RESEARCH ARTICLE

Long Non-coding RNA GAS5 Functions as a Tumor Suppressor in Renal Cell Carcinoma

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Abstract

Background: Renal cell carcinoma (RCC) is a malignancy with a poor prognosis. We aimed to explore whether the expression of Long Non-Coding RNA (LncRNA) growth arrest-specific transcript 5 (GAS5) is associated with RCC genesis. Methods: We selected twelve clinical samples diagnosed for renal clear cell carcinoma and found that the LncRNA GAS5 transcript levels were significantly reduced relative to those in adjacent unaffected normal renal tissues. Results: In addition, expression of GAS5 was lower in the RCC cell line A498 than that in normal renal cell line HK-2. Furthermore, using functional expression cloning, we found that overexpression of GAS5 in A498 cells inhibited cell proliferation, induced cell apoptosis and arrested cell cycling. At the same time, the migration and invasion potential of A498 cells were inhibited compared to control groups. Conclusion: Our study provided the first evidence that a decrease in GAS5 expression is associated with RCC genesis and progression and overexpression of GAS5 can act as a tumor suppressor for RCC, providing a potential attractive therapeutic approach for this malignancy.

Keywords: Renal cell carcinoma - Long non-coding RNAs - GAS5

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Introduction

Renal cell carcinoma (RCC) accounts for approximately 3% of adult malignancies and more than 90% of renal cancers (Chow et al., 1999). It is the third most common urological cancer after prostate and bladder cancer, but it has the highest mortality rate at over 40% (van Spronsen et al., 2005). Clear cell (conventional) carcinoma is the most common subtype of RCC and accounts for approximately 75-80% of these tumors (Soto-Vega et al., 2009). What is worse, the incidence and mortality rates of RCC all over the world are rising each decade (Hollingsworth et al., 2006). Apart from surgery, it is both chemotherapy and radiotherapy resistant (Cho and Chung, 2012). Among patients with RCC, at least one-third of patients are diagnosed with metastatic RCC and an additional 20-40% develop metastases after nephrectomy; however, less than 20% show a 5-year survival rate after surgical treatment (Mevorach et al., 1992; Linehan et al., 1993; Janzen et al., 2003; Ljungberg et al., 2006). Therefore, at present the treatment of RCC, especially metastatic RCC, remains a serious challenge and a major health problem. A more clear understanding of the pathogenesis of RCC is required for developing new target therapies and biomarkers that predict treatment efficacy.

Eukaryotic genomes encode numerous long non coding RNAs (LncRNAs), which is defined as endogenous cellular RNAs with length longer than 200 nucleotides, but lack open reading frames of significant length (less than 100 amino acids) (Gutschner and Diederichs). LncRNAs are initially thought to be the "dark matter" of the genome. In recent years, they have emerged as an integral function component of the mammalian transcriptome (Birney et al., 2007; Kapranov et al., 2010). LncRNAs play an important role in regulating gene expression at various levels, e.g., chromatin modification, transcription and post-transcriptional processing (Mercer et al., 2009; Wilusz et al., 2009). A large number of LncRNAs are specifically expressed during embryonic stem cell differentiation, pathogenesis or tumorigenesis (Nie et al., 2010). In recent years, due to the successful application of different new approaches such as genome-wide gene expression screen, genomewide association studies, region-targeted association assay and conventional linkage screen, designed LncRNA array, RIP-RNA sequencing as well as transgenic expression and gene knockdown/ knockout, the functions of LncRNAs in cancer are increasingly characterized. Accumulating data show that many identified LncRNAs are crucial players in a variety of tissue carcinogenesis, invasion, and metastasis (Huarte and Rinn, 2010; Tsai et al., 2011). According to their functions, LncRNAs can be roughly divided into oncogenic and tumor-suppressor groups.

GAS5 (Growth Arrest-Specific Transcript 5) is originally isolated from NIH3T3 cells using subtraction hybridization (Schneider et al., 1988). GAS5 transcripts

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Table 1. Patients Characteristics

Factor		Number
Age	mean	55
	range	41-69
Sex	male	6
	female	6
Stage	T1+T2	7
	T3	5
Fuhrman grade	G1	2
	G2	7
	G3	3
Early recurrence	YES	4
	No	8

display many different patterns of alternate splicing but its putative open reading frame is small and poorly conserved during even relatively short periods of evolution (Muller et al., 1998; Raho et al., 2000). It has therefore been deduced that any important biological activity of GAS5 must be mediated through the introns, which encode multiple small nucleolar RNAs (snoRNAs) (Smith and Steitz, 1998). A large number of studies demonstrate that GAS5 is a tumor-suppressor LncRNA. GAS5 expression can induce growth arrest and apoptosis independently of other stimuli in some prostate and breast cancer cell lines (Mourtada-Maarabouni et al., 2009). Down-regulation of GAS5 by RNA interference protects both leukemic and primary human T cells from the anti-proliferative effect of Rapamycin (Mourtada-Maarabouni et al., 2010). Breast cancers show a significantly lower GAS5 expression compared with normal breast epithelial tissues (Mourtada-Maarabouni et al., 2009). In addition, genetic aberrations of the GAS5 locus have been found in many types of tumors including melanoma, breast and prostate cancers but their functional significance still needs to be established (Smedley et al., 2000; Nupponen and Carpten, 2001; Morrison et al., 2007).

In a preliminary study, we found the expressional level of GAS5 in RCC specimens was obviously lower than that in adjacent normal tissues. This provides a clue that it may have some relationship between GAS5 and occurrence of RCC, and GAS5 may be used as a therapeutic target for RCC treatment. Thus, we introduced GAS5 into a human RCC cell line A498 cell and investigated its roles in tumor progression by in vitro assays. This study demonstrate that overexpression of the LncRNA GAS5 can function as a tumor suppressor in RCC.

Materials and Methods

Clinical samples

All human studies have performed in accordance with “Recommendations on the Establishment of Animal Experimental Guidelines” approved at the 80th General Assembly of the Japanese Science Council in 1980, and the principles set out in the Declaration of Helsinki 1964 as modified by subsequent revisions. Twelve patients diagnosed for renal clear cell carcinoma at Masaryk Memorial Cancer Institute (Brno, Czech Republic) between 2009 and 2012 were included in this study. Patients’ ages ranged from 41 to 69 years, with a median of 55. Histological diagnosis was established according to the guidelines of the World Health Organization. Cases were selected according to tissue availability and were not stratified for any known preoperative or pathological prognostic factor. Clinical follow-up data in the form of annually assessed survival time was available for all patients. The median follow-up time for all cases was 40 months (range, 3 -105 months). Clinical characteristics of the patients are summarized in Table 1.

Cell culture

We used a human RCC cell line A498 (derived from papillary RCC) and a nonmalignant renal cell line HK-2 obtained from the American Type Culture Collection (Manassas, VA, USA). The cell lines were incubated in RPMI 1640 medium supplemented with 10% fetal bovine serum and maintained in a humidified incubator (5% CO_2) at 37 °C.

Plasmid construction and cell transfection

Oligonucleotide primers containing BamHI or HindIII site were synthesized, respectively, for amplification of coding sequence (CDS) of GAS5 (Accession No. AF_314752). The two primers were: 5’-CGCGGATCCGTGCTGGGTGCAGATGCAGTGTG gc-3’ (sense) and 5’- CCGCTCGAGTTTTTTTTTTTTTT TTTTTTTTT-3’ (anti-sense). The PCR conditions were: 5 min at 94 °C for hot start, followed by 35 cycles of 45 s at 94 °C, 45 s at 60 °C, and 60 s at 72 °C, with a final extension of 10 min at 72 °C. The length of PCR product was 687 bp. PCR product was excised with BamHI and HindIII and cloned into pcDNA3.1 (+). The new vector was named pcDNA3.1-GAS5. The insert sequences were confirmed by DNA sequencing.

Cell transfection was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as described by the manufacturer. Briefly, A498 cells were plated and grown to 75-90% confluence without antibiotics. Then they were transfected with 1 ng pcDNA3.1-GAS5 for 24 h. In the following, A498-GAS5 group means A498 cells transfected with pcDNA3.1-GAS, and A498-pcDNA group means A498 cells transfected with vector pcDNA3.1 only.

RNA isolation and quantitative real-time PCR

Total RNAs from RCC tissues and cells were extracted as described (Liang and Pardee, 1992). Complementary DNA (cDNA) was synthesized according to the manufacturer’s protocol. The expression of GAS5 was measured by q-PCR, which was performed by the ABI7500 system (Applied Biosystems, CA, USA) and SYBR green premix (TaKaRa Biotechnology, Dalian, China). The expression of β-actin was also detected as the endogenous control, and all the samples were normalized to human β-actin according to the 2-ÄÄCT method. The primer sequences tested in this study were listed as follows: 5’- CTTCTGGGCTCAAGTGATCCT -3’ (sense) and 5’- TTGTGCCATGAGACTCCATCAG-3’ (antisense) for GAS5; 5’-TTGTTACAGGAAGTCCCTTGCC-3’ (sense) and 5’-ATGCTATCACCTCCCCTGTGTG-3’ (antisense) for β-actin. Each experiment was repeated three times.

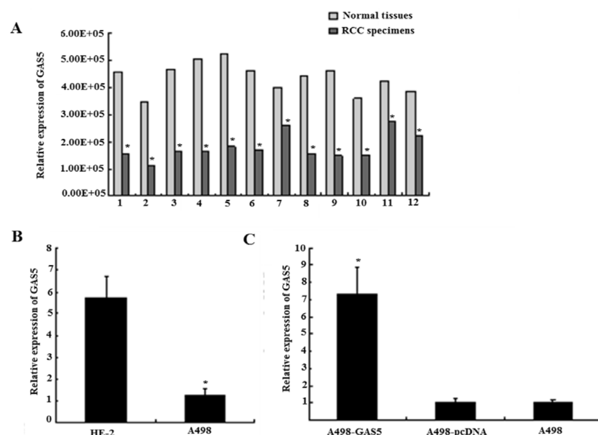

Figure 1. Real Time PCR Analysis of GAS5 Expression in RCC Tissues (A), A498 cells (B), and A498-GAS5 cells (C). A. Expression of GAS5 was significantly down-regulated in 12 RCC specimens compared with matched normal samples (*p<0.05). B. Expression of GAS5 was significantly lower in A498 cells than that in HE-2 cells (*p<0.05). C. Expression of GAS5 in A498 cells after transfected with pcDNA-GAS5 was drastically increased in comparison with A498-pcDNA group and non-transfected A498 group (*p<0.05). β-actin was used as an internal reference

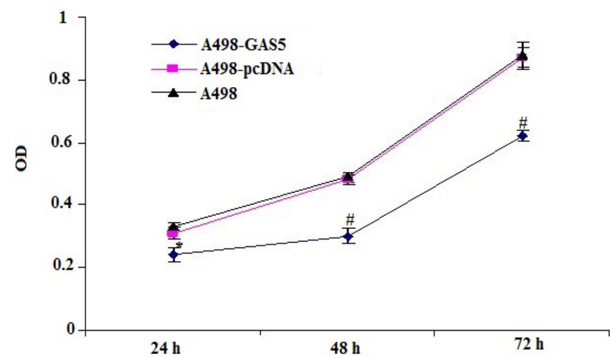

Figure 2. The Proliferation of A498 Cells after Transfected with pcDNA-GAS5. After incubation for 24 h, the value of OD in A498-GAS5 group was significantly lower than that in A498-pcDNA group and in A498 group (each, *p<0.05), and after incubation for 48 h and 72 h, the suppression were more obvious than 24 h when compared to A498-pcDNA group and A498 group (each, $^{\#}p$<0.01)

00.0
6
75.0
56
50.0
25.0
31
0

Cell proliferation and viability

Cell proliferation and viability of A498 cells were evaluated by a modified methythiazoletetrazolium (MTT) assay. Briefly, after 24 h transfected with pcDNA-GAS5, about 5×10^3 cells per well were seeded in 96-well culture plate at 37 °C. Each well was repeated there times. After further incubation with different times (24 h, 48 h and 72 h), medium were then removed. A 100 µl DMEM containing 100 µl MTT (5 mg/ml) was added to each well and further incubated for 4 h. After 4 h, the medium was aspirated and 150 µl of dimethyl sulfoxide (DMSO) was added to dissolve the MTT formazan crystals. The cell viability and proliferation was determined by OD450 value using an automatic microplate reader (BioRad, Model 680, USA).

Cytometric analysis of apoptotic cells

To explore the effect of GAS5 on A498 cells, detection of apoptosis in A498 was carried out after transfected with pcDNA3.1-GAS5 and pcDNA3.1 only for 48 h. Apoptosis cells were analyzed using a flow cytometer (CYTOMICS FC 500, Beckman Coulter) after incubating with reagent containing Annexin V-FITC and Propidium Iodide (BD Bioscience, San Jose, CA) for 15 min in darkness at room temperature. Each study was repeated four times.

Analysis of invasion and migration

A498 cells were transfected with pcDNA3.1-GAS5 as described above. Potentiality of cell invasion and migration were measured by an in vitro Transwell (Millipore, Billerica, MA) assay as previously described (Ito et al., 2007). After 24 h transfection, cells were serum starved overnight. About 5×10^4 cells of each suspended in 200 µl of serum-free DMEM were seeded in duplicate to invasion chamber whose porous membrane was coated with Matrigel (BD Bioscience). Serum (15%) was added to the lower chamber as a chemoattractant. After further 24 h incubation, cells remaining on the top side of membrane were removed using a cotton swab, and penetrated cells on the filters were fixed with methanol, stained in 0.1% crystal violet. In all assays, five fields per insert were photographed and quantified (400×) in each group.

Statistical analysis

All statistical data were analyzed by SPSS 16.0 software (SPSS, Chicago, IL). Chi-square test, two-tailed Student's t-test, Cox proportional hazards regression model, Kaplan–Meier method, and log-rank test were used as appropriate, and $P < 0.05$was considered statistically significant.

Results

Expression of GAS 5 in vivo and in vitro

Real time PCR showed that the expression level of GAS5 was significantly lower in clinical RCC specimens than that in adjacent normal tissues in 12 pairs ($p < 0.05$; Figure 1A). However, there was no significant relationship between the clinicopathological parameters (i.e. tumor stage, grade, recurrence) and the expression levels of GAS5 ($p > 0.05$, data not shown). Furthermore, compared with normal kidney HK-2 cells, GAS5 was also significantly down-regulated in A498 cell ($p < 0.05$; Figure 1B). Both in vivo and in vitro assays confirmed that the expression of GAS5 was at a comparatively low level in RCC. In addition, when A498 cells were transfected with pcDNA3.1-GAS5 for 24 h, we found that the expression of GAS5 in A498-GAS5 group was significantly increased than that in A498-pcDNA group and A498 group (each, $p < 0.05$, Figure 1C), indicating that pcDNA3.1-GAS5 transfection was successful and can express in A498 cells.

The effect of GAS5 on cell proliferation in RCC cells

The growth of A498 cell was markedly inhibited after transfected with pcDNA3.1-GAS5 for 24 h. After further incubation for 24 h, the value of OD in A498-GAS5 group (0.24 ± 0.0215) was much lower than that in A498-pcDNA group and A498 group (each, $p < 0.05$, 0.31 ± 0.0182 and 0.33 ± 0.0215, respectively), which means that cell

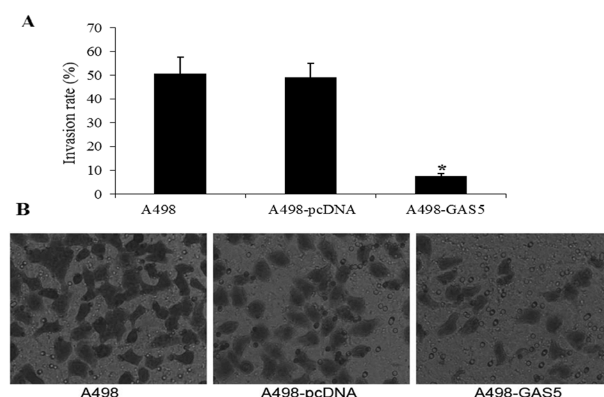

Figure 4. Invasion and Migration Detected with Transwell Assay. A. Statistical analysis of the invasion rate of A498 cell. The number of cells through Transwell was significantly less after transfected with pcDNA3.1-GAS5 compared to blank group (A498) and control group (A498-pcDNA) (each, **p*<0.05). B. Transwell assay view (400×magnification)

proliferation has been inhibited after transfected with pcDNA3.1-GAS5. It also can be seen and more obvious after incubation for 48 h (each, $P < 0.01$, 0.30 ± 0.0253 vs 0.48 ± 0.0170 and 0.49 ± 0.0156, respectively) and 72 h (each, $P<0.01$, 0.620 ± 0.0130 vs 0.870 ± 0.0340 and 0.880 ± 0.0412, respectively). While the ability of proliferation in A498-pcDNA group and A498 group were almost in the same level ($p > 0.05$), which implied that vector pcDNA3.1 itself had no effect on the growth of A498 cell (Figure 2).

Effect of GAS5 overexpression on apoptosis and cell cycle

Because the proliferation of RCC cells was significantly inhibited after treated with pcDNA3.1-GAS5, we presume that the increasing GAS5 may induce apoptosis and/or cell cycle arrest. The detection of apoptosis and cell cycle of A498 cells was performed with flow cytometry. As shown in Figure 3A, the rate of apoptotic and early apoptotic fraction (upper right and lower right in the quadrant images, respectively) were greater in A498-GAS5 (7.76 ± 0.82%, 10.61 ± 1.37%, respectively) group than that in A498-pcDNA (1.55 ± 0.93%, 6.12 ± 1.76%, respectively) group and A498 (1.87 ± 0.35%, 4.51 ± 1.12%, respectively) group. However, there was no significant difference between A498-pcDNA group and A498 group, which means that GAS5 can induce apoptosis in RCC cell in vitro.

As for the cell cycle distribution, the rate of cells in the G1 phase was significantly larger in A498-GAS5 group (58.97 ± 2.18%) in comparison with A498-pcDNA group and A498 group (each, $p < 0.05$, 46.12 ± 1.29%, 45.33 ± 2.54%, respectively). Conversely, the rate of cells in the G2/M phase was significantly smaller in A498-GAS5 group (12.54±3.72%) compared to A498-pcDNA group (24.03±2.20%) and A498 group (23.69±2.84%) (Figure 3B).

Effects of GAS5 on cell migration and invasion in RCC cell line

Our results indicated that A498 cells transfected with pcDNA3.1-GAS5 migrated significantly less (7.63

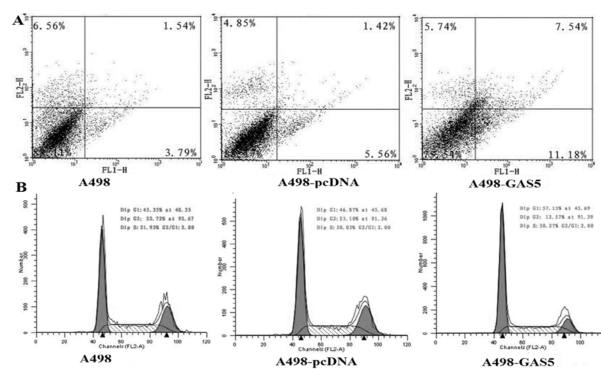

Figure 3. Transfected with pcDNA3.1-GAS5 Induced Apoptosis in A498 Cells and Cell Cycle Distribution. A. Apoptotic and early apoptotic cells increased significantly after 24 h transfection with pcDNA3.1-GAS5 compared to controls. B. Apoptotic and early apoptotic cells are mainly distribution in the G1 phase

± 1.40%) than non-transfected A498-pcDNA cells and A498 cells (50.69 ± 7.01%, 49.21 ± 5.82%, respectively), whereas A498-pcDNA cells showed almost no variation compared to A498 cells (Figure 4). These results suggest that GAS5 can resist the migration and invasion of A498 cells in vitro and the down-regulation of GAS5 may be a cause of RCC with high migratory and invasiveness.

Discussion

Long Non-coding RNAs are RNA transcripts of more than 200 nucleotides with no function of encoding proteins. More and more studies indicate that the molecular mechanisms of carcinogenesis are not only relevant to protein coding genes but also to non-coding regulatory RNAs. Some LncRNAs have been identified to play a pivotal role in the happening of cancers. Recent studies show that numerous LncRNAs are deregulated in various solid tumors and several LncRNAs can regulate cancer metastasis by directly targeting chromatin modification complexes, indicating that the abnormal expression of LncRNAs adds the chances to tumorigenesis and cancer development. However, at present only a few LncRNAs have been functionally studied in detail and many important questions remain to be addressed (Gibb et al., 2011).

Previous studies indicate that the genetic aberrations of GAS5 have relationship with many types of tumors including melanoma, breast and prostate cancers (Smedley et al., 2000; Nupponen and Carpten, 2001; Morrison et al., 2007). As a most common urological cancer, whether the abnormal expression of GAS5 is associated with RCC carcinogenesis has not yet been reported. In our study, it was the first to explore the role of LncRNA GAS5 in RCC, and the results indicated that the expression level of GAS5 was significantly down-regulated in clinical RCC specimens and RCC cell line A498 compared to adjacent normal tissues and nonmalignant renal cell line HK-2. What's more, over-expression of GAS5 in A498 cells inhibited cell proliferation, induced cell apoptosis and arrested cell cycle. Meanwhile, the migration and invasion potential of A498 cells were also suppressed compared to control groups. All of these suggest that

deregulation of GAS5 may play roles in the occurrence and development of RCC.

Based on our experimental results, we suppose that GAS5 has properties of tumor suppression in RCC. Hence, our study provides evidence that induction overexpression of GAS5 in RCC tissue in vivo would be an attractive strategy for RCC treatment. Therefore, finding an effective way to induce GAS5 expression in tumors or designing a vector that would induce the expression of GAS5 when injected into the tumor may provide an attractive therapeutic approach (Gutschner and Diederichs). For example, the expression of the LncRNAs H19 is increased in a broad range of human cancers. Intratumoral injections of BC-819 (DTA-H19) plasmid, which carries the gene for the A subunit of diphtheria toxin under the regulation of the H19 promoter, induce the expression of high levels of diphtheria toxin specifically in the tumor resulting in a reduction of tumor size in human trials. Recent studies have yielded encouraging results in a wide range of carcinomas including NSCLC, colon, bladder, pancreatic and ovarian cancers (Amit and Hochberg; Hasenpusch et al.; Smaldone and Davies; Sidi et al., 2008; Mizrahi et al., 2009). The successful trial undoubtedly gives us a faith and good method for the treatment of RCC through manipulating the expression of GAS5 in vivo.

In conclusion, this report described the role of LncRNAs GAS5 in the occurrence of RCC, , which provides an LncRNA point-of-view on tumor biology and will stimulate new research directions and therapeutic options for considering GAS5 as novel prognostic markers and therapeutic targets for RCC.

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