Accumulation of AGO2 facilitates tumor proliferation and metastasis through upregulating Survivin, Vimentin and Snail in human hepatocellular carcinoma

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Research article

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Abstract

Background Argonaute 2 (AGO2), a typical member of the Ago gene family, plays a pivotal role in hepatocellular carcinoma (HCC) tumorgenesis through regulating the short interfering RNA-mediated gene silencing. However, the underlined mechanism needs clarified. Herein, we found that AGO2 was frequently upregulated in human HCC cancerous tissues compared with non-cancerous tissues. Methods: Clinical analyses were performed to determine the relation between the expression level of AGO2 and prognosis in HCC patients. By using CRISPR/Cas9 approach in SMMC-7721 cells and establishing xenograft model in nude mice, we further identified the role of AGO2 in HCC. Gene expression microarray analysis was used to reveal the changes of gene expression profile mediated by AGO2 depletion in SMMC-7721 cells. Results: We observed that the overexpression of AGO2 was associated with poor prognosis in HCC patients. The knockout of AGO2 inhibited tumor cell proliferation and metastasis in vivo and in vitro. We also identified that AGO2 facilitates HCC tumorigenesis through modulating Survivin, Vimentin and Snail expression. Conclusions: Therefore, this study not only demonstrates that accumulation of AGO2 promotes cell proliferation and metastasis in HCC, but also provides a novel molecular mechanism in HCC progression.

Background

Hepatocellular carcinoma (HCC), a malignant epithelial tumor, is the third leading cause of cancer-associated death across the world[1]. The development of HCC is a multistep process and affected by many factors. It is associated with chronic liver injury, inflammation, hepatocellular degeneration/regeneration, necrosis, and small-cell dysplasia [2]. Intrinsically, the genetic and epigenetic aberrations of HCC are suggested to lead to HCC initiation and progression [2].

Argonaute (Ago) proteins are highly expressed in many species. In humans, the Argonaute family contains eight members, four of which belong to the eIF2C/AGO subfamily (EIF2C1/AGO1, EIF2C2/AGO2, EIF2C3/AGO3, and EIF2C4/AGO4). All of them are involved in the effector phase of RNA interference (RNAi) at the stage of translation initiation and elongation [3]. Among them, human AGO2 is the only member with an intrinsic endonuclease activity. Subsequently, AGO2 may function as a RNA-induced silencing complex (RISC) slicer to cleave target messenger RNA (mRNA), which bind to the small interference RNA (siRNA) or microRNA (miRNA) [4, 5]. In addition, AGO2 plays important roles in multiple biological or physical processes. AGO2 coordinates the hypoxic adaptation across cells through regulating circulating miR-210 [6]. AGO2 can also cooperates with KRAS to enhance cellular transformation [7]. Moreover, AGO2 expression has effects on testicular abnormalities, and fertilization bias [8].

Recently, the AGO2 is demonstrated as a potential oncogene in human tumors. For instance, AGO2 is overexpressed and involves in the malignant phenotypes in head and neck squamous cell carcinoma [9]. In nasopharyngeal carcinoma, the genetic polymorphism in AGO2 may be a risk factor for the advanced lymph node metastasis [10]. Furthermore, the elevated AGO2 expression is possibly involved in

tumorigenesis and development of bladder cancer [11]. It has also been reported that increased AGO2 expression is associated with tumor progression and poor prognosis in glioma [12]. Interestingly, AGO2 plays oncogenic functions in HCC. AGO2 up-regulates focal adhesion kinase expression to promote tumor metastasis in HCC [13]. AGO2 enhances angiogenesis through regulating the PTEN/VEGF signaling pathway in HCC [14]. Moreover, AGO2 interacts with PAPBC1 to regulate the microRNA mediated gene silencing in high grade HCC [15]. However, the downstream regulatory mechanisms of AGO2 in HCC remains ambiguous and needs further exploration.

In our study, we identified that AGO2 is upregulated in human HCC cancerous tissues and could act as an independent prognostic factor in HCC patients with poor outcome. Cultured cell experiments and animal studies further revealed the knockout of AGO2 inhibited cell proliferation and metastasis in HCC. Bioinformatic analyses showed that AGO2 regulated two clusters of genes involving in cell proliferation (eg. *Survivin*) and cell metastasis (eg. *Snail*, *Vimentin*), respectively. Our findings underline a new mechanism of AGO2 in HCC tumorigenesis, which may provide a novel insight into molecular mechanism in HCC progression.

Methods

Patients

On institutional review board approval, we identified 90 patients with hepatocellular carcinoma (HCC) treated with radical cystectomy between 2011 and 2019 at Renmin Hospital of Wuhan University and Tongji Hospital of Huazhong University of Science and Technology. None of the patients received adjuvant therapy. Data collected from each patient included gender, age at diagnosis, grade, stage, and overall survival time. Pairs of cancer tissues and adjacent epithelium tissues of the same HCC patients were obtained by radical cystectomy. The study was approved by the medical ethics committee of each participating institute. Informed consent (written or verbal) was obtained from the patients within this study. All the samples were anonymous.

Cell lines

Human colorectal cancer cell lines HepG2, HCC-1973, Hep3B, ZR-75-30, MCF-7, MDA-MB-231, T-47D, SMMC-7721 and HEK293T cell were grown in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; ScienCell, Carlsbad, CA). Cells were maintained in cell incubator with 5% CO_2 at 37°C. All cell lines were purchased from ATCC. Microsart® *Mycoplasma* Kit (Sartorius Inc., Gottingen, Germany) was used to monitor cells for *Mycoplasma* contamination routinely.

Antibodies

Primary antibody against AGO2 (ab186733) and Survivin (ab469) were purchased from Abcam Inc. (Cambridge, MA). Antibodies for detecting Snail (#3895) and Vimentin (#5741) were purchase from Cell Signaling Technology, Inc. (Danvers, MA). Primary antibody for GAPDH (sc-25778), and secondary antibodies including anti-rabbit IgG (sc-2004) and anti-mouse IgG (sc-2005), were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX).

Construction of AGO2 knock-out cell line

We knocked out the AGO2 in SMMC-7721 cells using CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats)–associated nuclease Cas9 method. sgRNA (5'-GCCACCATGTACTCGGGAGC, TSINGKE Inc., Beijing, China) was designed to target at genomic *AGO2* exon and cloned into plasmid lenti-CRISPR-v2 (Addgene plasmid # 52961). And then the construct was transfected into HEK293T cells with psPAX2 and psMD.2 (Addgene plasmid #) using Lipofectamine 2000 (Invitrogen). At 72 hours post transfection, the lentivirus was harvested and infected SMMC-7721 cells. At 48 hours post infection, the stable cell lines were generated by selection in 2 μg/ml puromycin for one week. Then, monoclonal cell was culture in 96-well plate until indicated cell population. Cells were harvested for genome DNA sequencing and western blot to identify AGO2 knocked out.

CCK8 assay

For the cell growth experiment, 2000 cells were cultured at each of the 96-well plate. And then we test the absorbance at 450 nm using CCK8 kit every day (100 μl fresh DMEM medium and 10 μl CCK8 solution were added into each 96-well plate. Subsequently, incubated at 37℃ for 2 hours). The absorbance was tested at 450nm using CCK8 kit as described above.

Cell focus colony formation and migration assay

In the colony formation assay, SMMC-7721 cells were grow in 6-well culture plates at a density of 600 cells per well. Then, the colonies were fixed by methanol and stained by crystal violet (Sigma–Aldrich, St. Louis, MO, USA) until the colonies were visible. The number of cells formed into a clone was calculated.

In the cell migration assay, 1×10^5 suspended SMMC-7721 cells in 600 μl with serum-free medium placed in the upper compartment of 8-μm transwells (Corning Inc., Acton, MA) in a 24-well plate. Additional 600 μl medium containing 10% FBS was added to the bottom wells. At 12 hours post culture, cells in bottom transwells were fixed by methanol and stained by crystal violet. The number of migration cells into bottom transwells was counted.

Mice and xenograft tumor model

BALB/c nude mice (4-6 weeks old female) were purchased from Beijing HFK Bioscience Co. Ltd. Laboratory Animal Center. The mice were housed under specific pathogen-free (SPF) conditions in separated ventilated cages. Mice were housed as 5 per cage and in a climate-controlled room (25 °C, 55% humidity and 12-h light/darkness cycle). All procedures involving mice and experimental protocols were approved by the ethics committee of Tongji Hospital of Tongji Medical College and in compliance with the NIH guidelines.

Mice were randomly assigned as control group and model group (n = 10 mice per group). In model group, SMMC-7721 cells (Control and AGO2 knock out) were subcutaneously injected in the flank of BALB/c nude mice with 2×10^6 cells. Mice in control group only received sterile PBS injection. About 7 days post injection, the tumor size was measured every other day. The tumor size was calculated as $(\text{length}\times\text{width}^2)/2$. At the indicated time, all mice were sacrificed with CO_2 inhalation and the tumors were photographed.

Reverse Transcription Reaction and Quantitative Real Time PCR

Total RNA was extracted with Trizol reagent (Invitrogen) and reverse transcribed into cDNA using the M-MLV reverse transcriptase (Promega). GAPDH was used as an internal control to normalize the amount of total mRNA in each sample.

Real time PCR was performed with a standard SYBR Green PCR kit (Takara Shuzo Co. Ltd, Kyoto, Japan) according to manufactor's instructions in a real-time PCR system (Applied Biosystems7500, Foster City, CA) as follows: 95°C for 3 min followed by 40 cycles of 95°C for 5 sec, 60°C for 20 sec and 72°C for 30 sec and then 94°C for 1 min, 60°C for 1 min, with addition of a cycle for every 0.5°C. The primers used in the study are shown as follow: AGO2 forward: 5'-CCTGTATGAGAACCCAATGTC; reverse: 5'-CAGCTAGTTTGAGCCCATCA. GAPDH forward: 5'-AAGGCTGTGGGCAAGG; GAPDH reverse: 5'-TGGAGGAGTGGGTGTCG.

Western Blot

Cells were lysed with radioimmunoprecipitation assay buffer (RIPA Buffer) containing protease inhibitors and centrifuged at 13000×g and 4ºC for 5 minutes. The protein concentration of the supernatants was determined with the BCA kit (Thermo Scientific). 100 μg of cell lysatea were resolved by 12% SDS-PAGE and transferred onto nitrocellulose membranes (Amersham, Piscataway, NY). After blocking with 5% non-fat milk in TBST containing 0.05% (V/V) Tween-20 at room temperature for 1 hour, membranes were

incubated overnight at 4ºC with the appropriate primary antibody. Blots were then incubated at room temperature for 1 hour with a horseradish peroxidase (HRP) conjugated secondary antibody and the peroxidase activity was detected with a chemiluminescent HRP substrate (Millipore, Billerica, MA) and imaged by a chemiluminescence system (Fujifilm LAS-4000, Tokyo, Japan).

Statistical Analysis

Survival curves were plotted using the method of Kaplan-Meier and the significance of observed differences was calculated with log-rank test. All other comparisons were determined by the Student's *t* test. All reporter assays were repeated for at least three times. Data are shown as average values (mean) ± SD (standard deviation) from one representative experiment. The *P* value < 0.05 was considered statistically significant.

Results

The AGO2 associates with poor prognosis in HCC patients

To explore AGO2 expression in HCC, we first checked the AGO2 expression in HCC micro-tissue array and showed that AGO2 expression was significantly increased in tumor tissues compared with normal tissues (Figure 1A and 1B). Next, AGO2 protein levels derived from matched pairs of HCC tissue and noncancerous tissue were quantified. AGO2 protein level in 80 of 85 (94%) matched tissue sets obviously enhanced in cancer tissue (Figure 1C).

Subsequently, the correlation between AGO2 expression and outcome of patients was assessed. Kaplan-Meier analysis revealed that high level of AGO2 was related to significantly poor overall survival (OS) (Figure 1C). Other clinicopathological variable, such as tumor size could also affect the OS, while age and gender showed no significant correlation with OS (Figure 1D-1F). Altogether, these data support that AGO2 associated with poor prognosis in HCC patients.

Knock-out of AGO2 inhibits cell proliferation and migration in HCC in vitro

To explore the biological function of AGO2 in HCC, we evaluated the AGO2 expression in several hepatocellular cell lines (Figure 2A), we found SMMC-7721 cells represented high expression of AGO2. Then, using CRISPR/Cas9 genome editing technology (Figure 2B), we conducted AGO2 knock-out ($AGR2^{-/-}$) SMMC-7721 cells. The effect of knock-out of AGO2 was confirmed (Figure 2C). In colony fomation assay, it revealed that knock-out of *AGO2* repressed cell proliferation (Figure 2D and 2E). Then, the cell cycle was assessed. We found that G2 phrase of SMMC-7721 $AGO2^{-/-}$ cells was decreased (Figure 2F and 2G), suggesting deletion of *AGO2* delayed the cell cycle. Furthermore, knock-out of *AGO2* inhibited

cell migration (Figure 2H and 2I). Taken together, these results demonstrated that knock-out of AGO2 inhibits cell tumorigenesis in HCC *in vitro*.

Knock-out of AGO2 suppresses HCC tumor growth and size in vivo

To further investigate AGO2 effect on xenograft tumor growth, nude mice were injected subcutaneously with identified number of SMMC-7721 cells. The body weights of mice bearing the tumors were not significantly changed (not shown) within 2 weeks. Tested nude mice (n = 10/group) developed subcutaneous tumors with a size of approximately 0.90 to 840 mm^3 after injection with SMMC-7721 or SMMC-7721 *AGO2*$^{-/-}$ cells (2 × 10^6 /mouse). SMMC-7721 control cells generated visible tumors at day 3 and formed continuously growing mass. However, SMMC-7721 *AGO2*$^{-/-}$ cells generated visible tumors at day 6, and the tumor growth rate of SMMC-7721 *AGO2*$^{-/-}$ cells was lower compared with control cells (Figure 3A). These findings suggested that AGR2 promoted tumor growth *in vivo,* while the knock-out of AGO2 appeared not affect the mice growth (Figure 3B). The tumor sizes from mice bearing with SMMC-7721 control cells were obviously smaller than those with SMMC-7721 *AGO2*$^{-/-}$ cells (Figure 3C). Accordingly, the average tumor weight in SMMC-7721 control group was heavier than the SMMC-7721 *AGO2*$^{-/-}$ group (Figure 3D). Therefore, knock out of *AGO2* suppressed tumor growth and size in HCC *in vivo.*

Analyses of gene expression microarray profiling mediated by AGO2

To explore the downstream of AGO2 involved in HCC tumorigenesis, we performed gene expression microarray profiling and gene set enrichment analysis (GSEA). We initially evaluated the global transcriptomic changes associated with expression of SMMC-7721 *AGO2*$^{-/-}$ cells. We generated a gene expression heatmap exhibiting a list of 2,327 genes showing > 2-fold differential expression (Figure 4A). Coherently with our previous data, several clusters of genes obviously differentially expressed involved in cell death and survival, cancer, cell growth and cell proliferation as well as cellular movement (Figure 4B). Conceivably, the genes associated with cell cycle and cell migration were expressed in the heatmap, which is accordance with our data (Figure 4C). Thus, these data supported the notion that knock-out of *AGO2* could block the cell proliferation and migration in a downstream-gene dependent manner.

AGO2 upregulates cell proliferation and migration related genes

It has been reported that Survivin, Vimentin, Snail plays an important role in cell proliferation and metastasis of hepatocellular carcinoma, respectively [16, 17]. To further determine the genes involved in AGO2 mediated cell proliferation and migration, we evaluated the Survivin, Vimentin and Snail expression

in AGO2 knock-out cells. In SMMC-7721 *AGO2*$^{-/-}$ cells, the Survivin expression was significantly reduced (Figure 5A), indicating AGO2 promotes cell proliferation through regulating Survivin in HCC. Then, we detected the expression of Vimentin and Snail in SMMC-7721 cells. The Western blot results showed that both of Vimentin and Snail expression dramatically declined in SMMC-7721 *AGO2*$^{-/-}$ cells compared to control cells (Figure 5B and 5C). Therefore, the data together demonstrated that AGO2 facilitated cell proliferation and migration by upregulating Survivin and Vimentin/Snail in HCC.

Discussion

Despite of environmental risk factors, intrinsic genetic changes including epigenetic alterations, genomic instabilities, as well as aberrant gene expression contribute to HCC development and progression [18, 19]. *AGO2*, one of the microRNA machinery genes, is originally identified as an oncogenic factor in renal cell carcinoma. It has been reported that the single nucleotide polymorphisms (SNP) of *AGO2* is related to tumorigenesis of renal cell carcinoma [20]. Notably, the typical oncogenic function of *AGO2* is the generation of microRNAs through microRNA machinery processing. The miRNA profiling along modulates tumour progression in carcinomas [21, 22]. Nevertheless, it is unclear whether AGO2 mediates mRNA profiling in HCC progression. Since the roles of AGO2 in HCC remain obscure, it is necessary to elucidate the function of AGO2 in HCC.

AGO2 is overexpressed in bladder carcinoma [11] and it functioned as an early prognostic factor in glioma and nasopharyngeal carcinoma [10, 12]. Here, we showed AGO2 highly expressed in tissue of HCC, which is consistent with the previous studies in HCC [13]. Additionally, our study showed AGO2 is associated with poor outcome of HCC patients. Therefore, we demonstrated that AGO2 served as an oncogene and an independent prognostic factor related with poor outcome in HCC patients. Moreover, using CRISPR/Cas9-based genome editing technology, we generated *AGO2* knock-out cell lines. Subsequently, we found the knock-out of *AGO2* obviously reduced cell proliferation and migration in SMMC-7721 cells, which agree with the findings as previously reported [9, 13]. Furthermore , in nude mice tumor-bearing model, we observed parallel results that knock-out of *AGO2* in SMMC-7721 cells delayed tumor growth and decreased tumor size, suggesting the positive role of AGO2 in HCC progression.

BAGO2, a microRNAs regulator, plays a role in tumorigenesis through regulation of microRNAs[23, 24]. However, it is unclear the regulatory pattern of AGO2 in HCC. Since the typical oncogenic function of AGO2 relies on the modulation of microRNA profiling, which mediate tumour progression in carcinomas [21, 22], we assessed the AGO2-associated gene expression microarray (mRNA) profiling. In the functional classification of differential expressed genes, several genes clusters were involved in the pathways of cell death and survival, cancer, cell growth and cell proliferation as well as cellular movement. These bioinformatic analysis showed potential regulatory pattern of AGO2 in cell proliferation and migration in HCC.

AGO2 was involved in a series of molecular pathways, we pay attention to cell proliferation and migration gene clusters. Then, we found knock-out of *AGO2* significantly reduced the expression of Survivin, Vimentin and Snail. Survivin is an important molecule in cell proliferation and survival of hepatocellular carcinoma [16, 17]. Vimentin and Snail are typical mesenchymal markers promoting HCC metastasis [25-27]. Thus, we speculated that AGO2 might facilitate cell proliferation and migration in HCC progression through modulating Survivin/Vimentin/Snail. However, the exact mechanisms that how AGO2 regulates Survivin/Vimentin/Snail need further exploration. AGO2 can not only mediate microRNA profiling [21, 22, 28] in tumor progression, but also activate some signaling pathways in tumor progression [9, 13, 14]. In our opinions, there might be two main regulatory ways of AGO2 on Survivin/Vimentin/Snail expression. On one hand, AGO2 enhanced Survivin/Vimentin/Snail by AGO2 related microRNAs targeting Survivin/Vimentin/Snail mRNAs, resulting in the enhancement of the. On the other hand, AGO2 stimulates signaling pathway involved in transcription of *Survivin/Vimentin/Snail*, leading to the accumulation of these genes.

Conclusions

In brief, we not only highlight the oncogenic role of AGO2 in promotion of tumorigenesis and tumor progression in HCC, but also reveal that AGO2 facilitates HCC tumorigenesis through modulating Survivin, Vimentin and Snail expression. Our study may provide a novel insight into regulatory way of AGO2 in HCC progression.

Abbreviations

AGO2 (Argonaute 2), HCC (Hepatocellular carcinoma)，CRISPR (clustered regularly interspaced short palindromic repeats), eIF2C (Eukaryotic translation initiation factor 2C), GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase), DMEM (Dulbecco's Modified Eagle Medium), CCK8 (Cell Counting Kit-8), BCA kit (bicinchoninic acid assay kit), TBST (Tris Buffered Saline with Tween 20).

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

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

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

Q.M. and Y.Y. designed the study; Y.Y. performed the experiments and drafted the manuscript; Q.M. participated in data analysis and was involved in discussion of the results. All authors read and approved the final manuscript.

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Figures

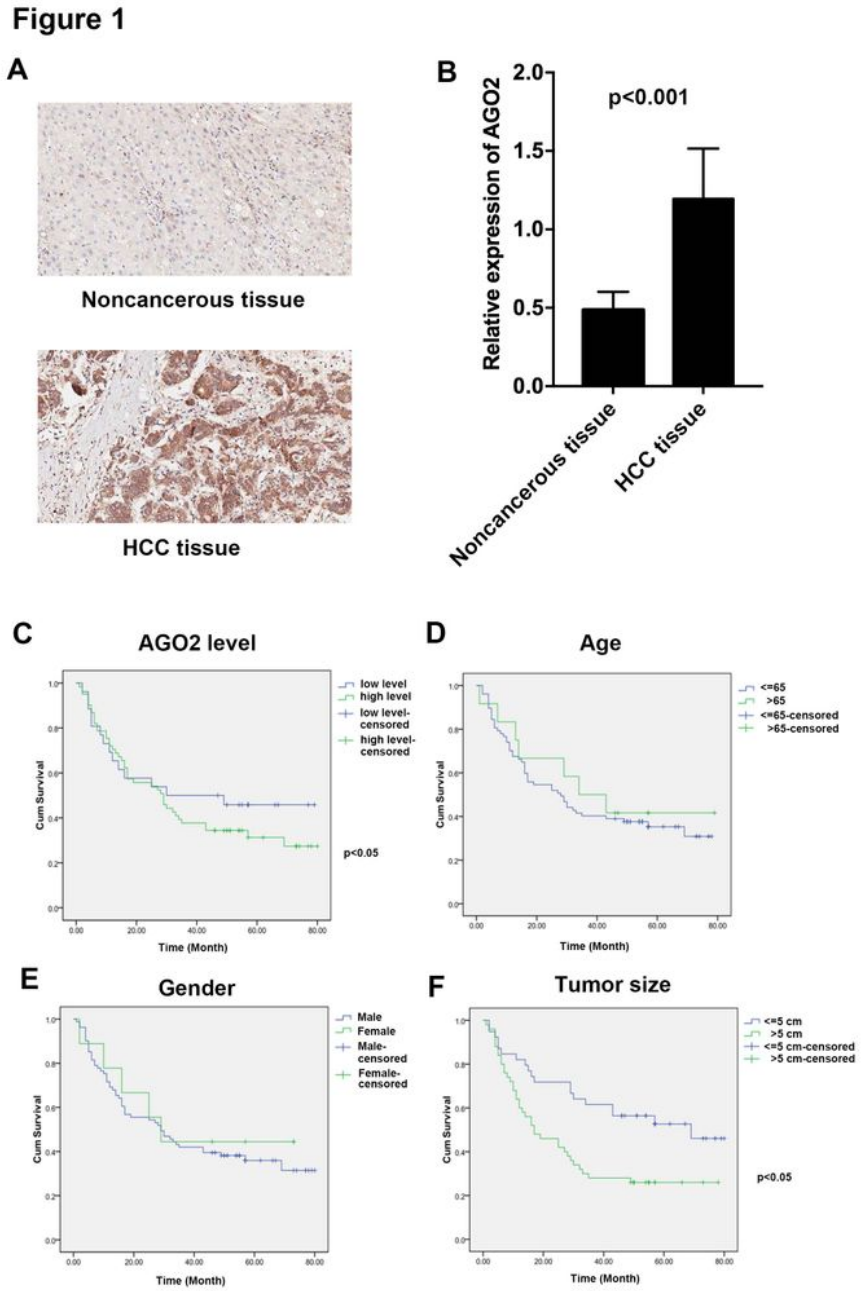

Figure 1

Clinical analysis of AGO2 expression in hepatocellular carcinoma patients. (A) Representative image of AGO2 IHC staining in noncancerous (epithelium) tissues and HCC tissues. (B) Relative expression levels of AGO2 in noncancerous and HCC tissues were determined by measruring the staining tensity (rating: 0, 1, 2, 3). Values represented as means ± S.D. n = 87. (C-F) Kaplan–Meier survival analysis according to AGO2 expression (C), age (D), gender (E), and tumor size (F) in 90 patients with HCC (log-rank test).

Probability of survival of patients: (C) low expression of AGO2, n = 26, v.s. overexpression of AGO2, n = 61 (P = 0.037); (D) age > 65, n = 12, v.s. age ≦ 65, n = 77 (P = 0.597); (E) male, n = 81, v.s. female, n = 9 (P = 0.625); (F) tumor size > 5 cm, n = 50, v.s. ≦ 5 cm, n = 39 (P = 0.005).

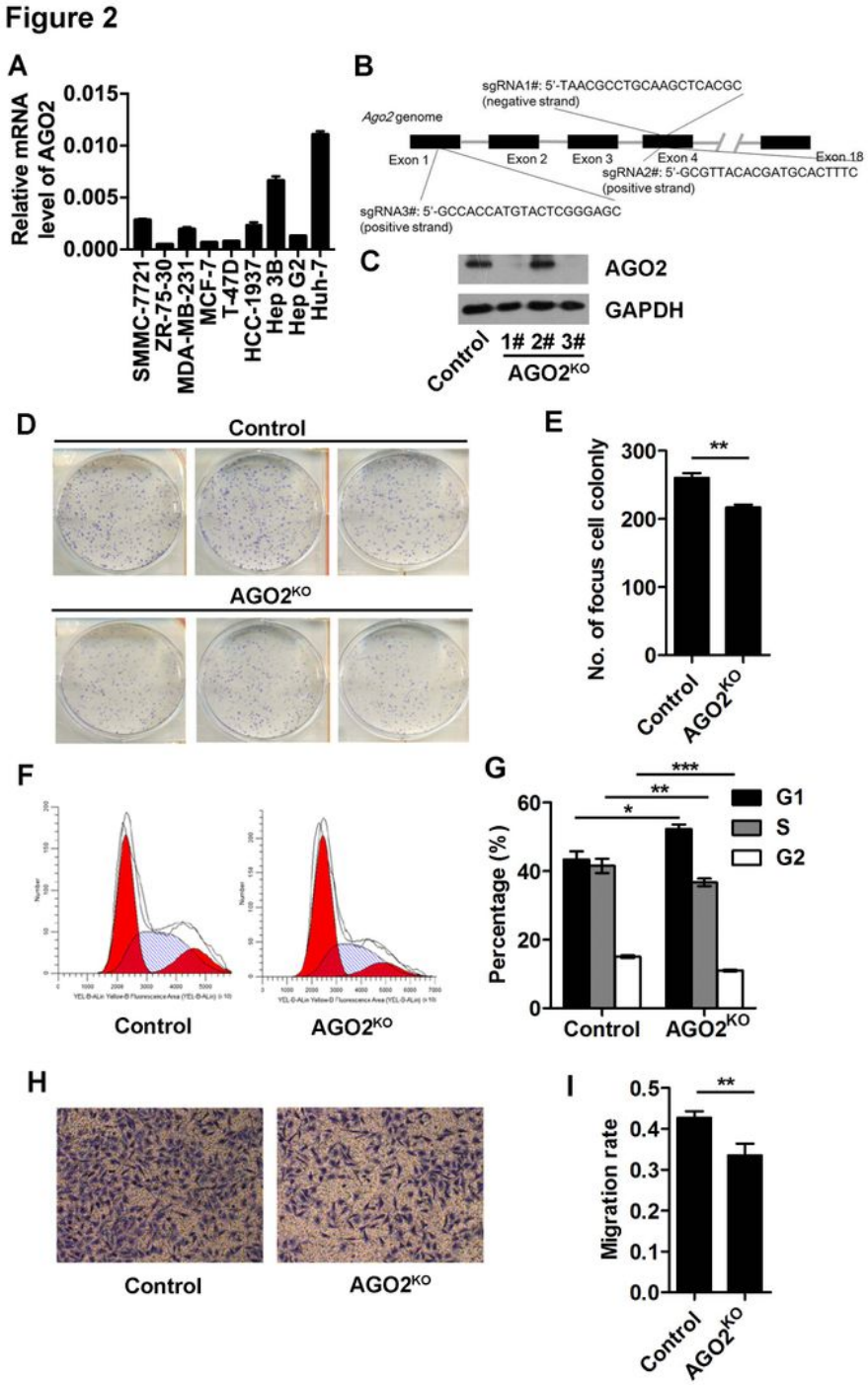

Figure 2

AGO2 promotes cell proliferation and migration in HCC in vitro. (A) Expression levels of AGO2 in different cell lines were measured using quantitative PCR and normalized by GAPDH mRNA, respectively. (B)

Designation of AGO2 gene knock out in SMMC-7721 cells using CRISPR/Cas9 strategy. (C) AGO2 expression in SMMC-7721 cells was detected by western blotting. (D) Colony formation detected by giemsa staining for normal and AGO2-/- SMMC-7721 cells. (E) AGO2 knock-out induced inhibition in colony formation as compared to control. (F) Diagram of the cell cycle analysis for normal and AGO2-/- SMMC-7721 cells based on flow cytometry. (G) The proportions of cells in G1, S, and G2 phases were calculated. (H) Representative images for cell migration assay. (E) Numbers of stained cells in migration assay were counted. Data are normalized and expressed as fold change relative to control values. Values represented as means ± S.D., n > 3 each group, * $P < 0.05$, ** $P < 0.01$, *** $p < 0.005$.

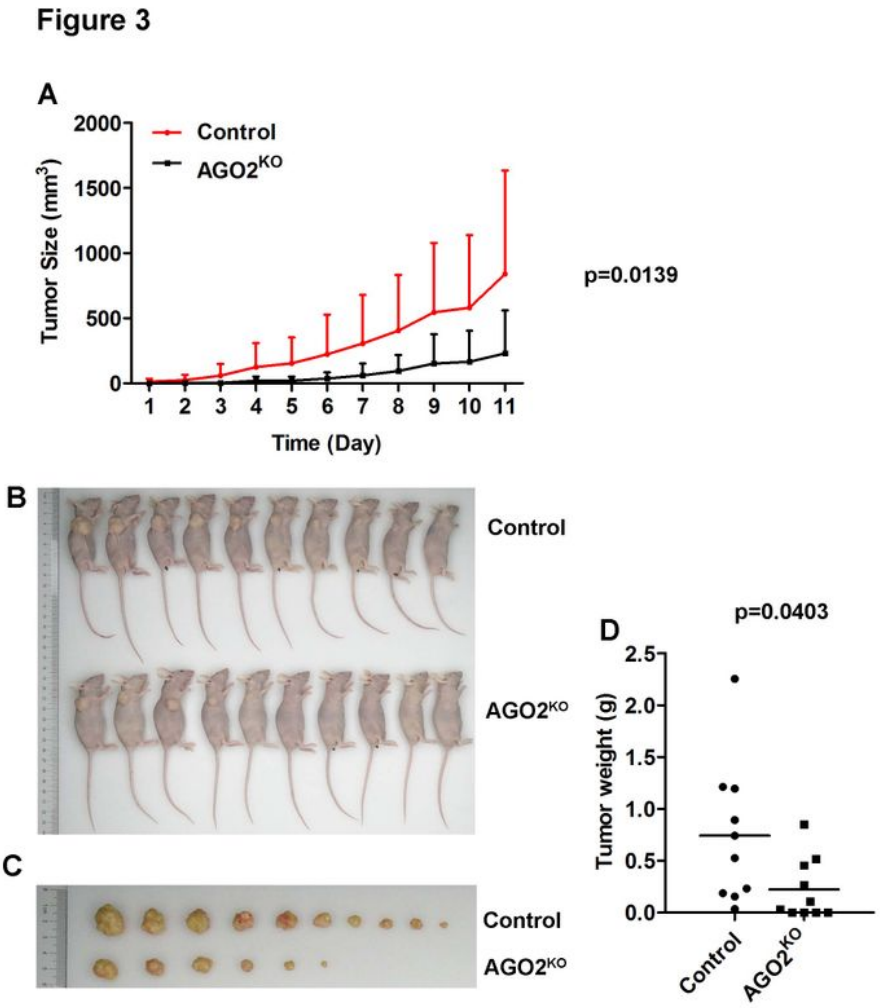

Figure 3

AGO2 enhances tumor growth and size in HCC in vivo. (A) Control and AGO2 knock-out SMMC-7721 cells were subcutaneously injected in the flank of BALB/c nude mice with 2×106 cells. About 7 days post injection, the tumor size was measured every other day. The tumor size was calculated as (length×width2)/2. At the indicated time, all mice were sacrificed and the tumors were photographed. (B)

The size of killed nude mice of the indicated groups. (C) The size of tumors isolated from nude mice. (D) The weight of tumors were measured. Values represented as means ± S.D.

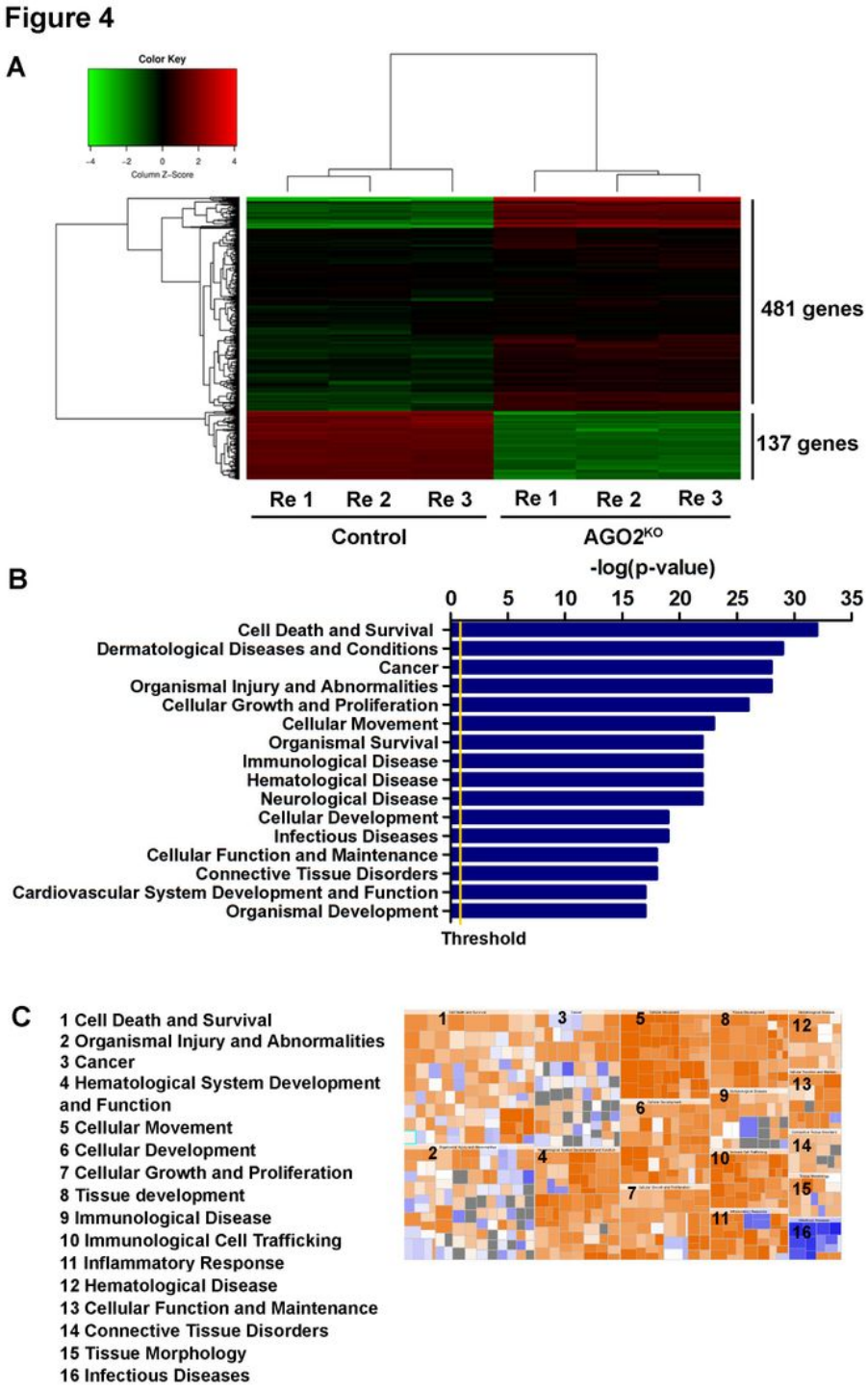

Figure 4

Bioinformatic analyses of gene expression microarray profiling mediated by AGO2. (A) The heatmap of the pairwise comparison in control and AGO2 knock-out SMMC-7721 cells. The colored bar shows the expression levels of genes. (B) The GO terms were shown in the bar chart of biological processes, cellular

components and molecular functions. (C) Heat maps show the up-regulation and down-regulation of differential expressed gene associated with disease. Orange: Z-score > 0, blue: Z-score < 0, gray: no Z-score value. Z-score>2 indicates that the function is significantly activated, while Z-score < -2 indicates that the function is significantly inhibited.

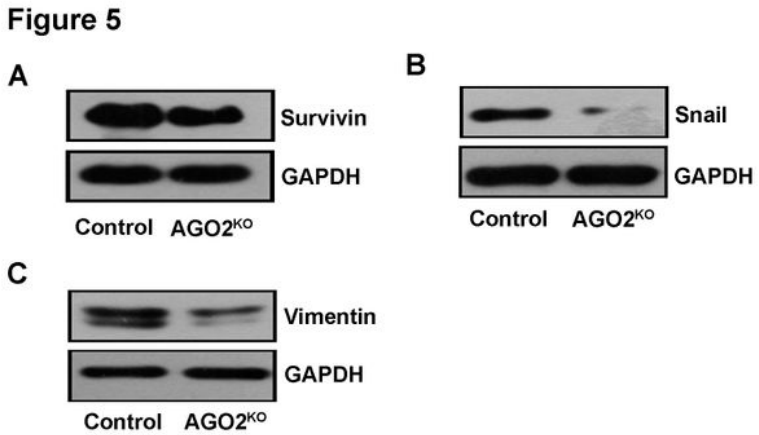

Figure 5

AGO2 upregulates Survivin, Vimentin and Snail expression. Western blot results showed that the expression of Survivin (A), Vimentin (B) and Snail (C) were significantly decrease in SMMC-7721 AGO2-/- cells compared to control cells.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ARRIVEChecklist.docx