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Effects of HMGA2 on the epithelial-mesenchymal transition-related genes in ACHN renal cell carcinoma cells-derived xenografts in nude mice

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Abstract:

Background: The architectural transcriptional regulator high-mobility group AT-hook 2 (HMGA2) is an oncofetal protein which has been reported to be ectopically expressed in a variety of cancers. A high expression of HMGA2 in human renal cell carcinoma (RCC) is related with tumor invasiveness and poor prognosis. In the in vitro studies, HMGA2 knockdown was found to lead to decreased cell proliferation, decreased migration and changes in gene expression related with the epithelial-mesenchymal transition. **Methods:** In order to understand HMGA2's effect *in vivo*, HMGA2 expression was knocked-down in ACHN cells using small hairpin RNA (shRNA). The HMGA2-deficient ACHN cells were xenografted into the BALB/c nude mice. The tumor growth was monitored and the expression of EMT-related genes was analyzed. **Results:** HMGA2 expression was confirmed to be knocked-down in the cultured and xenografted ACHN cells. The xenograft tumor of HMGA2-deficient cells demonstrated a retarded growth pattern compared with control. The expression of E-cadherin was increased, whereas N-cadherin and Snail were decreased in the HMGA2-deficient xenograft tumors. **Conclusions:** The present study suggested that the epigenetic regulation of EMT-related gene expression by HMGA2 exists both in the *in vitro* and *in vivo* conditions. It is likely that through this mechanism, HMGA2 regulates the cell growth in renal cell carcinoma.

Key Words: HMGA2, shRNA, epithelial-mesenchymal transition, ACHN cells, xenograft

Background:

High-mobility group AT-hook 2 (HMGA2), a member of the high mobility group protein family, is an epigenetic non-histone and architectural transcription regulator and one of the core components of the enhance some[1]. Through its binding to the AT-rich DNA sequences of promoter regions of target genes and modification of chromatin condensation, HMGA2 alters the transcription of target genes. HMGA2 plays an important role in the embryonic development[1]. Ectopic expression of HMGA2 has also been found in various cancers, participating in the process of carcinogenesis in adult. Tumors with aberrant expression of HMGA2 include renal clear cell carcinoma, lung adenocarcinoma, colon adenocarcinoma, stomach carcinoma and others[2]. HMGA2 overexpression has been significantly associated with advanced TNM stage, tumor local invasion and distal metastasis, tumor differentiation and unfavorable prognosis[2]. Mechanistically, studies have suggested that HMGA2 may participate in carcinogenesis by regulating the expression of critical genes involved in epithelial-mesenchymal transition, cell proliferation, DNA damage repair and cancer cell stemness[3]. Early studies from our lab showed that HMGA2 is significantly overexpressed in clear cell renal cell carcinoma specimens of patients[4] and RNAi silencing of HMGA2 gene in cultured renal cell carcinoma ACHN cells resulted in decreased cell ability of proliferation and invasion[5]. Another recent study reported that HMGA2 silencing in ACHN cells affected the expression of several epithelial-mesenchymal transition-related genes including E-cadherin, N-cadherin, Twist1 and Twist 2, as well as the expression of growth factor TGF-beta and smad2[6]. However, the studies of HMGA2's role in renal cancer remained incomplete since most studies were done using *in vitro* cultured cells and whether the same regulatory pattern also occurring *in vivo* remains

questionable. Aiming to fill this gap, in the present study, we performed an *in vivo* experiment with ACHN cells stably RNAi-silenced for HMGA2, which were grown as tumor xenograffts in nude mice. Our results show that knockdown of HMGA2 strongly impairs tumor growth of ACHN cells and that HMGA2 regulates in these cells the expression of EMT-related genes in vivo as previously demonstrated in vitro.

Methods:

Materials: ACHN cells, a renal cell carcinoma cell line, were obtained from the Cell Bank of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Peking Union Medical College (Beijing, China). The Trizol™ reagent was obtained from ThermoFisher Scientific (Waltham, MA, USA). Rabbit anti-HMGA2 antibody (Ab207301), Rabbit anti-E-cadherin antibody (Ab40772), Rabbit anti-N-cadherin antibody (Ab76011), Rabbit anti-Snail antibody (Ab216347) were purchased from Abcam (Boston, MA, USA). Goat anti-rabbit IgG-HRP was purchased from KeyGenBioTECH (Nanjing, China). The Reverse Transcription kit and One Step TB Green™ PrimeScript™ RT-PCR Kit II (SYBR Green) were purchased from TaKaRa-Bio (Dalian, China). The primers were ordered from Beijing Aoke Biotechnology Co., Ltd. (Beijing, China). The bicinchoninic acid (BCA) protein assay kit was purchased from Shenyang Wanlei Biological Technology Co. Ltd. (Shenyang, China). Three HMGA2 shRNAs and negative control scrambled shRNA were custom-designed and synthesized by KeyGenBioTECH (Nanjing, China).

Cell culture: The ACHN cells were grown in RPMI-1640 culture medium (Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA)

and 100 μg/ml streptomycin plus 100 U/ml penicillin (Invitrogen, Carlsbad, CA, USA), and cultured at 37° C under 5% CO2 atmosphere.

Lentiviral small hairpin RNA vector construction and transfection: Three independent oligoneucleotides for shRNAs specifically targeting HMGA2 gene together with the scrambled control shRNA were designed, synthesized and inserted into the shRNA expression vector U6-MCS-CMV-zsGreen-PGK-Puromycin. The three HMGA2 shRNA target sequences wereHMGA2-shRNA-1: 5'-AGTCCCTCTAAAGCAGCTCAA-3', HMGA2-shRNA-2: 5'-CCGAATTGGGTTTAGTCAATC-3' and HMGA2-shRNA-3: 5'-AGGAGGAAACTGAAGAGACAT-3'. For synthesis of the shRNAs, the following primers were used shRNA1 Forward: 5'-GATCAGTCCCTCTAAAGCAGCTCAACTCGAGTTGAGCTGCTTTAGAGGGACTTTTTTT-3', shRNA1 Reverse: 5'-AATTAAAAAAAGTCCCTCTAAAGCAGCTCAACTCGAGTTGAGCTGCTTTAGAGGGACT-3', shRNA2 Forward: 5'-GATCCCGAATTGGGTTTAGTCAATCCTCGAGGATTGACTAAACCCAATTCGGTTTTTT-3', shRNA2 Reverse: 5'-AATTAAAAAACCGAATTGGGTTTAGTCAATCCTCGAGGATTGACTAAACCCAATTCGG-3', shRNA3 Forward: 5'-GATCAGGAGGAAACTGAAGAGACATCTCGAGATGTCTCTTCAGTTTCCTCCTTTTTTT-3', shRNA3 Reverse: 5'-AATTAAAAAAAGGAGGAAACTGAAGAGACATCTCGAGATGTCTCTTCAGTTTCCTCCT-3'. After annealing, the oligonucleotides were incorporated downstream from the U6 promoter in the lentiviral vector pLenti-CMV-zsGreen–PGK-Puromycin (KeyGenBioTECH, Nanjing, China). The scrambled control shRNA was inserted into the lentivirus as non-specific control. For stable transfection, ACHN cells were treated with viral supernatant and Polybrene and incubated for 8

hours. The cells were selected with puromycin (2 ug/ml) for 7 days. The knockdown of HMGA2 in transient transfected cells (with HMGA2 siRNA1, siRNA2, siRNA2), in stable transfected cells (with HMGA2 shRNA2) and in xenograft tumor (with shRNA2) were confirmed with RT-PCR and western blotting.

Animal experiments: To obtain the *in vivo* tumorigenesis model, 4 X 10^6 ACHN cells, grown in culture medium and harvested in mixed population (Group 1: ACHN cells without transfection; Group 2: ACHN cells transfected with scrambled shRNA; Group 3: ACHN cells transfected with HMGA2 shRNA; 4 animals per group to satisfy the minimum sample size required for statistical analysis) in a volume of 0.2ml were injected subcutaneously into the right axillary region of 4 weeks old female BALB/c nude mice (SLAC Laboratory Animal Ltd, Shanghai, China). The xenograft tumors could be observed 18 days after injection. The tumor sizes were monitored every other day for 30 days and the tumor volume was calculated in mm^3 as $(width)^2$ x length/2. The mice were maintained with standard care and food/water supply and euthanized using CO_2 inhalation. The total body weight was recorded at the time of necropsy.

Reverse Transcription and Quantitative-Polymerase chain reaction: Total RNA extraction was performed using the Trizol™ method according to manufacturer's instruction. Subsequently, 2ug of total RNA was reverse transcribed into cDNA. For PCR amplification, the following primers were used: HMGA2 Forward：5'-ACAAGAGTCCCTCTAAAGCAGC-3'; HMGA2 Reverse: 5'-AGGCAACATTGACCTGAGC-3'; GAPDH Forward: 5'-CAAATTCCATGGCACCGTCA-3'; GAPDH Reverse: 5'- AGCATCGCCCCACTTGATTT-3'; E-cadherin Forward: 5'-GTTTACCTTCCAGCAGCCCT-3';

E-cadherin Reverse: 5’-TCCCAGATGAGCATTGGCAG-3’; N-cadherin Forward: 5’-GGCGTTATGTGTGTATCTTCACT-3’; N-cadherin Reverse: 5’-GCAGGCTCACTGCTCTCATA-3’; Snail Forward: 5’-TTCTGTGAGCAGGACATCCG-3’; Snail Reverse: 5’-GCAGCCGTCAATGGCTTTAG-3’. The quantitative PCR was performed using 2× RealTime PCR Master Mix (SYBR Green) on an ABI StepOne plus Real time-PCR system (ThermoFisher Scientific, Waltham, MA, USA). Quantitative gene expressions of HMGA2, E-cadherin, N-cadherin and Snail were normalized to the expression level of the house-keeping gene GAPDH.

Western blotting: Cell lysates were harvested from shRNA-transfected ACHN cells or from xenograft tumors using RIPA cell lysis buffer. Total protein concentration was determined using the bicinchoninic acid (BCA) method. Twenty micrograms of protein lysate were separated by 8-12% SDS-PAGE under a constant current of 200mA for 2 hours and protein was transferred onto a PVDF membrane. For blocking of non-specific epitopes, the membrane was incubated in 1X TBS-Tween-20 containing 5% (w/v) skimmed milk powder at room temperature for 1 hour. Then the membrane was incubated with primary antibodies at 4 °C overnight. The membrane was washed with 1X Tris-based saline-Tween 20 (0.05% v/v) and incubated with horseradish peroxidase-conjugated goat-anti-rabbit IgG at room temperature for 1 hour. The membrane was washed and developed using ECL reagent (KeyGeneBioTECH, Nanjing, China) and the pictures were captured and analyzed using the Chem Image5500 gel imaging system (Alpha Innotec, Santa Clara, CA). The protein expression of HMGA2, E-cadherin, N-cadherin and Snail were semi-quantitatively normalized to the protein expression of GAPDH and expressed as a ratio comparing the band intensities between the two.

Statistical analysis: Statistics were performed using the SPSS 17.0 statistical software (SPSS Inc, Chicago, IL, USA). In assessment of the significance, the experimental group and the control group were compared using a Student's t-test or (nonparametric one-way ANOVA) Kruskal-Wallis test. P value less than 0.05 was considered to be statistically significant. The power of study was set at 0.8. To set the sample size for the animal experiment, a thumb rule was following as "sample size= 2 SD^2 $(1.96 + 0.842)^2/d^2$". The criteria were set as type I error p=0.05 and type II error with power=0.8. Since the experiment used xenograft tumors with HMGA2-knockdown ACHN cells, the standard deviation was expected to be relatively small between 10-15% and therefore a sample size of 4 animals allows detection of observational difference (changes of EMT marker expression) (d)≧20% (if SD=10%), or ≧30% (if SD=15%).

Results:

Validation of HMGA2 silencing in ACHN cells: Upon the shRNA transfection in ACHN cells, the knockdown of HMGA2 expression was confirmed by RT-PCR and Western blotting. As shown in Figure 1A, twenty-four hours after transfection, HMGA2- shRNA1, shRNA2, shRNA3 significantly reduced HMGA2 mRNA expression level to 45%, 27% and 44% relative to the level in the untreated cells. The scrambled control shRNA caused no significant change. Therefore, it was confirmed that all the shRNAs reduced HMGA2 mRNA expression, with stronger effect by shRNA2. In order to further validate knockdown on the protein level, western blotting was used to measure HMGA2 protein expression in cells treated with shRNA2. As shown in Figure 1B and 1C, the protein level of HMGA2 in the treated cells was found to be decreased to 23% of

the level in the untreated cells. No significant change was found in the scrambled control shRNA-treated cells. Together, these results validated that the shRNA successfully silenced the HMGA2 expression in ACHN cells and the shRNA2 with the greatest effect was determined to be used in the further experiments.

Effect of HMGA2 silencing on xenograft tumor growth: The effect of HMGA2 silencing on tumorigenesis was studied by xenografting HMGA2 shRNA- or scrambled control shRNA-transfected ACHN cells (mass population) into the subcutaneous regions of 4 weeks old female BALB/c nude mice. Before injection, the knockdown of HMGA2 was further confirmed (Figure 1D). It was also confirmed that knockdown of HMGA2 led to decreased level of N-cadherin and Snail and increased level of E-cadherin in cultured ACHN cells (Figure 1 E-G). Three groups including one group using untreated ACHN cells (Group 1), one group using scrambled control shRNA-transfected cells (Group 2) and one group using HMGA2 shRNA2-transfected cells (Group 3) were designed. Four animals were included per group.

Eighteen days after injection of the ACHN cells (mass population) into the right axillary subcutaneous region, the xenograft tumors grew bigger enough to be observable. The tumor size was monitored for 4 weeks until necropsy. As shown in Figure 2A-C, in comparison with the tumors grown from the untreated or scrambled shRNA-treated ACHN cells, the xenograft tumors from the HMGA2 shRNA2-treated ACHN cells were significantly smaller in size ($p<0.001$). The average tumor measurements were 2.16 cm^3 in group 1, 2.08 cm^3 in group 2 and 0.26 cm^3 in group 3, with an inhibition rate being 88% by HMGA2 silencing (Figure 2C). This pattern of

growth inhibition was consistent throughout the entire observation period of 30 days (Figure 2D). Notably, there was no significant change in overall body weight among the three groups (Figure 2E), indicating that the difference in tumor size was not causatively correlated with the tumor burden or the cachexia condition. These results suggested that HMGA2 plays an important role in tumor cell proliferation both *in vivo* and *in vitro* [5] since HMGA2 silencing also retarded the growth of xenograft tumors in nude mice.

Effect of HMGA2 silencing on the expressions of EMT-related genes in xenograft tumor: The next effort was to address the question that whether silencing of HMGA2, as an epigenetic regulator, could cause changes in gene expression in xenografting ACHN cells. To this end, experiments were carried out to measure the mRNA and protein expression of the epithelial-mesenchymal transition-related genes. As shown in Figure 3A-C, the mRNA expression of E-cadherin was significantly increased by ~2.6 folds, whereas N-cadherin and Snail expressions were significantly decreased by ~2 folds in the HMGA2-silencing xenograft tumors than the level of the control xenograft tumors. The western blotting results also confirmed that the protein expression of E-cadherin was upregulated and N-cadherin and Snail expressions were down-regulated under the condition of HMGA2 knockdown (Figure 3D and 3E-G). Together, these results suggested that HMGA2 regulates the expression of genes involved in epithelial-mesenchymal transition in the xenograft RCC tumor model and plays an indispensable role for this critical molecular process.

Discussion:

HMGA2 is a non-canonical epigenetic transcription regulator physiologically expressed in the developing embryonic tissues but ectopically expressed in various human malignancies including renal cell carcinoma[1,4,7]. Interestingly, epigenetic regulators are known to play a crucial role in the development of renal cell carcinoma. While inactivation of Von Hippel-Lindau (VHL) occurs in majority of RCC, mutations of PBRM1, SETD2, BAP1 are also common contributors and they are all epigenetic factors [8]. It has been found that PBRM1 modulates HIF1a-VEGF signal and STAT3-Interferon signal [9, 10] and BAP1 promotes genomic instability and therefore accelerates tumor metastasis [11, 12]. Hence, it would be very interesting to examine whether and if so, how, another epigenetic factor HMGA2 participates in the pathogenesis of RCC. In our previous studies, the expression of HMGA2 has been found in the human RCC specimens, whereas very limited or no expression was found in benign tumors or adjacent normal tissues [4]. The expression of HMGA2 has been correlated with advanced TNM stage and lymph node metastasis but not age, gender, tumor size or histology subtypes [4]. Study by another group also confirmed HMGA2 expression is correlated with overall survival in RCC patients [6]. Therefore, it seemed that the aberrant expression pattern of HMGA2 gene has certain direct linkage with the malignant and metastatic behavior of the RCC carcinogenesis.

The target genes regulated by HMGA2 remained incompletely understood though previous studies have identified genes involved in cell proliferation, differentiation, apoptosis, DNA damage and repair and epithelial-mesenchymal transition[13,14]. To further explore the underlying mechanism, we and another research group used the ACHN renal cancer cell lines to

specifically knockdown the HMGA2 gene [5,6]. Results showed that the ACHN cells treated with HMGA2 siRNA impaired cell proliferation, metastasis behavior and tumor growth than the mock-treated cells [5]. Another study found that the ACHN cells with HMGA2 knockdown increased the expression of E-cadherin and decreased the expressions of N-cadherin, Twist1 and Twist2, whereas ACHN cells with HMGA2 overexpression exerted opposite effects[6]. These *in vitro* experimental data suggested that HMGA2 plays an important role regulating the growth and metastasis behavior of RCC cells through EMT, which biological process is of particular importance for its potential effect on local and distal dissemination of tumor cells.

Although in the present study, we only analyzed the tumor growth and a few EMT markers in the xenograft tumors, it provides a potential tool for future investigations involving more molecular pathways. For example, using the same model, high throughput RNA sequencing techniques would allow us to identify more genes being regulated by HMGA2 (e.g., by HMGA2 shRNA treatment) in cultured ACHN cells as well as in xenografted ACHN tumors. Another major limitation of the present study is that only one type of RCC cell line was used and a minimal number of mice with xenograft tumor were investigated. More insights could be gained by analyzing multiple cell lines as well as human specimens in relation to the expression of HMGA2 and EMT-related gene in our future prospective study.

Conclusions:

In the present study, we performed an *in vivo* experiment using ACHN cells treated with HMGA2 shRNA grown as xenografts in nude mice. Our results showed that silencing of HMGA2 gene led to decreased xenograft tumor growth and decreased EMT activation (increased E-cadherin and decreased N-cadherin and Snail expression) in xenograft tumors, suggesting that the regulation role by HMGA2 in vitro also exists *in vivo*.

List of Abbreviations: XXXXX

high-mobility group AT-hook 2 (HMGA2)

renal cell carcinoma (RCC)

small hairpin RNA (shRNA)

small interfering RNA (siRNA)

epithelial-mesenchymal transition (EMT)

RNA interference (RNAi)

Real Time Polymerase Chain Reaction (RT-PCR)

Bicinchoninic acid (BCA)

Tumor node metastasis (TNM)

Declarations:

Ethics approval and consent to participate

The animal procedures described in the study were approved and carried out following the guideline for the care and use of laboratory animals by the Ethics Committee of the Affiliated Zhongshan Hospital of Dalian University.

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article. Should there be any further requests and questions, the data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

GL, LS, GG performed experiment and data analysis. GL, LS and GG performed animal experiments. GL and GG performed in vitro experiments. GG wrote manuscript. QC, KL and XL contributed to data analysis and discussion. YD reviewed data analysis. YD and YL supervised research and wrote the final manuscript. All authors contributed to result discussion and approved final version.

Acknowledgements

Not applicable.

Figures and Figure Legends

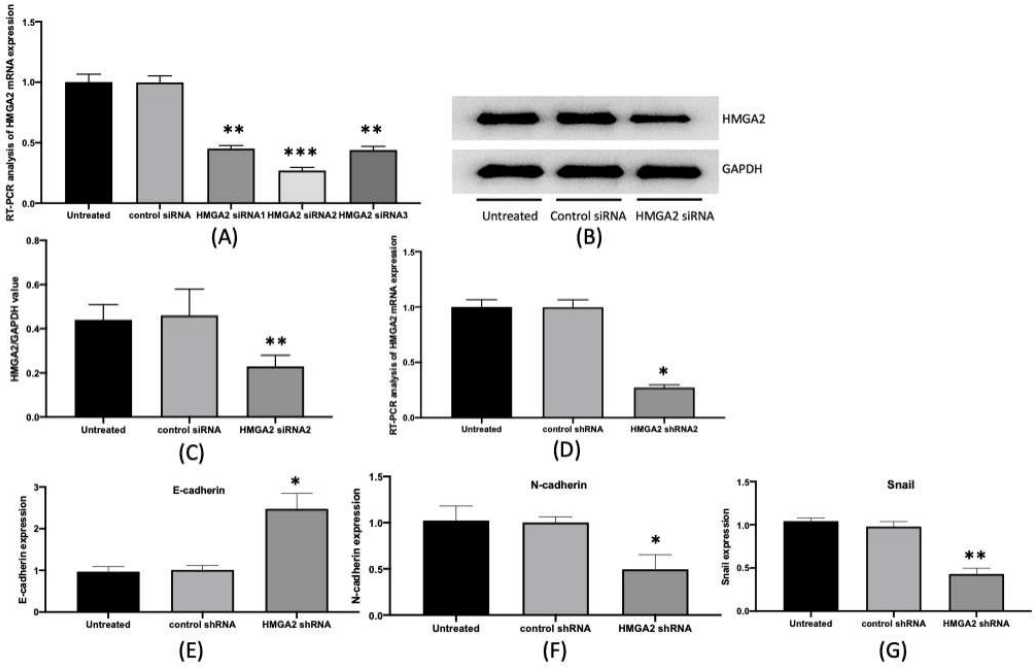

Figure 1: RT-PCR and western blotting results validated the efficacy of shRNAs to silence the expression of HMGA2 gene in ACHN cells. A: RT-PCR analysis of the HMGA2 mRNA expression in ACHN cells receiving either a scrambled control shRNA or a shRNA (#1-#3) specific to HMGA2 gene (statistical significance with $p<0.05$, $p=0.002$, $p<0.001$, $p=0.002$ for the three siRNA 1-3). B: Western blotting analysis of the HMGA2 and GAPDH protein expression in ACHN cells receiving HMGA2 siRNA2. C: quantification was performed by normalizing the protein expression level of HMGA2 against the level of the house-keeping gene GAPDH ($p=0.002$ **). D: the mRNA expression level of HMGA2 in stable-transfected and puromycin-selected ACHN cells ($p=0.0132$ *). This is done before xenograft modeling. E-G): the mRNA expression level of EMT markers E-cadherin, N-cadherin and Snail in cultured ACHN cells ($p=0.0132$ *, 0.0102 *, 0.0024 ** , for the three groups). Data were expressed as mean +/- standard deviation. For each group, data were obtained from three independent repeat experiments, with triplicate samples in each repeat. Statistical significance was calculated using Kruskal-Wallis test.

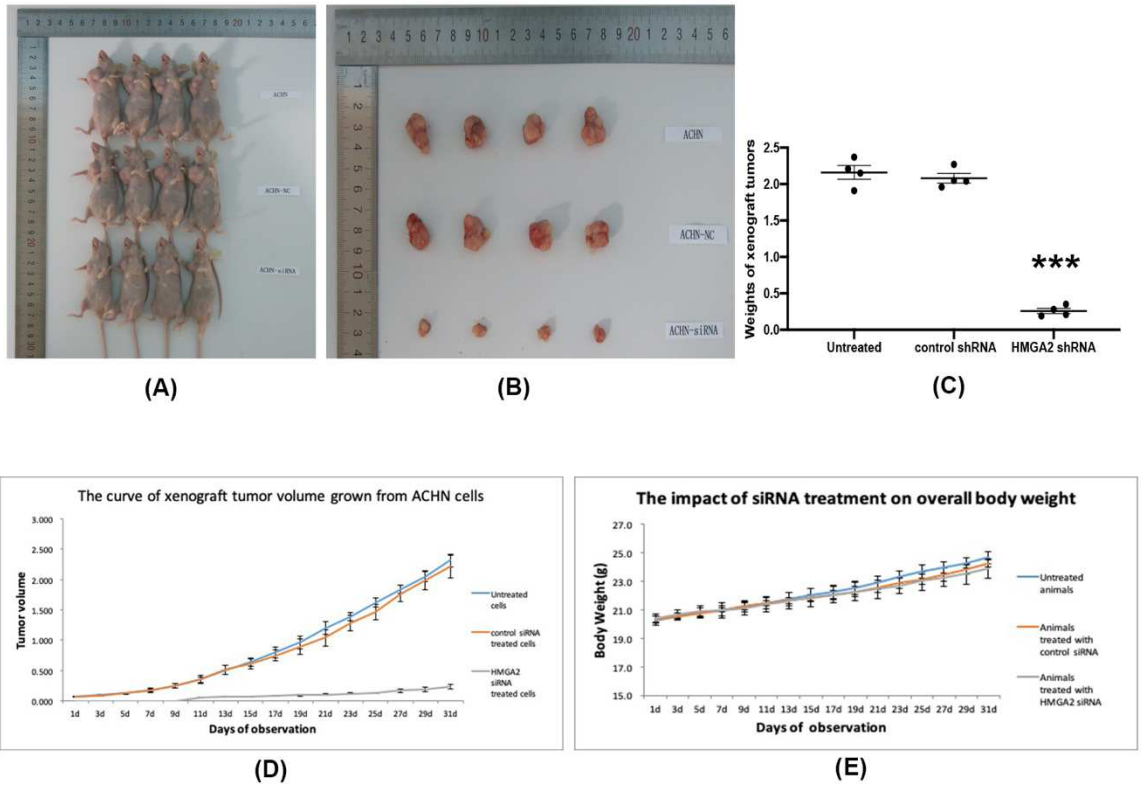

Figure 2: Xenograft tumors of untreated ACHN cells (group 1), ACHN cells treated with scrambled control shRNA (group 2) and cells treated with HMGA2-specific shRNA (group 3) in 4 weeks old female BALB/c nude mice. Tumors began to be observable 18 days after injection of 4 X 10^6 ACHN cells into the right axillary region of mice and the observation was maintained for 30 days until necropsy. A: pictures of nude mice receiving the above described treatments. B: pictures of xenograft tumors harvested at the time of necropsy. C: quantification of the average tumor sizes of three treatment groups. D: growth curves of xenograft tumors with size being recorded each other day for 30 days. E: curve of the body weight changes of the mice of the three treatment groups.

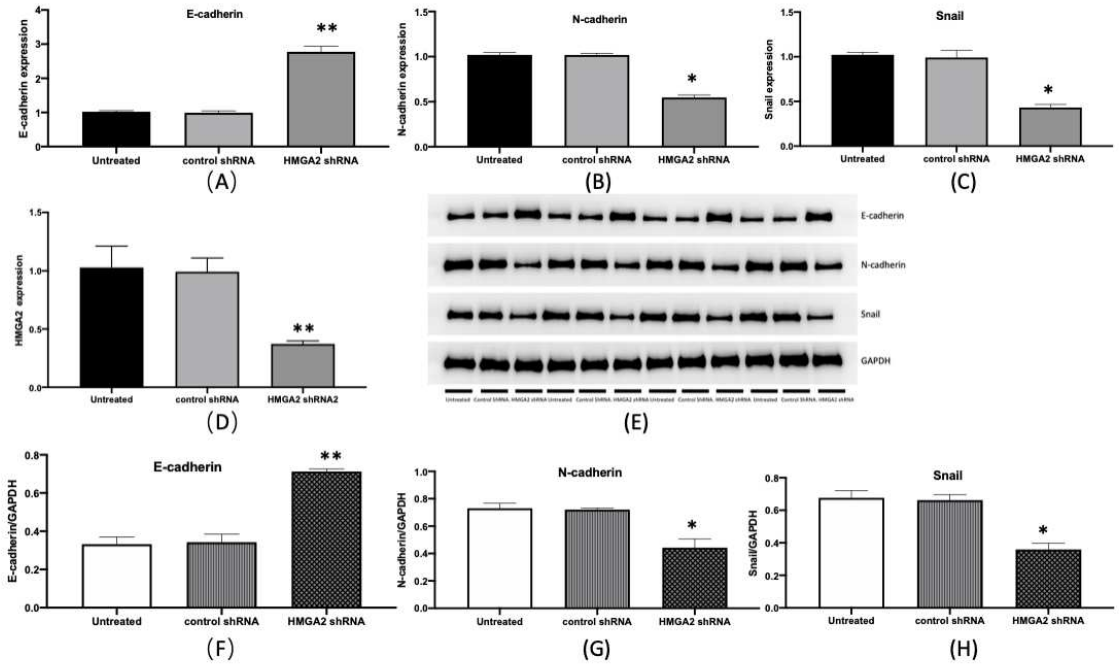

Figure 3: RT-PCR and western blotting analysis of E-cadherin, N-cadherin and Snail expression in xenograft tumors of untreated ACHN cells, ACHN cells treated with scrambled control shRNA and cells treated with HMGA2-specific shRNA. A-D: mRNA expression of E-cadherin (p=0.0024 **), N-cadherin (p=0.0132 *), Snail (p=0.0107 *) and HMGA2 (p=0.002 **). E: western blot gel pictures of E-cadherin, N-cadherin, Snail and GAPDH. F-H: quantification of E-cadherin (p=0.0066 **), N-cadherin (p=0.0107 *) and Snail (p=0.0127 *) protein expression under three treatment conditions. Normalization was performed by comparing the protein expression level of the above describe genes against the level of the house-keeping gene GAPDH (statistically significance with $p<0.05$). Data was present as mean value +/- standard deviation, Statistical significance was calculated using Kruskal-Wallis test.

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Figures

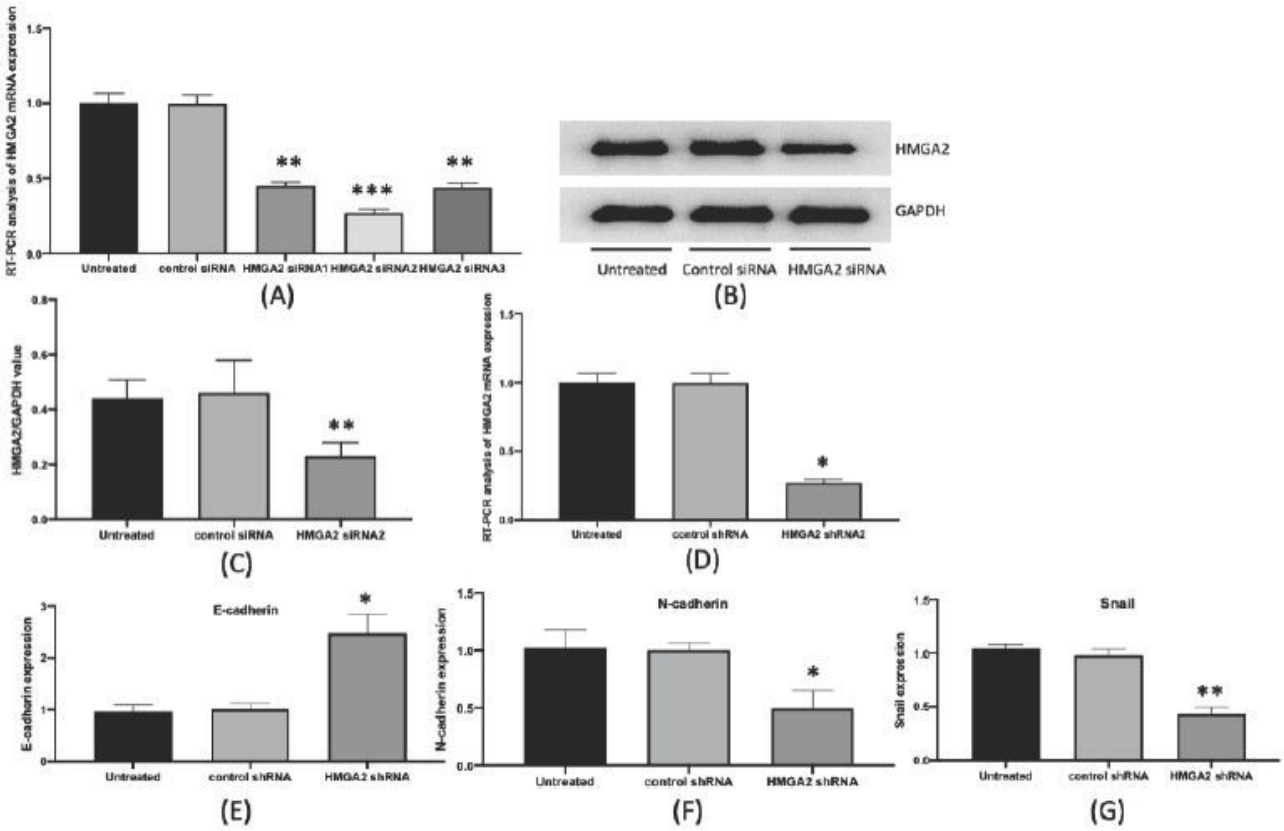

Figure 1

RT-PCR and western blotting results validated the efficacy of shRNAs to silence the expression of HMGA2 gene in ACHN cells. A: RT-PCR analysis of the HMGA2 mRNA expression in ACHN cells receiving either a scrambled control shRNA or a shRNA (#1-#3) specific to HMGA2 gene (statistical significance with $p<0.05$, $p=0.002$, $p<0.001$, $p=0.002$ for the three siRNA 1-3). B: Western blotting analysis of the HMGA2 and GAPDH protein expression in ACHN cells receiving HMGA2 siRNA2. C: quantification was performed by normalizing the protein expression level of HMGA2 against the level of the house-keeping gene GAPDH ($p=0.002$ **). D: the mRNA expression level of HMGA2 in stable-transfected and puromycin-selected ACHN cells ($p=0.0132$ *). This is done before xenograft modeling. E-G): the mRNA expression level of EMT markers Ecadherin, N-cadherin and Snail in cultured ACHN cells ($p=0.0132$ *, 0.0102 *, 0.0024 ** , for the three groups). Data were expressed as mean +/- standard deviation. For each group, data were obtained from three independent repeat experiments, with triplicate samples in each repeat. Statistical significance was calculated using Kruskal-Wallis test.

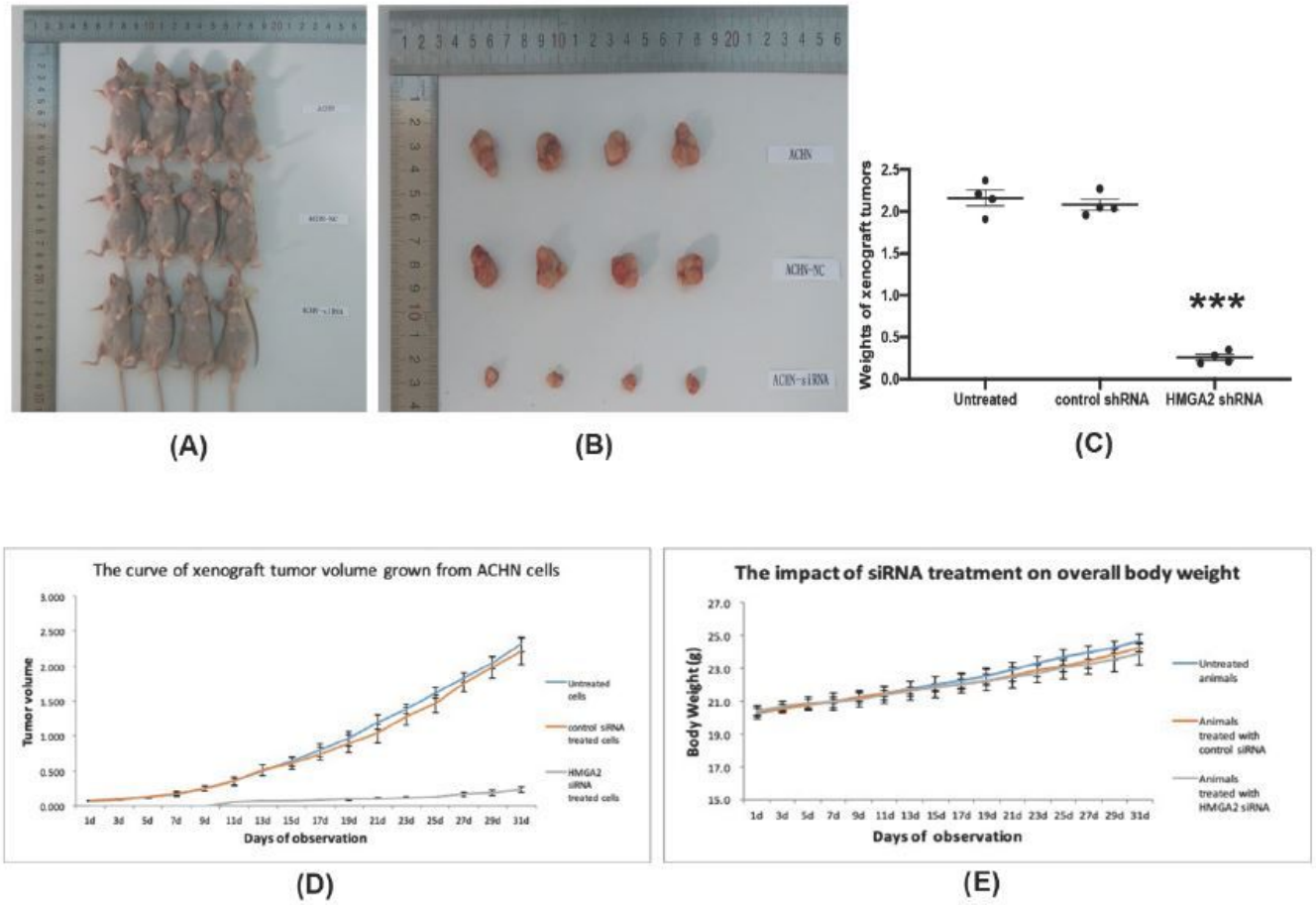

Figure 2

Xenograft tumors of untreated ACHN cells (group 1), ACHN cells treated with scrambled control shRNA (group 2) and cells treated with HMGA2-specific shRNA (group 3) in 4 weeks old female BALB/c nude mice. Tumors began to be observable 18 days after injection of 4 X 106 ACHN cells into the right axillary region of mice and the observation was maintained for 30 days until necropsy. A: pictures of nude mice receiving the above described treatments. B: pictures of xenograft tumors harvested at the time of necropsy. C: quantification of the average tumor sizes of three treatment groups. D: growth curves of xenograft tumors with size being recorded each other day for 30 days. E: curve of the body weight changes of the mice of the three treatment groups.

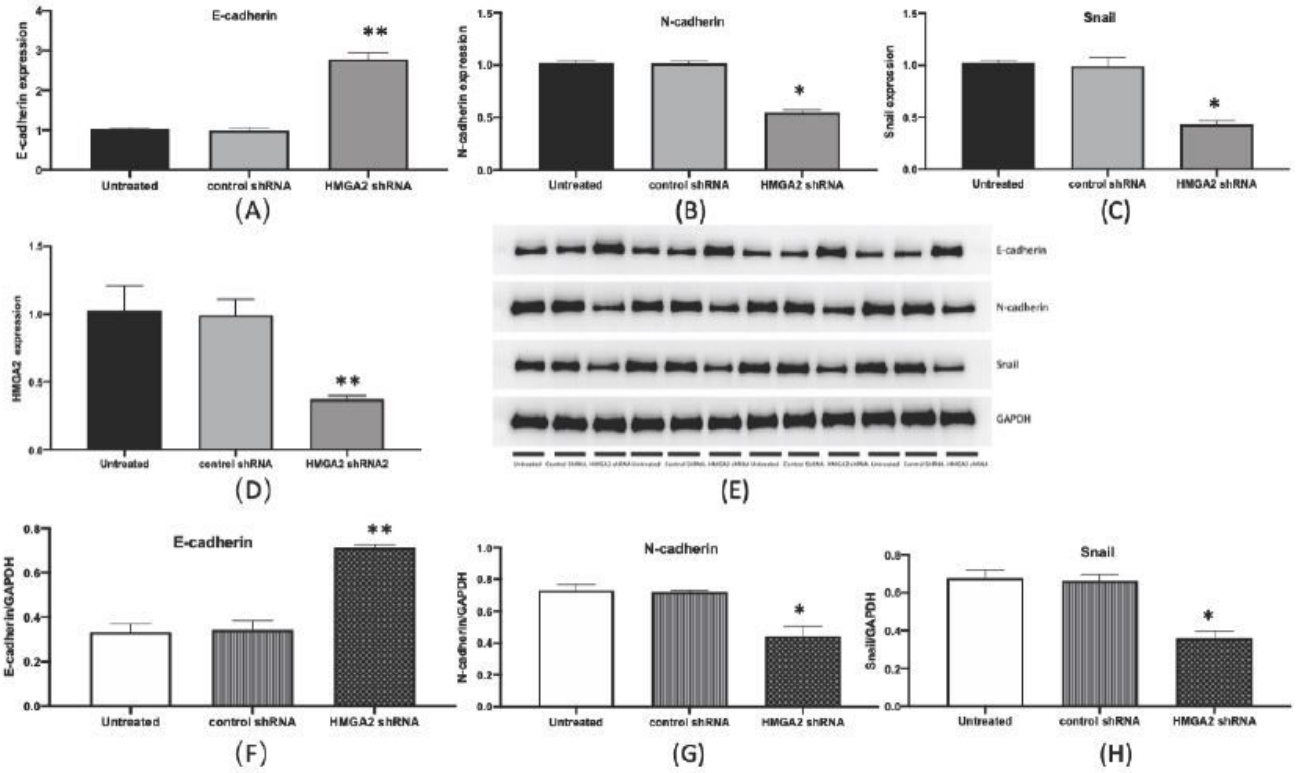

Figure 3

RT-PCR and western blotting analysis of E-cadherin, N-cadherin and Snail expression in xenograft tumors of untreated ACHN cells, ACHN cells treated with scrambled control shRNA and cells treated with HMGA2-specific shRNA. A-D: mRNA expression of E-cadherin (p=0.0024 **), N-cadherin (p=0.0132 *), Snail (p=0.0107 *) and HMGA2 (p=0.002 **). E: western blot gel pictures of E-cadherin, N-cadherin, Snail and GAPDH. F-H: quantification of E-cadherin (p=0.0066 **), N-cadherin (p=0.0107 *) and Snail (p=0.0127 *) protein expression under three treatment conditions. Normalization was performed by comparing the protein expression level of the above describe genes against the level of the house-keeping gene GAPDH (statistically significance with $p<0.05$). Data was present as mean value +/- standard deviation, Statistical significance was calculated using Kruskal-Wallis test.