. As reported by Chen *et al* (23) and Jin *et al* (24), LLH is associated with a shorter hospital stay, decreased morbidity and more importantly, expedited

post-operative recovery compared with an open procedure. The present study found that post-operative infective complication was still a problem affecting the patients. Data from the present report showed that although the immediate clinical outcome was fair for both groups of patients in this study, the elderly patients were more likely to develop infective complications, even after minimally invasive surgery such as LLH. The probability of peritonitis in elderly patients may be closely related to bile leakage from bile duct stones (25).

Several factors have been revealed to be related to elderly patient's vulnerability to infective complications after trauma, of which immune dysfunction after trauma is of importance (26). This effect is most pronounced in elderly patients due to immunosenescence (27). Immunosenescence

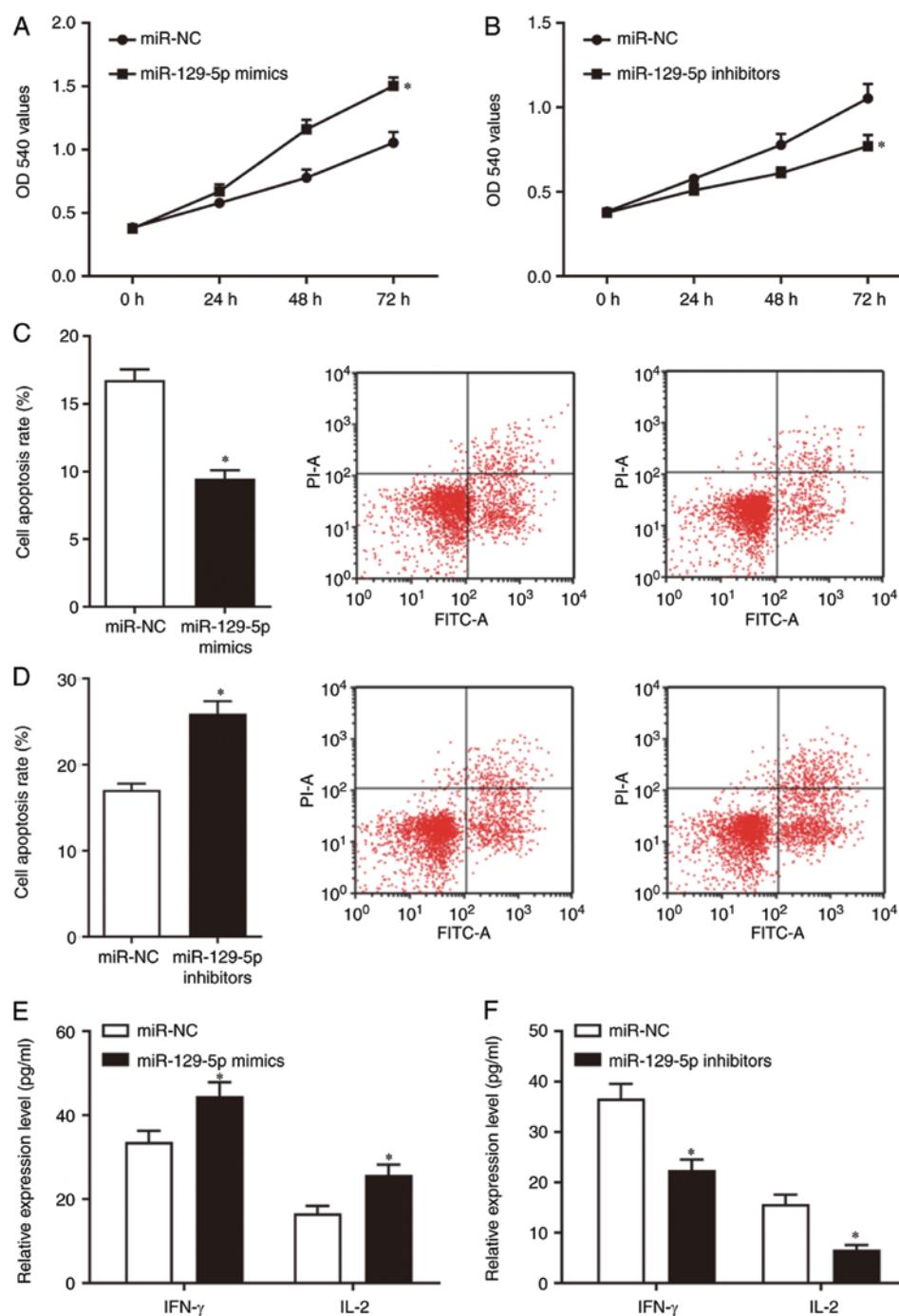


Figure 3. miR-129-5p is involved with the proliferation and apoptosis of CD3⁺/CD4⁺ T lymphocytes. The proliferation of CD3⁺/CD4⁺ T lymphocytes transfected with (A) miR-129-5p mimics or (B) miR-129-5p inhibitors was determined using MTT assays (Two-way ANOVA). The cell apoptosis rate of CD3⁺/CD4⁺ T lymphocytes transfected with (C) miR-129-5p mimics or (D) miR-129-5p inhibitors was determined using flow cytometry (Student's t-test). IL-2 and IFN- γ protein expression levels of CD3⁺/CD4⁺ T lymphocytes transfected with (E) miR-129-5p mimics or (F) miR-129-5p inhibitors were detected using ELISAs (Student's t-test). *P<0.05 vs. miR-NC. All assays were repeated three times in duplicates. FITC-A, anti-fluorescein isothiocyanate; miR, microRNA; NC, negative control; OD, optical density.

is the age-related decline in immune functions and affects various cell types such as neutrophils, monocytes, macrophages, natural killer cells and dendritic cells from the innate arm, as well as B and T cells from the adaptive arm of the immune system (26). Although aging alters both arms of the immune system, more severe detrimental changes occur in the adaptive immune system (28). The data from the present study showed that the basic quantity of peripheral lymphocytes in the elderly group was significantly lower than that in

the younger group pre-operatively. Secondly, after surgical trauma, the immune function of T lymphocytes went through a significant decline and lasted longer (no less than 2 weeks) when compared with that of the younger patients, which was reflected by perioperative changes to the T lymphocyte proliferative ability, levels of IL-2 secreted by T lymphocytes and the % of CD3⁺CD4⁺T lymphocytes in the peripheral blood. Finally, surgical trauma was found to further affect the number of T lymphocytes through altered levels of apoptosis.

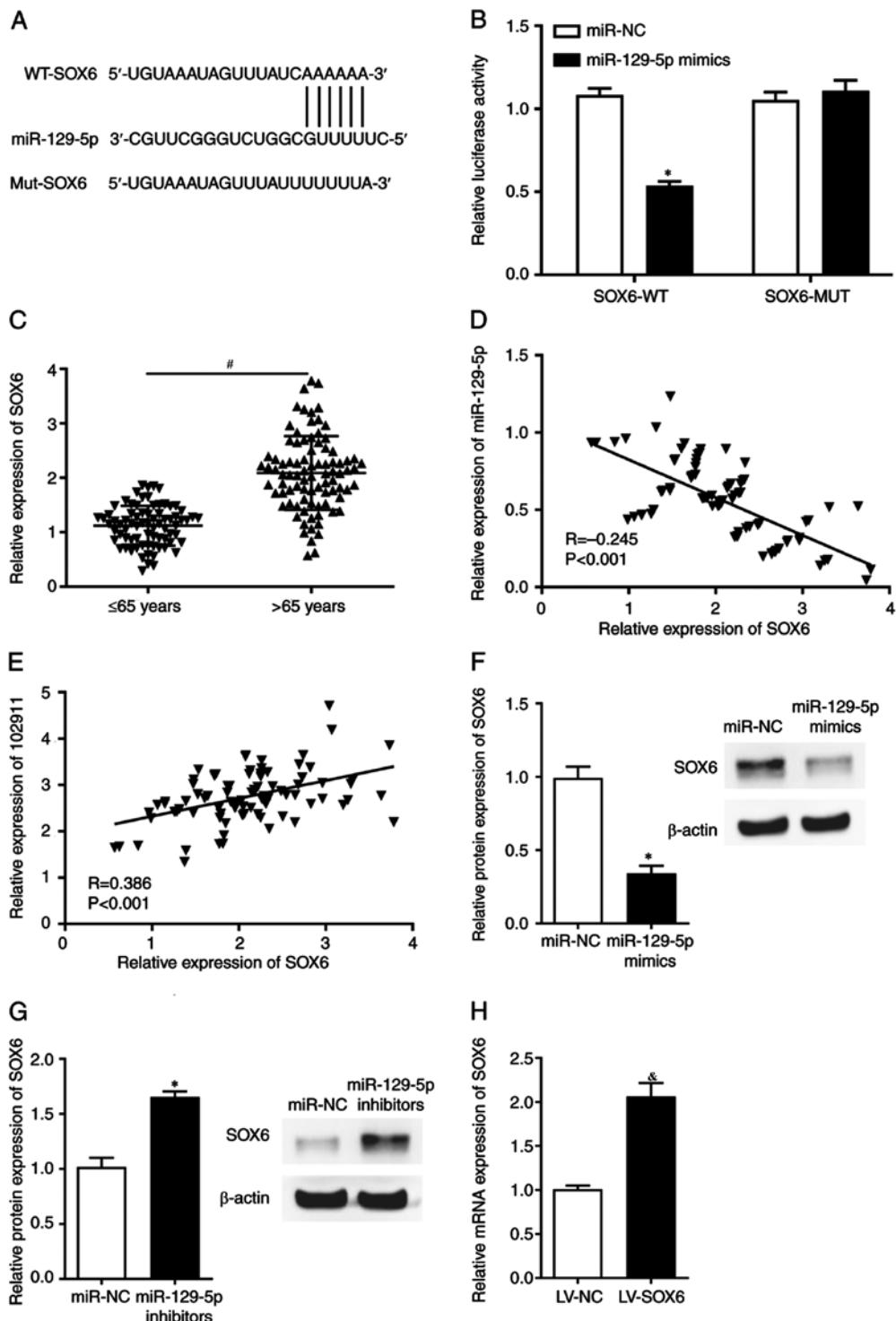


Figure 4. miR-129-5p directly binds with SOX6 in CD3⁺/CD4⁺ T lymphocytes. (A) The putative binding sites between the transcripts of miR-129-5p and SOX6. (B) Luciferase gene reporter assays demonstrated that miR-129-5p directly binds with SOX6 (Student's t-test). (C) The mRNA expression levels of SOX6 in CD3⁺/CD4⁺ T lymphocytes of two groups were determined using RT-qPCR (Student's t-test). (D) Spearman's correlation analysis indicated a negative correlation between miR-129-5p and SOX6 expression levels in CD3⁺/CD4⁺ T lymphocytes from elderly patients. (E) Spearman's correlation analysis indicated a positive correlation between 102911 and SOX6 expression in CD3⁺/CD4⁺ T lymphocytes from elderly patients. The protein levels of SOX6 of CD3⁺/CD4⁺ T lymphocytes transfected with (F) miR-129-5p mimics or (G) miR-129-5p inhibitors were determined using western blotting (Student's t-test). (H) The mRNA expression levels of SOX6 in CD3⁺/CD4⁺ T lymphocytes of transfected with LV-NC or LV-SOX6 were determined using RT-qPCR (Student's t-test). *P<0.05 vs. <65 years; *P<0.05 vs. miR-NC; *P<0.05 vs. LV-NC. All assays were repeated three times in duplicates. miR, microRNA; Mut, mutant; NC, negative control; RT-qPCR, reverse transcription-quantitative PCR; WT, wild-type.

The changes in T lymphocytes may be why elderly patients are more prone to post-operative complications.

It has been revealed that the immunosenescence-mediated decline in cell-mediated immune functions results in a chronic

inflammatory state (29). The age-related increases in cytokines and other components of inflammation are partially responsible for the increased prevalence and severity of infections (30). This pro-inflammatory response of aged patients

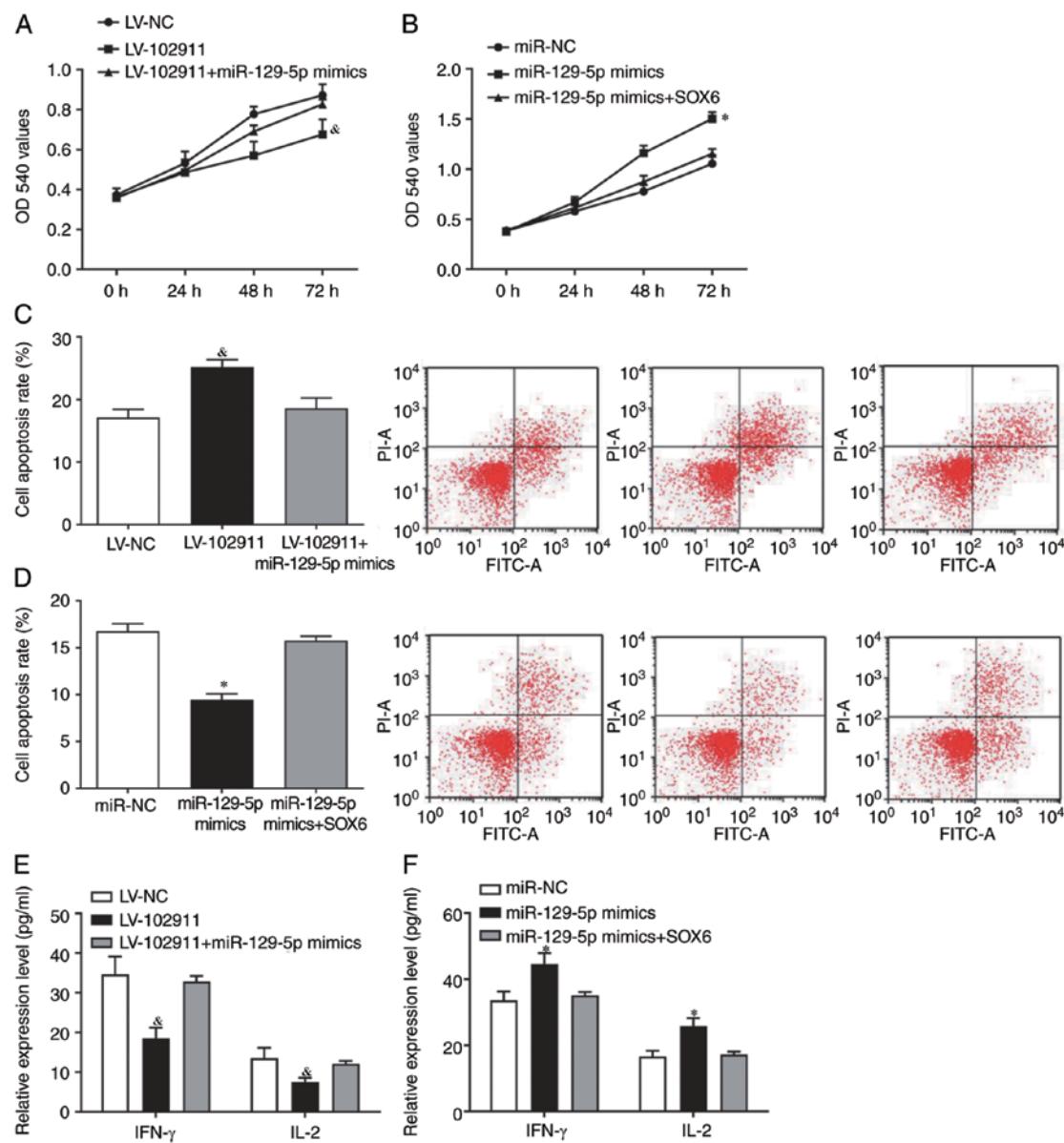


Figure 5. The 102911/miR-129-5p/SOX6 axis is involved with the proliferation and apoptosis of CD3⁺/CD4⁺ T lymphocytes. The proliferation of CD3⁺/CD4⁺ T lymphocytes co-transfected with (A) LV-102911 and miR-129-5p mimics, or (B) miR-129-5p and SOX6, was determined using MTT assays (Two-way ANOVA). The apoptosis of CD3⁺/CD4⁺ T lymphocytes co-transfected with (C) LV-102911 and miR-129-5p mimics, or (D) miR-129-5p and SOX6, was determined using flow cytometry (one-way ANOVA). IL-2 and IFN- γ protein expression levels of CD3⁺/CD4⁺ T lymphocytes co-transfected with (E) LV-102911 and miR-129-5p mimics, (F) or miR-129-5p and SOX6, were detected using ELISAs (one-way ANOVAs). *P<0.05 vs. miR-NC; *P<0.05 vs. LV-NC. All assays were repeated three times in duplicates. FITC-A, anti-fluorescein isothiocyanate; miR, microRNA; NC, negative control; OD, optical density.

is also believed to be responsible for the increased risk for developing complications following injury (31). The present study revealed that after trauma, the number and function of T lymphocytes in aged patients altered more drastically than that in the younger patients and took longer to return to the original levels. This theoretically worsens the immune function and increases the risk that elderly patients will suffer from infectious diseases. However, the exact mechanism of action has not yet been fully elucidated.

In the present study, 102911 was upregulated and miR-129-5p was downregulated in the CD3⁺CD4⁺ T lymphocytes from elderly patients with LLH for hepatolithiasis. 102911 overexpression inhibited CD3⁺CD4⁺ T lymphocyte proliferation and promoted cell apoptosis, with the opposite observations occurring following knockdown of 102911. miRNAs are a

class of non-coding small RNAs that are ubiquitous in the genome of an organism, which are ~20–24 nt (32). circRNA has the ability to recognize complementary sequences and bind to miRNAs (33). With the in-depth study of miRNAs, there is increasing evidence that they are closely related to the progression of various immune-related and age-related diseases (34,35).

The present study found that 102911 directly binds with miR-129-5p in CD3⁺CD4⁺ T lymphocytes and there was a significant negative correlation between expression levels of 102911 and miR-129-5p in CD3⁺CD4⁺ T lymphocytes from elderly patients. Overexpression of miR-129-5p significantly promoted T cell proliferation, inhibited T cell apoptosis and increased IL-2 and IFN expression levels. In contrast, reduced expression of miR-129-5p inhibited CD3⁺CD4⁺

T lymphocyte proliferation, promoted CD3⁺CD4⁺ T lymphocyte apoptosis and decreased IL-2 and IFN expression levels. These results showed that miR-129-5p could be a promising target of 102911 and that miR-129-5p is involved in the proliferation and apoptosis of CD3⁺CD4⁺ T lymphocytes.

SOX6 is a multifaceted transcription factor that participates in cell proliferation, apoptosis, differentiation and release of inflammatory factors (36). SOX6 also plays an important role in embryonic development (37). SOX6 has multiple cytotoxic T-lymphocyte and helper epitopes to induce antitumor activity and the effectiveness of SOX6-DNA vaccine for the prevention and treatment of glioma (38). In the present study, the expression levels of SOX6 in CD3⁺CD4⁺ T lymphocytes from elderly patients was significantly increased and SOX6 was found to be a downstream molecule of miR-129-5p.

In conclusion, the present study has demonstrated that the immune function and the number of T lymphocytes went through a significant decline and this decline lasted longer in elderly patients with LLH for hepatolithiasis after surgical trauma, compared with the younger patients. However, there were limitations to the present study. For example, *in vivo* studies should be performed to confirm the existing findings. Furthermore, other assays such as immunocytohistochemical staining should be carried out to evaluate the expression profile of the associated proteins. Despite the limitations, the present results suggested that the 102911/miR-129-5p/SOX6 axis was involved in T lymphocytes immune function, which provided a novel insight for potential treatments for elderly patients with hepatolithiasis.

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Availability of data and materials

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

Authors' contributions

WL designed the study. HZ, XS, HL, QL and JL performed the experiments and analyzed the data. HZ and XS drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of The Eighth Medical Center of the Chinese PLA General Hospital (Beijing, China). Written informed consents were obtained from all the patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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