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Original Article

The long noncoding RNA CASC2 inhibits pituitary adenoma progressionby inhibiting HMGA2 expression

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Abstract: Accumulating evidence has demonstrated that long non-coding RNAs (lncRNAs) actively participate in various biological processes through diverse mechanisms. Abnormal expression of lncRNAs is usually associated with oncogenesis. However, how Cancer Susceptibility Candidate 2 (CASC2) is involved in pituitary adenoma regulation remains largely unknown. In current study, we have found a tumor suppressive function of CASC2 in pituitary adenoma. CASC2 expression is downregulated especially in invasive pituitary adenoma. Patients with high CASC2 expression exhibited better overall survival. CASC2 also had a diagnostic value to differentiate invasive and non-invasive pituitary adenoma. Furthermore, increasing CASC2 expression may inhibit viability and invasion in AtT-20 and GT1-1 cells as well as xenograft tumor growth as indicated by Ki-67 staining. Mechanistic study argued that CASC2 possibly mediated tumor suppression by inducing apoptosis. CASC2 also decreased the expression of high-mobility group A2 (HMGA2), which is frequently upregulated in pituitary adenoma. Our results collectively suggested a tumor suppressive role of CASC2 in pituitary adenoma with promising diagnostic and prognostic values.

Keywords: CASC2, pituitary adenoma, HMGA2, diagnosis, prognosis

Introduction

The pituitary adenomas (PAs) belong to one of the most common intracranial tumors (~25%) [1]. Pituitary adenomas are usually benign neoplasms with invasive capacity but the progression to carcinomas is relatively rare [2]. It is well known that about one third of total pituitary adenomas are clinically non-functioning pituitary adenomas (NFPAs), with no significant signs of hormone hypersecretion [3]. The incidence rate on average of pituitary adenoma is reported to be around 80~90/100,000 in population [4]. However, this rate is rapidly escalated during the last decade. Recent data also argued that pituitary adenoma has been usually found during diagnosing nasopharyngeal disease or head injury and the incidencerate is over 20% [5]. Although pituitary adenoma is generally regarded as a benign tumor, some pituitary adenomas displayed invasive potential with malignant phenotypes capable of invading into such as bone, suprasellar, parasellar or sphenoid sinus and even nerves [5]. Such pituitary adenomas are regarded as invasive pituitary adenoma (IPA). Clinical treatment of invasive pituitary adenoma is extremely difficult possibly owing to incomplete removal during surgery. Furthermore, the recurrence rate is usually high (21~86%) [6]. Therefore, mechanistic studies for the progression of IPA are strongly required in an attempt to early diagnosis and effective pharmacological treatment.

Genomic data by sequencing have demonstrated that the human genome is characterized by large regions of non-coding RNAs. The long non-coding RNAs (lncRNAs) belong to series of RNAs ranging from 200 to 100,000 nucleotides with no protein coding capacities [7, 8]. The lncRNAs may play critical roles in diverse biological processes such as development, angiogenesis and differentiation while altered or aberrant expression of lncRNAs has been significantly correlated with oncogenesis [9-11]. Multiple studies have been performed to investigate the function of lncRNAs in various tumor types. For instance, the lncRNA MALAT1 can

promote the tumorigenesis in osteosarcoma [12]. Furthermore, a combinatorial use of lncRNA MEG3, HOTAIR and MALAT-1 can serve prognostic markers in non-functional pituitary adenomas (NFPA). The lncRNA PVT1 was reported to play a positive role in the progression of thyroid cancer by recruiting EZH2 [13]. Serum expression of lncRNA-AF085935 can serve as a diagnostic biomarker for discriminating hepatocellular carcinoma (HCC) patients from HBV infection and healthy ones [14].

The lncRNA Cancer Susceptibility Candidate 2 (CASC2), which is located at chromosome 10q26, is a recently found lncRNA with critical association with tumor development. Reduced expression of CASC2 has been demonstrated in various tumor types suggesting that CASC2 may display tumor suppressive function [15-18]. However, how CASC2 fulfill its role in pituitary adenoma remains largely elusive.

In current work, we evaluated the role of CASC2 in PA and found that CASC2 was markedly downregulated in PA samples. Meanwhile, CASC2 was also decreased in PA cell lines compared with normal pituitary. Furthermore, CASC2 expression had a high diagnostic value to differentiating between IPAs and non-IPAs. Meanwhile, the patients with lower CASC2 expression had a relatively poor prognosis. We also found that knocking down CASC2 expression in PA cell lines AtT-20 and GT1-1 can increase the viability and invasion. The tumor suppressive effect of CASC2 may partially be ascribed to its capacity of inducing apoptosis. We argued that CASC2 may inhibit PA progression by regulating HMGA2 expression. The *HMGA2* expression was also negatively correlated with CASC2 ($R = -0.7689$, $P < 0.0001$). The xenograft tumors using AtT-20 cells with si-CASC2 also showed increased Ki-67 staining. Collectively, these results suggested that-CASC2 might bea tumor suppressor and shed light on the diagnostic value of CASC2 in pituitary adenoma.

Materials and methods

Cell culture

The PA cell lines used in current study (αT3-1, AtT-20 and GT1-1) was purchased from Shanghai Institute of Cell Biology. The PA cells were cultured in Dulbecco's modified Eagle's medium (Sigma, Shanghai, China) with 5 mM L-glutamine, 5 g/L glucose supplemented with 0.01 mM nonessential amino acids and 5% fetal calf serum (FCS, Sigma, Shanghai, China) in 5% CO_2 at 20°C.

Collection of human specimens

Matched normal pituitary specimens and PA tissues were collected from 72 patients who have undergone resection at The People's Hospital of Leshan between February 2009 and March 2011. Immediately after surgical resection, human specimens were stored at -80°C until usage in current study. None of patients have received preoperative chemotherapy or radiotherapy. We have obtained formal consent forms from all patients. The research and protocols related to human specimens were formally approved by the Ethics Committee of The People's Hospital of Leshan.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was harvested from PA cell lines (AtT-20 and GT1-1) and human specimens with TRIzol reagent (Thermo Fisher Scientific, Inc.). 5 μg cDNA generated by reverse transcription was obtained using the miScript II RT kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The expression of CASC2 was measured using *Taq*Man microRNA RT-qPCR kit (Applied Biosystems, Shanghai, China). *GAPDH* was used as the control.The PCR was carried out using ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, Shanghai, China) according to the manufacturer's protocols. The primer sequences were as follows: *CASC2,* forward 5'-TGCACGACGACAGTT-3' and reverse 5'-TAGAGCCGCCCTGCT-3'; *GAPDH*, forward 5'-CTCGATGCATCGACATCGT-3' and reverse 5'-ACGCTTCGCGATCGTGTGAT-3'; *HMGA2*, forward 5'-CATACAATAGCTTGCTGAT-3' and reverse 5'-GTATCTCCTGCTACGAGAG-3'.

CASC2 knockdown and transfection

The CASC2cDNA was amplified by PCR and cloned into the pCDNA3.1 vector (TIANGEN, Shanghai, China). The empty pcDNA3.1 plasmidsand pcDNA3.1 plasmids containing CASC2 were synthesized by and purchased from Sigma (Shanghai, China). Nonspecific si-RNA was

used as a negative control (si-NC). The CASC2 small interfering RNA (si-CASC2) and si-NC were obtained from Sigma (Shanghai, China). Transfections were performed using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions.

Invasion assay

The upper chamber of plates was coated with Matrigel (Invitrogen; Thermo Fisher Scientific, Inc.) overnight. AtT-20 and GT1-1 cells were then re-suspended 36 hours after transfection and loaded into the upper chamber (10^5 cells/well) in RPMI-1640 medium (Sigma, Shanghai, China). The lower chambers were supplemented with RPMI-1640 medium with additional 5% FBS. Following a 24 h's incubation, the upper chambers were removed and those cells which migrated into lower chambers were fixed with 4% polytetrafluoroethylene (PFA) and stained with crystal violet. The Transwell assay wasmonitored under a Leica fluorescent microscope (DM-IRB; Leica Microsystems GmbH, Wetzlar, Germany). Experiments were performed in triplicates. Invasion was normalized to the results under the control condition.

Cell viability assay

The Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used. Following transfection for 36 h, AtT-20 and GT1-1 cells were re-suspended and seeded into a 12-well plate (10^5 cells/well). The viability was monitored once a day for a total of 5 days. Crystalline formazan was dissolved in 100 μl 20% sodium dodecyl sulfate (SDS) solution for 12 h and the optical densityat 490 nm (O.D. 490) was measured using the Spectramax M5 microplate monitor (Molecular Devices, Sunnyvale, CA, USA) following the manufacturer's instructions.

Apoptosis analysis

After transfection for 36 hours, AtT-20 and GT1-1 cells were washed with cold PBS twice. Cells were then fixed with 70% ethanol at 4°C overnight. For apoptosis assay, the transfected cells were examined with a double staining method with FITC-labeled Annexin V/PI Apoptosis Detection Kit (Beyotime, Shanghai, China) following the manufacturer's protocols. Flow cytometry was performed immediately after double staining. Data analysis was carried out usingfluorescence-activated cell sorting (FACS) (BD Bioscience, Mansfield, MA, USA).

Western blot

The AtT-20 and GT1-1 cells were harvested using lysis buffer (15% glycerol and 3% NP-40) from Sigma (Shanghai, China). The protein extracts were electrophoresed using 10% SDS-PAGE and migrated to nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules CA, USA). The membrane was coated with anti-GAPDH antibody (Sigma, Shanghai, China) 4°C overnight. The horseradish peroxidase-conjugated secondary antibodies (Sigma, Shanghai, China) were then added and incubated at 20°C for 1.5 h. Immunoblots were visualized with an Image Quant™ LAS 4000 mini-biomolecular imager (Fujifilm, Tokyo, Japan) and quantified using Image J (NIH).

In vivo implantation and immunohistochemistry

Transfection of AtT-20 cells was performed using lenti-virus transfection system. The system was initially maintained for 12 h. Then, cells were re-suspended and totally ~5×10^5 cells were injected subcutaneously into the nude mice (Age, 5-6 weeks; average weight, 16.5 g). Mice were housed at ~20°C, 50-55% humidity, light-dark cycle of 12 h. A*d libitum* access to food and water was provided. 30 days later, mice were sacrificed by sodium amobarbital overdose (300 mg/kg with intraperitoneal injection). Ki-67 immunostaining was performed using the Ki-67 ELISA kit (Sigma, Shanghai, China). The animal procedures conformed to the Guidelines for Animal Experiments and were approved by the Animal Research Committee of The People's Hospital of Leshan.

Statistical analysis

Results were shown as mean ± SD. Statistical significance was determined using Student's *t*-test (SPSS, version 16.0, Inc., Chicago, IL, USA). Kaplan-Meier survival curve was tested using log-rank test. The receiver operating characteristic (ROC) curve was used to evaluate the diagnostic value of CASC2 on IPA/non-IPA. Fisher exact test was used to evaluate the

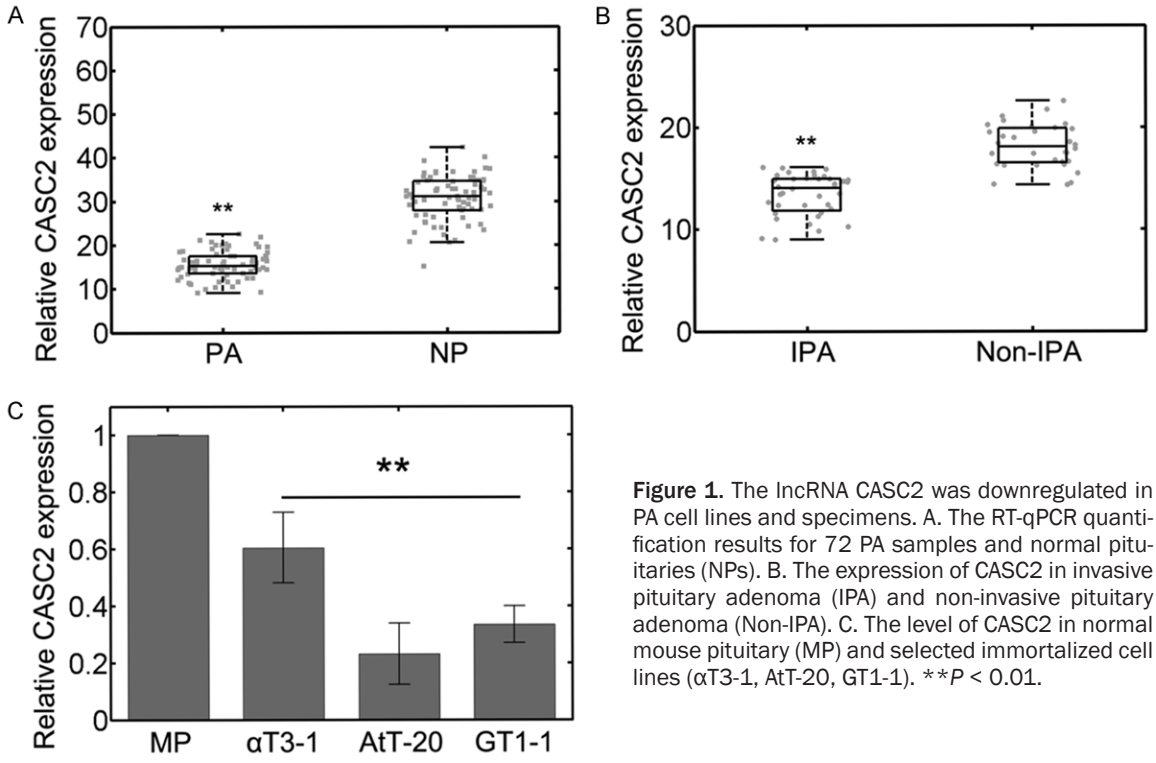

Figure 1. The lncRNA CASC2 was downregulated in PA cell lines and specimens. A. The RT-qPCR quantification results for 72 PA samples and normal pituitaries (NPs). B. The expression of CASC2 in invasive pituitary adenoma (IPA) and non-invasive pituitary adenoma (Non-IPA). C. The level of CASC2 in normal mouse pituitary (MP) and selected immortalized cell lines (αT3-1, AtT-20, GT1-1). $^{**}P < 0.01$.

Table 1. Correlation between CASC2 and clinicopathological factors

Clinicopathological factor	No.	CASC2 expression		*P*
		Low (N, %)	High (N, %)	
Age				
< 50	38	21 (55.3%)	17 (44.7%)	0.479
≥ 50	34	15 (44.1%)	19 (55.9%)	
Gender				
Male	29	16 (55.2%)	13 (44.8%)	0.631
Female	43	20 (46.5%)	23 (53.5%)	
Biological behavior				
Invasive	40	26 (65.0%)	14 (35.0%)	0.009
Non-invasive	32	10 (31.2%)	22 (68.8%)	
Tumor size (cm)				
< 3	42	15 (35.7%)	27 (64.3%)	0.008
≥ 3	30	21 (70.0%)	9 (30.0%)	
Adenoma type				
ACTH	20	13 (65.0%)	7 (35.0%)	0.122
PRL	17	6 (35.3%)	11 (64.7%)	
GH	15	6 (40.0%)	9 (60.0%)	
UF	20	6 (30.0%)	14 (70.0%)	

NF: nonfunctional; ACTH: adrenocorticotrophic hormone; PRL: prolactin; GH: growth hormone.

correlation between CASC2 and clinicopathological factors. All experiments were performed in at least triplicate. Statistical significance was evident if $P < 0.05$.

Results

The lncRNA CASC2 was downregulated in specimens and PA cell lines

To quantify the expression of CASC2, we performed RT-qPCR. We found decreased expression of CASC2 in 72 PA samples compared with that in normal PAs (NP, **Figure 1A**, $P < 0.01$). To discriminate IPAs from non-IPAs, we further classified the PA into IPAs (n = 40) and non-IPAs (n = 32) based on following criteria: (1) destruction of cavernous sinus; (2) Grade III-IV in Hardy-Wilson guideline; (3) infiltration into sellar diaphragm or floor bones. We found that the CASC2 expression was significantly lowered in IPAs compared with that in non-IPAs (**Figure 1B**, $P < 0.01$). Examination in cell lines also confirmed that CASC2 expression was attenuated in cancerous cell lines (**Figure 1C**). These results suggested that CASC2 expression was reduced in PA specimens and cell lines. Since AtT-20 and GT1-1 cells showed relatively lower CASC2 lev-

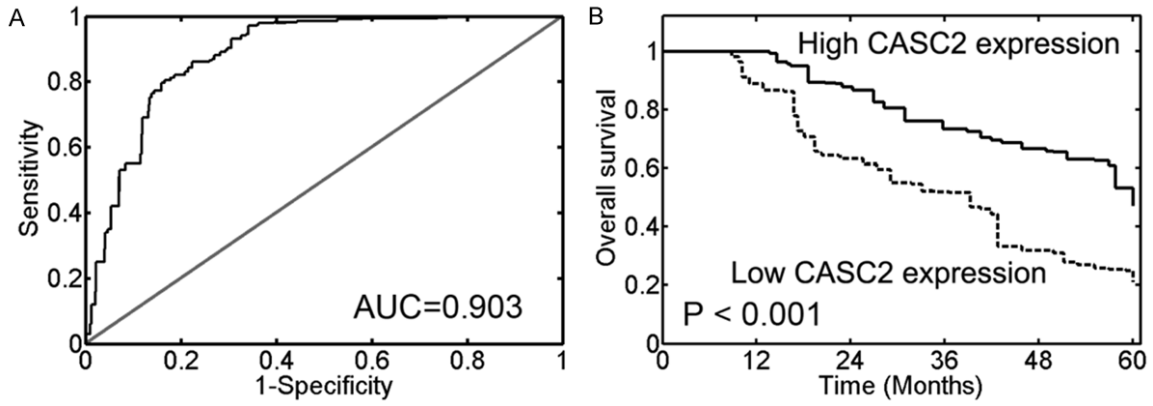

Figure 2. CASC2 inhibits PA progression and may serve as a prognostic marker. A. ROC analysis for diagnostic values of CASC2. The area under curve (AUC) is 0.903. The cutoff value was 20.0596. The sensitivity and the specificity were 81.24% and 83.25%, respectively. B. The Kaplan-Meiersurvival curves for PA patients. The patients with higher CASC2 expression exhibited better survival compared to those withlower CASC2 expression ($P < 0.001$, log-rank test). The median value was used as the cutoff (15.2402).

els, we chose these two cell lines for further study.

Correlation between CASC2 expression and clinical features

We divided the PA specimens into low/high expression groups using the median level as the cut-off line (15.2402). Results suggested that there existed significant correlation between CASC2 expression and IPA/non-IPA group (**Table 1**, $P = 0.009$). We also observed significant association between tumor size and CASC2 levels (**Table 1**, $P = 0.008$). However, we could not find significant relationship between age, gender or adenoma types and CASC2 expression (**Table 1**). These results implied that CASC2 may serve as a critical factor during pituitary adenoma development.

Diagnosis and prognosis value of CASC2 in pituitary adenoma

We calculated the classification accuracy of CASC2 in IPA/non-IPA discrimination in pituitary adenomas. We constructed the ROC curves and the results showed that CASC2 had an AUC value of 0.903 (**Figure 2A**). The cut-off value, positive predictive value (ppv) and the negative predictive value (npv) were 20.0596, 92.11% and 88.24%, respectively. The sensitivity was 81.24% while the specificity was 83.25%. We further evaluated the prognostic value of CASC2 in pituitary adenoma. The survival was evaluated from the day of primary surgery to death or to the last follow-up. The Kaplan-Meier survivalcurve showed that patients with higher CASC2 expression had an increased survival (**Figure 2B**, $P < 0.001$, cutoff value = 15.2402). These results suggested CASC2 may serve as a potential diagnostic and prognostic marker.

CASC2 inhibits viability and invasion by inducing apoptosis

We next performed series of *in vitro* experiments to investigate the role of CASC2 in PA. The overexpression and knockdown efficiency was evident in AtT-20 and GT1-1 cells (**Figure 3A**, $P < 0.01$). We noticed that CASC2 transfection can substantially inhibit viability of AtT-20 cells (**Figure 3B**). The viability was markedly elevated when CASC2 level was knocked down (**Figure 3B**). Qualitatively similar results can be found in GT1-1 cells (**Figure 3C**). CASC2 transfection can further diminish the invasive capacity of AtT-20 cells while lowering CASC2 expression instead promoted the invasion in AtT-20 cells (**Figure 3D**). We also evaluated the effect of CASC2 overexpression or knockdown in GT1-1 cells and we found consistent results (**Figure 3D**). To identify the potential mechanism of CASC2 mediated PA suppression, we investigated the effect of CASC2 on apoptosis in AtT-20 cells. We found that pcDNA-CASC2 transfection greatly enhanced the apoptosis of AtT-20 cells (**Figure 3E**). Meanwhile, reduced CASC2 expression by si-RNA significantly lowered the apoptosis (**Figure 3E**). We

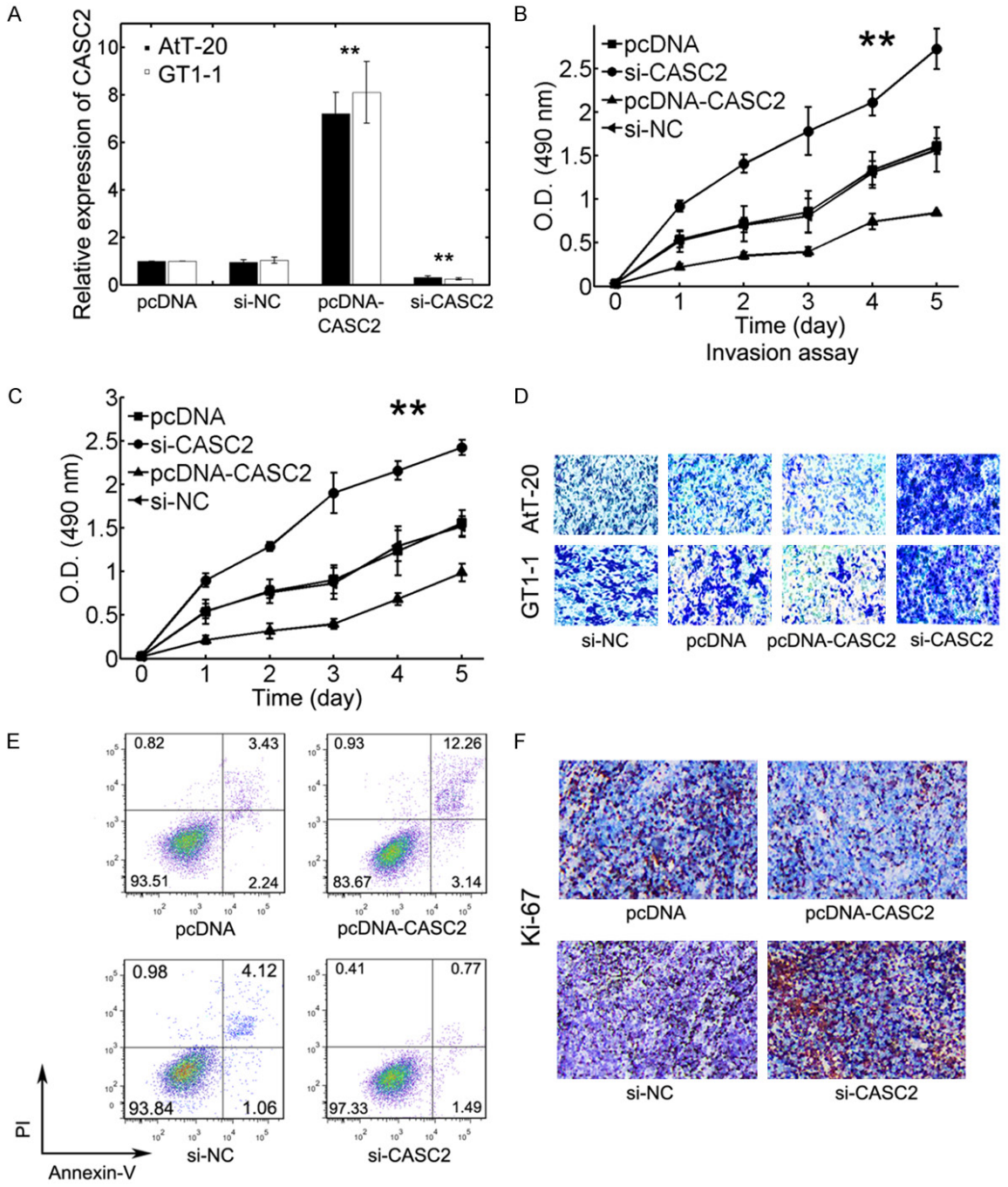

Figure 3. The inhibitoryeffect of CASC2 on PA progression *in vitro* and *in vivo*. (A) The AtT-20 and GT1-1 cell lines were transfected with empty pcDNA plasmid, pcDNA-CASC2 plasmid, si-NC or si-CASC2. Then, the CASC2 expression was quantified using RT-qPCR. **: $P < 0.01$. The comparison was performed with corresponding control group. A five-day viability assay for (B) AtT-20 and (C) GT1-1 cells were transfected with empty pcDNA plasmid, pcDNA-CASC2 plasmid, si-NC or si-CASC2. **: $P < 0.01$. (D) Transwell invasion assays for AtT-20 and GT1-1 cells transfected with empty pcDNA plasmid, pcDNA-CASC2 plasmid, si-NC or si-CASC2. (E) The apoptosis was measured using flow cytometry in AtT-20 cells transfected with empty pcDNA plasmid, pcDNA-CASC2 plasmid, si-NC or si-CASC2. Apoptosis was evaluated 24 hours post transfection. (F) The Ki-67 immunostaining for AtT-20 cells *in vivo* implantation. The AtT-20 cells were transfected with empty pcDNA plasmid, pcDNA-CASC2 plasmid, si-NC or si-CASC2.

next explored whether similar effects can be obtained in *vivo*. The AtT-20 cells were transfected with empty pcDNA, pcDNA-CASC2, si-NC or si-CASC2 for 36 hours. Then, AtT-20 cells

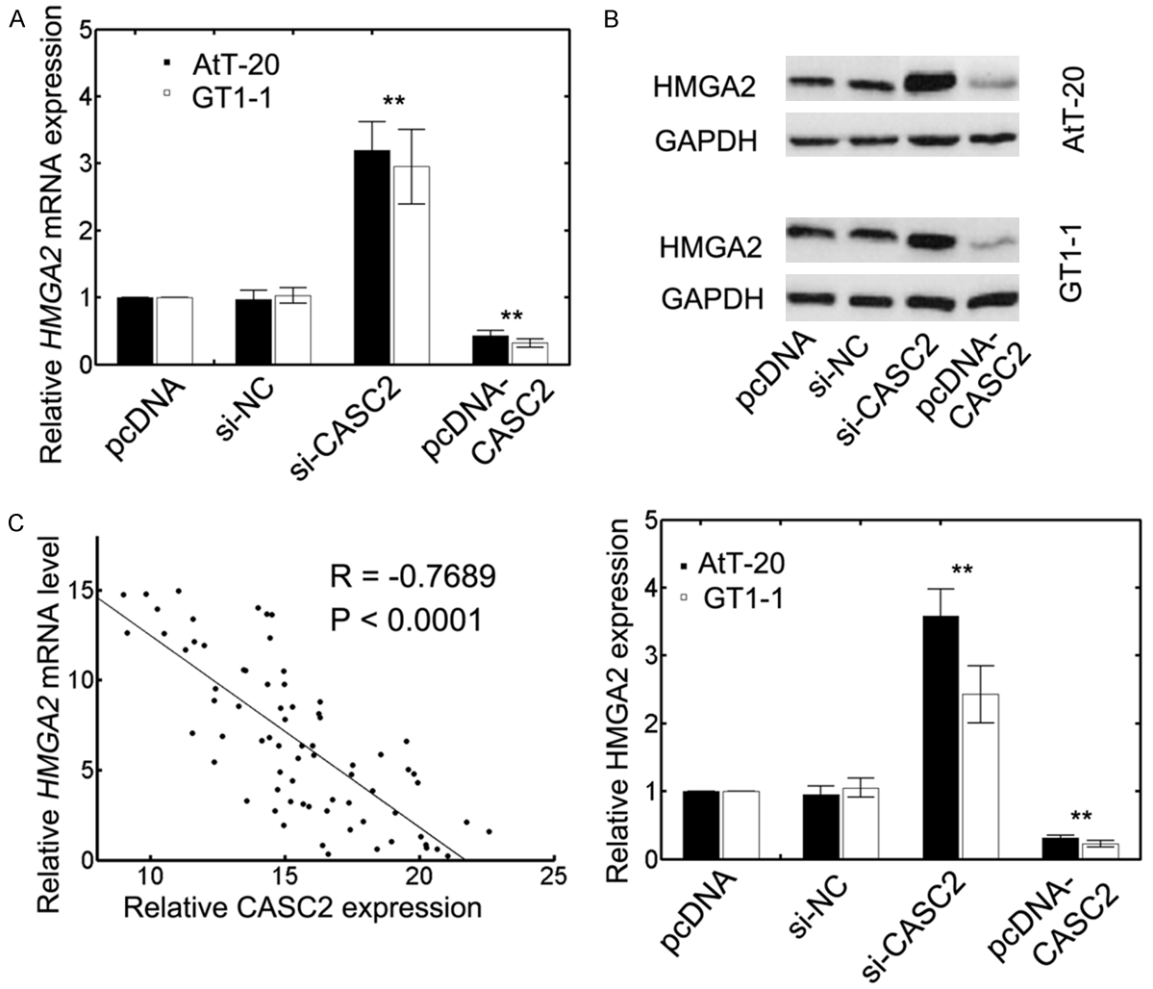

Figure 4. CASC2 inhibits HMGA2 expression. A. Relative *HMGA2* mRNA expression in AtT-20 and GT1-1 cells transfected with empty pcDNA plasmid, pcDNA-CASC2 plasmid, si-NC or si-CASC2. $**P < 0.01$. B. Western blot results for HMGA2 protein expression in AtT-20 and GT1-1 cells transfected with empty pcDNA plasmid, pcDNA-CASC2 plasmid, si-NC or si-CASC2. Quantification results were shown at bottom. $**P < 0.01$. The comparison was performed with corresponding control group. C. Correlation between *HMGA2* mRNA expression and CASC2 in 72 specimens. R = -0.7689, $P < 0.0001$.

were subcutaneously injected into nude mice. After 30 days, the tumor xenograft was resected and subject to Ki-67 staining. We found that-CASC2 overexpression markedly decreased the Ki-67 staining (**Figure 3F**). Instead, CASC2 knock down significantly increased the Ki-67 positive fraction compared with that under control condition (**Figure 3F**). These results suggested that CASC2 may inhibit malignant phenotypes of PA cells both *in vitro* and *in vivo* partially by inducing apoptosis.

CASC2 attenuates pituitary adenoma progression by inhibiting HMGA2 expression

Since HMGA proteins are involvedin cell cycle dysfunction and development of PA [19], we then investigated whether CASC2 can affect HMGA2 expression. Transfection into AtT-20 and GT1-1 cells with pcDNA-CASC2 substantially inhibited *HMGA2* transcript levels (**Figure 4A**). Lowering CASC2, however, markedly enhanced *HMGA2* expression in AtT-20 and GT1-1 cells (**Figure 4A**). The protein level of HMGA2 was consistently upregulated when CASC2 expression was knocked down (**Figure 4B**). Instead, HMGA2 protein expression was abolished with pcDNA-CASC2 transfection (**Figure 4B**). We could also find a significantly negative correlation between *HMGA2* mRNA and CASC2 expression in specimens (**Figure 4C**, R = -0.7689, $P < 0.0001$). These results suggested that CASC2 may contribute to tumor suppression in pituitary adenomaby inhibiting HMGA2 expression.

Discussion

Recently, tremendous advances in biological technology have greatly enriched our understanding about lncRNA profiles. Numerous lncRNAs have been identified to exert diverse functions in various tumors. Owing to the difficulty in curative radical surgery in invasive pituitary adenoma and the deficiency in effective drugs for NFPAs, investigating the molecular mechanisms of pituitary adenoma development has been in urgent need.

In current study, we found that CASC2 was frequently downregulated in pituitary adenoma tissues as well as cell lines. Low CASC2 expression predicted poor overall survival. Meanwhile, higher CASC2 expression displayed multiple *in vitro* effect such as inhibiting viability and invasion. The tumor suppressive role of CASC2 was also confirmed *in vivo*. The inhibitory role of CASC2 on pituitary adenoma was partially ascribed to apoptosis induction. Furthermore, we verified that CASC2 can decrease HMGA2 expression at both mRNA and protein levels. We also observed a significantly negative correlation between CASC2 and *HMGA2* transcripts in specimens. These data support a tumor suppressive role for CASC2 and argue that CASC2 may also serve as a prognostic or prognostic marker.

The lncRNA CASC2 was originally found to be downregulated in endometrial cancer in 2004 implying that CASC2 may suppress tumor progression [20]. Shortly afterwards, there is a report demonstrating that CASC2 expression is markedly reduced in glioma [16]. Recent findings suggest that CASC2 interacts with miR-181a to exert its tumor inhibitory functions in Temozolomide (TMZ) resistant glioma [21]. Cao et al. recently found that the lncRNA CASC2 can directly target miR-21 and suppress the progression of renal cell carcinoma [22]. CASC2 overexpression also inhibits non-small cell lung cancer (NSCLC) development suggesting that CASC2 can act as a prognostic factor [18]. Li et al. consistently found that CASC2 attenuates the proliferation of gastric cancer [23]. Decreased CASC2 expression in colon cancer was also associated with poor survival and advanced TNM stages [17]. In current work, we have identified a tumor suppressive role for CASC2 in pituitary adenoma. It seems that CASC2 might function as a universal tumor suppressor in various tumors. The function and clinical significance of CASC2 in pituitary adenoma has never been investigated in previous work [24]. Our current work may therefore unravel a novel facet for CASC2. Further studies are required to explore the potential function of CASC2 in other types of tumors.

Previous studies found that the high-mobility group A2 (HMGA2) exerts diverse functions in numerous biological processes including differentiation and neoplastic transformation [25]. *HMGA2* transfection can dictate a terminal fate towards developing pituitary adenomas implying that HMGA2 is critically involved in pituitary adenoma progression [19]. Consistently, HMGA2 is usually upregulated in many types of pituitary adenomas and displays negative correlation with tumor suppressor microRNAs [26]. Meanwhile, the clinical significance of HMGA2 overexpression has been described by Qian et al. [27]. Therefore, overexpressing HMGA2 contributes largely to various carcinomas [19, 26]. Given the important role of HMGA2 dysregulation in pituitary adenoma, we hypothesized that CASC2 may affect the expression of HMGA2 at both mRNA and protein levels, which were then verified in experiments (**Figure 4**). However, the underlying mechanism about how CASC2 mediates HMGA2 inhibition remains obscure. Previous studies have implied that CASC2 may interact with either microRNA-18a or microRNA-21a to regulate target gene expression [16, 24]. CASC2 can also function as a competing RNA to sponge miR-18a in colon cancer [17]. Therefore, CASC2 may regulate HMGA2 induction by series of indirect effects possibly being as competing endogenous RNAs. The exact function and mechanism for CASC2 mediated HMGA2 regulation should be explored in future.

In summary, our study has revealed the tumor suppressive function of CASC2 in pituitary adenoma. Our data suggest that CASC2 may function as a prognostic and diagnostic factor at least in pituitary adenoma. Loss of CASC2 expression evidently results in tumor progression while increased CASC2 expression is associated with tumor inhibition. Collectively, we have uncovered a novel function of CASC2 in pituitary adenoma possibly by regulating HMGA2 expression and may shed light on the pharmacological intervention targeting CASC2.

Disclosure of conflict of interest

None.

Authors' contribution

SC, SZ and NDZ conceived the study. SC, SZ, HGW, LY and BL performed the experiments. BL, YX and ZHW analyzed the data. SC and NDZ wrote the paper. All authors have read and approved the final manuscript.

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