

RESEARCH ARTICLE

Influence of Curcumin on HOTAIR-Mediated Migration of Human Renal Cell Carcinoma Cells

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Abstract

Background: This study investigated the influence of curcumin on HOX transcript antisense RNA (HOTAIR)-mediated migration of cultured renal cell carcinoma (RCC) cells. **Materials and Methods:** Five RCC cell lines (769-P, 769-P-vector, 769-P-HOTAIR, 786-0, and Kert-3) were maintained *in vitro*. The expression of HOTAIR mRNA was determined by quantitative real-time PCR and cell migration was measured by transwell migration assay. The effects of different concentrations of curcumin (0 to 80 $\mu\text{mol/L}$) on cell proliferation was determined by the CCK-8 assay and influence of non-toxic levels (0 to 10 μM) on the migration of RCC cells was also determined. **Results:** Comparison of the 5 cell lines indicated a correlation between HOTAIR mRNA expression and cell migration. In particular, the migration of 769-P-HOTAIR cells was significantly higher than that of 769-P-vector cells. Curcumin at 2.5-10 μM had no evident toxicity against RCC cells, but inhibited cell migration in a concentration-dependent manner. **Conclusions:** HOTAIR expression is correlated with the migration of RCC cells, and HOTAIR may be involved in the curcumin-induced inhibition of RCC metastasis.

Keywords: Curcumin - renal cell carcinoma - migration - HOX transcript antisense RNA

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Introduction

Renal cell carcinoma (RCC) is the most common kidney cancer, with an annual incidence of 5-10/100,000 among Europeans (Ferlay et al., 2007) and an increasing incidence of about 2% annually (Moskvina et al., 2011; Volpe et al., 2010). RCC accounts for 3% of malignancies in humans (Mohan et al., 2009) and 85% of primary renal carcinomas in adults, and its mortality rate is as high as 40% (Ficarra et al., 2006; Lam et al., 2009). Twenty percent of patients with early-stage RCC may develop metastasis after surgery (Rouviere et al., 2006). Patients with RCC metastasis usually have poor prognoses, with a median survival time of 6-12 months, and a 5-year survival rate of only 9% (Chow et al., 1999). Invasion and metastasis of RCC are the major causes of therapeutic failure, so effective control of these is key to improving patient prognosis.

HOX transcript antisense RNA (HOTAIR) is the first identified long non-coding RNA (lncRNA). This gene is trans-acting and is upregulated in numerous tumors (Zhang et al., 2014). Recent studies indicated that HOTAIR mediates the migration of breast cancer cells, liver cancer cells, and colon cancer cells via epigenetic modifications (Gupta et al., 2010; Kogo et al., 2011; Yang et al., 2011), but there have been no studies on the effect of HOTAIR on migration of RCC cells.

Curcumin is a phenolic pigment extracted from rhizoma curcumae longae, the rhizome of turmeric (*Curcuma longa*). This compound has multiple biological activities including anti-coagulation, lipid-lowering, anti-oxidative, anti-inflammatory, anti-atherosclerotic, anti-tumor, anti-rheumatic, liver-protecting, cholagogic, and stomach-protecting (Balasubramanian et al., 2007; Guo et al., 2008; Zhao et al., 2008). In addition, it inhibits the invasion and metastasis of certain malignancies (Boonrao et al., 2010; Zhang et al., 2013). However, the mechanism of curcumin-mediated inhibition of cancer is poorly understood. The present study investigated the effect of curcumin on the migration of RCC cells *in vitro* and explored the potential role of HOTAIR-mediated epigenetic modification in this effect.

Materials and Methods

Cell lines and drugs

Several types of cultured human RCC cells were used: 769-P cells with low metastatic activity and low HOTAIR expression (Shanghai Cell Bank of Chinese Academy of Sciences), 769-P-HOTAIR cells with high and stable HOTAIR expression (Hebron Technology Co., Hangzhou), 769-P-vector cells with expression of a blank vector (Hebron), 786-0 cells with high migration and high HOTAIR expression, and Kert-3 cells with high migration

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and low HOTAIR expression (both from Shanghai Cell Bank of Chinese Academy of Sciences). Curcumin was purchased from Sigma (USA), and a 100 mM curcumin solution was prepared in DMSO.

Reagents

The following reagents were used: MEM (GIBCO), RPMI 1640 (GIBCO), CCK-8 Kit (Dojindo, Japan), fetal bovine serum (FBS; Hyclone), 0.25% Trypsin (GIBCO), and 100×penicillin/streptomycin (PS; Hangzhou Haotian Bio-Technology Co., Ltd.)

Cell culture

Cells were maintained in MEM containing 10% FBS and 1×PS in an environment with 5% CO₂ at 37°C. After washing in PBS, cells were digested with 0.25% trypsin and 0.02% EDTA, followed by cell passaging. The cell density was adjusted, and cells in the logarithmic growth phase were harvested for experiments.

HOTAIR mRNA expression

RNA extraction: Total RNA was separately extracted from the different types of cells with Trizol reagent and then stored at -80°C before use.

Reverse transcription: Two samples of total RNA were used for reverse transcription on ice. In brief, total RNA (about 1 µg, the template) was mixed with 1 µL of 0.5 µg/µL Oligo (dT) primers and DEPC-treated water (up to 12 µL), followed by incubation at 65°C for 5 min. After addition of 1 µL of 20 U/µL RiboLock Ribonuclease inhibitor, 4 µL of 5×Reaction Buffer, and 2 µL of 10 mmol/L dNTP, the sample was incubated at 37°C for 5 min. Then, 1 µL of 200 U/µL RevertAid M-Mulv Reverse Transcriptase was added and the final volume was adjusted to 20 µL. Then, the sample was incubated at 25°C for 5 min, 42°C for 60 min and then 70°C for 5 min to terminate the reaction. The resulting cDNA was stored at -70°C.

Fluorescence-based quantitative real-time PCR: The primers were as follows: Homo actin: 5' TGCCATCCTAAAAGCCACC 3' (forward), 5' CAATGCTATCACCTCCCCTGT 3' (Reverse); Homo HOTAIR: 5' CAAACAGAGTCCGTTTCAGTGTCA 3' (forward), 5' GGTGGATTCTGGGTGGGT 3' (Reverse). Detection of mRNA expression used the SBGreen Mix kit according to the manufacturer's instructions (Roche, USA). In brief, 12.5 µL of IQ SYBR Green Supermix, 1 µL of 10 µmol/L forward primer, 1 µL of 10 µmol/L reverse primer, 1 µL of cDNA, and up to 24 µL of distilled water (as appropriate) were mixed, followed by incubation at 95°C for 5 min for pre-denaturation. Then, this mixture was incubated at 95°C for 10 s, 59°C for 20 s, and 72°C for 20 s for 40 cycles. Then, fluorescence was measured every 0.5°C as the temperature of the sample increased from 70 to 95°C (melt curve). Data were analyzed with BIO-RAD CFX Manager software.

Toxicity of curcumin

In brief, cells in the logarithmic growth phase were harvested by digestion with 0.25% trypsin+0.02% EDTA, followed by centrifugation. Following cell counting, cells were seeded into 96-well plates at 5×10³ cells/well. In a

blank control, solution without cells was added. Cells were incubated at 37°C in an environment with 5% CO₂ for 24 h. Then, different concentrations of curcumin (0, 1.25, 2.5, 5, 10, 20, 40 and 80 µM) was added, followed by incubation for 24 h. Cell viability was determined by CCK-8 assay.

Influence of curcumin on the migration cells

Cell migration: A transwell chamber assay was used to measure the migration of the different cells. In brief, cells in the logarithmic growth phase were harvested by digestion with 0.25% trypsin+0.02% EDTA, followed by centrifugation. Following cell counting, cells were seeded into 96-well plates at a density of 2.5×10⁴ cells/well. Cells were incubated at 37°C in an environment with 5% CO₂ for 24 h, digested with 0.25% trypsin, and harvested by centrifugation. Then, viable cells were counted after trypan blue staining.

In addition, cells were re-suspended in serum-free RPMI1640 and cell density was adjusted to 1×10⁵ cells/mL. Then, 200 µL of cell suspension was added to the upper chamber, and 600 µL of medium containing 10% FBS was added to the lower chamber. Then, cells were incubated at 37°C in an environment with 5% CO₂ for 24 h. Cells from the chambers were collected, and were fixed in 4% paraformaldehyde for 20 min. The non-migrated cells in the upper chamber were removed with a swab, and the Transwells were placed upside down and dried. Then, 200 µL of 0.1% crystal violet was added to 24-well plates, and the chambers were placed in these wells, followed by incubation for 60-90 min at room temperature. Cells from the chambers were collected, washed with PBS, and observed by light microscopy (50×). Six fields were randomly selected, and cells were counted, followed by statistical analysis.

Influence of curcumin: The transwell invasion assay was used to assess the effect of curcumin on cell migration. In brief, cells in the logarithmic growth phase were harvested by digestion with 0.25% trypsin+0.02% EDTA and centrifugation. After cell counting, cells were seeded into 6-well plates at 2.5×10⁴ cells/well, followed by incubation at 37°C in an environment with 5% CO₂. When confluence reached about 100%, cells were treated with curcumin at different concentrations (0, 1.25, 2.5, 5 and 10 µM) for 24 h. Cell viability was determined as described above.

Statistical analysis

SPSS version 17.0 was used for statistical analysis. Data are expressed as means±standard deviations (SDs), and comparisons employed the t-test. A *p*-value less than 0.05 was considered statistically significant.

Results

HOTAIR mRNA expression in 5 RCC cell lines

Figure 1 shows the HOTAIR mRNA expression in 5 RCC cell lines. The 769-P-HOTAIR cells and 786-0 cells had significantly higher HOTAIR expression than Kert-3 cells (*p*=0.002 and *p*=0.003, respectively), and HOTAIR expression was the lowest in 769-P cells and 769-P-vector

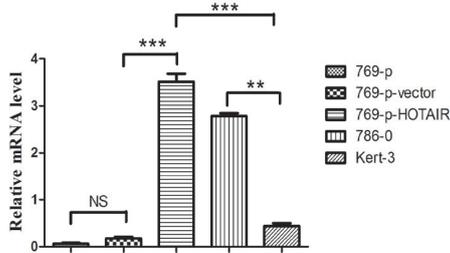


Figure 1. Expression of HOTAIR mRNA in the 5 RCC Cell Lines. Expression of HOTAIR mRNA was detected in 5 RCC cells lines of 769-p, 769-p-vector, 769-p-HOTAIR, 786-0 and kert-3 by q RT-PCR. Values in assays were expressed as mean±SD. *** $p < 0.001$, ** $p \leq 0.01$. NS=non-significant

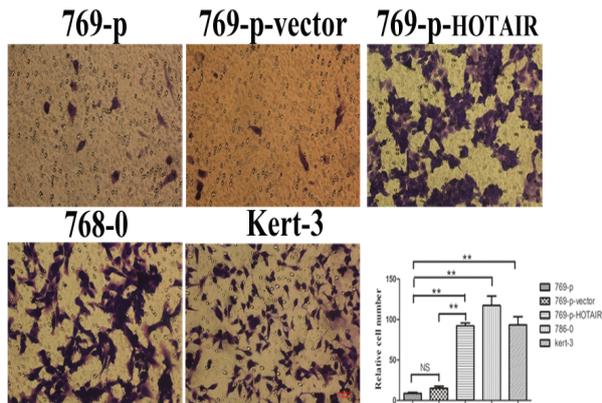


Figure 2. Migration of Cells in the 5 RCC Cell Lines. RCC cell migration was assessed in 769-p, 769-p-vector, 769-p-HOTAIR, 786-0 and kert-3 by transwell migration assays after 24h. Representatives were from three independent experiments. Values in assays were expressed as mean±SD. * $p \leq 0.05$, ** $p \leq 0.01$. NS=non-significant

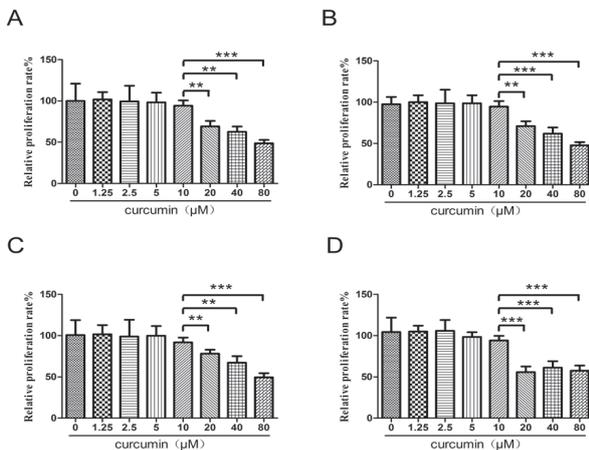


Figure 3. Toxicity of curcumin in 4 RCC cell lines. Evaluation of effects of curcumin on cell proliferation of 4 RCC cell lines by CCK-8 assays. A) 769-P; B) 769-P-HOTAIR; C) 786-0; D) Kert-3; Representatives were from three independent experiments. Values in assays were expressed as mean±SD. *** $p < 0.001$, ** $p < 0.01$, * $p \leq 0.05$, NS=non-significant

cells. In addition, expression was significantly greater in 769-P-HOTAIR cells than 769-P-vector cells ($p=0.0002$).

Migration of 5 RCC cells

Figure 2 shows the results of the Transwell migration

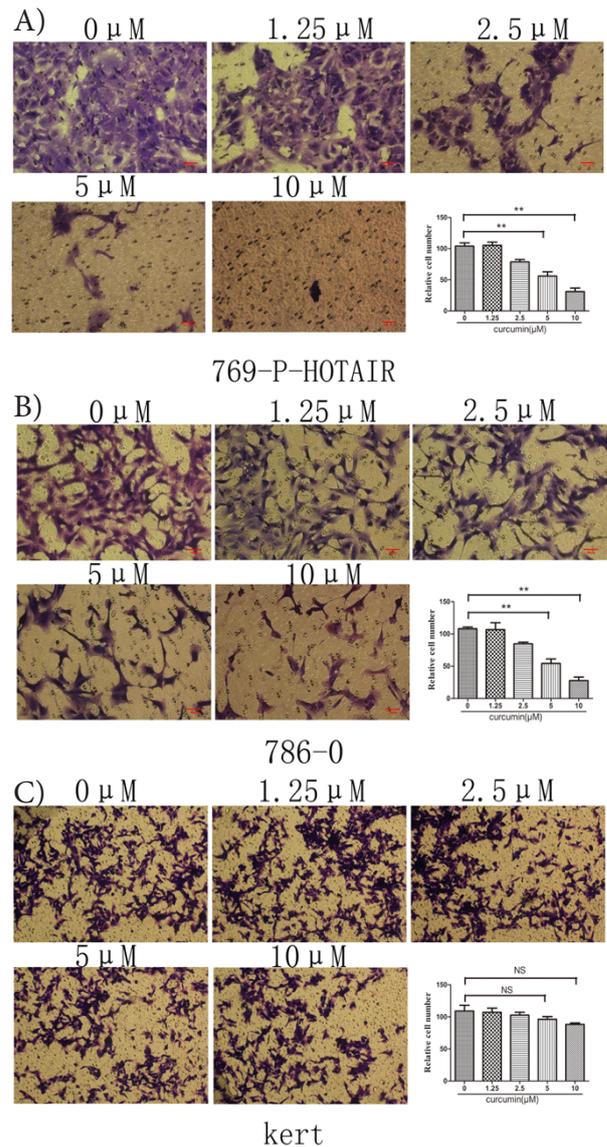


Figure 4. Effect of Curcumin on the Migration of 3 RCC Cell Lines. Evaluation of effects of curcumin on cell migration of 3 RCC cell lines by transwell migration assays after 24h. A) 769-P-HOTAIR; B) 786-0; C) Kert-3. Representatives were from three independent experiments. Values in assays were expressed as mean±SD. * $p < 0.05$, ** $p < 0.01$, NS=non-significant

assays. The 769-P-HOTAIR cells, 786-0 cells, and Kert-3 cells exhibited more migration than the 769-P cells (p -values of 0.002, 0.00131 and 0.00183, respectively). In addition, migration was also greater in 769-P-HOTAIR cells than in 769-P-vector cells ($p=0.0015$).

Toxicity of curcumin against 4 RCC cell lines

Figure 3 shows the toxicity of curcumin in the 4 RCC cell lines. The results indicate that curcumin inhibited the growth of all RCC cell lines in a concentration-dependent manner. In particular, there was significant inhibition of cell growth when the curcumin concentration was greater than 10 µM ($p=0.001-0.0083$ for all tested cell lines). When the curcumin concentration was 10 µM or less, curcumin had no significant effect within 24 h. Thus, we used a curcumin concentration of 10 µM or less (the approximate threshold for toxicity) in the subsequent migration experiments.

Effect of curcumin on the migration of 3 RCC cell lines

The results of the Transwell migration invasion assay showed that curcumin inhibited the migration of 769-P-HOTAIR cells and 786-0 cells in a concentration dependent manner (Figure 4). In particular, a curcumin concentration of 5 μM or greater markedly suppressed cell migration compared with the control group (p -value of 0.017-0.0030). However, under the same conditions, curcumin at 5 -10 μM had no obvious effect on the migration of Kert-3 cells (p -values of 0.831-0.1341).

Discussion

Recent studies of the pathogenesis of tumors have shown that the occurrence and development of tumors are related to genetic as well as epigenetic factors, such as DNA methylation, histone modification, chromatin remodeling, and expression of non-coding RNAs (Esteller, 2007). In addition, there is evidence showing that epigenetic events play important roles in the metastasis of RCC (Bae et al., 2002; Bernstein et al., 2005; Carninci et al., 2005; Drewell et al., 2002; Kern et al., 2007; Sessa et al., 2007). It has been confirmed that expression of LncRNAs is closely related to the occurrence and metastasis of multiple malignancies (van Herpen et al., 2002; Yap et al., 2013). HOTAIR is a type of LncRNA that mediates numerous processes in cancer metastasis via epigenetic modifications, and thus is closely associated with the occurrence and development of cancers (Zhang et al., 2014). Recent studies determined that HOTAIR is over-expressed in the metastatic foci of breast cancers, liver cancers, and colon cancers (Gupta et al., 2010; Kogo et al., 2011; Yang et al., 2011). Studies of cancer pathogenesis showed that HOTAIR binds to PRC2 and LSD1/COREST/REST and mediates the binding of both complexes to specific genomic loci. This causes the trimethylation of H3K27 and de-dimethylation of H3K4, resulting in closed state of chromosome and gene silencing, a process that is believed to mediate the metastasis of at least 3 different cancers (Gupta et al., 2010; Kogo et al., 2011; Yang et al., 2011). The results of the present study showed that 768-0 cells (which had high HOTAIR expression) and 769-P-HOTAIR cells (which had high and stable HOTAIR expression) had more potent migration than 769-P cells (which had low HOTAIR expression). This suggests that HOTAIR may have a role in mediating the migration of RCC cells, as has been suggested for several other cancers.

Invasion and metastasis are the major causes of therapeutic failure in RCC patients, so control of these processes is key to controlling RCC. There is increasing attention on the development of drugs with low toxicity to inhibit the metastasis of RCC. Studies have shown that curcumin can significantly inhibit the growth and migration of multiple cancer cells (Balasubramanian et al., 2007), although the exact mechanisms are still unclear. There is evidence that curcumin disrupts the invasion and metastasis of cancer cells by triggering epigenetic events (Balasubramanian et al., 2007). This study investigated whether curcumin affects the HOTAIR-mediated migration of cultured RCC cells. Our results showed that

curcumin at a concentration of 2.5 to 10 μM inhibited the HOTAIR-mediated invasion and metastasis of RCC cells in a concentration-dependent manner, but had no influence on cell proliferation at these concentrations. Our results suggest that HOTAIR may be involved in the curcumin-induced inhibition of RCC metastasis and provide a basis for study of the treatment of metastatic RCC with curcumin as a targeted therapy.

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