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Downregulation of long non-coding RNA ENSG00000241684 is associated with poor prognosis in advanced clear cell renal cell carcinoma

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ABSTRACT

Objective: In order to identify potential novel biomarkers of advanced clear cell renal cell carcinoma (ccRCC), we re-evaluated published long non-coding RNA (lncRNA) expression profiling data.
Materials and methods: The lncRNA expression profiles in ccRCC microarray dataset GSE47352 were analyzed and an independent cohort of 61 clinical samples including 21 advanced and 40 localized ccRCC patients was used to confirm the most statistically significant lncRNAs by real time PCR. Next, the relationships between the selected lncRNAs and ccRCC patients' clinicopathological features were investigated. The effects of LncRNAs on the invasion and proliferation of renal carcinoma cells were also investigated.
Results: The PCR results in a cohort of 21 advanced ccRCC and 40 localized ccRCC tissues were used for confirmation of the selected lncRNAs which were statistically most significant. The PCR results showed that the expression of three LncRNA (ENSG00000241684, ENSG00000231721 and NEAT1) were significantly downregulated in advanced ccRCC. Kaplan–Meier analysis revealed that reduced expression of LncRNA ENSG00000241684 and NEAT1 were significantly associated with poor overall survival. The univariate and multivariate Cox regression indicated LncRNA ENSG00000241684 had significant hazard ratios for predicting clinical outcome. LncRNA ENSG00000241684 expression was negatively correlated with pTNM stage. Overexpression of ENSG00000241684 significantly impaired cell proliferation and reduced the invasion ability in 786-O and ACHN cells.
Conclusion: lncRNAs are involved in renal carcinogenesis and decreased lncRNA ENSG00000241684 expression may be an independent adverse prognostic factor in advanced ccRCC patients.

Introduction

Renal cell carcinoma (RCC) is the third most common genitourinary cancer and accounts for about 2% of all cancers, of which the clear cell renal cell carcinoma (ccRCC) is the most common subtype of RCC, accounting for an estimated 70% of all patients [1–3]. While the resection of localized RCC (stage I and II) is usually curative, the prognosis of advanced ccRCC (stage III and IV) is poor [4]. In addition, 20%–40% of the patients with localized ccRCC would experience recurrence and metastasis [5]. Patients with advanced ccRCC have a median survival of only 6–12 months and only 9% survive 5 years due to strong resistance to chemotherapy and radiotherapy [6]. In addition, it is difficult to differentiate precisely advanced and localized ccRCC without tumor biopsy and histopathological classification. As a result, identifying novel biomarkers and a better estimation of tumor aggressiveness would be helpful for the management of the patients with renal carcinoma, especially for advanced ccRCC.

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Long noncoding RNAs (LncRNA) are key regulators of cellular function and dysregulated lncRNAs occur in a variety of cancers and play significant roles in oncogenic or tumor-suppressive pathways [7,8]. Several expression profiling studies have reported dysregulation of several lncRNAs (MALAT1, NBAT-1) may be substantial contributors in cancer development and serve as potential diagnostic biomarkers and/or therapeutic targets [9,10]. However, expression patterns and their characteristics of lncRNA in advanced ccRCC remain largely unexplored.

In order to identify the relationship of novel lncRNAs with prognostic relevance in advanced ccRCC, we re-analyzed lncRNA earlier published microarray expression data and performed quantitative real-time PCR to confirm the findings in an enlarged cohort of ccRCC patients, including localized and advanced ccRCC. This study would provide novel information on lncRNAs expression profiles in advanced ccRCC, which might be beneficial to the precise diagnosis, subcategorization and ultimately, the individualized therapy of advanced ccRCC.

Materials and methods

Patients and samples

Total 61 cases of ccRCC patients were collected from patients who had underwent radical or partial nephrectomy in the department of Urology, Fudan University Shanghai Cancer Center (FUSCC) between 2008 and 2010, including 21 advanced (stage III and IV) and 40 localized (stage I and II) ccRCC. None of these patients received chemotherapy or radiotherapy. The ccRCC tissues were confirmed by postoperative pathologic analysis and these specimen tissues were stored at RNA later.

Tumor staging was redefined according to the AJCC/UICC TNM 2010 classification by reviewing the gross description, gross pictures, and representative slides. Patients with pN0/pNx (cN0) and M0 disease and pT1 and pT2 RCC were grouped as localized RCC, and analyzed separately compared to the group of advanced RCC defined as pN1 and M1 disease and pT3 and pT4 tumours, which was line with the previous study [11,12]. Patients were regularly followed up by telephone or in the clinic every 3 months until December 2014 or the date of death. Tumor recurrence, progression, metastasis, cause and date of death were recorded. Overall survival (OS) was defined as the time from the date of surgery to the date of death or final clinical follow-up. The study protocol was approved by the Ethics Committee of FUSCC and patients provided written informed consent prior to enrollment.

Microarray analysis

The ccRCC microarray datasets and corresponding clinical data in this study were directly downloaded from Gene expression omnibus (GEO) database:GSE47352, including the expression of 2448 lncRNA transcripts in localized (n = 5) and advanced (n = 4) ccRCC tissues. Differentially expressed lncRNA transcripts were identified using the Arraytool software.

GSE47352 fulfilled the following criteria: (i) a comparison between advanced and localized ccRCC tissues was performed; (ii) the same chip platform (Affymetrix HG-U133 Plus 2.0 Array) was used in the analysis.

Screening for aberrantly expressed RNA transcripts from GSE47352

In order to discover potential biomarkers of advanced clear cell renal cell carcinoma, we analyzed the lncRNA microarray data and stringent filtering criteria (fold change > 2, $P < .05$) were used to explore the different expression between the advanced and localized ccRCC. Schematic of the workflow and the heatmap of lncRNA expression profile was separately showed in Supplementary Fig. 1 and Supplementary Fig. 2. Since the upregulated lncRNAs were studied in another article (unpublished data), we focused on downregulated lncRNAs in this article. Compared with localized cohort, eight LncRNAs were downregulated in the advanced ccRCC cohort, including ENSG00000241684, ENSG00000235501, ENSG00000231721, ENSG00000214145, NEAT1, ENSG00000244020, PSORS1C3, H19 (Supplementary Table 1). In our previous study, ENSG00000244020 was decreased in ccRCC compared to adjacent normal tissue ($P < .05$) and had no predictive value for prognosis [13]. Wang et al. have found LncRNA H19 might be considered as a potential prognostic indicator and a target for gene therapy of ccRCC [14]. So we choose ENSG00000241684, ENSG00000235501, ENSG00000231721, ENSG00000214145, NEAT1 and PSORS1C3 as the main research subjects.

RNA extraction and quantitative real-time PCR

Total RNA was extracted by TRIzol reagent (Invitrogen). The RNA quantity was determined using a NanoDrop 2000 spectrophotometer and 1 μg of total RNA was reverse-transcribed using the PrimeScriptPTMP RT Reagent Kit (Perfect Real Time; Takara, Shiga, Japan). The amplified transcript level of each specific lncRNA was normalized to that of GAPDH. The primers were provided by Shenggong Company, Shanghai. The sequences of forward and reverse primers were shown in Supplementary Table 2.

Cell culture and treatment

Human RCC cell lines (786-O) were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) and ACHN were cultured in DMEM medium (Gibco, Carlsbad, CA, USA) and Caki-1 and Caki-2 were cultured in McCoy's 5A medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum at 37 °C in a 5% CO2 atmosphere. All the cells were purchased from American Type Culture collection (ATCC, Manassas, VA, USA). Transfection of the ENSG00000241684 and non-specific negative control plasmids was performed using the FuGENE transfection reagent (Life Technologies, Shanghai, China).

Cell proliferation assay and transwell assay

Cell proliferation was assessed by the BrdU incorporation assay (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, control and treated RCC cells were seeded onto the 96-well plates at an initial density of 4×10^3 cells per well. BrdU labeling solution (10 μl/well) was added to the cells at specified time points. After incubating for 2 h, culture medium was removed and the cells were fixed. Then DNA was denatured by adding FixDenat (200 μl/well) and then anti-BrdU-POD working solution (100 μl/well) was added to the cells and incubated for 90 min. The immune complexes were detected by the subsequent substrate reaction. The reaction product was quantified by measuring the absorbance at 370 nm (reference wavelength: approx. 492 nm).

Transwell assays were performed using 24-well transwells (8-μm pore size; Millipore) precoated with Matrigel (BD Biosciences, USA). Cells at logarithmic phase were transfected with ENSG00000241684 or control plasmid. Transfected cells were then harvested, and 1×10^5 cells were seeded in serum-free medium into the upper chamber, whereas medium supplemented with 20% FBS was applied to the lower chamber as a chemoattractant. After

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