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CRISPR-Cas9 mediated AGO2 knockout inhibits tumorigenesis in human colorectal cancer cells

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Abstract

AGO2 plays a vital role in small RNA-guided gene silencing, which has been implied in the tumorigenesis of different types of tumors. Fundamentally, increased expression of AGO2 protein is associated with cancer progression and metastasis. This study aims to investigate the molecular mechanism by which AGO2 promotes tumorigenesis in colorectal cancer (CRC). Databases were used to analyze the expression levels of AGO2 in CRC and confirmed by a quantitative reverse transcriptase-PCR (qRT-PCR) assay in CRC tissues and normal adjacent tissues collected from 25 CRC patients. CRISPR/Cas9-mediated genome editing was used to knockout the AGO2 in HCT116 cells as a model system for colorectal cancers. The cell proliferation, migration and invasion ability of HCT116 cells were detected by CCK-8 assay, Wound scratch assay and Transwell assay. Moreover, the quantities of miRNA binding with AGO2 were detected by RNA-Binding Protein Immunoprecipitation (RIP-Assay). We demonstrated that AGO2 was aberrantly high-expressed in 25 matched-tissue pairs of colorectal cancer and para-carcinoma tissue. The following functional experiments verified that knockout of AGO2 suppressed cell proliferation, migration and tumorigenesis to hamper the aggressiveness of CRC. Our study also suggests a possible link between AGO2 and miRNA in RISC. AGO2 was elevated in CRC and knockout of AGO2 suppressed proliferation and tumorigenicity of CRC cells. Moreover, RISC formation and the function of miRNAs are also subject to AGO2. AGO2 may be a meaningful target for CRC therapy.

Keywords: AGO2; Colon cancer; CRISPR/Cas-9; miRNA

1. Introduction

Colorectal cancer (CRC) stands out as a prevalent gastrointestinal malignancy on a global level. As per the International Agency for Research on Cancer (IARC) of the World Health Organization's 2023 report on global cancer statistics, it is projected that approximately 1.9 million fresh incidents of CRC will emerge across the world, making up roughly 10% of the total cases. This ranks colorectal cancer as the third most prevalent cancer with 930,000 new deaths [1]. Clinical data analysis indicates that patients with stage I colorectal cancer have an impressive five-year survival rate of 90%. However, patients with advanced colorectal cancer and metastasis exhibit a dismal five-year survival rate of less than 10% [2]. Therefore, it is of utmost importance to predict and identify biomarkers that influence metastasis, elucidate the pathogenic mechanism of colorectal cancer cell migration and metastasis, and establish a theoretical foundation for early diagnosis and therapeutic targets of colorectal cancer.

Fundamentally, the AGO2 protein belongs to the AGO family of proteins. It is prevalent and highly conserved among mammals. Among the human AGO family members, only AGO2 has been found to be catalytically active and plays a crucial role in the RISC complex. The RISC complex regulates the process of miRNA-induced gene silencing [3]. Unlike other AGO family members, such as AGO1, AGO3, and AGO4, the knockout of the AGO2 gene in mouse embryonic development leads to fatality [4]. As an integral component of RISC, the AGO2 protein regulates the synthesis and function of miRNA, which in turn binds to target messenger RNA, resulting in the inhibition of its expression. As a result, the possible enhancement of RNA interference efficacy and reduction of side effects in RNA interference drugs could be achieved by gene regulation through AGO2 overexpression. AGO2, a crucial regulator of miRNA function and maturation, has also been found to be overexpressed in various tumors such as colon cancer, glioma, bladder cancer, myeloma, stomach cancer, and liver cancer. Furthermore, the overexpression of AGO2 has been linked to the occurrence, prognosis, and overall survival rate of cancer patients [5-7].

The GW182 family proteins are well-known for their important role in the process of miRNA-mediated gene silencing in animal cells. This role is made possible because these proteins are able to join the miRNA repression complex by directly interacting with Argonaute proteins. Their function is integral to the overall mechanism of gene silencing in miRNA-mediated processes [8]. Interestin-

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gly, there is a higher affinity between the miRNA-bound Ago2 protein and the GW182 protein, which promotes the formation of the RISC complex. Additionally, it is noteworthy that the miRNA-mediated RISC, involving the GW182 protein, is independent of mRNA translation status [9]. GW182-mediated mRNA degradation can occur even when the mRNA is not actively in translation, as evidenced by its ability to function under the action of actinomycin [10].

Over the past few decades, RNAi technology has gained wide acceptance in studying various gene functions. However, the CRISPR/Cas9 technology possesses a distinct advantage due to its capability to eliminate off-target effects [11]. Furthermore, the CRISPR/Cas9 system utilizes synthetic guide RNA (gRNA) along with cas9 nuclease to precisely identify and cut specific DNA strands at cluster sites. Consequently, this technique is commonly employed to permanently silence target genes at the genomic DNA level in cells and organisms [12].

In this particular study, we set out to investigate the biological roles of AGO2 in colorectal cancer. Using CRISPR-Cas9 technology, we obtained AGO2 knockout (AGO2-KO) cell clones. Our findings indicate that AGO2 plays a crucial role in HCT116 cells and is necessary for migration and invasion. Additionally, through RNA-immunoprecipitation (RIP) experiments, we discovered that AGO2 potentially influences the silencing of miRNAs.

2. Materials and methods

2.1. Patients

We selected a cohort of 25 patients diagnosed with colorectal cancer and treated via surgical intervention at the Affiliated Changzhou Second People's Hospital of Nanjing Medical University between the years 2021 and 2022. None of the patients underwent any form of additional therapy following surgery. Subsequently, tissue samples consisting of cancerous tissues and adjacent epithelium were surgically collected from the same set of patients with CRC. In order to carry out this research, the patients provided their informed consent, either in written or verbal form, ensuring their anonymity throughout the study.

2.2. Cell culture

The HCT116 and HEK 293T cells were cultured in a moist CO_2 incubator with a high-sugar DMEM medium comprising 10% fetal bovine serum (FBS) and penicillin/streptomycin. Subsequently, the HCT116 AGO2-/- cells were treated with 2 μg/ml of puromycin every three days.

2.3. Construction of AGO2 Knockout Cell Line

The sgRNA sequences for CRISPR/Cas9 were determined using the CRISPR design website (http://crispr.mit.edu/), developed by the Feng Zhang Lab. The sgRNA sequences employed were as follows: forward: 5'-CACCGCTCCACCTAGACCCGACTTT-3' reverse: 5'-AAACAAAGTCGGGTCTAGGTGGAGC-3'. In the first step, we synthesized the gRNA by annealing two specific target sequences artificially. Next, we used BsmB1 to digest the pLenti CRISPR v2 plasmid (obtained from Addgene, USA) followed by ligating the gRNA into the plasmid as per the manufacturer's guidelines. To serve as a negative control, we employed an empty vector. Subsequently, using Lipofectamine 2000 (from Thermo Fisher Scientific, Waltham, MA, USA), we transfected the constructed plasmid into HEK293T cells along with psPAX2 and psMD.2. After 72 hours of transfection, we collected the lentivirus and infected HCT116 cells. Following a 48-hour infection period, stable cell lines were generated by exposing the cells to 2 μg/ml puromycin for one week. Additionally, we selected a monoclonal cell and cultured it in a 96-well plate. Lastly, we harvested the cells and performed genome DNA sequencing and western blot analysis to validate the depletion of AGO2 expression.

2.4. CCK8 assay

Cell Counting Kit-8 (CCK8, Beyotime, Shanghai, China) was utilized to perform CCK8 assay. The examination involved seeding cells into the 96-well plates, with each well containing 4000 cells. Next, 10 μL of CCK8 reagents were added to each well. It is crucial to keep the entire process away from light. Following this, the 96-well plates were incubated at a temperature of 37°C and with a CO_2 concentration of 5% for 2 hours. Finally, we used a microplate reader to observe the optical densities of the wells at a wavelength of 450 nm. It should be noted that all experiments were conducted three times.

2.5. Wound scratch assay

We conducted the migration assay using the wound scratch assay technique. In addition, HCT116 and HCT116 AGO2-/- cells were seeded at a density of 7.5×10^5 cells per well in 6-well plates and incubated at 37°C in a 5% CO_2 atmosphere for 24 hours. Subsequently, a wound was created in the confluent cells using a 200 μl pipette tip when the cells reached 90% confluency. To document the progress of wound healing, photographs were taken at 0 hours, 24 hours, and 48 hours after the wound was made. We repeated all experiments three times to ensure consistency and reliability.

2.6. Invasion Assay

In the Matrigel invasion assay, cells were prepared for testing by seeding 5×10^4 cells on the upper chamber with a coated membrane provided by BD Biosciences (Franklin Lakes, NJ, USA). These cells were suspended in 200 μl of medium without FBS. In the lower chamber, 500 μl of medium containing 20% FBS was added. After an incubation period of 16 hours at 37°C in 5% CO_2, the cells were fixed using a solution of 4% paraformaldehyde. To prepare the cells for analysis, non-migrated cells on the top surface of the membrane were gently removed using a cotton swab. On the other hand, the migrated cells were stained with 0.5% crystal violet. Subsequently, these stained cells were photographed and counted using a light microscope with a magnification of 200×.

2.7. Real time quantitative PCR

Firstly, total RNA was extracted from cells with TRIzol reagent in conformity with the manufacturer's protocol (TRIzol™ Reagent, Invitrogen, Carlsbad, CA, USA). Secondly, we conducted quantitative reverse transcription-PCR (RT-PCR) analyses utilizing the SYBR green quantitative PCR (qPCR) master mix. To ensure sample normalization, we included the GAPDH gene as a reference. Finally, a standard amplification protocol was applied consistent with the manufacturer's instructions, wherein the primers were as follows:

AGO2 forward-5′-CCTGTATGAGAACCCAATGTC-3',

reverse-5′-CAGCTAGTTTGAGCCCATCA-3’; GAPDH forward-5′-AAGGCTGTGGGCAAGG-3’, reverse-5′-TGGAGGAGTGGGTGTCG-3’. miRNA-141: forward-5'-TCCATCTTCCAGTGCAGTGTTG-3', reverse-5'-GCTGTCAACGATACGCTACGTAA-3'. miRNA-21: forward-5'- CAAAGATCAATATCCCAATCATC-3’, reverse-5'- GCGGTCTTTCAATCTAAGTC-3’.

2.8. RNA-Binding Protein Immunoprecipitation (RIP-Assay)

It is customary to approximate the initial cell count and growth rate in order to achieve 80% - 90% cell confluence within 10 cm dishes on the day of the experiment. Following the completion of the culture, the cells were twice washed with ice-cold 1×PBS while on ice. Subsequently, an appropriate volume of 1×PBS was added to cover the cells at the bottom of the 10 cm dishes, which were then exposed to a single 400 mJ/cm2 ultraviolet cross-linking session using a cross-linker. The 1×PBS was removed from the culture dishes, and a solution of Triton×100 containing RNase inhibitor was directly introduced to the dishes. The resulting lysate was then transferred to a microcentrifuge tube that was free of RNase. Subsequently, the lysate was centrifuged at 16,000×g for 10 minutes in a 4°C benchtop centrifuge. Using a 200 µL pipette tip, the supernatant enriched with protein and RNA was carefully transferred to another RNase-free tube. To maintain consistent protein concentrations in all samples for western blotting, 5 µL of the supernatant was reserved for BCA assay. GW182 antibody was added to the remaining supernatant and incubated at 4°C for 2-3 hours, followed by the addition of suitable Protein A/G agarose beads for an overnight incubation at 4°C. The mixture was then centrifuged at 3,000×g for 5 minutes in a 4°C benchtop centrifuge. The supernatant was discarded, and the pellet containing protein and RNA was retained. The pellet was subjected to three subsequent washes using Triton×100 containing RNase inhibitors. When performing the third wash, 15% of the suspension was transferred to a new RNase-free tube for the western blot assay. The remaining suspension was then centrifuged and precipitated to facilitate RNA extraction.

2.9. Protein extraction and Western blot

At the beginning of the experiment, the dish-contained cells underwent a three-time wash with cold 1×PBS and then added RIPA lysis buffer for a duration of 10 minutes. Subsequently, the scraped lysate was placed in a 1.5 mL EP tube and centrifuged at 12000g for 10 minutes at a temperature of 4°C. Later, the protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). In order to conduct western blot analysis, the supernatant was supplemented with 5x loading buffer (composed of 200 mM pH 6.0 Tris-HCl, 10% SDS, 0.05% BPB, and 50% Glycerol). The protein samples were subjected to SDS-PAGE to separate them based on their distinct molecular weights. Following separation, the samples were transferred to a PVDF membrane (Millipore, Billerica, MA, USA). Subsequently, the PVDF membrane was incubated overnight with primary antibodies and subsequently with HRP-conjugated secondary antibodies. To visualize the target protein, a chemiluminescence system from Tanon (180-5001) was employed.

2.10. Statistical analysis

The statistical analysis was conducted using GraphPad Prism 5 software (La Jolla, CA, USA). The measurement data were expressed as the mean ± standard error. Comparisons were made for homogeneity variance and analysis of variance in each group. The differences were analyzed using one-way ANOVA, and multiple comparisons were examined using the Sidak test. A P-value less than 0.05 was deemed significant.

3. Results

3.1. AGO2 is over-expressed in human colon cancers

In order to gain a comprehensive understanding of AGO2 expression in colon cancer, we turned to the Human Protein Atlas (HPA) database for analysis. The results showed that both AGO2 mRNA and protein were highly expressed in colon cancer, confirming the importance of AGO2 in CRC (Figure 1A, 1B). To further validate these findings, Quantitative reverse transcription-PCR (RT-PCR) was employed to evaluate the transcript levels of AGO2 in a total of 25 matched-tissue pairs consisting of colorectal cancer and para-carcinoma tissue. The results revealed significantly increased levels of AGO2 mRNA expression in colon carcinoma tissues compared to the para-tumor tissue.

3.2. Successful knockout of AGO2 in HCT116 cells using CRISPR/Cas9

In the interest of further underscoring the role of AGO2 in colon cancer, we generated HCT116 cells with AGO2 knockout (HCT116 AGO2 −/−) using the CRISPR/Cas9 methodology. Accordingly, techniques such as Sanger sequencing and Western analysis confirmed the knockout of the genes and proteins, respectively (Figure 2A, 2B).

3.3. AGO2 deficient cells exhibit decreased proliferation, mobility, and invasion

When we generated the HCT116 AGO2-/- cell lines, we observed an overall reduction in cell proliferation in AGO2 knockout cells compared with wildtype. Consequently, we examined cell proliferation using CCK-8. Based on

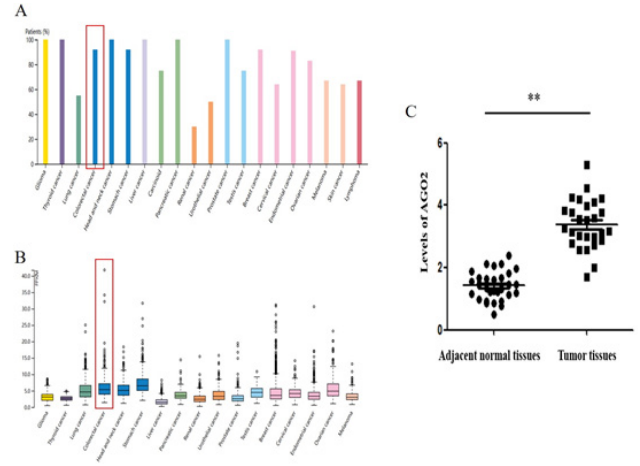

Fig. 1. AGO2 expression in normal and cancerous tissues. (A) AGO2 protein expression in different cancer tissues from the HPA database. (B) AGO2 mRNA expression in different cancer tissues from the HPA database. (C) RT-qPCR analysis revealed that AGO2 was overexpressed in tumor tissues compared to normal tissues from 25 patients with colorectal cancer. **P< 0.01.

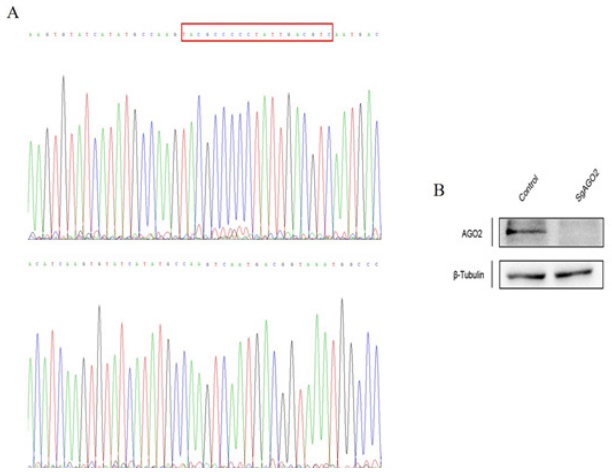

Fig. 2. Identification of AGO2 knockout using the CRISPR/Cas9 system in HCT116 cells. (A) DNA sequence identification of targeted genomic region in AGO2 wildtype and AGO2 knockout cells; (B) AGO2 knockout effect was analyzed by Western blotting with antibodies against AGO2 and β-Tubulin.

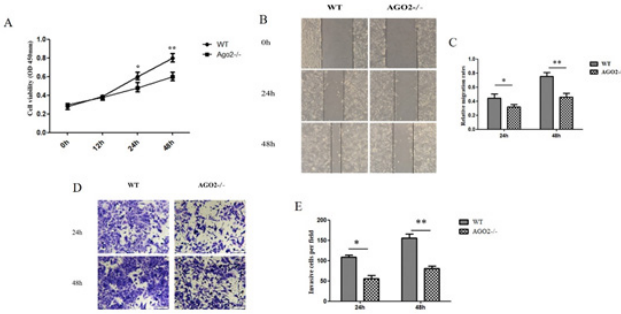

Fig. 3. Knockout of AGO2 inhibited HCT116 cell proliferation, mobility, and invasion in vitro. (A) Cell Counting Kit 8 (CCK8) assay of the proliferation of HCT116 (WT or AGO2-/-) cells. (B) Motility of HCT116 (WT or AGO2-/-) cells was determined by scratch wound healing assay. (C) Statistical results of scratch wound healing assay. (D) Invasion of HCT116 (WT or AGO2-/-) cells were determined by transwell invasion assay. (E) Statistical results of transwell invasion assay. * $P < 0.05$, ** $P < 0.01$.

our inference, we discovered that the knockout of AGO2 remarkably inhibited HCT116 cell viability, thereby indicating that AGO2 plays a crucial role in cell survival ($P < 0.05$, Figure 3A). With an eye to diligently assess the mechanisms of AGO2 in promoting tumor progression, scratch and invasion assays were employed to evaluate both the cell mobility and invasion ($P < 0.05$, Figure 3B, 3C). Both results highlighted that HCT116 cell migration and invasion were considerably suppressed in the AGO2 knockout cells.

3.4. Knockout of AGO2 affects RISC function through diminishing RNA binding

GW182-mediated RNA immunoprecipitation (RIP) was carried out to compare the quantities of RNA associated with RISC in HCT116 WT or HCT116 AGO2-/-. As shown in Figure 4A, dramatically less RNA was co-immunoprecipitated with GW182 in AGO2-/- than in HCT116 WT cells. For further study, we detected the amount of miR-141 and miR-21 binding to GW182 by RT-PCR, which have been identified as metastasis-associated miRNAs in CRC[13]. As expected, both miR-141 and miR-21 binding to GW182 were significantly reduced in HCT116 AGO2-/- ($P < 0.05$, Figure 4B, 4C).

4. Discussion

Despite the critical role of miRNA in gene regulation, studies have shown that the extracellular environment and stress state can influence the functions of miRNA and determine whether it inhibits or activates mRNA translation [14]. The AGO2 protein has been identified as a crucial element in regulating miRNA silencing function, and its expression level, activity, and intracellular localization can affect the activity of the RISC complex, which plays a significant role in miRNA regulation [15].

Several solid tumors have reported AGO2 as an oncogene, and its single nucleotide polymorphism (SNP) has been linked to the development of renal cell carcinoma [16]. Furthermore, abnormal overexpression of AGO2 has been observed in various human tumors, including breast cancer [17], urothelial carcinoma of the bladder [18], glioma [19], and nasopharyngeal carcinoma [20], and has been linked to higher tumor grading and poor prognosis. Based on the aforementioned studies, we can deduce that the overexpression of AGO2 is generally associated with higher tumor grading and inadequate prognosis. The overexpression of AGO2 has also been shown to affect the expression of let-7, c-myc, and FAK mRNAs, which are implicated in tumorigenesis and tumor metastasis [21-23]. However, in human lung adenocarcinomas, AGO2 expression was found to be lower compared to non-cancerous tissue [24]. With the goal of substantiating the functional roles of AGO2 in CRC, we demonstrated that AGO2 greatly increased in colorectal cancer tissues. AGO2 is known to play a vital role in various developmental processes, such as neural tube closure and embryonic lethality [25]. In addition, the downregulation of AGO2 has been demonstrated to trigger programmed cell death in myeloid leukemia cells and hinder the suppression of transfected cancer-causing genes in HEK-293T cells [26,27]. We conducted a study to create AGO2 knockout HCT116 cells using CRISPR/Cas9-based gene editing technology. The depletion of AGO2 in these cells resulted in a notable decrease in both cell proliferation and migration, which aligns with prior findings. These findings provide additional evidence that AGO2 might serve as a dependable marker for determining the outlook of colorectal cancer (CRC). Additionally, our findings also suggest that AGO2 plays a crucial role in the formation of the RNA-induced silencing complex (RISC) and the silencing of miRNAs.

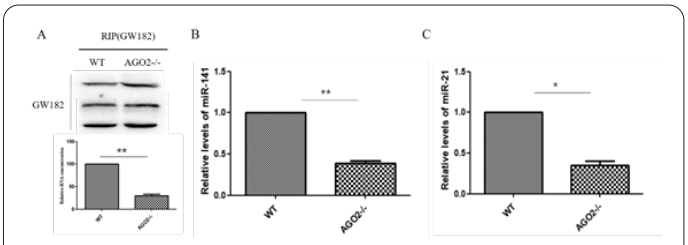

Fig. 4. AGO2 deficiency affects the formation and function of RISC in HCT116 cells. (A) The quantities of RNA associated with GW182 were detected by GW182-mediated RNA immunoprecipitation. (B) The relative expression of miR-141 binding to GW182 in HCT116 WT or AGO2-/-. (C) The relative expression of miR-21 binding to GW182 in HCT116 WT or AGO2-/-. * $P < 0.05$, ** $P < 0.01$.

Our study focused on investigating the effects of AGO2 on the progression of colorectal cancer. However, there are a few shortcomings that should be noted. First, we did not have any in vivo evidence to support our findings. In future studies, we aim to overcome this limitation by conducting experiments on live organisms. By doing so, we will be able to gather more reliable evidence to support our conclusions. Another restriction of our investigation is the comparably limited size of the sample. To acquire more accurate outcomes, it is imperative to enlarge the sample size and encompass a greater number of participants. This will allow for a more representative analysis of the effects of AGO2 on colorectal cancer progression. Further investigations are necessary to obtain a comprehensive understanding of the mechanism of AGO2 in colorectal cancer. By addressing these deficiencies and conducting additional research, we will be able to gain deeper insights into the role of AGO2 in the progression of colorectal cancer.

5. Conclusion

In summary, our investigation revealed that CRC tumorigenesis is significantly influenced by the presence of AGO2, which actively participates in the silencing of miRNA. Thus, AGO2 has the potential to serve as a comprehensive indicator for both the prognosis of CRC and the development of targeted molecular therapy.

Conflict of Interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

This study was approved by the ethics committee of the Affiliated Changzhou Second People's Hospital of Nanjing Medical University.

Informed Consent

Signed written informed consents were obtained from the patients and/or guardians.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

Yizhi Zhu and Yuan Guo designed the study and performed the experiments, Gengfang Wang collected the data, Haoran Xu analyzed the data, Yizhi Zhu and Yuan Guo prepared the manuscript. All authors read and approved the final manuscript.

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