

Long Noncoding RNA MALAT1 Promotes Aggressive Renal Cell Carcinoma through Ezh2 and Interacts with miR-205

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Abstract

Recently, long noncoding RNAs (lncRNA) have emerged as new gene regulators and prognostic markers in several cancers, including renal cell carcinoma (RCC). In this study, we investigated the contributions of the lncRNA MALAT1 in RCC with a specific focus on its transcriptional regulation and its interactions with Ezh2 and miR-205. We found that MALAT1 expression was higher in human RCC tissues, where it was associated with reduced patient survival. MALAT1 silencing decreased RCC cell proliferation and invasion and increased apoptosis. Mechanistic investigations showed that MALAT1 was transcriptionally activated by c-Fos and that it

interacted with Ezh2. After MALAT1 silencing, E-cadherin expression was increased, whereas β -catenin expression was decreased through Ezh2. Reciprocal interaction between MALAT1 and miR-205 was also observed. Lastly, MALAT1 bound Ezh2 and oncogenesis facilitated by MALAT1 was inhibited by Ezh2 depletion, thereby blocking epithelial–mesenchymal transition via E-cadherin recovery and β -catenin downregulation. Overall, our findings illuminate how overexpression of MALAT1 confers an oncogenic function in RCC that may offer a novel theranostic marker in this disease. *Cancer Res*; 75(7); 1322–31. ©2015 AACR.

Introduction

Renal cell carcinoma (RCC) is one of the ten most common cancers, and it is estimated that there were 63,920 new cases of RCC in the United States in 2013 (1). The incidence of RCC has increased for over two decades (2), and patients with advanced RCC (stage IV) have a significantly reduced 5-year survival rate (less than 30%; ref. 3). Compared with other cancers, there are very few tumor and biomarkers for renal cancer (4). Also, patients with renal cancer respond poorly to conventional chemotherapy and radiotherapy treatment (5); thus, early detection and treatment are very important for patients with RCC.

Human genome sequence data indicate that more than 90% of the DNA sequence is actively transcribed, but only 2% of it encodes protein, thus the majority of transcripts are referred to as noncoding RNAs (ncRNA; refs. 6, 7). Based on size, ncRNAs are divided into two groups, small noncoding RNAs and long noncoding RNAs (lncRNA) greater than 200 nt. Small noncoding

RNAs, such as miRNAs, have been studied extensively, and their roles in gene regulation and cell function have been elucidated in numerous cancers (7). Recent studies have shown that lncRNAs play important roles in both normal development and diseases including cancer (8). lncRNAs participate in several different biologic processes, including epigenetic regulation, nuclear import, cell cycle control, nuclear and cytoplasmic trafficking, imprinting, cell differentiation, alternative splicing, RNA decay, transcription, and translation (9, 10). Thus, lncRNAs have emerged as new players in cancer research, and several studies have shown that some lncRNAs function as oncogenes, tumor suppressor genes, or both, depending on the circumstance (11).

Several lncRNAs [GAS5, MEG3 (= GTL2), HIF1 α -AS1, H19, KCQN1OT1, MALAT1 (alpha gene), and HOTAIR] have been reported to be involved in renal cancer (12–19). MALAT1 (alternative name "alpha gene") is located on chromosome 11q13, and the expression of MALAT1 has been reported to be upregulated in several cancers, including lung, breast, pancreas, liver, colon, uterus, cervix, and prostate (10). Previous articles showed that MALAT1 expression is an independent prognostic parameter for survival in several cancers (10). Davis and colleagues (17) have reported that chromosomal translocation of t(6;11)(p21;q13) was observed in pediatric renal cancer, resulting in the fusion of the alpha gene (MALAT1) with TFEB, a basic helix-loop-helix leucine zipper (bHLH-LZ) transcription factor. Kuiper and colleagues (18) have reported that the bHLH-LZ transcription factor TFEB gene on chromosome 6 is fused to the anonymous non protein encoding alpha gene (MALAT1) on chromosome 11 through chromosomal translocation, t(6;11)(p21;q13) in papillary RCC.

In this study, we focused on lncRNA MALAT1 in clear cell RCC because the majority of adult renal cancers are of the clear cell type, and many studies have focused on clear cell RCC. The aim of this study was to (i) determine the role of MALAT1 in renal cancer,

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(ii) determine the reason for MALAT1 overexpression in renal cancer tissues, (iii) determine the downstream effects of MALAT1 in renal cancer, and (iv) determine the association between MALAT1 and miR-205 in renal cancer.

Patients and Methods

Clinical samples

A total of 50 patients (34 male and 16 female) with pathologically confirmed clear cell RCC (cc-RCC) were enrolled in this study (Toho University Hospital, Tokyo, Japan). The mean age of the patients was 60.2 (range, 37–77). They were classified according to the WHO criteria and staged according to the tumor–node–metastasis classification. Information about the patients is shown in Supplementary Table S1. Samples were obtained from the patients after written informed consent was obtained at Toho University Hospital.

Cell culture

Normal renal epithelial cells (HK-2; ATCC number: CRL-2190) and renal cancer cell lines (A-498, ATCC number: HTB-44; 786-O, ATCC number: CRL-1932; Caki-2, ATCC number: HTB-47; Caki-1, ATCC number: HTB-46) were purchased from the ATCC. The cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS. HK-2 cells were cultured in keratinocyte-SFM (GIBCO/Invitrogen). When purchased, permanent stocks of cells were prepared and all cells were stored at –80°C until use. Cells were used for experiments within 6 months.

Total RNA, DNA, and protein extraction

RNA (miRNA and total RNA) was extracted from formalin-fixed, paraffin-embedded (FFPE) human renal cancer and adjacent noncancerous normal kidney tissues using a miRNeasy FFPE kit (QIAGEN) after microdissection based on a pathologist's reviews. To digest DNA, the Qiagen RNase-Free DNase Kit was used. RNA (miRNA and total RNA) was also extracted from human cell lines using an miRNeasy mini kit (QIAGEN). Genomic DNA was extracted from cell lines using a QIAamp DNA mini kit (QIAGEN). Cells were lysed with RIPA buffer (Thermo Fisher Scientific) containing protease inhibitors (Sigma-Aldrich). Protein quantification was done using a BCA protein assay kit (Thermo Fisher Scientific).

Knockdown of MALAT1 mRNA in renal cancer cells

Of four renal cancer cell lines, the expression of MALAT1 was higher in 786-O and A-498 than Caki-2 and Caki-1 cells. Thus, we used 786-O and A-498 cells for further experiments in this study. Renal cancer cells (786-O and A-498) were transfected with two MALAT1 siRNAs [si-MALAT1 (No.1 and No.2); Life Technologies] or negative control siRNA [si-negative control (si-NC); Life Technologies] according to the manufacturer's instructions. Briefly, cells were grown in 6-well plates and transfected individually with two si-MALAT1 siRNAs [No.1 (custom order) and No.2 (product#n272234)] at a concentration of 50 pmol per well. Knockdown effect of si-MALAT1 was examined by real-time RT-PCR using RNA extracted 48 hours after transfection (si-NC, si-MALAT) in two RCC cell lines (A-498 and 786-O). Cell viability (24, 48, and 72 hours after transfection), invasion (48 hours after transfection), and apoptosis analysis (48 hours after transfection) were performed using si-NC or si-MALAT1-transfected RCC cells. The two

siRNA sequences are shown in Supplementary Table S2. Transfection was performed with Lipofectamine RNAiMAX Transfection Reagent (Life Technologies) accordingly.

Cell viability, cell invasion, and apoptosis assay

Cell viability was measured 72 hours after transfection with MTS (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega). Cell invasion assays were performed with the CytoSelect 24-well cell invasion assay Kit (Cell BioLab) according to the manufacturer's instructions. FACS analysis for apoptosis was done using Annexin V–FITC solution and 7-AAD kit after 48-hour transfection according to the manufacturer's protocol (Beckman Coulter). All experiments were performed in triplicate.

Search for transcription factors binding to the MALAT1 promoter

To determine why MALAT1 is overexpressed in renal cancer tissues, we focused on transcription factors binding to the MALAT1 promoter. Based on several computer algorithms (PROMO, http://algggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3; ChIPbase, <http://deepbase.sysu.edu.cn/chipbase>; and TFSEARCH, <http://www.cbrc.jp/research/db/TFSEARCH.html>), we identified 22 transcription factors involved in cancer pathways as potential transcription activators for MALAT1 (Supplementary Table S3). Of the 22 transcription factors, we selected c-Fos because An and colleagues (20) found that inactivation of the *von Hippel–Lindau* (*VHL*) tumor suppressor gene induces activation of JNK via phosphorylation of members of the AP1 family of transcription factors such as c-Jun and c-Fos, and Twist expression is upregulated, resulting in induced epithelial–mesenchymal transition (EMT) in RCC. Clear cell RCC is the major histologic subtype (about 80% of RCC cases), and inactivation of the *VHL* tumor suppressor gene is typical in ccRCC (20). We also found that high c-Fos overexpression was associated with shorter overall survival of patients with RCC (data not shown). Thus, we focused on c-Fos as a potentially important transcription factor in MALAT1 activation.

Construction of luciferase-expressing plasmid vector containing the MALAT1 promoter region

Based on several computer algorithms as described above, we identified potential binding sites for transcription factor c-Fos in the promoter region of *MALAT1* gene. Using 786-O genomic DNA, the identified MALAT1 promoter DNA region was amplified, and the PCR products were cloned into the pGEM-T easy vector System (Promega; Supplementary Table S2). Then the MALAT1 promoter DNA region was incorporated into the pGL4 luciferase expression vector (Promega). Luciferase activity was assessed using the Dual-Luciferase Reporter Assay System (Promega; 48 hours after their transfection), and the ratio of Firefly/*Renilla* luciferase activity was determined.

Overexpression of transcription factor genes

To construct transcription factor gene (c-Fos) over expressing plasmids, the gene was amplified with total RNA from human adult normal kidney tissues (catalog#: R1234142-50; Biochain Institute) by RT-PCR as described previously (21). The sequences of primers for cloning are shown in Supplementary Table S2.

TCF/LEF reporter assay (TOPflash luciferase assay)

TOPflash luciferase assays were performed to assess the knock-down effect of MALAT1 on the Wnt/ β -catenin signaling pathway as described previously (21).

RNA immunoprecipitation assay

RNA immunoprecipitation was performed to investigate whether ribonucleoprotein complex contained lncRNA MALAT1 and its potential binding protein (Ezh2) in renal cancer cells. We used an Imprint RNA Immunoprecipitation Kit according to the manufacturer's instructions (Sigma-Aldrich). The RNA immunoprecipitation fraction was digested by DNase, and cDNA was generated using the PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio Company). Final analysis was performed using qRT-PCR and shown as fold enrichment of MALAT1. The RNA immunoprecipitation fraction C_t value was normalized to the input RNA fraction C_t value.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed on cell line DNA using an Imprint Chromatin Immunoprecipitation Kit (Sigma-Aldrich). Antibody for trimethyl-Histone H3 (Lys27) (H3K27me3; catalog#07-449, EMD Millipore) was obtained from vendors. The immunoprecipitated DNA was analyzed using quantitative real-time PCR with SYBR Green PCR Master Mix (Applied Biosystems). Quantitative PCR was performed in triplicate with an Applied Biosystems Prism 7500 Fast Sequence Detection System (Applied Biosystems) using the

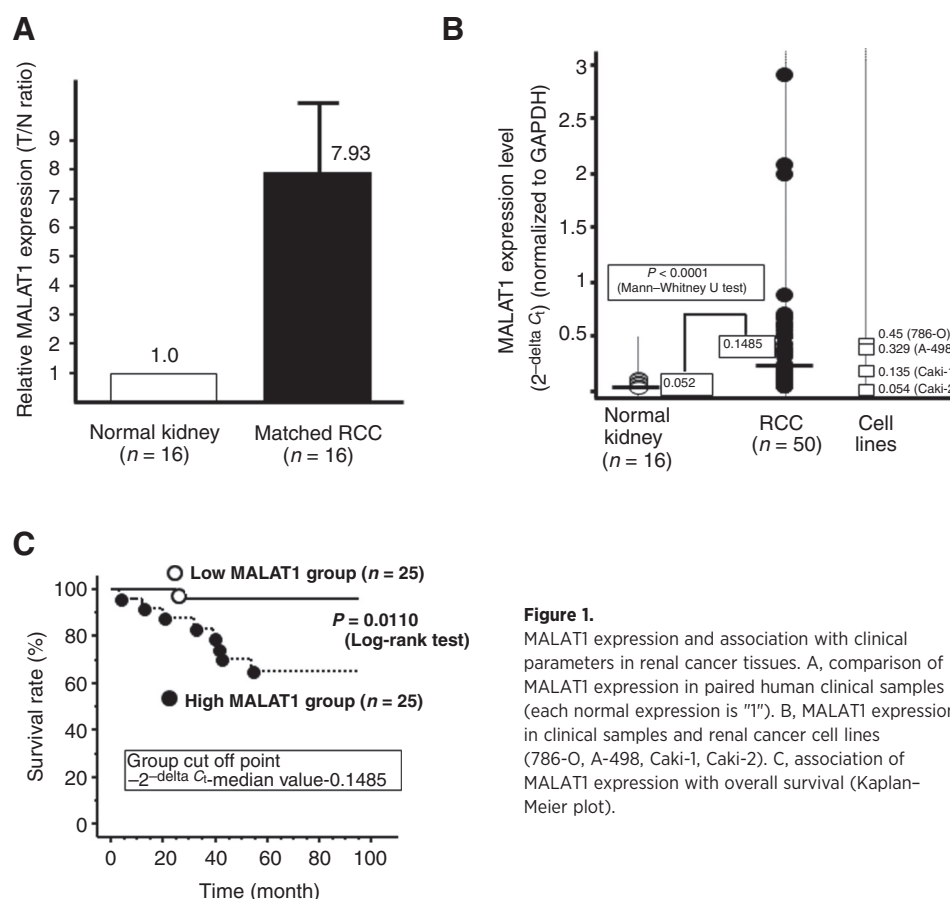
following conditions: 10 minutes, 95°C; followed by 40 cycles of 30 seconds; 95°C, 60 seconds; 60°C. Delta- Δ cycle threshold (C_t) method was used to calculate real-time PCR results. The fold changes related to 10% input ΔC_t were calculated as $2^{-(\Delta\Delta C_t)}$. All experiments were performed in triplicate. The primer pairs used for ChIP assays are shown in supporting information of Supplementary Table S2.

Immunohistochemistry study

Immunostaining of E-cadherin was performed on FFPE specimens using rabbit polyclonal antibody against human E-cadherin (#3195; Cell Signaling Technology). The staining procedure was according to a commercial kit (Lab vision). The sections were counterstained with Harris' hematoxylin. Immunohistochemical staining was evaluated by visually assessing staining intensity (0–3) using a microscope at 200 \times . The criteria of intensity are as follows: 0, negative expression; 1+, weakly positive expression; 2+, moderately positive expression; 3+, strongly positive expression. All specimens were scored blindly by two observers.

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR was performed in triplicate with an Applied Biosystems Prism 7500 Fast Sequence Detection System using TaqMan Universal PCR Master Mix according to the manufacturer's protocol (Applied Biosystems Inc.). The TaqMan probes and primers were purchased from Applied Biosystems. Human GAPDH and RNU48 were used as endogenous controls.

**Figure 1.**

MALAT1 expression and association with clinical parameters in renal cancer tissues. A, comparison of MALAT1 expression in paired human clinical samples (each normal expression is "1"). B, MALAT1 expression in clinical samples and renal cancer cell lines (786-O, A-498, Caki-1, Caki-2). C, association of MALAT1 expression with overall survival (Kaplan-Meier plot).

Levels of RNA expression were determined using the 7500 Fast System SDS software version 1.3.1 (Applied Biosystems).

Western blot analysis

Western blot was performed as described previously. The antibodies used were specific for c-Myc (#551101; BD Biosciences), Ezh2 (#5246; Cell Signaling Technology), E-Cadherin (#3195; Cell Signaling Technology), β -catenin (#9562; Cell Signaling Technology), PCNA (#13110; Cell Signaling Technology), and β -tubulin (#2128; Cell Signaling Technology). Proteins were enhanced by chemiluminescence (Amersham ECL Plus Western Blotting Detection System or SuperSignal West Pico chemiluminescent substrate; Thermo Scientific) for visualization. The ChemiDoc XRS+ system was used for chemiluminescent detection (BIO-RAD Laboratories). The protein expression levels were expressed relative to β -tubulin or PCNA levels.

Statistical analysis

All statistical analyses were performed using StatView (version 5; SAS Institute Inc.). Error bars in figures represent SD. The Mann-Whitney *U* test was used to compare median value between two groups. The Fisher exact test or χ^2 test was used to determine whether there is significant difference between two groups. Correlation between cFos mRNA expression and MALAT1

mRNA or MALAT1 mRNA and Ezh2 mRNA in human renal cancer tissues was examined with two-sided Pearson correlation (22). Statistical significance was determined using the Student *t* test or ANOVA for functional analysis. A *P* value of <0.05 was regarded as statistically significant.

Results

Relationship between MALAT1 expression and clinical characteristics

Initially, we compared MALAT1 expression in 16 matched normal kidney and renal cancer tissues (all cc-RCC) using real-time RT-PCR. MALAT1 expression was significantly higher in all renal cancer tissues compared with matched normal kidney tissues (Fig. 1A). We then used 50 renal cancer samples and divided the patients with RCC into two groups using a median MALAT1 $2^{-\Delta C_t}$ value of 0.1485 (Fig. 1B). Next, we investigated the association of MALAT1 expression with several clinical parameters, including grade, pathologic tumor classification (pT), pathologic lymph node status (pN), pathologic metastatic status (pmol/L), stage, and outcome (overall survival). The expression of MALAT1 was significantly higher in pT (pT3+pT4), pN (+), pmol/L (+), and high-stage (3+4) patients compared with lower-stage patients (Supplementary Table S1). Overall survival was also significantly shorter in the high MALAT1 expression group compared with the low MALAT1 group (Fig. 1C).

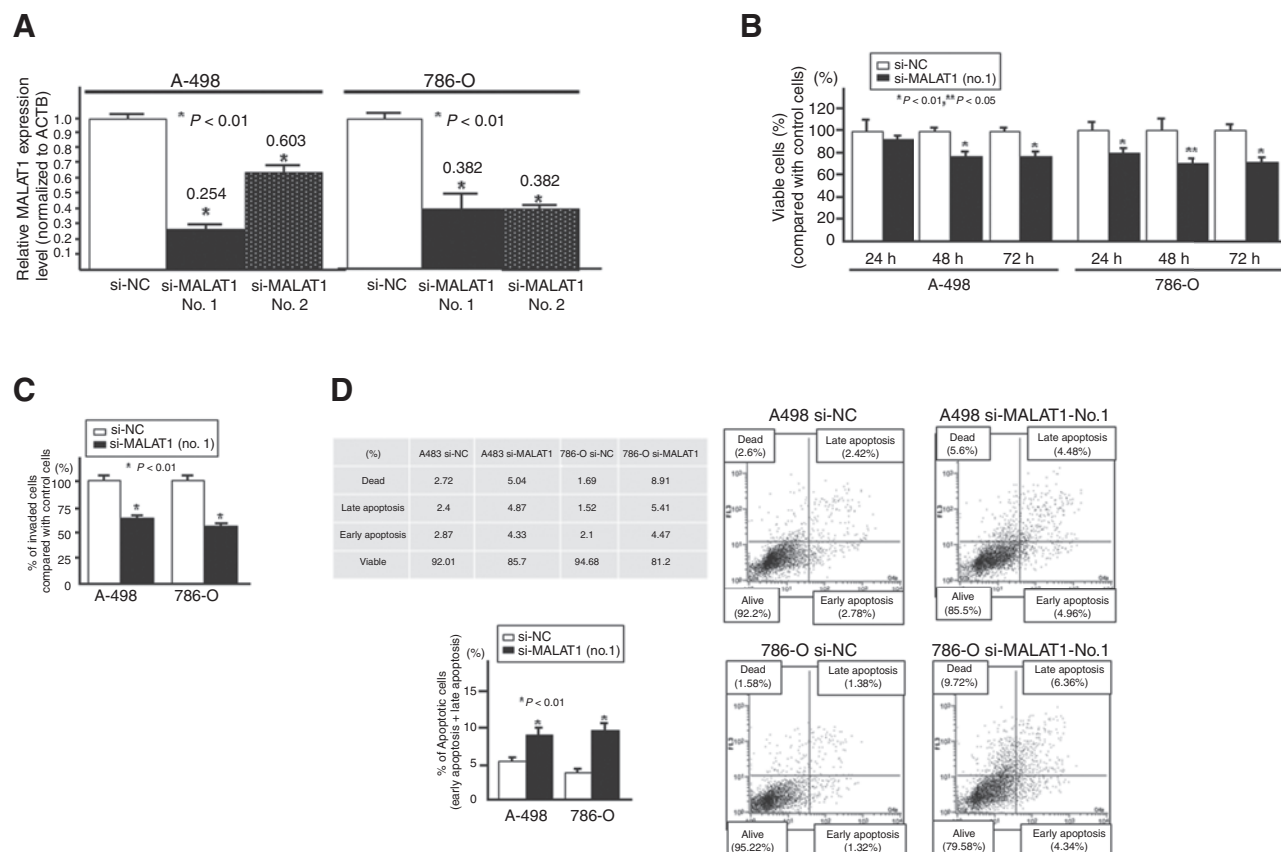


Figure 2.

Effect of MALAT1 knockdown on renal cancer cell function (A-498, 786-O). Two renal cancer cell lines (A-498 and 786-O) were transiently transfected with either si-MALAT1 (No. 1, No. 2) or control (si-NC). A, validation of si-MALAT1 knockdown in RCC cell lines. B, cell viability assay. C, invasion assay. D, flow cytometric analysis of apoptosis in si-NC or si-MALAT1-transfected renal cancer cells. Error bars, \pm SD.

Effect of MALAT1 on renal cancer cell viability, invasion, and apoptosis

As shown in Fig. 2A, the knockdown effect was best using si-MALAT1 (No. 1) compared with si-MALAT1 (No. 2). Thus, we used si-MALAT1 (No. 1) for further experiments. As shown in Fig. 2, we observed significantly decreased cell viability (Fig. 2B) and invasion (Fig. 2C) in si-MALAT1-transfected renal cancer cells compared with si-NC-transfected cells. The percentage of apoptotic cells was significantly increased in si-MALAT1-transfected cells (Fig. 2D).

c-Fos expression in normal kidney and renal cancer tissues

Expression of transcription factor c-Fos mRNA was significantly higher in renal cancer tissues compared with those in matched normal kidney tissues (Fig. 3A).

Positive correlation between c-Fos and MALAT1 expression

As shown, there was significant positive correlation between c-Fos mRNA and MALAT1 mRNA expression (two-sided Pearson correlation, $r = 0.6643$, $P < 0.0001$; Fig. 3B). MALAT1 expression was also significantly increased after c-Fos overexpression in 786-O cells (Fig. 3C and D). To investigate the direct binding of c-Fos to the MALAT1 promoter, we constructed luciferase vectors containing potential c-Fos binding sites (clone 1; clone 2; Fig. 3E). The clone 1 luciferase vector contained two potential c-Fos binding sites, and clone 2 contains one binding site. As shown in Fig. 3F, luciferase activity was significantly increased in c-Fos-trans-

fected cells compared with control vector with the two constructs (Fig. 3F).

Signaling cross-contribution of transcription factors, MALAT1, and Ezh2 in RCC

Some lncRNAs are activated transcriptionally (23–26), and several reports have shown that lncRNA HOTAIR binds to polycomb repressive complex2 (PRC2; Ezh2, Suz12, EED) enhancing methylation of H3K27me3, resulting in gene silencing (27). Several lncRNAs, such as H19 and EBIC, have been found to bind to Ezh2 in other cancers (28, 29). MALAT1 has been reported to bind to Suz12, a component of PRC2 in bladder cancer cell lines (30). These articles indicated that the interaction between MALAT1 and Ezh2 might be important for this study. We have made a schematic diagram illustrating MALAT1 signaling, transcription factor activator, and downstream effectors via Ezh2 in promoting renal cancer growth (Fig. 4A).

Ezh2 expression in renal cancer and normal kidney tissues

Ezh2 mRNA expression was significantly higher in renal cancer tissues compared with normal kidney tissues (Fig. 4B).

Positive correlation between MALAT1 mRNA and Ezh2 mRNA expression

We compared the mRNA expression levels for both MALAT1 and Ezh2 in renal cancer tissues and found that there was significant positive correlation between the two groups (two-sided Pearson correlation, $r = 0.595$, $P < 0.0001$; Fig. 4C).

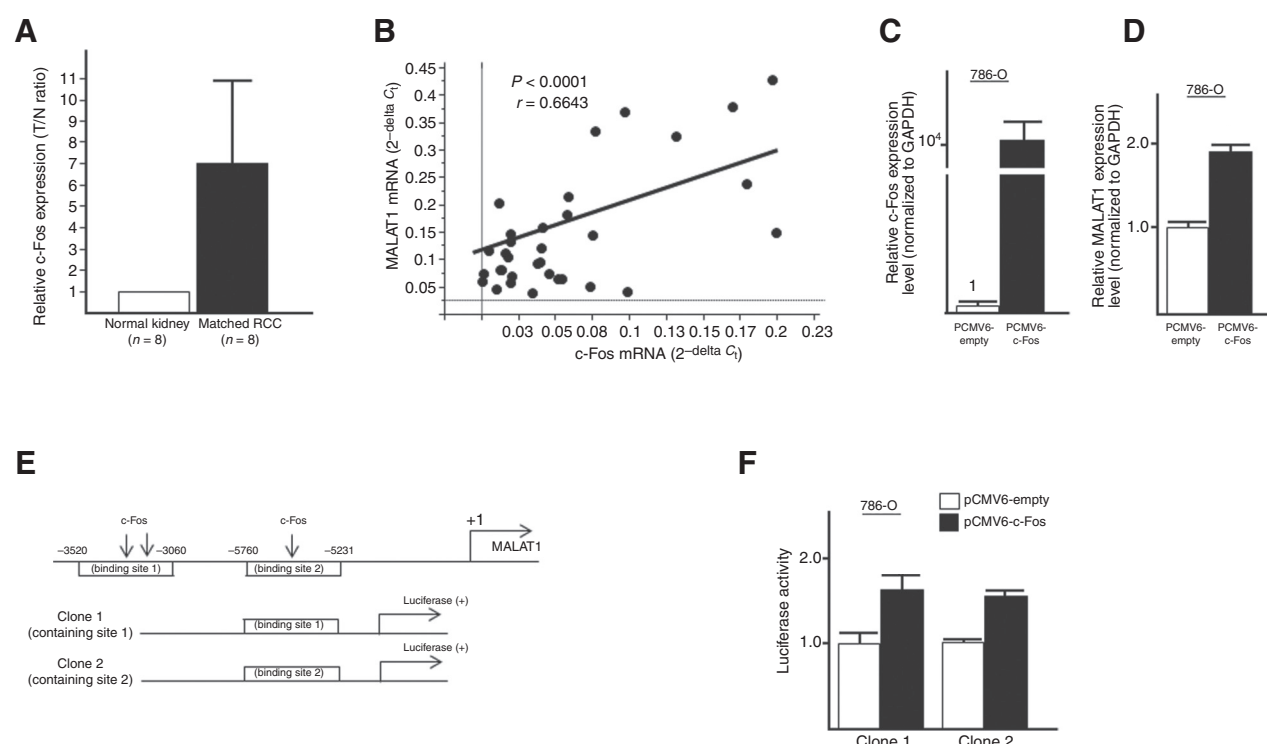
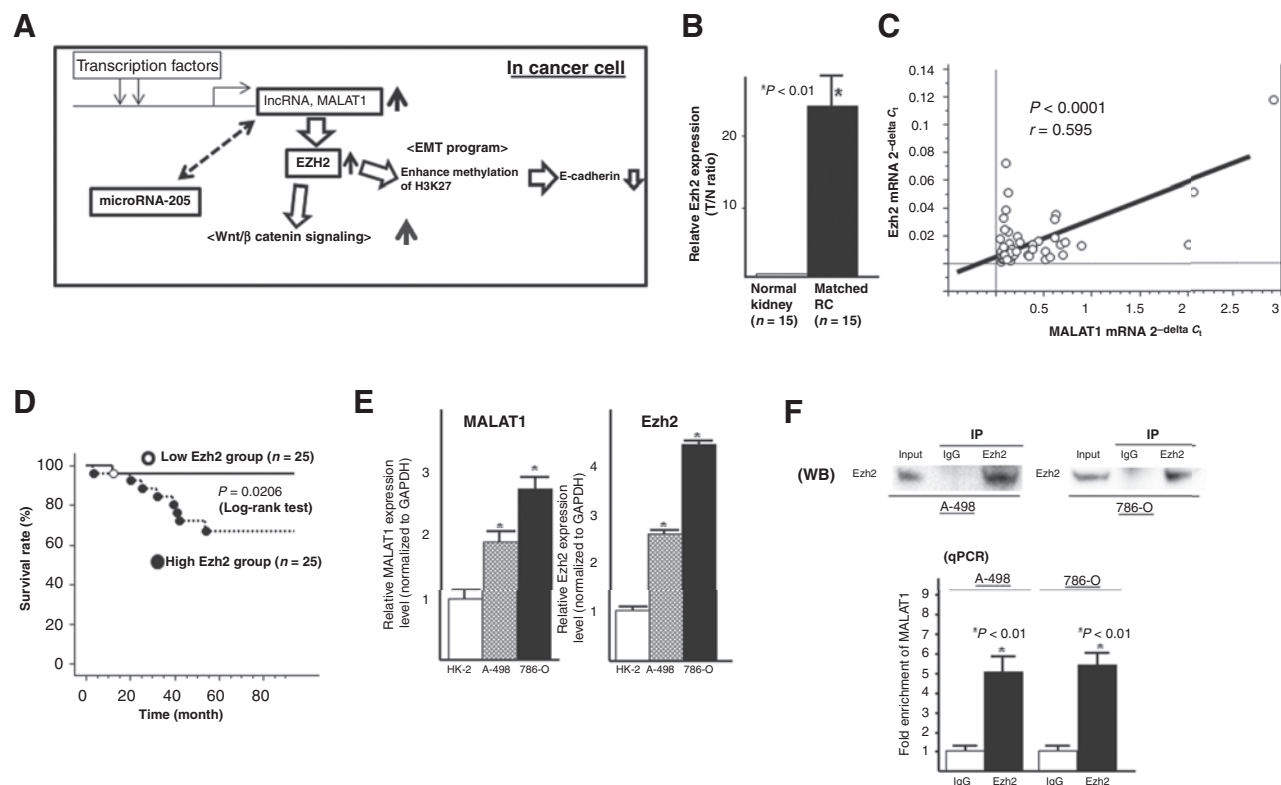


Figure 3.

Transcriptional activation of MALAT1 by c-Fos in 786-O cells. A, c-Fos expression level in renal cancer and matched normal kidney tissues. B, Pearson correlation between c-Fos mRNA and MALAT1 mRNA. C, validation of c-Fos overexpression in 786-O cells after c-Fos vector transfection. D, MALAT1 mRNA expression level after transfection of c-Fos overexpressing vector plasmid and control. E, potential c-Fos binding region in the promoter region of MALAT1 used for construction of luciferase vector containing the binding region. F, luciferase reporter assay (pCMV6-empty vs. pCMV6-c-Fos). Error bars, \pm SD.

**Figure 4.**

MALAT1 interacts with Ezh2 in renal cancer. A, schematic diagram illustrating signaling of MALAT1 and its upstream activator and its downstream effectors in RCC. B, Ezh2 mRNA expression in normal kidney and matched renal cancer tissues. C, Pearson correlation between MALAT1 mRNA and Ezh2 mRNA. D, high Ezh2 was associated with shorter overall survival of patients with RCC. E, expression of MALAT1 (left) and Ezh2 (right) mRNA in cell lines (HK-2, A-498, 786-O). F, RNA immunoprecipitation (A-498 and 786-O). F, top, Western blot (input, rabbit IgG, anti-Ezh2 antibody; immunoprecipitation followed by Western blot with Ezh2 antibody). Bottom, qPCR showing MALAT1 is significantly enriched with the Ezh2 antibody compared with IgG (control antibody) in two renal cancer cell lines (A-498 and 786-O).

Association of Ezh2 with clinicopathologic parameters in RCC tissues

The expression of Ezh2 was significantly higher in pT (pT3+pT4) and pN (+) patients compared with those in pT1+pT2 and pN (–) patients (Supplementary Table S4). Overall survival was also significantly shorter in the high Ezh2 expression group compared with the low Ezh2 group (Fig. 4D).

MALAT1 and Ezh2 expression in renal cancer cells and a normal kidney cell line

MALAT1 expression was significantly higher in renal cancer cell lines (A-498 and 786-O) compared with that in normal kidney cell line HK-2 (Fig. 4E). Ezh2 mRNA expression was also significantly higher in renal cancer cell lines (Fig. 4E) compared with normal kidney cells.

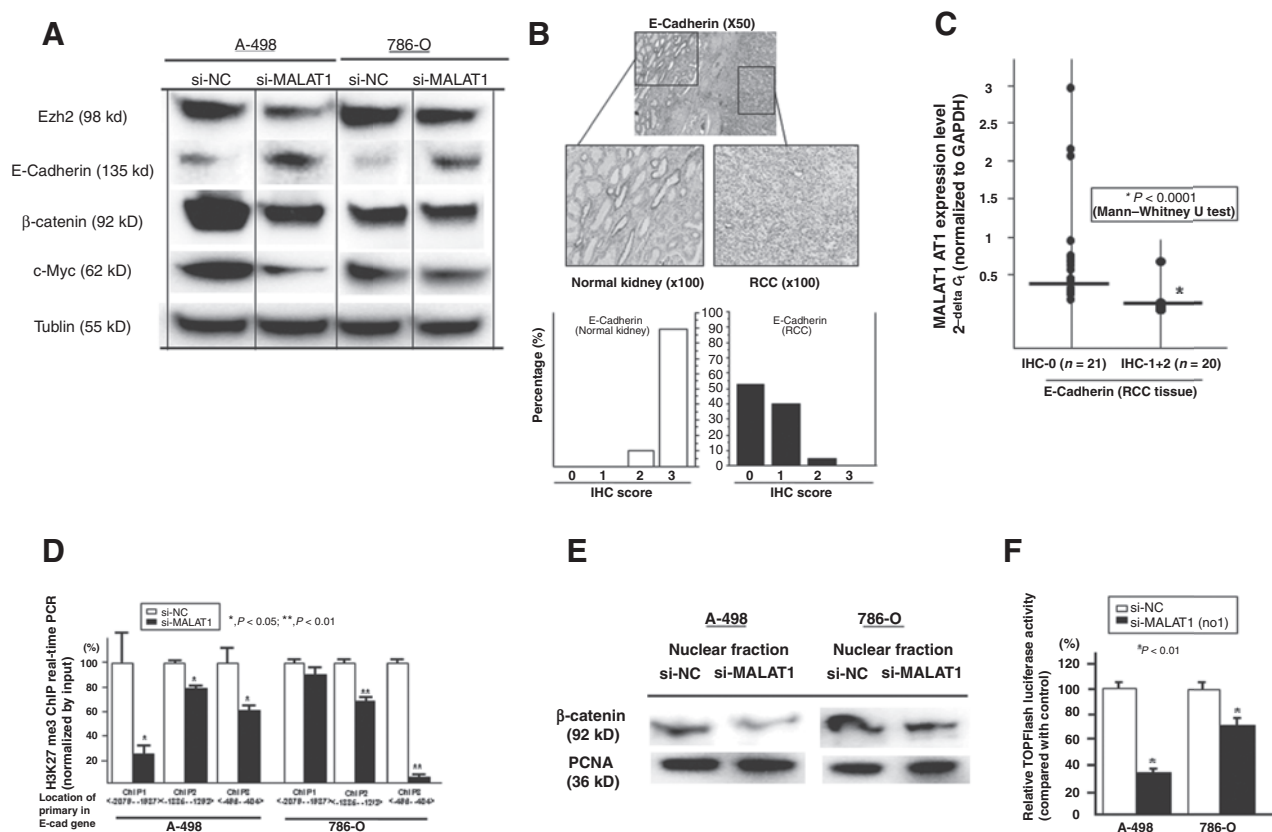
RNA immunoprecipitation

Some lncRNAs affect expression of protein coding genes by interacting with PRC2 subunit Ezh2 (27). To investigate potential interaction between lncRNA MALAT1 and Ezh2, RNA immunoprecipitation was performed. We found that MALAT1 was significantly enriched with the Ezh2 antibody compared with IgG (control antibody) in two renal cancer cell lines (A-498 and 786-O; Fig. 4F).

Effect of MALAT1 on downstream effectors in renal cancer cell

After knockdown of MALAT1, expression of Ezh2, β -catenin, and c-Myc protein was significantly decreased in RCC cell lines, whereas in contrast E-cadherin protein expression was significantly increased (Fig. 5A). E-cadherin mRNA expression was low in renal cancer cells (A-498 and 786-O) compared with HK-2 cells (Supplementary Fig. S1), and E-cadherin protein expression was also significantly lower in renal cancer tissues (Fig. 5B). There was also inverse correlation between E-cadherin protein and MALAT1 mRNA expression in renal cancer tissues (Fig. 5C). Because Ezh2 is an enhancer of H3K27 methylation, we used ChIP analysis using anti-H3K27me3 antibody to determine the effect of si-MALAT1 on histone modification in the *E-cadherin* gene promoter. The histone-associated DNAs that were immunoprecipitated with antibody against H3K27me3 were individually amplified with primers sets covering the *E-cadherin* gene promoter regions. The level of H3K27me3 was significantly decreased by MALAT1 knockdown compared with control cells (Fig. 5D). Because total β -catenin expression was significantly decreased by knockdown of MALAT1, we looked at the nuclear fraction and found that nuclear β -catenin was also significantly decreased (Fig. 5E). TCF reporter activity (TOPflash) was also significantly decreased by MALAT1 knockdown in both renal cancer cell lines (Fig. 5F).

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**Figure 5.**

Downstream effect of MALAT1 on Ezh2-mediated pathways in renal cancer. A, Western blot results (si-NC vs. si-MALAT1). B, immunohistochemistry of E-cadherin in renal cancer and normal kidney tissues. C, association of MALAT1 mRNA with E-cadherin protein expression. D, quantification by ChIP real-time PCR of H3K27me3 in the *E-cadherin* gene promoter region using si-NC and si-MALAT1-transfected cells (A-498 and 786-O). E, β-catenin protein expression (Western blot) in the cell nuclear fraction. F, TOPflash luciferase assay.

Interaction between MALAT1 and miR-205 in renal cancer

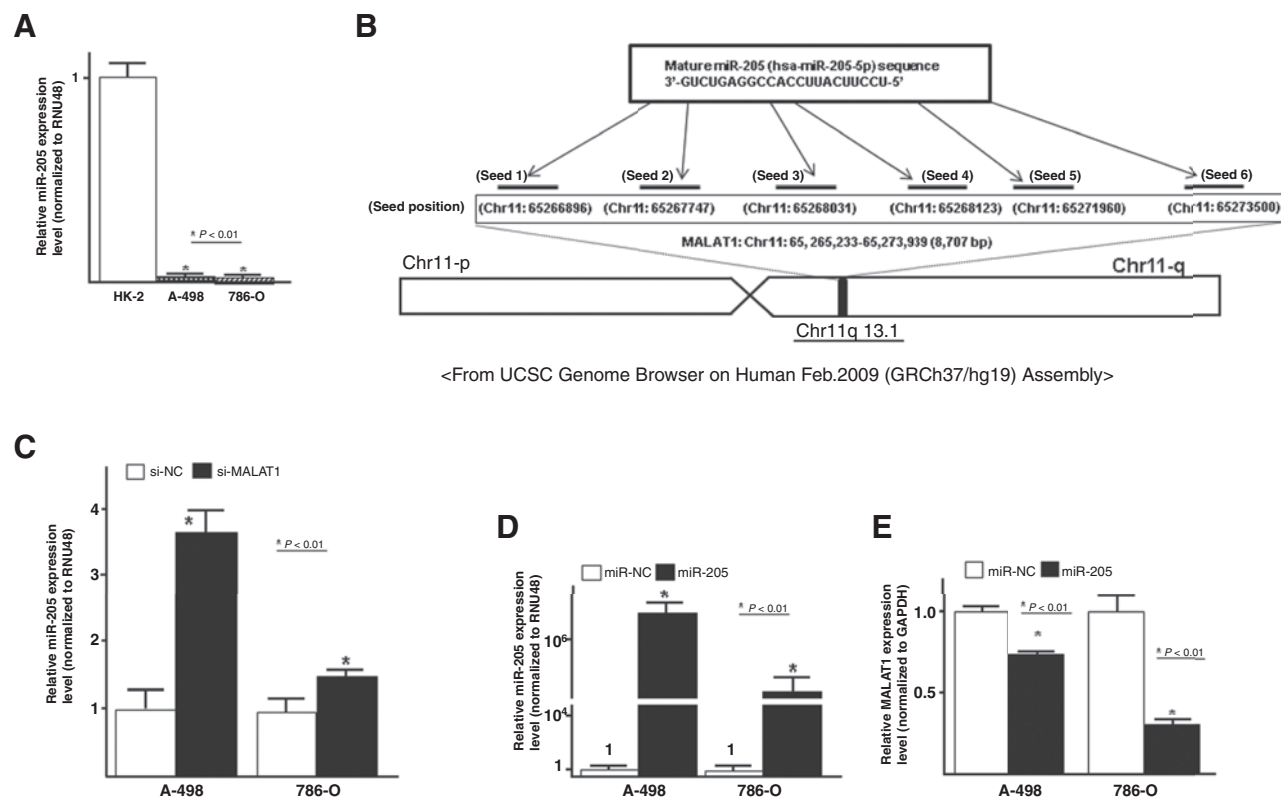
We also investigated the potential relationship between lncRNA MALAT1 and miRNA. Using miRcode (<http://www.mircode.org/mircode>), a computer algorithm to identify miRNA target genes including lncRNAs (31), we identified several miRNAs MALAT1 binding sites. Of these miRNAs, our laboratory has previously reported that miR-205 functions as tumor suppressor gene in renal cancer (32). Thus, we focused on miR-205 and investigated the interaction between MALAT1 and miR-205 in renal cancer cell lines. Although the expression of MALAT1 was high in renal cancer cell lines compared with normal HK-2 cells, miR-205 expression was significantly lower in renal cancer cell lines (Fig. 6A). Knockdown of MALAT1 significantly increased miR-205 expression in renal cancer cells (Fig. 6B); however, after overexpression of miR-205 (Fig. 6C), MALAT1 expression was significantly decreased (Fig. 6D). These results suggest that interaction between MALAT1 and miR-205 has reciprocal effects.

Discussion

lncRNA MALAT1 (metastasis associated in lung adenocarcinoma transcript 1) has been reported to be upregulated in many cancers (33). Initially, we looked at MALAT1 mRNA expression in matched normal human kidney and renal cancer tissues and found that MALAT1 expression was significantly higher in renal

cancer tissues (all samples: ccRCC). High MALAT1 expression was also associated with high stage, metastasis, and shorter overall survival after radical nephrectomy in patients with RCC. After siRNA knockdown of MALAT1, cell proliferation and invasion were significantly decreased, whereas the percentage of apoptotic cells was significantly increased in renal cancer cells. These results suggest that MALAT1 functions as an oncogene in renal cancer.

Based on previous reports, many transcription factors are highly expressed in cancer tissues and several (c-Myc, B-MYB, E2F, PU.1) induce upregulation of lncRNAs (23–26). To determine the reason for high MALAT1 expression in renal cancer tissues, we focused on the proto-oncogenic transcription factor, c-Fos (34) because inactivation of the *VHL* tumor suppressor gene induces activation of JNK via phosphorylation of members of the AP1 family of transcription factors, such as c-Jun and c-Fos, and Twist expression is upregulated, resulting in induced EMT in RCC (20). We also found that c-Fos expression was significantly higher in renal cancer compared with normal kidney tissues, and high c-Fos expression was associated with shorter overall survival in patients with RCC (data not shown). We observed significant positive correlation between c-Fos and MALAT1 mRNA expression in renal cancer tissues and found c-Fos binding sites in the MALAT1 promoter region. Luciferase assay showed that c-Fos directly binds to lncRNA MALAT1 in 786-O cells, suggesting that c-Fos regulates MALAT1 expression in renal cancer. Future studies

**Figure 6.**

Interaction between MALAT1 and miR-205 in A-498 and 786-O cells. A, miR-205 expression in cell lines (HK-2, A-498, 786-O). B, representation of the miR-205 binding sites in MALAT1 based on miRcode (<http://www.mircode.org/mircode/>). Potential miR-205 binding sites in MALAT1 based on miRcode and UCSC Genome Browser. C, MALAT1 knockdown and effect on miR-205 expression (qPCR) in RCC cells (A-498 and 786-O). D, overexpression of miR-205 in A-498 and 786-O transfected cells (qPCR). E, effect of miR-205 overexpression on MALAT1 in transfected RCC cell lines (A-498 and 786-O; qPCR).

will be essential to understand the role of other transcription factors in the activation of MALAT1.

We sought to determine the underlying molecular mechanisms by which MALAT1 regulated downstream effectors in renal cancer. To examine this, we focused on Ezh2, a PRC2 subunit, because some lncRNAs regulate downstream effectors via Ezh2-driven H3K27 methylation (27). It was first discovered that lncRNA HOTAIR mediates its effect by interacting with PRC2, enhancing methylation of histone H3 lysine 27 (H3K27), leading to silencing of tumor suppressor genes (35). Previous reports have shown that Ezh2 expression is high in renal cancer tissues (36) promoting renal cancer progression, invasion, and metastasis (37). Ezh2 expression is also associated with metastasis and adverse clinical outcome in patients with RCC (37). Thus, we investigated Ezh2 expression in renal cancer and normal kidney tissues as well as cell lines (HK-2, A-498, 786-O). Similar to a previous report (38), we observed that Ezh2 mRNA expression was significantly higher in renal cancer cell lines and renal cancer tissues compared with normal cells and tissues. Also, Ezh2 expression in patients with kidney cancer was associated with higher stage and shorter overall survival. Other lncRNAs (lncRNA EBIC, lncRNA H19) have been shown to interact with Ezh2 in cancer cells based on RNA immunoprecipitation experiments (28, 29). Our RNA immunoprecipitation results also showed that MALAT1 interacts with Ezh2 in renal cancer cell lines, and there was significant positive correlation between MALAT1 and Ezh2 mRNA in renal cancer

tissues. Together, these results indicate that MALAT1 regulates downstream effectors through Ezh2 in renal cancer cells.

Ezh2 enhances methylation of H3K27, leading to gene silencing involved in cancer progression and metastasis (35). Of the various metastasis-related pathways, EMT has been extensively studied in numerous cancers including renal cancer (39). EMT is an important step in cancer progression and metastasis and downregulation of tumor suppressor gene *E-cadherin* (*CDH1*) is typically observed during EMT in renal cancer (39). Recently, Liu and colleagues (37) found that silencing of Ezh2 with sh-Ezh2 inhibited renal cancer cell invasion and migration as well as upregulation of E-cadherin. Their ChIP assay results showed that H3K27me3 binds to the *CDH1* gene promoter, and knockdown of Ezh2 significantly decreased the occupancy of H3K27me3 on the *CDH1* promoter in two RCC cell lines (ACHN and 786-O; ref. 37). In the present study, Ezh2 protein expression was significantly decreased, whereas E-cadherin protein expression was significantly increased after knockdown of MALAT1. In addition, E-cadherin protein expression was found to be significantly lower in renal cancer cells and renal cancer tissues, and there was inverse correlation between E-cadherin protein expression and MALAT1 mRNA expression. To determine the effect of H3K27me3 on E-cadherin, we performed ChIP analysis and found that the level of H3K27me3 was significantly decreased in si-MALAT1-transfected cells compared with control cells, suggesting that MALAT1 may decrease E-cadherin expression through Ezh2-mediated

H3K27me3 in renal cancer. Ezh2 also induces Wnt/ β -catenin signaling hyperactivation (40). The Wnt pathway is usually activated in cancer cells causing un-phosphorylated β -catenin to accumulate in the cytoplasm, move to the nucleus, and bind to TCF/LEF, thereby transcriptionally regulating Wnt target genes such as c-Myc that drive tumor formation and promote tumorigenesis (41). Thus, we investigated β -catenin protein expression after knockdown of MALAT1. As expected, β -catenin expression was significantly decreased in si-MALAT1-treated renal cancer cells. In addition, the expression of c-Myc, a downstream effector in the Wnt/ β -catenin signaling pathway was also significantly decreased in si-MALAT1-treated cells. β -catenin in the nuclear fraction was significantly decreased as was TCF reporter activity (TOPflash activity) with MALAT1 knockdown in RCC cell lines (A-498 and 786-O). Thus, MALAT1 exerts its oncogenic effect via Ezh2, thereby regulating EMT and β -catenin signaling pathways in renal cancer cells.

Because MALAT1 and β -catenin exist mainly in nucleus, Ji and colleagues (42) investigated the direct interaction between the two. However, there was no direct interaction between MALAT1 and β -catenin based on an RNA-binding protein immunoprecipitation technique in a colon cancer cell line (42). Future study will be needed to elucidate any direct interaction between MALAT1 and β -catenin.

Finally, we looked to see if MALAT1 interacts with other ncRNAs such as miRNA. As a newly described regulatory mechanism, lncRNA can influence posttranscriptional regulation and interfere with miRNA pathways by competing for shared miRNA response elements (43). In some cases, lncRNAs have miRNA response elements and act as a natural miRNA sponge to reduce binding of endogenous miRNAs to target genes. Thus, lncRNA may modulate derepression of miRNA's target gene expression (10, 43–45). Liu and colleagues have shown that lncRNA HOTAIR functions as a ceRNA to regulate expression of human epithelial growth factor receptor 2 (HER2) through competition for miR-331-3p (46). In contrast, miR-125b bound to lncRNA MALAT1 and decreased expression of MALAT1 (47). Our results showed reciprocal repression of MALAT1 and miR-205, which may be a new regulatory mechanism between two ncRNAs, a finding similar to recent results (23).

In conclusion, this is the first report documenting that MALAT1 is highly expressed in clear cell renal cancer tissues and is associated with clinicopathologic parameters including patient outcome. After MALAT1 knockdown, cell invasion was decreased and apoptosis increased in RCC cell lines, suggesting that MALAT1 functions as a renal cancer oncogene. Transcriptional activation of MALAT1 by c-Fos contributed to oncogenesis, whereas knockdown of MALAT1 inhibited oncogenic function and depleted Ezh2, resulting in inhibition of EMT via recovery of E-cadherin and downregulation of β -catenin.

This study shows how overexpression of the lncRNA MALAT1 confers a potent oncogenic signal in renal cancers and may be a novel functional biomarker or therapeutic target in RCC. MALAT1 expression is significantly higher in renal cancer tissues and could be a novel biomarker in blood or urine from patients with RCC. Silencing of lncRNAs including MALAT1 via siRNAs could be a useful therapeutically, but is complicated because of lncRNAs extensive secondary structure or intracellular localization. Recently, Gutschner (48) developed a highly effective silencing method using genomic integration of RNA-destabilizing elements, which may be useful to silence lncRNA expression in patients with cancer. A limitation of the current study is lack of *in vivo* experiments such as an orthotopic animal model using siRNA or shRNA for MALAT1 knockdown, which may be useful to help the molecular mechanism involved in RCC invasion and metastasis. Future studies using a large cohort of samples from patients with renal cancer will be required to establish a strong correlation between MALAT1 expression and renal cancer clinicopathologic characteristics as well as help identify potential clinical application in patients with RCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: H. Hirata, N. Ishii, R. Dahiya

Development of methodology: H. Hirata

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): V. Shahryari, K. Nakajima, Z.L. Tabatabai

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Hirata, Y. Hinoda, Z.L. Tabatabai

Writing, review, and/or revision of the manuscript: H. Hirata, Y. Hinoda

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Hirata, G. Deng, N. Ishii

Study supervision: H. Hirata, R. Dahiya

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References

1. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. *CA Cancer J Clin* 2014;64:9–29.
2. Olshan AF, Kuo TM, Meyer AM, Nielsen ME, Purdue MP, Rathmell WK. Racial difference in histologic subtype of renal cell carcinoma. *Cancer Med* 2013;2:744–9.
3. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, et al. Cancer statistics, 2008. *CA Cancer J Clin* 2008;58:71–96.
4. Linehan WM. Genetic basis of kidney cancer: role of genomics for the development of disease-based therapeutics. *Genome Res* 2012;22:2089–100.
5. Walsh N, Larkin A, Kennedy S, Connolly L, Ballot J, Ooi W, et al. Expression of multidrug resistance markers ABCB1 (MDR-1/P-gp) and ABCG1 (MRP-1) in renal cell carcinoma. *BMC Urol* 2009;9:6.
6. Djebali S, Davis CA, Merkel A, Dobin A, Lassmann T, Mortazavi A, et al. Landscape of transcription in human cells. *Nature* 2012;489:101–8.
7. Martens-Uzunova ES, Böttcher R, Croce CM, Jenster G, Visakorpi T, Calin GA. Long noncoding RNA in prostate, bladder, and kidney cancer. *Eur Urol* 2014;65:1140–51.

8. Ponting CP, Oliver PL, Reik W. Evolution and functions of long noncoding RNAs. *Cell* 2009;136:629–41.
9. Wapinski O, Chang HY. Long noncoding RNAs and human disease. *Trends Cell Biol* 2011;21:354–61.
10. Shi X, Sun M, Liu H, Yao Y, Song Y. Long non-coding RNAs: a new frontier in the study of human diseases. *Cancer Lett* 2013;339:159–66.
11. Zhou S, Wang J, Zhang Z. An emerging understanding of long noncoding RNAs in kidney cancer. *J Cancer Res Clin Oncol* 2014;140:1989–95.
12. Qiao HP, Gao WS, Huo JX, Yang ZS. Long non-coding RNA GAS5 functions as a tumor suppressor in renal cell carcinoma. *Asian Pac J Cancer Prev* 2013;14:1077–82.
13. Kawakami T, Chano T, Minami K, Okabe H, Okada Y, Okamoto K. Imprinted DLK1 is a putative tumor suppressor gene and inactivated by epimutation at the region upstream of GTL2 in human renal cell carcinoma. *Hum Mol Genet* 2006;15:821–30.
14. Bertozzi D, Iurlaro R, Sordet O, Marinello J, Zaffaroni N, Capranico G. Characterization of novel antisense HIF-1 α transcripts in human cancers. *Cell Cycle* 2011;10:1389–97.
15. Frevel MA, Sowerby SJ, Petersen GB, Reeve AE. Methylation sequencing analysis refines the region of H19 epimutation in Wilms tumor. *J Biol Chem* 1999;274:29331–40.
16. Chiesa N, De Crescenzo A, Mishra K, Perone L, Carella M, Palumbo O, et al. The KCNQ1OT1 imprinting control region and non-coding RNA: new properties derived from the study of Beckwith-Wiedemann syndrome and Silver-Russell syndrome cases. *Hum Mol Genet* 2012;21:10–25.
17. Davis JJ, Hsi BL, Arroyo JD, Vargas SO, Yeh YA, Motyckova G, et al. Cloning of an Alpha-TFEB fusion in renal tumors harboring the t(6;11)(p21;q13) chromosome translocation. *Proc Natl Acad Sci U S A* 2003;100:6051–6.
18. Kuiper RP, Schepens M, Thijssen J, van Asseldonk M, van den Berg E, Bridge J, et al. Upregulation of the transcription factor TFEB in t(6;11)(p21;q13)-positive renal cell carcinomas due to promoter substitution. *Hum Mol Genet* 2003;12:1661–9.
19. Chiyomaru T, Fukuhara S, Saini S, Majid S, Deng G, Shahryari V, et al. Long non-coding RNA HOTAIR is targeted and regulated by miR-141 in human cancer cells. *J Biol Chem* 2014;289:12550–65.
20. An J, Liu H, Magyar CE, Guo Y, Veena MS, Srivatsan ES, et al. Hyperactivated JNK is a therapeutic target in pVHL-deficient renal cell carcinoma. *Cancer Res* 2013;73:1374–85.
21. Hirata H, Ueno K, Nakajima K, Tabatabai ZL, Hinoda Y, Ishii N, et al. Genistein downregulates onco-miR-1260b and inhibits Wnt-signalling in renal cancer cells. *Br J Cancer* 2013;108:2070–8.
22. Liu PY, Enriquez D, Marshall GM, Tee AE, Polly P, Wong M, et al. Effects of a novel long noncoding RNA, lncUSMycN, on N-Myc expression and neuroblastoma progression. *J Natl Cancer Inst* 2014;106:pii: dju113.
23. Ma MZ, Li CX, Zhang Y, Weng MZ, Zhang MD, Qin YY, et al. Long non-coding RNA HOTAIR, a c-Myc activated driver of malignancy, negatively regulates miRNA-130a in gallbladder cancer. *Mol Cancer* 2014;13:156.
24. Tripathi V, Shen Z, Chakraborty A, Giri S, Freier SM, Wu X, et al. Long noncoding RNA MALAT1 controls cell cycle progression by regulating the expression of oncogenic transcription factor B-MYB. *PLoS Genet* 2013;9:e1003368.
25. Feldstein O, Nizri T, Doniger T, Jacob J, Rechavi G, Ginsberg D. The long non-coding RNA ERIC is regulated by E2F and modulates the cellular response to DNA damage. *Mol Cancer* 2013;12:131.
26. Wang P, Xue Y, Han Y, Lin L, Wu C, Xu S, et al. The STAT3-binding long noncoding RNA lnc-DC controls human dendritic cell differentiation. *Science* 2014;344:310–3.
27. Benetatos L, Voulgaris E, Vartholomatos G, Hatzimichael E. Non-coding RNAs and EZH2 interactions in cancer: long and short tales from the transcriptome. *Int J Cancer* 2013;133:267–74.
28. Sun NX, Ye C, Zhao Q, Zhang Q, Xu C, Wang SB, et al. Long noncoding RNA-EBIC promotes tumor cell invasion by binding to EZH2 and repressing E-cadherin in cervical cancer. *PLoS ONE* 2014;9:e100340.
29. Luo M, Li Z, Wang W, Zeng Y, Liu Z, Qiu J. Long non-coding RNA H19 increases bladder cancer metastasis by associating with EZH2 and inhibiting E-cadherin expression. *Cancer Lett* 2013;333:213–21.
30. Fan Y, Shen B, Tan M, Mu X, Qin Y, Zhang F, et al. TGF- β -induced upregulation of malat1 promotes bladder cancer metastasis by associating with suz12. *Clin Cancer Res* 2014;20:1531–41.
31. Jeggari A, Marks DS, Larsson E. miRcode: a map of putative microRNA target sites in the long non-coding transcriptome. *Bioinformatics* 2012;28:2062–3.
32. Majid S, Saini S, Dar AA, Hirata H, Shahryari V, Tanaka Y, et al. MicroRNA-205 inhibits Src-mediated oncogenic pathways in renal cancer. *Cancer Res* 2011;71:2611–21.
33. Gutschner T, Hämmerle M, Diederichs S. MALAT1 – a paradigm for long noncoding RNA function in cancer. *J Mol Med (Berl)* 2013;91:791–801.
34. Milde-Langosch K. The Fos family of transcription factors and their role in tumorigenesis. *Eur J Cancer* 2005;41:2449–61.
35. Tsai MC, Manor O, Wan Y, Mosammaparast N, Wang JK, Lan F, et al. Long noncoding RNA as modular scaffold of histone modification complexes. *Science* 2010;329:689–93.
36. Wagener N, Holland D, Bulkescher J, Crnković-Mertens I, Hoppe-Seyler K, Zentgraf H, et al. The enhancer of zeste homolog 2 gene contributes to cell proliferation and apoptosis resistance in renal cell carcinoma cells. *Int J Cancer* 2008;123:1545–50.
37. Liu L, Xu Z, Zhong L, Wang H, Jiang S, Long Q, et al. EZH2 promotes tumor cell migration and invasion via epigenetic repression of E-cadherin in renal cell carcinoma. *BJU Int* 2014 Feb 25. [Epub ahead of print].
38. Xu B, Aboubih S, Sircar K, Kassouf W, Mansure JJ, Aprikian A, et al. Enhancer of zeste homolog 2 expression is associated with metastasis and adverse clinical outcome in clear cell renal cell carcinoma: a comparative study and review of the literature. *Arch Pathol Lab Med* 2013;137:1326–36.
39. He H, Magi-Galluzzi C. Epithelial-to-mesenchymal transition in renal neoplasms. *Adv Anat Pathol* 2014;21:174–80.
40. Jung HY, Jun S, Lee M, Kim HC, Wang X, Ji H, McCrea PD, et al. PAF and EZH2 induce Wnt/ β -catenin signaling hyperactivation. *Mol Cell* 2013;52:193–205.
41. Ahmad I, Morton JP, Singh LB, Radulescu SM, Ridgway RA, Patel S, et al. β -Catenin activation synergizes with PTEN loss to cause bladder cancer formation. *Oncogene* 2011;30:178–89.
42. Ji Q, Liu X, Fu X, Zhang L, Sui H, Zhou L, et al. Resveratrol inhibits invasion and metastasis of colorectal cancer cells via MALAT1 mediated Wnt/ β -catenin signal pathway. *PLoS ONE* 2013;8:e78700.
43. Salmena L, Poliseno L, Tay Y, Kats L, Pandolfi PP. A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language? *Cell* 2011;146:353–8.
44. Li JH, Liu S, Zhou H, Qu LH, Yang JH. starBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data. *Nucleic Acids Res* 2014;42:D92–7.
45. Tay Y, Rinn J, Pandolfi PP. The multilayered complexity of ceRNA crosstalk and competition. *Nature* 2014;505:344–52.
46. Liu XH, Sun M, Nie FQ, Ge YB, Zhang EB, Yin DD, et al. Lnc RNA HOTAIR functions as a competing endogenous RNA to regulate HER2 expression by sponging miR-331–3p in gastric cancer. *Mol Cancer* 2014;13:92.
47. Han Y, Liu Y, Zhang H, Wang T, Diao R, Jiang Z, et al. Hsa-miR-125b suppresses bladder cancer development by down-regulating oncogene SIRT7 and oncogenic long noncoding RNA MALAT1. *FEBS Lett* 2013;587:3875–82.
48. Gutschner T. Silencing long noncoding RNAs with genome-editing tools. *Methods Mol Biol* 2015;1239:241–50.

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Long Noncoding RNA MALAT1 Promotes Aggressive Renal Cell Carcinoma through Ezh2 and Interacts with miR-205

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