Elevated circASCC3 Suppresses Antitumor Immunity by Sponging miR-432-5p to Upregulate C5a in Non-small-cell Lung Cancer

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Abstract

**Background:** Tumor invasion and immune evasion are the main mechanisms underlying the progression of non-small-cell lung cancer (NSCLC). In addition, abnormally expressed circular RNAs (circRNAs) contribute to the malignant phenotype of NSCLC. Thus, further investigation of the mechanism of dysregulated circRNAs may provide new insight into the treatment of NSCLC.

**Methods:** circRNA sequencing was used to explore the different expression profiles of circRNAs in 4 NSCLC tissues and paired normal tissues. Then, the expression of key circRNAs in NSCLC tissues and matched normal tissues was further evaluated via in situ hybridization and in cell lines using quantitative real-time polymerase chain reaction (qRT-PCR). Next, in vitro and in vivo models of NSCLC were employed to uncover the functions and mechanisms of key circRNAs in NSCLC progression and treatment.

**Results:** circASCC3 (hsa_circ_0077495) was overexpressed in NSCLC tissues compared to paired normal tissues, and the upregulation of circASCC3 indicated a dismal prognosis in patients with NSCLC. Overexpressed circASCC3 enhanced the malignant phenotype of NSCLC cells in vitro and led to an immunosuppressive microenvironment in vivo. Mechanistically, circASCC3 sponged miR-432-5p to increase the expression of complement C5a, which induced the progression and dysfunctional immune status of NSCLC. Moreover, the combination of the C5aR inhibitor PMX-53 and anti-programmed cell death 1 (PD-1) antibody achieved synergistic effects in NSCLC models overexpressing circASCC3.

**Conclusion:** These results uncover the contributions of circASCC3 to NSCLC progression and immunosuppression and provide a potential strategy for overcoming resistance to anti-PD-1 therapy.

**Background**

Lung cancer is still the leading cause of cancer-related death and non-small-cell lung cancer (NSCLC) ranks as the main pathological type, accounting for approximately 80–85% of cases[1]. In addition to conventional treatments such as surgery and chemoradiotherapy, immunotherapy has gradually arisen and represents a promising strategy for the clinical treatment of NSCLC patients. However, the progression of NSCLC is still inevitable, and acquired resistance to immune therapy presents another challenge for immune treatment[2]. Thus, it is critical to uncover the molecular mechanism underlying tumor progression and immune resistance and develop an effective treatment strategy for NSCLC.

Circular RNA (circRNA), characterized by a continuous covalent closed loop structure, is a type of noncoding RNA or protein-encoding RNA derived from exons and is present in the cytoplasm[3, 4]. It regulates extensive biological functions in eukaryotes via different mechanisms[5]. Various studies have revealed the potential impact of circRNAs on oncogenesis and the progression of cancer[6]. For instance, circFGFR1 acts as a competitor for miR-381-3p, enhancing the upregulation of chemokine receptor 4 and further promoting the immune evasion of NSCLC[7]. The sponging of miR-582-3p by circSHKBP1 induces the expression of the downstream mRNA HUR to facilitate growth and invasion in gastric cancer[8]. While
the biological functions of dysregulated circRNAs have been gradually uncovered, the underlying mechanisms of circRNAs that regulate the biological functions of NSCLC remain to be further explored.

Immune evasion is a hallmark of cancer and the major barrier to developing effective antitumor therapy. It is characterized by the loss of cancer cell immunogenicity and dysfunction of the immune system in the tumor microenvironment (TME)[9]. The crosstalk between immune cells and tumors in the TME drives the progression of cancer[10]. Immune checkpoints refer to a series of immune inhibitory pathways within the immune system that are vital for the balanced regulation of physiological immune cells[11]. Thus, immune checkpoint blockade has been a promising strategy for antitumor therapy, and programmed cell death 1 (PD-1) blockade has been approved for NSCLC patients. However, only 45% of patients achieve a major pathological response; in addition, some patients have no response to the inhibitor, and adverse events have been observed, which suggests that investigation of the immunosuppressive microenvironment in NSCLC is warranted[12].

In this study, circRNA sequencing was used to compare the expression profiles of circRNAs in NSCLC tissue samples with those in matched peritumor tissue samples, and the results identified significant upregulation of circASCC3 (hsa_circ_0077495), which is located on chromosome 6q13. Moreover, the expression of circASCC3 was found to be an independent predictor for the prognosis of NSCLC patients. In addition, circASCC3 sponged miR-432-5p and further induced the upregulation of the downstream target complement C5, which participates in the progression of NSCLC and immune evasion in the TME. Taken together, these findings suggest that circASCC3 might be a new target for NSCLC treatment.

**Methods**

**Cells and compound reagents**

Five NSCLC cell lines (A549, H1299, H460, H1703 and LLC/LUC) and the HEK-293T and THP-1 cell lines were obtained from the American Type Culture Collection. The cells were cultured in DMEM or RPMI-1640 mixed with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin and streptomycin in a cell incubator.

**Transfection of lentiviral vectors**

Lentiviral vectors containing circASCC3 and shcircASCC3 were designed and constructed (Genomeditch, Shanghai). The miR-432-5p inhibitor was purchased from Thermo Fisher (USA). After 48 h of transfection, the expression of the potential target genes was confirmed by quantitative real-time polymerase chain reaction (qRT-PCR). The sequences of shcircASCC3 are presented in Supplementary Table 4.

**Patients and postoperative follow-up**

A tissue microarray (TMA) containing tumor tissues and matched normal tissues that were collected from 188 patients who were identified to have NSCLC by pathologists and underwent radical resection at Fudan University Zhongshan Hospital from 2008 to 2010 was used to perform immunohistochemistry
(IHC) and in situ hybridization (ISH). The last follow-up was in May 2016. The Zhongshan Hospital Research Ethics Committee approved the study, and each patient signed informed consent forms.

**Transcriptome sequencing and bioinformatics analysis**

Transcriptome sequencing and relevant bioinformatics analysis were conducted by the Cloud-Seq Biotech platform (Shanghai, China). The differentially expressed circRNAs and mRNAs with fold changes $\geq 1.5$ and $p < 0.05$ between different groups were analyzed. The heatmap is a visualization tool used for presenting the discrepant expression of circRNAs and mRNAs. Reactome, KEGG and PANTHER were applied to determine the functions of these differentially expressed mRNAs in biological pathways.

**RNase R digestion**

One microgram of total RNA was digested by 5 U/μg RNase R (Epicentre, USA) for 30 min. Next, the digested RNA was purified by a RNeasy MinElute cleaning Kit (Qiagen) and used for qRT-PCR.

**Western blot, RT-qPCR, IHC, ISH, fluorescence in situ hybridization (FISH) and multicolor immunofluorescence (mIF) assays**

The western blot, qRT-PCR and IHC procedures were performed according to previous reports and are summarized in the Supplementary Materials and Methods[13, 14]. The primers and antibodies are presented in Supplementary Tables 2 and 3. For the ISH assay, the TMA slide was deparaffinized in dimethylbenzene, hydrated with alcohol and digested with proteinase K (Thermo Fisher, USA) for 5 minutes. The digestion was neutralized by Tris-HCl buffered saline plus Tween (TBST), incubated with ISH buffer for approximately 2 h at 42°C and then hybridized at 37°C for 12 h. Then, the sections were incubated with BSA for 30 minutes and subsequently with anti-digoxigenin-labeled peroxidase for 40 minutes at 37°C. 3,3'-Diaminobenzidine was used for color development, and the sections were counterstained with hematoxylin. The TMA was scored under a microscope at a magnification of 200× by two pathologists. The score was calculated according to the staining intensity (0, negative; 1, weak; 2, moderate; and 3, strong staining) and extent of staining (1, $\leq 25%$; 2, 25%-50%; 3, 50%-75%; and 4, $> 75$%). The total score was calculated as follows: total score = intensity score $\times$ extent of staining score.

For the FISH assay, probes specific to circASCC3 and miR-432-5p were designed and constructed by GenePharma (Shanghai, China). The probe signals were detected according to the FISH kit protocol (GenePharma, Shanghai, China). The images were captured with a fluorescence microscope. The probe sequences of circASCC3 and miR-432-5p are presented in Supplementary Table 5. For the mIF assays, WiSee Biotechnology (Shanghai, China) performed mIF staining of CD4, CD8, CD163 and PD-L1 in 15 NSCLC tissues from patients receiving anti-PD-1 monotherapy.

**Cell counting kit-8 (CCK-8), colony formation, woundhealing, and Matrigel invasion assays**

The procedures of these experiments have been reported in a previous study and are summarized in the Supplementary Materials and Methods.
In vivo circRNA precipitation (circRIP)

For the in vivo circRIP assay, biotin-labeled circASCC3 and control probes were designed and constructed (GenePharma, China). A total of $1 \times 10^7$ A549 cells were harvested, fixed with 1% formaldehyde, lysed, sonicated and centrifuged. Then, Dynabeads M-280 Streptavidin (Invitrogen) were added to the supernatant and incubated for approximately 12 h to sponge microRNA (miRNA). Next, the mixture was washed and incubated with 200 μl lysis buffer and proteinase K to reverse the crosslinking caused by formaldehyde. Finally, RNA was extracted with TRIzol reagent (Invitrogen).

Dual luciferase reporter gene assay

The luciferase reporter vectors were constructed following the protocol of a mutagenesis kit (Agilent, USA). Plasmids were transiently transfected into HEK-293T cells. After 48 h, the cells were lysed and collected, and the relative firefly and Renilla luciferase activities were detected with the Dual-Luciferase Reporter Assay System (Promega, USA).

RNA pulldown assay

For the miR-432-5p pulldown assay, a total of $1 \times 10^7$ A549 cells were harvested, lysed, sonicated and incubated with a biotinylated miR-432-5p probe (GenePharma, China) at 4°C for 4 h. Then, the mixture was further incubated with Dynabeads M-280 Streptavidin (Invitrogen, USA) at 4°C for 4 h. Finally, the RNA complexes sponged to the beads were extracted with a RNeasy Mini Kit (QIAGEN, USA) and detected by qRT-PCR and RNA sequencing (RNA-seq).

Coculture assay

The coculture assay was performed in 6-well Transwell chambers with a 0.4 μm-pore membrane (Corning, USA) with $5 \times 10^5$ H1299-control and H1299-circASCC3 cells seeded in the lower chamber 24 h before $1 \times 10^6$ THP-1 cells stimulated with PMA were added to the upper chamber. After 48 h, H1299 cells, macrophages and supernatant were collected separately for analysis.

Flow cytometry analysis and CD8+ T cell isolation

Flow cytometry analysis was performed according to a previous study[15]. For surface marker staining, the cells were resuspended in DMEM and washed with 2 ml of staining buffer (PBS with 1% FBS and 0.2% EDTA). Next, $1 \times 10^6$ cells were stained with the Fc blocker and antibodies. For the surface marker staining of subcutaneous tumors, fresh mouse tumors were processed into single-cell suspensions, which were resuspended in DMEM. After cell counting with red cell lysis and trypan blue, $1 \times 10^6$ cells were washed with 2 ml of staining buffer and stained with the Fc blocker and antibodies. For PD-L1, IFN-γ, and GZMB staining in the cytoplasm, after stimulation with 10 ng/ml phorbol myristate acetate (PMA), 1 μg/ml ionomycin and 10 μg/ml brefeldin A for 4 h and surface marker staining for 30 minutes, cells were fixed with 4% paraformaldehyde for 15 minutes and permeabilized with 0.2% saponin buffer for 20
minutes. Then, the cells were stained with the Fc blocker and antibodies for 30 minutes. Specimens were analyzed using a Fortessa (BD). The Fc blocker and antibodies are listed in Supplementary Table 3. For the isolation of CD8+ T cells, peripheral blood mononuclear cells were isolated from the blood of healthy donors using Ficoll. Next, the CD8+ T cells were purified with anti-CD8 antibody-coupled immunomagnetic beads.

**Human cytokine chip assay and enzyme-linked immunosorbent assay (ELISA)**

The secreted cytokines in the supernatants of the coculture experiments were detected by a human cytokine panel according to the manufacturer's protocol (Luminex, USA). The concentrations of complement C5a, IFN-γ and GZMB in the supernatant and C5a and PD-L1 in the plasma of mice and NSCLC patients were detected by corresponding ELISA kits according to the manufacturer's protocol.

**In vivo subcutaneous xenograft and lung metastasis model**

C57BL/6 and nude mice were used to construct an in vivo subcutaneous xenograft and lung metastasis model. The mice were purchased from the Shanghai JieSiJie Laboratory Animal Co., Ltd. and were fed in a pathogen-free environment. The Animal Experimentation Ethics Committee of Zhongshan Hospital Fudan University approved the relevant experiments involving these animals, and detailed procedures are described in the Supplementary Materials and Methods.

**Statistical analysis**

Statistical analysis was performed with SPSS software (23.0, Chicago, IL). The values are presented as the mean ± standard deviation. Student's t test was applied for comparisons between two groups. Categorical variables were analyzed by chi-squared or Fisher's exact tests. Spearman correlation analysis was performed to determine associations between circASCC3 and CD8, CD163, and C5. In terms of survival analysis, the Kaplan-Meier method and the log-rank test were used to analyze cumulative recurrence and overall survival (OS). Cox's proportional hazards regression model was applied to identify independent prognostic factors. All p values were two tailed, and p <0.05 was regarded as statistically significant.

**Results**

**Clinical implications of abnormally expressed circASCC3 in NSCLC**

First, we conducted circRNA sequencing to explore differentially expressed circRNAs between NSCLC and matched normal tissues. A total of 32 circRNAs were obviously upregulated, while 19 circRNAs were downregulated in NSCLC tissues (Supplementary Fig. 1a). Among these differentially expressed circRNAs, the expression of circASCC3 (hsa_circ_0077495) was the most significantly different, and it was consistently upregulated in 4 NSCLC tissues; moreover, the back splice site of circASCC3 was confirmed by Sanger sequencing with divergent primers (Fig. 1a, b). In addition, the detection of
circASCC3 in the 4 pairs of NSCLC tissues and matched nontumor tissues by qRT-PCR further confirmed the reliability of circRNA sequencing (Supplementary Fig. 1b). The PCR results showed that circASCC3 could be detected by divergent primers in cDNA reverse transcribed by random hexamers rather than oligo dT primers (Fig. 1c). Further experiments with RNase R exonuclease confirmed the covalent closed-loop structure of circASCC3 (Fig. 1d). Since circASCC3 is located on chromosome 6q13, a location known to carry chromosomal aberrations that contribute to tumor progression, we speculated that circASCC3 might potentially regulate the biological function of NSCLC. Next, circASCC3 expression was detected in 35 NSCLC tissues and matched normal tissues using qRT-PCR, and the results showed that circASCC3 was highly expressed in 23/35 NSCLC tissues (Fig. 1e).

To identify the clinical relevance of circASCC3, the expression of circASCC3 was evaluated in 188 pairs of NSCLC tissues and matched peritumor tissues in a TMA by ISH. The expression of circASCC3 was obviously elevated in NSCLC tissues (Fig. 1f). The results of the analysis of circASCC3 expression and clinical characteristics of 188 NSCLC patients are presented in Table 1. The results suggested that a high level of circASCC3 was associated with a larger tumor size, the appearance of lymph node metastasis and an advanced TNM stage. Therefore, we further explored the potential impact of circASCC3 expression on the prognosis of NSCLC patients. Consistently, the expression of circASCC3 was correlated with recurrence and OS. Patients with high expression of circASCC3 had an obviously higher postoperative recurrence rate and shorter OS (Fig. 1g, h). Moreover, the Cox regression analysis showed that the expression of circASCC3 was an independent predictor for recurrence and OS (Tables 2, 3). In short, these results reveal that circASCC3 may exert an influence on the progression of NSCLC.

**circASCC3 enhances invasion, metastasis and proliferation in NSCLC**

To further investigate the biological function of circASCC3 in NSCLC, the expression of circASCC3 was measured in 4 NSCLC cell lines, A549, H1299, H460, and H1703, via qRT-PCR. CircASCC3 expression was highest in A549 cells and lowest in H1299 cells (Fig. 2a). Therefore, we designed and synthetized two lentiviral vectors for the knockdown and overexpression of circASCC3 to establish two stable cell lines (A549-shcircASCC3 and H1299-circASCC3, respectively) (Fig. 2b). Matrigel Transwell assays showed that the invasive potential was significantly enhanced after overexpression of circASCC3 (Fig. 2c). Consistently, the wound healing assays also suggested a similar tendency; the wound healing ability was reduced in A549-shcircASCC3 cells but enhanced in H1299-circASCC3 cells (Fig. 2d). In terms of the proliferation ability, the colony formation assay and CCK-8 assay showed that overexpression of circASCC3 enhanced the proliferation of NSCLC cells, while knockdown of circASCC3 led to the opposite effect (Fig. 2e, f). Furthermore, we verified the in vitro results in nude mice. The results showed that the mice implanted with H1299-circASCC3 cells had a larger tumor burden and were more prone to lung metastasis than those implanted with control cells (Fig. 2g, h). Thus, these results indicate that circASCC3 promotes a malignant phenotype in NSCLC.

**circASCC3 functions as a sponge for miR-432-5p**

To further investigate the biological function of circASCC3 in NSCLC, the expression of circASCC3 was measured in 4 NSCLC cell lines, A549, H1299, H460, and H1703, via qRT-PCR. CircASCC3 expression was highest in A549 cells and lowest in H1299 cells (Fig. 2a). Therefore, we designed and synthetized two lentiviral vectors for the knockdown and overexpression of circASCC3 to establish two stable cell lines (A549-shcircASCC3 and H1299-circASCC3, respectively) (Fig. 2b). Matrigel Transwell assays showed that the invasive potential was significantly enhanced after overexpression of circASCC3 (Fig. 2c). Consistently, the wound healing assays also suggested a similar tendency; the wound healing ability was reduced in A549-shcircASCC3 cells but enhanced in H1299-circASCC3 cells (Fig. 2d). In terms of the proliferation ability, the colony formation assay and CCK-8 assay showed that overexpression of circASCC3 enhanced the proliferation of NSCLC cells, while knockdown of circASCC3 led to the opposite effect (Fig. 2e, f). Furthermore, we verified the in vitro results in nude mice. The results showed that the mice implanted with H1299-circASCC3 cells had a larger tumor burden and were more prone to lung metastasis than those implanted with control cells (Fig. 2g, h). Thus, these results indicate that circASCC3 promotes a malignant phenotype in NSCLC.
Serving as a miRNA sponge has been identified as an important function of circRNAs. Thus, we hypothesized that circASCC3 might promote the progression of NSCLC by binding to miRNAs. We found 34 miRNAs that were potential targets for circASCC3 in StarBase. To further screen the miRNAs that were possibly sponged by circASCC3, a circASCC3 probe was designed to purify the circASCC3-related miRNAs using circRIP in A549 cells, and then, the predicted miRNAs were evaluated by RT-qPCR. The results showed an obvious enrichment of miR-432-5p in complex with circASCC3 in contrast to the negative control, which suggested that miR-432-5p might be one of the critical miRNAs interacting with circASCC3 to regulate its biological function in NSCLC (Fig. 3a). To further prove that circASCC3 serves as a sponge for miR-432-5p, a dual-luciferase reporter gene assay was performed in HEK-293T cells. The wild-type (WT) and mutant circASCC3 sequences were copied into the vector pLG3 (Fig. 3b). The miR-432-5p mimic obviously weakened the luciferase activity of WT circASCC3 but not mutant circASCC3 (Fig. 3c). In addition, the pulldown assay with a biotinylated miR-432-5p probe resulted in significant enrichment of circASCC3 in A549 and H460 cells (Fig. 3d). Moreover, further knockdown of miR-432-5p with miR-432-5p inhibitor rescued the migration, invasion and proliferation ability of A549-shcircASCC3 and H460-shcircASCC3 cells (Fig. 3e-h). In FISH analysis, circASCC3 and miR-432-5p were colocalized in the cytoplasm of NSCLC cells (Fig. 3i). Taken together, these findings indicate that circASCC3 sponges miR-432-5p in NSCLC.

Sponging of miR-432-5p by circASCC3 enhances the expression of C5a

To further identify the downstream mechanism of circASCC3 in promoting the progression of NSCLC, we performed RNA-seq to explore the differentially expressed genes between H1299-control and H1299-circASCC3 cells and discovered the upregulated expression of 84 genes and downregulated expression of 65 genes in H1299-circASCC3 cells (Fig. 4a). Reactome analysis suggested that these genes showed significant enrichment of processes involved in the immune system. KEGG analysis showed enrichment of pathways in cancer, cell cycle, PI3K-Akt signaling pathway, T cell receptor signaling pathway and leukocyte transendothelial migration, and PANTHER analysis showed enrichment of the TGF-beta signaling pathway, EGF receptor signaling pathway and Wnt signaling pathway (Fig. 4b). We also predicted the potential targets of miR-432-5p by overlapping the 149 differentially expressed genes identified from the RNA-seq experiments with the predicted targets from TargetScan and the miR-432-5p pulldown experiment results, and the results showed that ZNF579, STK16, C5, ANTXR2, ANKRD42 and AMD1 were potential candidate genes (Fig. 4c). Since previous studies have reported an oncogenic role of C5, ANTXR2 and AMD1[16–18], we assessed these three genes in separate dual luciferase assays to determine whether they are targets of miR-432-5p. The luciferase activity was dramatically reduced in HEK-293T cells carrying the C5 WT sequence compared to HEK-293T cells carrying the mutant sequence of C5 (Fig. 4d, e, Supplementary Fig. 2a). Moreover, the expression of C5 mRNA was decreased in A549 cells with knockdown of circASCC3 and increased in H1299 cells after overexpression of circASCC3 (Fig. 4f). Previous studies have identified C5a (a hydrolysate of C5) as an important mediator in the progression of cancer[19, 20]. Thus, we also detected the expression of C5a in the supernatant using ELISAs, and the results showed that the concentrations of C5a were increased in the supernatant of H1299 cells after forced expression of circASCC3 and decreased in the supernatant of A549 cells with
knockdown of circASCC3 compared with the relative values in the supernatant of control cells (Fig. 4g). To further identify the role of circASCC3/C5a in NSCLC cells, we stimulated A549-shcircASCC3 cells with C5a (50 ng/ml) and inhibited the C5a/C5aR axis in H1299-circASCC3 cells with the C5aR inhibitor PMX-53 (20 nM). The Matrigel Transwell assay showed enhanced invasion after C5a stimulation, while PMX-53 treatment had the opposite effect (Fig. 4h). In addition, the relationship between circASCC3 and C5 was evaluated in 30 NSCLC patient tissues using qRT-PCR. The results showed that the expression of C5 was positively correlated with that of circASCC3 (Fig. 4i). Previously, we discovered that the C5a/C5aR axis was involved in the progression of NSCLC through epithelial–mesenchymal transition (EMT)[21]. Here, we identified the EMT phenotype in H1299-circASCC3- and C5a-induced A549-shcircASCC3 cells, and this phenotype could be reversed by PMX-53 (Fig. 4j, Supplementary Fig. 2b). Collectively, these results confirm the contribution of the circASCC3/miR-432-5p/C5a axis to the malignant phenotype of NSCLC cells.

**circASCC3 induces an immunosuppressive phenotype of macrophages through C5a and further suppresses CD8+ T cell function via PD-L1**

Since C5a has been identified as a critical regulator in tumor-associated macrophages (TAMs) and is downstream of circASCC3 in NSCLC[22], we speculated that circASCC3 may have a potential impact on the TME. Therefore, we established an LLC-circASCC3 subcutaneous xenograft model in C57BL/6 mice and further analyzed the immune profile of the tumor tissues by flow cytometry. The results showed that the infiltration of CD8+ T cells was dramatically reduced, while no obvious change in other subpopulations was observed (Fig. 5a). To further verify the in vivo results, we also detected the relationship between circASCC3 and CD8+ T cells in a TMA containing 188 NSCLC tissues by IHC (Fig. 5b). The results showed that the expression of circASCC3 was negatively correlated with CD8+ T cells, which was in line with the in vivo results. To identify the impact of circASCC3 on CD8+ T cells, we isolated CD8+ T cells from 5 healthy donors and cultured them with supernatants from H1299-circASCC3/control cells. However, the overexpression of circASCC3 in NSCLC cells did not impair the cytotoxic function or induce the apoptosis of CD8+ T cells, but the C5a level in the supernatant of H1299-circASCC3 cells was significantly upregulated (Fig. 5c-e). Thus, the results suggest that there is a mediator between circASCC3 and CD8+ T cells.

Since macrophages are a critical target of C5a and M2-type macrophages exert immune inhibition[23, 24], we established a 48-h coculture system of H1299-circASCC3 cells and PMA-stimulated THP-1 cells and further cultured CD8+ T cells with the supernatant from the coculture system for 72 h. Flow cytometry analysis showed that the macrophages cocultured with H1299-circASCC3 cells exhibited a CD163-high phenotype, which was not seen for macrophages cocultured with H1299-control cells (Fig. 5f). In addition, the qRT-PCR results showed significantly increased mRNA levels of CD163 and other M2 markers, such as CD206 and Arg-1, in macrophages cocultured with H1299-circASCC3 cells (Fig. 5g, Supplementary Fig. 3a). Moreover, the cytotoxic function of CD8+ T cells was obviously impaired, with a remarkable decline in IFN-γ and GZMB in the supernatant (Fig. 5h). The apoptosis of CD8+ T cells was
also significantly elevated (Fig. 5i). The IHC staining of CD163 in the TMA containing 188 NSCLC tissues revealed that the infiltration of CD163+ macrophages was positively correlated with the expression of circASCC3 (Fig. 5j).

To determine whether there were differences in the levels of secreted cytokines in the supernatant of the circASCC3 coculture system, the levels of secreted cytokines in supernatants from the H1299-control cell/macrophage and H1299-circASCC3 cell/macrophage coculture systems were detected by a Luminex Human Cytokine Antibody Panel. The levels of IL-2 and IL-12 were significantly reduced, while those of M-CSF, IL-6, IL-8, PD-L1, TNF-a and VEGF were obviously increased in the H1299-circASCC3 cell/macrophage coculture system (Fig. 5k). Considering the critical role of the PD-L1/PD-1 axis in CD8+ T cell dysfunction and immune evasion, we detected the expression of PD-L1 in macrophages and H1299-circASCC3 cells in the coculture system by western blot and qRT-PCR. The results showed that circASCC3 overexpression drastically elevated the expression of PD-L1 in macrophages, while the expression of PD-L1 was only mildly increased in H1299 cells (Fig. 5l, Supplementary Fig. 3b). In addition, the impaired function of CD8+ T cells could be rescued when the C5aR inhibitor PMX-53 was introduced into the circASCC3 coculture system or when CD8+ T cells were cultured with the PD-L1 inhibitor durvalumab and the supernatant from the coculture system (Fig. 5m). The apoptosis of CD8+ T cells was also inhibited by these two agents (Fig. 5n). Notably, PMX-53 reversed M2-like polarization and reduced PD-L1 expression in macrophages cocultured with H1299-circASCC3 cells (Fig. 5o, p, Supplementary Fig. 3c). In summary, these results indicate that circASCC3 promotes an immunosuppressive phenotype of macrophages through the upregulation of C5a, which further induces the upregulation of PD-L1 and the dysfunction of CD8+ T cells.

**PMX-53 improves the effects of anti-PD-1 immunotherapy in a xenograft model with circASCC3 overexpression**

Considering the promising effects of PMX-53 in inhibiting the PD-L1 expression of macrophages and reversing CD8+ T cell dysfunction, we speculated that the combination therapy of PMX-53 and anti-PD-1 antibody could inhibit the progression of NSCLC driven by circASCC3 and improve the efficacy of anti-PD-1 therapy. We performed an in vivo investigation of the anti-PD-1 antibody and PMX-53 combination therapy in an LLC-circASCC3 xenograft subcutaneous model in C57BL/6 mice. The anti-PD-1 antibody was administered on days 1, 4, 7, 10, and 13, and PMX-53 was administered daily when the tumor size reached 100 mm³ (Fig. 6a). The in vivo results showed that the combination therapy significantly inhibited tumor growth compared to PD-1 or PMX-53 monotherapy (Fig. 6b, c). In addition, the combination therapy also increased survival and reduced lung metastasis compared to either single agent (Fig. 6d, e). The C5a and PD-L1 level in the plasma of mice were significantly reduced after the combination therapy of anti-PD-1 monoclonal antibody and PMX-53 (Supplementary Fig. 4a, b).

Moreover, flow cytometry analysis showed a significant reduction in M2-type macrophage infiltration and the expression of PD-L1 in macrophages in the tumors of mice treated with anti-PD-1 monoclonal antibody and PMX-53 compared to the respective levels in the tumors of mice treated with monotherapy.
In addition, the tumor-infiltrating CD8\(^+\) T cells were apparently increased, and the cytotoxic function of the CD8\(^+\) T cells was also obviously improved, as reflected by the increased infiltration of IFN-\(\gamma^+\)CD8\(^+\) T and GZMB\(^+\)CD8\(^+\) T cells in tumors after the combination therapy of anti-PD-1 antibody and PMX-53 (Fig. 6h). Thus, the in vivo results in an immunocompetent mouse model suggest that the abnormal expression of circASCC3 is involved in the immune evasion of NSCLC cells and that the C5aR1 inhibitor PMX-53 can improve the efficacy of anti-PD-1 immunotherapy.

**Expression of circASCC3 correlates with the efficacy of anti-PD-1 therapy in NSCLC patients**

To further validate our results in NSCLC patients, we retrospectively analyzed the data from 15 NSCLC patients in clinical stage III or IV according to the 8th TNM staging system who received anti-PD-1 immunotherapy (Opdivo). Following the RECIST1.1 evaluation, 7 patients had progressive disease (PD), 6 patients achieved stable disease (SD), and 2 patients were classified as having partial remission (PR). Next, the expression of circASCC3 was evaluated in biopsied tissues using qRT-PCR. The results showed higher expression of circASCC3 in patients with PD than in patients with SD or PR (Fig. 7a). Among the patients with high circASCC3 expression, only 2 patients had SD, and 5 patients were classified as having PD. In patients with low circASCC3 expression, 2 patients had PD, 4 patients had SD, and 2 patients were classified as having PR (Fig. 7b). In addition, the expression of circASCC3 was positively correlated with the expression of C5 (Fig. 7c and Supplementary Fig. 5a). While no relationship was found between the PD-L1 in the plasma and the expression of circASCC3, the concentration of PD-L1 was higher in patients with high circASCC3 expression (Supplementary Fig. 5b, c). The mIF results of tumor tissues showed lower infiltration of CD8\(^+\) T cells but higher infiltration of CD163\(^+\)PD-L1\(^+\) macrophages in tumors with high expression of circASCC3 (Fig. 7d).

Moreover, the imaging results also showed a similar pattern. The results of contrast CT imaging of two representative patients are shown in Fig. 7e. In the high circASCC3 group, 5 patients showed tumor growth, and only 2 patients had slight tumor shrinkage; in contrast, 6 patients in the low circASCC3 group had tumor shrinkage, and only 2 patients had tumor progression after treatment (Fig. 7f). In addition, a negative correlation was presented between the expression of circASCC3 and shrinkage of tumor size (Supplementary Table 1). Next, we further investigated the relationship between circASCC3 and the prognosis of 15 patients. PFS and OS were prolonged in the low circASCC3 group (Fig. 7g). Taken together, these preclinical results reveal an association between circASCC3 and immune resistance and provide a potential strategy for overcoming resistance to anti-PD-1 immunotherapy in patients with NSCLC.

Figure 8 shows a schematic summarizing the results of this study. In NSCLC, highly expressed circASCC3 can sponge miR-432-5p, which further upregulates the expression of downstream C5a and induces EMT to promote the progression of NSCLC. On the other hand, the activation of the C5a/C5aR axis in macrophages in the TME induces macrophages to transition toward an immunosuppressive phenotype and elevates the expression of PD-L1, which further leads to the dysfunction of CD8\(^+\) T cells.
Discussion

To date, a large number of studies have identified the critical role of circRNAs in regulating the development of many cancer types due to advances in high-throughput deep sequencing[25]. Some circRNAs have been verified to regulate biological functions in NSCLC[26, 27]. However, the mechanisms behind the biological processes of circRNAs remain unclear[28]. Here, we detected circRNAs that were differentially expressed between NSCLC tissues and matched normal tissues and further identified the critical role of an ASCC3-derived circRNA (hsa_circ_0077495) in the progression and immune suppression of NSCLC. The expression of circASCC3 was significantly higher in NSCLC tissues, and patients with high levels of circASCC3 expression were characterized by a larger tumor size, lymph node metastasis, a higher clinical stage and poor RFS and OS. Moreover, we demonstrated that circASCC3 promotes NSCLC progression and an immunosuppressive environment by sponging miR-432-5p, which in turn induces the upregulation of C5a. In addition, the C5aR1 inhibitor PMX-53 synergized with PD-1 blockade to produce an enhanced antitumor effect in an immunocompetent xenograft model bearing tumors with high expression of circASCC3 by inhibiting the immunosuppressive phenotype of macrophages and further improving the infiltration and cytotoxic function of CD8⁺ T cells in the tumor.

Alterations of chromosome 6q13 have been reported to be involved in cancer susceptibility and aggressive biological functions in NSCLC and other cancers[29–31]. Moreover, this chromosomal region contains many cancer-related genes and several oncogenes, such as HTR1B and CoL12A1[32, 33]. Since circRNA-seq suggested that circASCC3 was significantly upregulated in NSCLC tissues and located on chromosome 6q13, we further explored the potential role and biological mechanism of circASCC3 in NSCLC. The results showed that circASCC3 plays a critical role in NSCLC progression and immune suppression in the TME. Thus, this study may have identified another NSCLC-related oncogene on chromosome 6q13.

Sponging miRNAs and acting as competing endogenous RNAs are important mechanisms by which circRNAs induce the dysregulation of their downstream targets, leading to the progression of cancers, including NSCLC[25, 34, 35]. Here, we demonstrated that circASCC3 contributes to the progression of NSCLC by sponging miR-432-5p. miR-432-5p has been identified as a tumor suppressor in several cancers[36–38]. The tumor suppressive role of miR-432-5p in NSCLC found in this study is consistent with the findings of previous studies.

Complement is a critical component of the innate immune system implicated in cancer progression[19]. Previous studies have confirmed that anaphylatoxin C5a generated from the TME contributes to the progression of cancer[39, 40]. The activation of C5aR on the membrane of tumor cells via complement promotes tumor growth and generates an antiapoptotic response[19]. However, previous studies have mainly focused on the biological contributions of C5a released from the immune system to immune dysfunction and tumor progression. The impact of tumor-derived C5a on the TME has not been studied in depth. In addition, the PD-L1/PD-1 axis has been verified as a critical pathway for immune evasion in the TME[41, 42]. TAMs serve as a double-edged sword in the progression of cancer and can be stimulated in
opposite directions: M1- and M2-like polarization. While M1-type macrophages show a proinflammatory phenotype and suppress the progression of cancer, M2-type macrophages have anti-inflammatory characteristics and facilitate the progression of cancer[43]. The upregulation of PD-L1 in macrophages is another potential mechanism underlying immune suppression[44]. Thus, the depletion of M2-type macrophages and their phenotypic conversion to M1-type macrophages represent promising strategies in the treatment of cancer. Previous studies have discovered the contribution of the C5a/C5aR axis to the imbalance in M1- and M2-like polarization of macrophages, and the C5aR inhibitor PMX-53 was found to reverse the M2-like polarization of macrophages and improve the efficacy of chemotherapy[20]. Here, we discovered that highly expressed circASCC3 could induce the upregulation of C5a, which promoted the M2 phenotype and the expression of PD-L1 in macrophages and further led to immune suppression. The C5aR inhibitor PMX-53 impeded the C5a/C5aR axis, which inhibited the malignant phenotype of NSCLC cells and reversed the immunosuppressive propensity of macrophages, leading to the regression of NSCLC.

Since the presence of immune suppression within the TME hampers immunotherapy from combating tumors, an increasing number of studies are investigating the synergistic effects of strategies combining immune checkpoint inhibitors with other therapies[45]. Anti-PD-1/PD-L1 therapies are still the main agents used for these combinations. A previous study observed that the synergistic effects of anti-PD-1 antibody and C5a blockade in a NSCLC xenograft model occurred as a result of the activation of CD8+ T cells[46]. Thus, we investigated whether the combined use of the C5aR1 inhibitor PMX-53 and anti-PD-1 treatment in an immunocompetent xenograft model bearing tumors with high expression of circASCC3 could overcome immune suppression. This combination strategy achieved substantially improved efficacy and restored the antitumor immune response compared to monotherapy. Finally, we further verified the in vitro and in vivo results in a retrospective study of 15 NSCLC patients treated with PD-1 blockade monotherapy. Therefore, the inhibition of C5aR in NSCLC with high expression of circASCC3 is a new strategy for treating tumors with resistance to PD-1 blockade immunotherapy.

Conclusions

In summary, this study revealed the oncogenic role of circASCC3 in NSCLC. Overexpressed circASCC3 induced the progression of NSCLC and immune resistance through the miR-432-5p/C5a axis. In addition, the C5aR inhibitor PMX-53 improved the efficacy of anti-PD-1 therapy in NSCLC with high expression of circASCC3, providing a new strategy in the treatment of NSCLC.

Abbreviations

NSCLC, non-small-cell lung cancer; circRNA, circular RNA; qRT-PCR, quantitative real-time polymerase chain reaction; PD-1, programmed cell death 1; TMA, tissue microarray; IHC, immunohistochemistry; ISH, in situ hybridization; FISH, fluorescence in situ hybridization; mIF, multicolor immunofluorescence; CCK-8, cell counting kit-8; circRIP, circRNA precipitation; miRNA, microRNA; PMA, phorbol myristate acetate; ELISA, enzyme-linked immunosorbent assay; OS, overall survival; PFS, progression-free survival; WT, wild-
Declarations

Acknowledgments

None.

Authors’ contributions

JG, J-YD and J-HJ conceived and designed the experiments; JG and L-XZ performed the experiments; P-FZ, Y-QO, CJ, H-KW, SW and ML analyzed the data; JG, J-HJ and J-YD wrote the paper. All authors have read and approved the final manuscript.

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Availability of data and materials

All data in our study are available upon request.

Ethics approval and consent to participate

The ethical approval was provided by the Ethics Committee of Zhongshan Hospital, and written informed consent was obtained from each patient.

Consent for publication

Not applicable.

Competing interests

None.

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Tables
Table 1
The clinicopathology characteristics of NSCLC patients with different circASCC3 expression.

| Factors                     | circASCC3 expression | P     |
|-----------------------------|----------------------|-------|
|                             | low  | high |       |
| Sex                         |      |      | 0.558 |
| Male                        | 40   | 45   |       |
| Female                      | 54   | 49   |       |
| Age                         |      |      | 0.764 |
| ≤ 60                        | 60   | 57   |       |
| > 60                        | 34   | 37   |       |
| Smoking status              |      |      | 0.883 |
| Smokers                     | 43   | 41   |       |
| Nonsmokers                  | 51   | 53   |       |
| Histological type           |      |      | 0.507 |
| Adenocarcinomas             | 72   | 67   |       |
| SCC                         | 22   | 27   |       |
| Tumor size (cm)             |      |      | 0.003 |
| ≤ 3                         | 60   | 39   |       |
| >3                          | 34   | 55   |       |
| Differentiation             |      |      | 1     |
| Well and moderate           | 60   | 61   |       |
| Poor                        | 34   | 33   |       |
| Lymph node metastasis       |      |      | 0.019 |
| Yes                         | 35   | 52   |       |
| No                          | 59   | 42   |       |
| TNM                         |      |      | <0.001|
| I-II                        | 76   | 51   |       |
| III-IV                      | 18   | 43   |       |

Note: SCC, squamous cell carcinoma
### Table 2
Univariate and multivariate Cox analysis of predictors for recurrence

| Factors                              | Cumulative recurrence |        |        |        |    |
|--------------------------------------|-----------------------|--------|--------|--------|----|
|                                      |                       | Univariate | Multivariate | P | HR | 95%CI | P |
| Sex (male vs female)                 |                       | 0.232   | NA     |        |    |
| age (≤ 60 vs > 60)                   |                       | 0.482   | NA     |        |    |
| Smoking status (Smokers vs. Nonsmokers) |                       | 0.629   | NA     |        |    |
| Histological type (Adenocarcinomas vs SCC) |               | 0.975   | NA     |        |    |
| Tumor size (> 3 vs. ≤ 3cm)           |                       | 0.002   | NS     |        |    |
| Differentiation (Well and moderate vs. Poor) |        | 0.958   | NA     |        |    |
| Lymph node metastasis (Yes vs. No)   | < 0.001               | 2.484  | 1.34–4.604 | 0.004 |
| Tumor stage (III-IV vs. I-II)        | < 0.001               | NS     |        |        |    |
| CircASCC3 expression (High vs. Low)  | 0.001                 | 1.584  | 1.012–2.459 | 0.041 |

Note: SCC, squamous cell carcinoma
# Table 3
Univariate and multivariate Cox analysis of predictors for overall survival

| Factors                                      | OS                  |
|----------------------------------------------|---------------------|
|                                              | Univariate | Multivariate |
|                                              | P   | HR   | 95%CI    | P     |
| Sex (male vs female)                         | 0.149 | NA   |          |       |
| age (≤ 60 vs > 60)                            | 0.473 | NA   |          |       |
| Smoking status (Smokers vs. Nonsmokers)      | 0.368 | NA   |          |       |
| Histological type (Adenocarcinomas vs SCC)   | 0.648 | NA   |          |       |
| Tumor size (> 3 vs. ≤ 3cm)                   | 0.001 | NS   |          |       |
| Differentiation (Well and moderate vs. Poor) | 0.416 | NA   |          |       |
| Lymph node metastasis (Yes vs. No)          | < 0.001 | 2.06 | 1.079–3.935 | 0.029 |
| Tumor stage (III-IV vs. I-II)                | < 0.001 | NS   |          |       |
| CircASCC3 expression (High vs. Low)          | 0.001 | 1.63 | 1.035–2.557 | 0.033 |

Note: SCC, squamous cell carcinoma

# Figures
The expression of circASCC3 is elevated in NSCLC patients and is an indicator of a poor prognosis. a. The expression profiles of circRNAs were detected by circRNA sequencing in 4 NSCLC tissues and matched normal tissues. b. Schematic illustrations of the structure of circASCC3 and the back splice site confirmed by Sanger sequencing with divergent primers. c. circASCC3 was detected by PCR in cDNA reverse transcribed by random hexamers or oligo dT primers and gDNA. d. The closed-loop structure of
circASCC3 was confirmed by RNase R exonuclease experiments. The expression of circASCC3 was evaluated in 35 pairs of NSCLC tissues and matched normal tissues. circASCC3 was detected by in situ hybridization in a TMA containing 188 pairs of NSCLC tissues and matched peritumor tissues. Kaplan-Meier analysis of postoperative recurrence and OS in 188 NSCLC patients according to circASCC3 expression. ns, no significance, \( p > 0.05, \ast p < 0.05, \ast\ast p < 0.01, \ast\ast\ast p < 0.001 \).

Figure 2
Overexpressed circASCC3 promotes a malignant phenotype of NSCLC cells. a. The expression of circASCC3 in 4 NSCLC cell lines was detected by qRT-PCR. b. circASCC3 was successfully silenced in A549 cells and overexpressed in H1299 cells. c. The invasion ability of NSCLC cell lines with knockdown or overexpression of circASCC3 was evaluated by Matrigel Transwell assay. d. The migration ability of NSCLC cell lines with knockdown or overexpression of circASCC3 was evaluated by a wound healing assay. e-f. The proliferation of NSCLC cells with knockdown or overexpression of circASCC3 was evaluated by CCK-8 and colony formation assays. g. The growth of tumors derived from NSCLC cells with forced circASCC3 expression was evaluated in nude mice. h. Lung metastasis of NSCLC tumors derived from cells with forced circASCC3 expression was evaluated in nude mice.
circASCC3 acts as a competitor for miR-432-5p. a. circRIP was performed to determine whether circASCC3 sponges miRNAs in A549 cells. b. The binding sites of circASCC3 and miR-432-5p predicted by StarBase and the design of the mutant circASCC3 construct. c. circASCC3 sponging of miR-432-5p was evaluated by luciferase reporter gene assays. d. RNA pulldown assays were performed in A549 and H460 cells using biotin-labeled miR-432-5p mimics. e and f. The invasion and migration abilities of miR-432-5p-
silenced NSCLC cells with circASCC3 knockdown were evaluated by Matrigel Transwell assays (e) and wound healing assays (f). The proliferation of miR-432-5p-silenced NSCLC cells with circASCC3 knockdown was evaluated by colony formation (g) and CCK-8 (h) assays. The localization of circASCC3 and miR-432-5p in A549 and H1299 cells was explored using FISH.

**Figure 4**
circASCC3 regulates the biological function of NSCLC cells through the miR-432-5p/C5a axis. a. The differentially expressed genes between H1299-control and H1299-circASCC3 cells were investigated by RNA sequencing and are shown in the volcano plot. b. Reactome, KEGG and PANTHER analyses of the differentially expressed genes. c. Overlapping differentially expressed genes from the RNA-seq analysis and gene targets of miR-432-5p predicted by TargetScan, in addition to overlapping genes from the miR-432-5p pulldown experiments. d. The putative binding sites of C5 and miR-432-5p and the mutant miR-432-5p construct. e. The luciferase activity of C5 in HEK-293T cells after transfection with miR-432-5p. f. The mRNA levels of C5 in NSCLC cells after silencing or overexpression of circASCC3 were detected using qRT-PCR. g. The C5a concentration in the supernatant of NSCLC cells after silencing or overexpression of circASCC3 was detected by ELISA. h. The impact of C5a and PMX-53 on the invasion ability of A549-shcircASCC3 and H1299-circASCC3 was evaluated by Matrigel Transwell assays. i. The relationship between circASCC3 and C5 in 30 NSCLC patients was evaluated by qRT-PCR. j. The EMT phenotype of NSCLC cells transfected with circASCC3 or shcircASCC3 and the impact of C5a and PMX-53 on the EMT phenotype of NSCLC cells with shcircASCC3 and circASCC3 were assessed by western blot.
Figure 5

circASCC3 induces a dysfunctional immune status in NSCLC. a. Flow cytometry analysis of the immune profile in a subcutaneous xenograft model overexpressing circASCC3. b. Investigation of the relationship between circASCC3 and CD8+ T cells in NSCLC tissues. c. IFN-γ and GZMB in the supernatant were detected by ELISA after CD8+ T cells were cultured with the supernatant from H1299-control/circASCC3 cells. d. Flow cytometry analysis of the apoptosis profile of CD8+ T cells cultured with the supernatant
from H1299-control/circASCC3 cells. e. C5a in the supernatant of CD8+ T cells was detected by ELISA. f. Flow cytometry analysis of CD163 expression on macrophages cocultured with H1299-control/circASCC3 cells. g. The mRNA level of CD163 on macrophages cocultured with H1299-control/circASCC3 cells was detected by qRT-PCR. h. IFN-γ and GZMB in the supernatant were detected by ELISA after CD8+ T cells were cultured with the supernatant from the coculture system. i. Flow cytometry analysis of the apoptosis profile of CD8+ T cells cultured with the supernatant from the coculture system. j. Investigation of the relationship between circASCC3 and CD163+ M2-type macrophages in NSCLC tissues. k. Detection of secreted cytokines in the supernatant of H1299-control/circASCC3 cells was performed using a Luminex human cytokine antibody panel. l. The expression of PD-L1 in H1299 cells and macrophages was detected by western blot. m. IFN-γ and GZMB in the supernatant were detected by ELISA after CD8+ T cells were cultured in the supernatant from the coculture system and treated with PMX-53 or durvalumab. n. Flow cytometry analysis of the apoptosis profile of CD8+ T cells cultured in supernatant from the coculture system and treated with PMX-53 or durvalumab. o. Flow cytometry analysis of CD163 expression in macrophages cocultured with H1299-control/circASCC3 cells after treatment with PMX-53. p. The expression of PD-L1 in macrophages of the coculture system treated with PMX-53 was detected by western blot.
Figure 6

Combination use of PMX-53 and anti-PD-1 monoclonal antibody overcomes anti-PD-1 monoclonal antibody resistance in a xenograft model with forced circASCC3 expression. a. Schematic of the PMX-53 and anti-PD-1 antibody administration scheme. b. Typical images of LLC tumors from each group taken using a noninvasive in vivo imaging system. c. The tumor volume change in each group was measured every 3 days. d. The overall survival of mice bearing LLC tumors from each group. e. Representative H&E
images of lung metastases and the statistical analysis results for each group. 

f. Flow cytometry analysis of infiltrating M2-type macrophages (gated on CD45+/F4/80+/CD11b+ cells) for each group.

g. Flow cytometry analysis of PD-L1 expression in macrophages (gated on CD45+F4/80+/CD11b+ cells) for each group.

h. Flow cytometry analysis of CD8+ T cells, IFN-γ+CD8+ T cells and GZMB+CD8+ T cells infiltrated into the tumor tissues for each group.

**Figure 7**

![Graphs and images related to Figure 7]
Expression of circASCC3 is correlated with the efficacy of PD-1 blockade in NSCLC patients. a. The expression of circASCC3 in the biopsied tissues was detected using qRT-PCR. b. The efficacy of anti-PD-1 monotherapy in NSCLC patients classified by the expression of circASCC3. c. The relationship between C5 and circASCC3 in 15 NSCLC patients treated with anti-PD-1 monotherapy. d. mIF analysis of CD4, CD8, CD163 and PD-L1 in tumor tissues from the 15 NSCLC patients. e. The change in tumor size based on CT imaging for two representative patients is annotated with a blue line. f. The tumor size change in all NSCLC patients treated with anti-PD-1 monotherapy. g. Kaplan-Meier survival curves of PFS and OS for NSCLC patients treated with anti-PD-1 monotherapy classified by the expression of circASCC3. The log-rank test was used to analyze the survival difference.

Figure 8

A schematic summarizing the results of this study.

Supplementary Files

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