The role of CRP and ATG9B expression in clear cell renal cell carcinoma

Running title: The role of CRP and ATG9B expression in CCRCC

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Funding: None
Abstract

**Background:** The purpose of the study is to investigate the correlation between the expression of C-reactive protein (CRP) and autophagy-related 9B (ATG9B) and pathological features of clear cell renal cell carcinoma (CCRCC) patients. We also intended to explore the effects of manipulated expression of CRP and ATG9B on the apoptosis and cell cycle progressing of CCRCC cell line.

**Material and Methods:** ATG9B expression in CCRCC tissues and adjacent renal tissues was analyzed by immunohistochemistry (IHC). Gene expression was determined at transcription and translational levels using real-time quantitative polymerase chain reaction (RT-qPCR) and western blot. The association between CRP/ATG9B expression and clinical-pathological parameters including age, gender, pathological grades, TNM stage and distant metastasis of the patients was assessed by correlation analysis. Small interfering RNA (siRNA) and overexpression plasmids construction were used to manipulate the expression of CRP in human CCRCC cell line 786-O. Cell apoptosis and cell cycle progressing were determined using flow cytometry and Hoechst 33258 staining.

**Results:** CRP expression correlates with ATG9B expression. The expression of CRP and ATG9B is significantly correlated with TNM staging, distant metastasis and survival time of CCRCC patients. A high-level of CRP indicates a poor overall survival (OS). In addition, CRP expression influences cell cycle and apoptosis of CCRCC cells.

**Conclusion:** The study reveals that CRP might be a CCRCC development promoter. In addition, there is a close relationship between CRP and ATG9B in CCRCC carcinogenesis.
**Key words:** clear cell renal cell carcinoma (CCRCC), CRP, ATG9B, cell apoptosis
**Introduction**

Renal cell carcinoma (RCC) is the most common substantial lesion within kidney (1), constituting 2%-3% of adult malignancies (2) and 85% of primary renal tumors (3). It commonly spreads to lungs, liver, bones, brain, adrenals, and lymph nodes, but seldom to skin, thyroid, and pancreas (4). Clear cell renal cell carcinoma (CCRCC), as a subtype of RCC, accounts for around 75% of RCC (5). 40% of CCRCC patients would eventually die of carcinoma development (6). CCRCC shows the highest fatality rate among the common urologic malignancies (7). Over 10,000 patients die from kidney cancer each year, however, systemic treatment of RCC has been improved vastly in the last two decades (8). The pathogenesis of the most common type of RCC, CCRCC has been better understood. Therapies with high rates of response, longer progression-free survival such as anti-angiogenic drugs targeting vascular endothelial growth factor (VEGF) and its receptors and mechanistic target of rapamycin (mTOR) inhibitors have been used (9).

The C-reactive protein (CRP) is located on chromosome 1q23.2 and encodes protein that belongs to the pentaxin family. CRP is a plasma protein mainly generated in liver and activated by interleukin 6 (IL-6) (10). It is a prognostic factor for survival and recurrence of different types of cancers including mammary, prostatic, colonic, hepatocellular, bone and upper aerodigestive tract (UADT) tumors (11-14). Additionally, a previous meta-analysis has shown that high serum level of CRP (>1.0 mg/dl) is correlated with increased hazard of lung cancer and possibly breast, prostate and colorectal cancers (15). Furthermore, recent studies have revealed that CRP expression is significantly associated with overall survival (OS) time.
of patients with RCC (16-19). However, whether abnormal CRP expression is associated with CCRCC pathogenesis, metastasis and OS remains to be clarified.

The autophagy-related 9B (ATG-9B), locating on chromosome 7q36.1, belongs to the ATG family. A previous study has shown that ATG9B expression is tissue-specific, that is, ATG9B is abundant in organs such as placenta and ovary but minimum in lung, testis, liver, muscle, brain and pancreas (20). The methylation of ATG9B promoter may interrupt the autophagy signal pathway and influence the invasive ductal carcinoma (IDC) development (21). Similarly, Kang et al. discovered that the mutation of ATG9B is common in human gastric and colorectal cancers and it can be closely related to stomach and colorectal carcinogenesis, suggesting that ATG9B mutation may promote neoplasm development by deregulating autophagy (22). What’s more, ATG9B was found to be interacted with p38IP and regulated by p38α mitogen-activated protein kinase (MAPK) pathway, which then influenced the trafficking of ATG9B and therefore the autophagy process in a mammalian system (23, 24).

Autophagy is closely related to cancer development including CCRCC (25, 26). However, the relationship between ATG9B expression and CCRCC pathogenesis, metastasis and OS remains to be clarified as well.

Previous studies have shown us the aberrant expression of CRP and ATG9B and their relationship with various human diseases especially cancer development including CCRCC. Yet, the influence of their expression on CCRCC progression remains further elaborated. Our study here aimed to explore the relationship between CRP and ATG9B expression with CCRCC pathogenesis, metastasis and survival as well as the role they play in CCRCC using
Materials and Methods

Tissue specimens

185 CCRCC tissues and normal adjacent tissues were collected from CCRCC patients in the Urology Center of Liaocheng People's Hospital between 2013 and 2016. All tissues were frozen in liquid nitrogen immediately and were stored at -80°C. No patients had received any adjuvant treatments, such as radiotherapy or chemotherapy before surgery. Written informed consents were obtained from all participants. The study had been approved by the Ethics Committee of Liaocheng People's Hospital.

Cell culture and siRNA transfection

CCRCC cell line (786-O) was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were placed in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Life Technologies, Darmstadt, Germany) which contains 10% fetal bovine serum (FBS; HyClone, Logan, UT), penicillin (100 U/ml) and streptomycin (100 mg/ml). All were stored at 37°C in a humidified atmosphere containing 5% CO₂. SiRNA-1 and siRNA-2 were synthesized by Suzhou GenePharma Co., Ltd. (Suzhou, China). 24 h before transfection, the 786-O cells were seeded in the DMEM media with 10% FBS without antibiotics so the cells grew to 90% confluence. siRNA-Lipofectamine 2000 complexes were prepared. Briefly, siRNA-1 and siRNA-2 were resuspended in 1x siRNA buffer to reach a final concentration of 1 μM. 1 μl siRNA solution was added to 100 μl serum-free media to mix. 0.5 μl
Lipofectamine 2000 reagent was added to 25 μl serum-free media. siRNA media and diluted Lipofectamine 2000 reagent were incubated together for 20 min at room temperature to allow complex formation. The old media were removed after 4-6 h. The complexes were added to each well. Cells were then harvested 24 h after transfection. The siRNA sequences were provided in Table S1. SiRNA-1 and siRNA-2 were both used to knock down CRP, yet they had different sequences.

**enzyme-linked immunosorbent assay (ELISA)**

CRP Human ELISA Kit (ab99995, Abcam, Boston, MA, USA) was purchased for the conduction of ELISA. All materials were prepared at room temperature prior to use. 100 μl of standards and tissue samples were added to wells. The wells were covered ad incubated for 2.5 h. The solution was discarded and the wells were washed three times by adding 300 μl 1× wash solution into each well. Any remaining liquid was removed completely. 100 μl 1× biotinylated anti-human CRP detector antibody to each well. The antibody was incubated for 1 h. Then the solution was discarded and the wells were washed three times by adding 300 μl 1× wash solution into each well. 100 μl 1× HRP-Streptavidin solution to each well and incubated for 45 min. The solution was removed completely. 100 μl TMB One-Step substrate reagent was added to each well and incubated for 30 min in the dark. Lastly, 50 μl of Stop Solution was added to each well. The optimal density was read at 450 nm immediately.

**RNA extraction and real-time quantitative polymerase chain reaction (RT-qPCR)**

Total mRNAs of stored human CCRCC tissues and cells were extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. Reverse
transcribed complementary DNA (cDNA) synthesis was performed with PrimeScript RT reagent Kit (TaKaRa, Dalian, China). RT-qPCR was conducted using miSYBR-Green PCR kit (TransGen Biotech, China) according to manufacturer’s protocols. Data were evaluated using SDS 2.2 software. GAPDH acted as the internal control. The expressions of CRP and ATG9B were quantified using $2^{-\Delta\Delta Ct}$ method. The primer sequence was listed in Table 1.

**Immunohistochemical (IHC) staining**

Three µm-thickness paraffin-embedded histotomy was soaked in xylene to dewax twice for 20 min, hydrated in gradient ethanol (100%, 90%, 80% and 70%) for 7 min. The specimens were then rinsed in the tap water three times, 3 min each time. The tissue sections were boiled with sodium citrate buffer for 5 min. The sections were then cultured in 3% H2O2 for 10 min to prevent endogenous peroxidase activities. The slices were then rinsed using phosphate-buffered saline (PBS) three times (5 min each time) and sealed in 10% serum for 10 min at room temperature to avoid non-specific binding. The tissue sections were subsequently incubated with primary antibodies anti-CRP antibody (ab31156, 5µg/ml, Abcam, Boston, MA, USA) and anti-ATG9B antibody (ab117591, 5 µg/ml, Abcam, Boston, MA, USA). After incubation at 4°C overnight with Galectin-3, the serum was discarded and the sections were mixed with biotinylated secondary antibodies for 10 min after being washed in PBS three times (5 min each time). Afterwards, the tissue sections were cultured with Streptavidin streptavidin-horseradish peroxidase (SA-HRP) for another 10 min and rinsed with PBS for 5 min, three times. Finally, after diluted with 3, 3’-diaminobenzidine (DAB), the sections were blended with Mayer’s hematoxylin (Merck, Darmstadt, Germany), gradient
ethanol (95%, 85% and 75%) for 3 min respectively as well as absolute ethanol for 10 min and dehydrated in xylene twice, each time 5 min. The criteria IHC staining records were shown as follows: no expression (0 represents without staining), low expression (1 represents more than 30 % of cells weakly stained), or high expression (2 represents more than 30 % of cells strongly stained).

**Western Blot**

Cells were washed with PBS, scraped from the dishes and centrifuge at 12000 r.p.m., 4 °C for 15 min. Cell lysates were prepared with radioimmunoprecipitation assay (RIPA) buffer. The supernatants were collected and protein concentration was determined using the bicinchoninic acid BCA assay (Beyotime, Shanghai, China). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad, Hercules, CA, USA), transferred to a polyvinylidene difluoride (PVDF) membranes (Invitrogen, Gaithersburg, MD, USA) for western blot analysis following the manufacturer’s guidelines. PVDF membranes were sealed using 5% skim milk for one hour at room temperature, and then incubated with primary antibodies anti-CRP (ab31156, 5µg/ml, Abcam, Boston, MA, USA) and Anti-ATG9B (ab117591, 2 µg/ml, Abcam, Boston, MA, USA). After cultured with primary antibodies overnight at 4°C, secondary antibodies (1:1000) were added for another one-hour incubation at room temperature. The membranes were washed three times with tris buffered saline-tween (TBST). The immunoreactive protein bands were visualized using G: Box XR5 and the membranes were subsequently exposed.

**Hoechst 33258 staining**
786-O cells were seeded on cover slips in 24-well plates. After transfection for 48 hours, cells were mixed with 4% paraformaldehyde at room temperature for 5 min. The cells were then permeabilized using 0.2% Triton X-100 solution with PBS for another 5 min, and rinsed with PBS. Then 5% BSA was used to block the cells at room temperature for 2 h, after which they were rinsed with PBS three times (5 min each time). The cells were then stained with 0.5 ml Hoechst 33258 in dark for 5 min and rinsed in PBS. Finally, they were coverslipped with aqueous mounting medium (Dako Faramount, Shanghai, China) for later observation.

**Apoptosis assay**

Flow cytometry (FCM) was employed to analyze the apoptosis of CCRCC cells. Cells were collected in logarithmic growth phase, placed in a 96-well plate and pre-incubated for 24h in a CO₂ incubator. Single-cell suspensions were fixed with 70% alcohol and then rinsed twice with PBS and binding buffer was employed to re-suspend cells. For Annexin V staining, 5 μl Annexin V-PE, 5 μl 7-AAD staining and binding buffer of 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)/ NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl₂ were added to samples, which were incubated for 15 min at room temperature in dark and analyzed by a flow cytometer (FACSCanto II, BD Biosciences). The data were analyzed using FlowJo software (LLC, Ashland, OR, USA). Three experiments were performed in triplicate. Apoptotic cells that were positive of Annexin V-PE but not 7-AAD were early-apoptotic, whereas those were Annexin V-PE and 7-AAD positive were late-apoptotic or necrotic.

**Statistical Analysis**
SPSS 22 (Chicago, Illinois, USA) was used for statistical analyses and data were all presented as mean ± standard deviation (SD). Correlations between expressions of CRP and ATG9B and the clinic pathological characteristics were analyzed using two-sided Fisher’s exact test. The correlation between the protein expressions of CRP and ATG9B was assessed using Spearman’s rank correlation coefficient test. The Kaplan–Meier method was applied to draw overall survival (OS) curves and the expression comparisons distinction between groups was evaluated using the log-rank test. \( P<0.05 \) was considered statistically significant.

**Results**

**The expression of CRP and ATG9B in serum and tissues**

The concentration of CRP in human serum was evaluated using ELISA method. The results showed that the expression of CRP in CCRCC group was significantly higher than that in control group (<10 mg/L, Figure 1A). Meanwhile, among 185 CCRCC serum samples, there were high expressions of serum CRP in 159 cases (> 30 mg/L), moderate expression of CRP in 18 cases (11-30 mg/L) and lowly expression of CRP in 8 cases (< 10 mg/L).

The IHC staining results showed that compared with adjacent tissues, CCRCC tissues demonstrated more positive expression of ATG9B. In CCRCC tissues, low expression of ATG9B was detected in 82 patients and highly expressed ATG9B was observed in 103 patients (Figure 1B). The positive signal was in the cytoplasm of cancer cells.

**The correlation between gene expression and the pathological characteristics of CCRCC patients**
The relationship between CRP and ATG9B expression levels and CCRCC patients’ characteristics were analyzed using two-sided Fisher’s exact test. As was shown in Table 2, high expression of CRP or ATG9B was positively related to advanced TNM stage and distant metastases. In addition, there was also a positive correlation between CRP expression and ATG9B expression (Table 3).

**The correlation between CRP expression and patients’ prognosis**

Survival curves were generated by means of the Kaplan–Meier method and the distinctions among groups with different expressions were detected using the log-rank test. The correlation between CRP expression level and overall survival (OS) of CCRCC patients was shown in Figure 1C. The results indicated that patients with high CRP expression level had a lower OS and poorer prognostic result than those who with low CRP expression level.

**The correlation between CRP expression and ATG9B expression**

The effect of small interfering RNA (siRNA) transfection on the expression of CRP was confirmed by RT-qPCR (Figure 2A). Compared with the negative control group, siRNAs (siRNA-1 and siRNA-2) could significantly inhibit CRP mRNA expression. ATG9B expression dramatically decreased after the transfection of siRNAs. In addition, the expression level of ATG9B decreased more quickly than that of CRP (Figure 2B). According to western blot results, the expression levels of CRP and ATG9B proteins were remarkably downregulated.

**The inhibitive effects of CRP on the apoptosis of 786-O cells**

The results of Hoechst 33258 staining of the tumor cell cytoplasm showed that the
transfection of siRNAs significantly promoted the apoptosis of 786-O cells (Figure 3A). Annexin-V and 7-AAD dual-staining flow cytometry results demonstrated that the apoptosis rate of cells in siRNA-1 or siRNA-2 group was much higher than that in the control group (Figure 3B). This also indicated that the apoptosis of 786-O cells could be induced through silencing CRP expression. On the other hand, cell cycle assay also demonstrated that cells were arrested in G1 phase in the two siRNA groups (Figure 3C).

The synchronous expression of CRP expression and ATG9B expression

As shown in Figure 4, mRNA expression levels of CRP and ATG9B in overexpressed group was upregulated by more than twice in comparison with the control group. Western blot results revealed that overexpression of CRP could significantly up-regulate the expression of ATG9B.

The inhibitive effects of CRP expression on the apoptosis in 786-O cells

The apoptotic rate of the overexpression group presented a drastic decline in comparison with negative control group, which also suggested CRP overexpression could inhibit apoptosis (Figure 5A). Moreover, CRP expression group displayed much fewer apoptotic cells than the negative control group, shown by Annexin-V and 7-AAD dual-staining flow cytometry test results. CRP overexpression could suppress cell apoptosis (Figure 5B). S and G2/M phases increased in 786-O cells of overexpression group compared to the negative control group (Figure 5C). This showed that CRP expression level could exert influence on the cell cycle of 786-O cells.
Discussion

In this study, we have discovered that the expression of CRP and ATG9B is significantly correlated with CCRCC TNM staging, metastasis and OS. A higher level of CRP indicates a poorer OS of CCRCC patient. The inhibition of CRP expression significantly increased the apoptosis and cell cycle arrest of CCRCC cell line 786-O. CRP expression is positively correlated with ATG9B, and its overexpression led to less apoptosis and less cell cycle arrest of 786-O cells. CCRCC is the most common kind of RCC with poor prognosis with 5-year survival rate of 0%~10% (27). To understand the relationship of CRP and ATG9B expression and CCRCC is significant to the development of CCRCC prognosis and treatment.

In our study, we found that CRP expression was associated with ATG9B expression. The inhibition of CRP expression was accompanied by decreased expression of ATG9B and the overexpression of CRP was accompanied with increased expression of ATG9B. Serum CRP protein was low-expressed in normal patients. Among CCRCC patients, 159 were found with high-level CRP (> 30 mg/L), 18 medium-level (11-30 mg/L) and 8 low-level (< 10 mg/L). On the other hand, ATG9B was found overexpressed in most of CCRCC patients. CRP was found significantly associated with OS of CCRCC patients. Based on the above results, we speculated that the expression of CRP and ATG9B was positively correlated with CCRCC and it may indicate a poorer status of CCRCC. Our results are consistent with previous studies, in which identical findings were demonstrated in regards of RCC OS and aberrant CRP expression (17, 28-30). Thus, we speculated that the aberrant overexpression of CRP and ATG9B could possibly promote CCRCC development.
The *in vivo* experiment results support our hypothesis that the aberrant overexpression of CRP and ATG9B could promote CCRCC development, possibly by influencing the cell cycle and apoptosis. The inhibition of CRP expression promoted whereas the promotion of CRP expression inhibited 786-O cell cycle arrest and apoptosis. As a stable downstream inflammation marker, it can be stimulated by IL-1 and tumor necrosis factors (TNFs) (31). Chronic inflammation can be predictor of cancer initiation, progression, metastasis and survival. CRP-level measurements can be clinically significant for RCC prognosis (32, 33). Hence, we inferred that CRP could be involved in CCRCC development via some inflammation pathway that involves ILs and TNFs and affect CCRCC cell proliferation and apoptosis.

On the other hand, CRP overexpression was found accompanied/associated with ATG9B overexpression. ATG9B is involved in autophagy process that delivers cytoplasmic constituents degraded by autophagosomes to lysosomes for digestion (34), which is a common scene during carcinogenesis (26). Autophagy can play a protective role in tumor cell survival, and can also contribute to cancer progression by preventing tumor cell from programing death (35). Zhang et al. found aberrant promoter methylation of ATG9B in sporadic breast carcinoma (21). Oncogenic autophagy in clear cell renal cell carcinoma was reported to be regulated by some molecules, which could contribute to the CCRCC progression (25). ATG genes as well as ATG-interacted genes have been reported to be related to the development of diverse carcinomas such as lung cancer and CCRCC etc. (36). Based on the previous findings as well as ours, we speculated that aberrant ATG9B expression might
contribute to the aberrant autophagy of CCRCC cells, which then induced CCRCC progression.

Certain limitations exist in this study. First of all, in vivo studies need to be done to elaborate the role of CRP and ATG9B expression during CCRCC development. Secondly, ATG9B-related autophagy pathway can be further studied for a better comprehension of the ATG9B-related mechanism of CCRCC pathogenesis. Thirdly, the influence between CRP-related inflammation pathways and CCRCC need to be further explored. Fourthly, in vivo experiments need to be further studied to verify the in vitro discoveries. Lastly, detailed correlation between ATG9B and CCRCC pathogenesis need further investigation as well.

In conclusion, our study showed that CRP and ATG9B were both aberrantly overexpressed in CCRCC tissues and cells, and they were closely correlated with CCRCC TNM staging, metastasis and OS. The suppression of CRP expression led to the cancer cell cycle arrest and apoptosis. The results indicate that CRP and ATG9B could be significant predictors of CCRCC and can be a valuable target for CCRCC therapy.
Declarations

Ethics approval and consent to participate: All procedures of this research were in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Liaocheng People's Hospital. All patients participated have given consent to this study.

Conflicts of interest: All authors declare that they have no conflict of interest.

Acknowledgements: Not applicable.

Author contributions: Research conception and design: Zheng Ma and Zengguang Qi; Data analysis and interpretation: Jiangsong Li and Jing Yang; Drafting of the manuscript: Zheng Ma and Zengguang Qi; Critical revision of the manuscript: Zhonghua Xu; Approval of final manuscript: all authors.
References


Figure Legends

Figure 1  *CRP* and *ATG9B* expression in tissues and the relationship between *CRP* expression and overall survival rate of patients. **A:** Serum *CRP* protein was low-expressed in patients of the normal group, but high-expressed (> 30 mg/L) in 159 CCRCC cases, medium-expressed in 18 CCRCC cases (11-30 mg/L) and low-expressed (< 10 mg/L) in 8 CCRCC cases. **B:** *ATG9B* expression in all normal kidney and tumorous tissues was positive, with 82 low-expressed and 103 high-expressed in CCRCC patients group. The positive signal is in the cancer cell cytoplasm. **C:** Patients with high *CRP* expression level had worse survival outcomes compared to low expression group. * *P* < 0.05 compared to control group.

Figure 2 *CRP* silences effects and its correlation with *ATG9B* expression **A:** siRNAs could significantly down-regulate mRNA expressions of *CRP* and *ATG9B* protein. mRNA levels of *CRP* and *ATG9B* were both significantly lower in siRNA-1 and siRNA-2 groups than in control group. **B:** *CRP* protein level as well as *ATG9B* protein level in siRNA-1 and siRNA-2 groups was much lower than in control group. *P* < 0.05, # *P* < 0.05 compared to control group.

Figure 3 Inhibition of *CRP* expression induces cell cycle arrest and the apoptosis of 786-O cells **A:** The apoptosis of 786-O cells increased by either siRNA by approximately 2.5 times. Scale bar: 30 μm. **B:** Dual-staining flow cytometry test results showed that apoptotic cells transfected with siRNAs were much more than those in the control group. * means *P* < 0.05 compared to the control group. **C:** The cell cycle was significantly arrested in G0/G1 phase in siRNA groups. * *P* < 0.05 compared to control group.

Figure 4 The effects of *CRP* overexpression and its correlation with *ATG9B* expression **A:**
CRP mRNA expression increased by approximately 2.5 times, while ATG9B expression increased by approximately 2.2 times significantly in CRP overexpression group than in Scr group. **B:** CRP level increased significantly because of its overexpression, which also improved ATG9B level. *P*<0.05, # *P*<0.05 compared to Scr group.

**Figure 5 CRP overexpression promotes the apoptosis of 786-O cells and reduces the cell cycle**

A: 786-O cell apoptosis decreased by approximately two-fold. Scale bar: 30 μm. **B:** the number of apoptotic cells was much less in CRP overexpression group than that in Scr group. **C:** The percentage of cells that were arrested in G0/G1 phase in CRP overexpression group was reduced significantly compared to Scr group. *P*<0.05 compared to Scr group.
**A**

\[
\text{mRNA expression} = \begin{cases} 
1.5 & \text{control} \\
1.0 & \text{SiRNA-1} \\
0.5 & \text{SiRNA-2}
\end{cases}
\]

\* and \# indicate significant differences.

**B**

ATG9B, CRP, and GAPDH protein expression levels for control, SiRNA-1, and SiRNA-2 conditions.

\[
\text{Relative protein expression} = \begin{cases} 
1.5 & \text{control} \\
1.0 & \text{SiRNA-1} \\
0.5 & \text{SiRNA-2}
\end{cases}
\]

\* and \# indicate significant differences.
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primers (5’-3’)</th>
</tr>
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</table>
| CRP       | F: TGTGAGCCAGAAAAACAAGCAAA  
|           | R: GGTATGGGGGTGGGTCTCTAA   |
| ATG9B     | F: TGTGAGCCAGAAAAACAAGCAAA  
|           | R: GGTATGGGGGTGGGTCTCTAA   |
| GAPDH     | F: TGTGAGCCAGAAAAACAAGCAAA  
<p>|           | R: GGTATGGGGGTGGGTCTCTAA   |</p>
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<th>Characteristics</th>
<th>Volume (%)</th>
<th>CRP expression (N = 185)</th>
<th>p</th>
<th>ATG9B expression (N = 185)</th>
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<tr>
<td></td>
<td></td>
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<td></td>
<td>Low (N=82) (%) High (N=88) (%)</td>
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<td>36 (49)</td>
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</tr>
<tr>
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<td>50 (46)</td>
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<td>60 (61)</td>
<td>38 (39)</td>
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<td>37 (43)</td>
<td>50 (57)</td>
<td>50 (57)</td>
<td>37 (43)</td>
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<td>75 (59)</td>
<td>52 (41)</td>
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<td>22 (38)</td>
<td>36 (62)</td>
<td>19 (33)</td>
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<td>Spearman’s rho</td>
<td>Correlation Coefficient</td>
<td>Sig. (2-tailed)</td>
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<td>---------------</td>
<td>-------------------------</td>
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<td>1.000</td>
<td>0.000</td>
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<tr>
<td>ATG9B</td>
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Table 3: Correlations between CRP and ATG9B
Table S1 SiRNA sequences that were used in the experiments.

<table>
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<th>Site</th>
<th>DNA sequence</th>
<th>Sense 5’-3’</th>
<th>Antisense 3’-5’</th>
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