Silencing HCCR2 expression inhibits the proliferation of leukemia cells by inducing apoptosis and promoting cell cycle arrest

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Received July 8, 2013; Accepted September 30, 2013

DOI: 10.3892/ijmm.2013.1518

Abstract. The human cervical cancer oncogene (HCCR2) has been found to be overexpressed in a variety of human malignant tumors cells, and its function is related to cell cycle progression and survival. However, the molecular mechanisms of action of HCCR2 in leukemia remain unclear. In this study, we used the RNA interference strategy to investigate the effects of HCCR2 knockdown in the K562 leukemia cell line, and to explore the potential mechanisms involved. Following transfection with small interfering RNA (siRNA) targeting HCCR2 (HCCR2-siRNA), we examined the effects of HCCR2 knockdown on cell morphology, cell proliferation, cell cycle progression and apoptosis in K562 cells. Morphological changes were evaluated by Wright-Giemsa staining. Cell cycle progression and apoptosis were measured by flow cytometry. The expression levels of genes related to the cell cycle and apoptosis were detected by quantitative RT-PCR (qRT-PCR) and western blot analysis. HCCR2 expression at the mRNA and protein level was significantly decreased following transfection with plasmids expressing HCCR2-siRNA. Silencing HCCR2 expression significantly suppressed cell proliferation, induced G1 cell cycle arrest and promoted the apoptosis of K562 cells. Additionally, we found that the expression of Bax, p53 and p21 was significantly increased, while Bcl-2 expression was significantly decreased in the HCCR2-siRNA-transfected cells. However, the expression of p27 was not affected. These results suggest that the HCCR2 gene plays an important role in the tumorigenesis of leukemia, thus making it an attractive therapeutic target for acute leukemia.

Introduction

Acute leukemia is the most common hematological malignancy that is characterized by an uncontrolled expansion of clonal leukemia cells in the bone marrow and/or peripheral blood often accompanied with anemia, bleeding and frequent infectious complications (1-4). Acute leukemia is generally classified as either acute myelogenous leukemia (AML) or acute lymphoblastic leukemia (ALL) and the primary treatments are remission induction therapy and post-remission chemotherapy (1,2). In recent years, with the introduction of high-dose chemotherapy followed by allogeneic hematopoietic stem cell transplantation (allo-HSCT) and the discovery of novel therapeutic agents, considerable progress has been made in the outcomes of patients with acute leukemia (3-5). However, acute leukemia currently remains an incurable malignancy due to the complex molecular mechanisms behind its tumorigenesis (6,7). Thus, the identification of novel molecular targets is mandatory for the early diagnosis and long-term management of acute leukemia.

The human cervical cancer oncogene (HCCR) was first identified from human cervical tissue through the differential display RT-PCR method by Ko et al. (8). The HCCR gene maps to the long arm of the chromosome 12 and is classified into two types, HCCR1 and HCCR2 according to their molecular characteristics. A comparison of both sequences has revealed that HCCR2 lacks the exon 1 of HCCR1 (8,9). Previous studies have demonstrated that HCCR2 is not only overexpressed in human cervical cancer tissues, but is expressed at high levels in a variety of human malignancies, including breast, kidney, stomach, colon, liver and ovarian cancer (8-13). The functional role of HCCR2 in tumorigenesis may involve the negative regulation of the p53 tumor suppressor gene (8,13,14). Animal experiments have demonstrated that HCCR2-transgenic nude mice form spontaneous breast tumors, which further confirms the crucial role of HCCR2 in tumorigenesis (10). In our previous study, we observed that HCCR2 mRNA was overexpressed in patients with acute leukemia and leukemia cell lines, such as K562 and HL-60 cells, but not in normal leukocytes (15). Therefore, HCCR2 shows great promise as an important molecular target for the diagnosis and treatment of acute leukemia. Although accumulating data have shown that HCCR2 expression in many solid tumors correlates with clinical outcome has been confirmed as a good biomarker for monitoring disease progression, its specific mechanisms of action in acute leukemia remain elusive (10-13).

In the present study, we examined whether silencing HCCR2 expression using small interfering RNA (siRNA) exerts significant anti-proliferative effects in K562 cells. Furthermore, we
also wished to investigate the potential molecular mechanisms behind these effects, which may help identify HCCR2 as a novel therapeutic target for acute leukemia.

Materials and methods

Cell line and cell culture. The K562 cell line, established from human chronic myelogenous leukemia (CML) cells in blastic crisis, was purchased from the Cell Culture Center in Chinese Academy of Medical Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 µg/ml penicillin, 10 µg/ml streptomycin and 2 mmol/l L-glutamine. The cells were maintained in log phase growth at 37°C in a humidified atmosphere containing 5% CO₂.

Silencing HCCR2 expression by siRNAs. We used the K562 cells to perform the HCCR2 silencing experiment, as a high expression level of HCCR2 in K562 cells was demonstrated in our previous study (15). The siRNAs used for the silencing of HCCR2 were designed and synthesized from Invitrogen Life Technologies according to previous literature (13). The oligonucleotide sequences used to generate the three synthesized HCCR2-targeting siRNA are listed in Table I. The siRNAs used for the silencing experiment of HCCR2 were designed and synthesized from Invitrogen Life Technologies according to previous literature (13). The oligonucleotide sequences used to generate the three synthesized HCCR2-targeting siRNA are listed in Table I. The siRNA-H1, targeting HCCR2 mRNA coding sequence 475-493 bp; siRNA-H2, targeting HCCR2 mRNA coding sequence 611-629 bp; and siRNA-H3, targeting HCCR2 mRNA coding sequence 854-872 bp. A scrambled sequence ACTACCGTTGTATAGGTGT was used as the negative control siRNA that is absent in human, mouse, and rat genomes. Each oligonucleotide pair was annealed by incubation at 95°C for 5 min and cooled slowly, and was ligated separately into plasmid vector pcDNA3 (Invitrogen Life Technologies), which had been digested with BamHI and EcoRI. These HCCR2-siRNA vectors were transfected into the K562 cells using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. The ability of the three transfected HCCR2-siRNAs to silence HCCR2 expression was investigated by quantitative RT-PCR (qRT-PCR) and western blot analysis.

Cell proliferation assay. Cell proliferation was determined in cells in 96-well plates using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)/phenazine ethosulfate (PES) assay (Promega Corp., Madison, WI, USA) according to manufacturer's instructions. In brief, the blank control cells, and the negative control- and HCCR2-siRNA-transfected K562 cells were seeded in 96-well plates at densities of 5x10⁴ cells/well. Cultures were set up in triplicate and maintained at 37°C in a humidified atmosphere with 5% CO₂. At 24, 48, 72, 96 and 120 h after transfection, 10 µl MTS-PES (10 mg/ml) (Promega Corp.) were added into each well for an additional 6 h of incubation. A microplate reader was then used to measure the absorbance value at 490 nm for each well, which represented K562 cell proliferation.

Morphological observation of K562 cells. After transfection with HCCR2-siRNA, the K562 cells were smeared on microscope glass plates and stained with Wright-Giemsa. The morphological changes in the K562 cells was observed under a light microscope.

Cell cycle analysis and assessment of apoptosis. Cell cycle distribution was determined using DNA staining with propidium iodide (PI) (BioLegend, San Diego, CA, USA) followed by flow cytometry. Apoptosis assay was performed according to the directions contained in the manual provided with the Annexin V/PI apoptosis assay kit (BioLegend). In brief, the cells were harvested and washed twice with cold phosphate-buffered saline (PBS), and then the cells were resuspended in 1X binding buffer at a concentration of 1x10⁶ cells/ml. A total of 100 µl of the solution was then transferred to a 5-ml culture tube with 5 µl of Annexin V-FITC and 10 µl of PI (20 mg/ml). The cells were gently vortexed and incubated for 15 min at room temperature in the dark. After the addition of 400 ml of 1X binding buffer, the cells were analyzed by flow cytometry. Annexin V⁻ and PI⁻ cells were considered as viable cells. Annexin V⁺ and PI⁻ cells were considered as early-stage apoptotic cells. Annexin V⁺ and PI⁺ cells were considered as late-stage apoptotic cells.

qRT-PCR. Total RNA was isolated from the K562 cells using the TR Izol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions, and then cDNA was synthesized from 2 µg total RNA using a first-strand cDNA synthesis kit (Invitrogen Life Technologies). The PCR amplification protocol was as follows: denaturation at 94°C for 10 min, 40 cycles of 94°C for 15 sec, 58°C 30 sec, and 72°C for 40 sec. The mRNA expression levels of HCCR2, Bcl-2, Bax, p21, p27 and β-actin were detected using ABI 7900 (Applied Biosystems, Foster City, CA, USA) and SYBR-Green chemistry. The relative quantification of the target genes was calculated using the 2⁻ΔΔCt formula by the comparative cycle threshold (Ct) value method (16). The PCR primers were designed based on the corresponding gene structure, and the sequences are listed in Table II. qRT-PCR assays were performed in triplicate.

Western blot analysis. The K562 cells were harvested and subsequently protein was separated by 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) before being transferred onto a polyvinylidene difluoride (PVDF) membrane. The blots were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) at 37°C for 2 h, followed by incubation with primary antibodies against HCCR2, Bcl-2, Bax, p21, p27 and β-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 4°C overnight. The blots were washed with TBST and incubated with a horseradish peroxidase-conjugated secondary antibody at 37°C for 2 h. After several washes with TBST, the immunoreactive bands were visualized on X-ray film and scanned. All experiments were performed in triplicate. The quantitative data from bands were expressed as the target protein/β-actin ratio using BandScan analysis software.

Statistical analysis. All statistical data are presented as the mean ± standard deviation (SD). The effects of treatment among the different groups were compared using a one-way analysis of variance. All analyses were performed using GraphPad Prism software version 5.0 (GraphPad Software Inc., San Diego, CA,
USA) and a value of $P<0.05$ was considered to indicate a statistically significant difference.

**Results**

*Inhibition of HCCR2 expression by siRNAs in K562 cells.* We analyzed the ability of the three HCCR2-siRNAs to suppress HCCR2 mRNA expression by qRT-PCR. The results revealed that transfection with the three HCCR2-siRNAs resulted in varying degrees of reduced HCCR2 mRNA expression in the K562 cells. The quantification analysis revealed that siRNA-H1 ($P<0.01$), siRNA-H2 ($P<0.05$) and siRNA-H3 ($P<0.01$) significantly inhibited HCCR2 expression at the mRNA level compared with the negative control cells (Fig. 1A). The western blot analysis results also indicated that HCCR2 protein expression was significantly suppressed following transfection with the three HCCR2-siRNAs (Fig. 1B). The HCCR2 mRNA and protein levels were not altered in the blank group cells or in the negative control siRNA-transfected cells. As siRNA-H3 showed the highest efficiency in silencing HCCR2 expression, we thus selected siRNA-H3 for the subsequent experiments.

**HCCR2 knockdown suppresses the proliferation of K562 cells.** Our results revealed that silencing the HCCR2 gene affected the proliferation of K562 cells. As shown in Fig. 2, cell proliferation was markedly inhibited in the HCCR2-siRNA-transfected cells from day 4 to 6 after transfection when compared with the blank control and the negative control cells ($P<0.01$). However, there was no statistically significant difference in cell proliferation during the first three days among the three groups of cells.

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**Table I. Sequences used to generate the three synthesized HCCR2-targeting siRNA.**

<table>
<thead>
<tr>
<th>No.</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>siRNA-H1</td>
<td>5’-GATCCGACAGATCTGTGCAACAAGATCGTCTTGTTGTCAGCAGATCTGTTTTTTA-3’</td>
</tr>
<tr>
<td></td>
<td>3’-GCTGTCTAGACGGTCTGGTCTGAGAAGACGCTTCAGTACAAACTTTAATTTCTGA-5’</td>
</tr>
<tr>
<td>siRNA-H2</td>
<td>5’-GATCCGTAAGATGTGAAGCATGTTCTCAGTACAAACTTTAATTTCTGA-3’</td>
</tr>
<tr>
<td></td>
<td>3’-GCATTCTACACTCTCTCTCTCTCTGACAAATTTTATTGA-5’</td>
</tr>
<tr>
<td>siRNA-H3</td>
<td>5’-GATCCGTTTGTGCGAGAAAGGACTACAAAGGTCTTCAAGGACGCTTTAATTTTATTAATTTCTGA-3’</td>
</tr>
<tr>
<td></td>
<td>3’-GCAACACGTGTTCTCTTCTCTTGTAGTCTGACAGAGAAACAGCTGTTTAAAAATTCTGA-5’</td>
</tr>
</tbody>
</table>

HCCR2, human cervical cancer oncogene 2; siRNA, small interfering RNA.

**Table II. Primers used for quantitative RT-PCR.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
</tr>
</thead>
</table>
| HCCR2   | Sense 5’-GGAGGCAGAGAGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG-
|         | 3’                                           |
| Bcl-2   | Sense 5’-ATCCCGCTGTTAGATCTGAGTCTGAGTCTGAGTCTGAGTCTGAGTCTGAGTCTGAGTCTGAGTCTGAGTCTGAG-
|         | 3’                                           |
| Bax     | Sense 5’-GGACGAAGTGGATGATGCTGAGTCTGAGTCTGAGTCTGAGTCTGAGTCTGAGTCTGAGTCTGAGTCTGAGTCTGAG-
|         | 3’                                           |
|         | Antisense 5’-GGAGCCAGGAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAG-
|         | 3’                                           |
| p21     | Sense 5’-GGAGGCTACCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT-
|         | 3’                                           |
| p27     | Sense 5’-GGAGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT-
|         | 3’                                           |
| p53     | Sense 5’-GGAGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT-
|         | 3’                                           |
| β-actin | Sense 5’-GGAGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT-
|         | 3’                                           |

**Figure 1.** Effects of transfection with small interfering RNA (siRNA) targeting the human cervical cancer oncogene (HCCR2). (A) HCCR2 mRNA expression in the blank control, and negative control- and the three HCCR2-siRNA-transfected cells was detected by quantitative RT-PCR after transfection. (B) HCCR2 protein expression was measured by western blot analysis after transfection with HCCR2-siRNA. The upper lane indicates that the HCCR2 protein expression was markedly inhibited in the HCCR2-siRNA-transfected cells compared with the blank control cells. Data represent the means ± standard deviation (SD) of three independent experiments. **P<0.05**, *P<0.01 compared with the the blank control or negative control cells.
Morphological changes in K562 cells following transfection with HCCR2-siRNA. After transfection for 96 h, a morphological examination of the HCCR2-siRNA-transfected K562 cells revealed the typical appearance of apoptotic cells, which included the appearance of condensed nuclear chromatin, nuclei shrinkage, nuclear fragmentation and the formation of apoptotic bodies. However, these morphological changes were not observed among the blank control and negative control cells (Fig. 3).

HCCR2 knockdown inhibits cell cycle progression and induces apoptosis. To determine whether HCCR2 knockdown results in cell cycle changes, the cellular DNA content was measured by flow cytometry. The results revealed that the percentage of K562 cells in the G0/G1 phase was significantly increased when compared with the blank control (P<0.01). By contrast, the percentage of K562 cells in the S phase was significantly decreased as compared with the blank control (P<0.01). However, the number of K562 cells in the G2/M phase was relatively unaffected. These data demonstrated that HCCR2 knockdown by siRNA induced a significant arrest of cell cycle progression in the G1 phase, resulting in a decreased number of cells in the S phase with a corresponding accumulation of cells in the G0-G1 phase (Fig. 4A). Fig. 4B presents a representative example of apoptotic cells in the blank control, and the negative control- and HCCR2-siRNA-transfected group. The results revealed that the ratio of early-stage and late-stage apoptotic cells in the HCCR2-siRNA-transfected cells was significantly higher than that in the blank control and negative control cells (P<0.01) (Fig. 4C).

Effect of HCCR2 knockdown on the expression of apoptosis- and cell cycle-related genes. To further investigate the molecular mechanisms behind the suppression of cell proliferation and G0/G1 cell cycle arrest induced by transfection with HCCR2-siRNA, we analyzed the mRNA and protein expression levels of Bcl-2, Bax, p21, p27 and p53 in the K562 cells at 96 h after transfection by qRT-PCR and western blot analysis. Fig. 5A represents the relative mRNA quantification of these genes in the blank control group, and negative control- and HCCR2-siRNA-transfected K562 cells. The results indicated that Bcl-2 mRNA expression significantly decreased (P<0.01), while Bax mRNA expression markedly increased (P<0.01) when compared with the blank control. Furthermore, the Bax/Bcl-2 ratio was also significantly increased in the HCCR2-siRNA-transfected cells. In addition, we also observed that the mRNA expression levels of p21 and p53 were markedly elevated in the HCCR2-siRNA-transfected K562 cells as compared with the blank control and negative control (P<0.01); however, p27 mRNA expression was not markedly altered in the three groups of cells. Representative immunoblots are shown in Fig. 5B. The western blot analysis results confirmed a marked decrease in Bcl-2 protein expression (P<0.01), and a significant increase in Bax, p21 and p53 protein expression following transfection with HCCR2-siRNA (P<0.01). No significant difference was observed in the p27 protein expression level among the three groups (Fig. 5C).

Discussion

siRNA is a powerful tool for gene knockdown, in which double-stranded RNA (dsRNA) is processed into siRNA, inducing the activation of cellular pathways, leading to selective sequence-specific silencing of target genes with homology to dsRNA (17).
A large number of studies have indicated that siRNA is an ideal method in molecular biology research and a very attractive therapeutic alternative to chemical drugs (17-19). In the present study, in order to investigate the function role of the HCCR2 gene in the tumorigenesis of acute leukemia, we silenced the expression of HCCR2 in K562 cells using siRNA. It is well known that K562 cells are derived from human CML cells in blastic crisis and are often used as an in vitro model of leukemia cells. Our results demonstrated that the mRNA and protein expression levels of HCCR2 were markedly decreased following transfection with HCCR2-targeting siRNA in K562 cells.

A previous report by Yoon et al (11) identified that HCCR2 was overexpressed in patients with hepatocellular carcinoma and thus may be recognized as a novel biomarker for hepatocarcinogenesis. The same study indicated that the HCCR2 assay has an advantage over the alpha-fetoprotein (AFP) assay in the early diagnosis of hepatocellular carcinoma. In our previous study, we found that the HCCR2 gene was abnormally overexpressed in newly diagnosed AML and ALL patients when compared with the normal control group. Furthermore, the patients who achieved complete remission after chemotherapy presented a significant decrease in HCCR2 expression; however, the patients with no remission showed not decrease in expression. In addition, the moment a patient relapsed, the level of HCCR2 expression once again increased (15). These results indicate that HCCR2 may be used as biomarker for monitoring minimal residual disease in patients with acute leukemia.
In this study, we examined whether silencing HCCR2 expression by siRNA affects cell proliferation, cell cycle progression and apoptosis in K562 cells. We observed that the cell growth of HCCR2-knockdown clones was significantly attenuated compared with the blank or negative control clones, while the cell numbers were comparable among all the groups at 4 days after transfection. Additionally, we measured the apoptosis of K562 cells using Annexin V/PI staining and found that the rate of early-stage and late-stage apoptotic cells was markedly increased in the HCCR2-siRNA-transfected cells. In the cell cycle analysis, a decreased number of cells in the S phase and an increased number of cells in the G0/G1 phase were observed in the HCCR2-siRNA-transfected cells; however, the number of cells in the G2/M phase was relatively unaffected. Taken together, our results demonstrated that silencing HCCR2 expression effectively suppressed the proliferation and survival of K562 cells, inducing marked antitumor effects, which were associated with the induction of apoptosis and the inhibition of cell cycle G1/S phase transition.

p53 is a putative tumor suppressor gene, which plays a crucial role in controlling cell proliferation, and suppressing the growth and transformation of cells primarily through the induction of cell cycle arrest or apoptosis (20). The function of the p53 protein is to maintain the integrity of the genome and to repair damaged DNA. Before the initiation of damaged DNA repair, the p53 protein first induces the cells into G1 phase arrest. If DNA repair is not completed, p53 may induce the damaged cells into programmed cell death (21). In addition, the p53 protein is well known as the master regulator of the p21 gene (22,23). In agreement with previous reports demonstrating the function of HCCR2 as a negative regulator of p53 in tumorigenesis (8,13,14), we found that HCCR2 was overexpressed and accompanied with an almost null expression of p53 in K562 cells. Furthermore, p53 expression was markedly upregulated following transfection with HCCR2-siRNA, which was consistent with the increased number of apoptotic K562 cells. However, there have been conflicting reports, in which it was found that siRNA targeting HCCR2 did not alter the p53 protein expression level in HepG2 cells (24). This difference in the role of p53 may be explained by the fact that cells derived from various tissues may have different genetic backgrounds.

Cell cycle progression is regulated by cyclin-dependent kinases (CDKs), which are activated by cyclins binding or inhibited by CDK inhibitors (CKIs) (25). The p21 proteins belong to the first family of CKIs, which inhibit cell cycle progression mainly through the suppression of CDK2 activity and block the transition from the G1 phase into the S phase after DNA damage (26-28). Although this activity is shared by other CKIs, such as p27 and p57, a number of clinical and experimental studies have suggested that p21 plays a crucial role in tumorigenesis (29-31). In this study, our data demonstrated that silencing HCCR2 expression upregulated the expression of p21, but did not affect p27 expression in K562 cells.

Bcl-2, as an anti-apoptotic protein, inhibits apoptotic cell death and its overexpression is considered to promote survival in tumor cells (32). On the contrary, Bax is a pro-apoptotic protein, whose overexpression can inhibit the malignant progression of a tumor (33). The upregulation of the Bax/Bcl-2 ratio directs the cells more towards apoptosis than survival (34). In this study, we found that the knockdown of HCCR2 decreased Bcl-2 expression and increased Bax expression. Furthermore, the Bax/Bcl-2 ratio was significantly increased in the K562 cells following the knockdown of HCCR2 using siRNA. These results demonstrated that silencing HCCR2 expression induced
a greater number of K562 cells into irreversible apoptosis by regulating the expression of Bcl-2 and Bax.

In conclusion, the results from the present study suggest that silencing HCCR2 gene expression by siRNA suppresses cell proliferation, promotes cell cycle G1 phase arrest and induces apoptosis in K562 cells. The data presented in this study also provide a mechanistic explanation for the anti-proliferative and anti-apoptotic effects induced by silencing HCCR2 expression in K562 cells. Although the details of the functional mechanisms of HCCR2 in vivo require further investigation, HCCR2 may serve as an attractive molecular target for the treatment of leukemia.

References