RESEARCH ARTICLE

Down-regulation of EZH2 by RNA Interference Inhibits Proliferation and Invasion of ACHN Cells via the Wnt/β-catenin Pathway

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Abstract

Although enhancer of zeste homolog 2 (EZH2) has been reported as an independent prognostic factor in renal cell carcinoma (RCC), little is known about the exact mechanism of EZH2 in promoting the genesis of RCC. However, several studies have shown that dysregulation of the Wnt/β-catenin signaling pathway plays a crucial role. Therefore, we determined whether EZH2 could affect ACHN human RCC cell proliferation and invasion via the Wnt/β-catenin pathway. In the present study, we investigated the effects of short interfering RNA (siRNA)-mediated EZH2 gene silencing on Wnt/β-catenin signaling in ACHN cells. EZH2-siRNA markedly inhibited the proliferation and invasion capabilities of ACHN, while also reducing the expression of EZH2, Wnt3a and β-catenin. In contrast, cellular expression of GSK-3β (glycogen synthase kinase-3β), an inhibitor of the Wnt/β-catenin pathway, was conspicuously higher after transfection of EZH2 siRNA. These preliminary findings suggest EZH2 may promote proliferation and invasion of ACHN cells via action on the Wnt/β-catenin signaling pathway.

Keywords: EZH2 - invasion - proliferation - renal cell carcinoma - RNA interference - Wnt/β-catenin signaling

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Introduction

The enhancer of zeste homolog 2 (EZH2), as a homolog of the Drosophila Enhancer of zeste (E (z)), is the catalytic subunit of the Polycomb-Repressive Complex 2/3 (PRC2/3), which has been found to contribute to the initiation of gene repression through lysine 27 of histone H3 (H3K27) trimethylation (Ciarapica et al., 2011). EZH2 has been reported to be involved in several key regulatory mechanisms in human cells, such as embryogenesis, lymphocytes and central nervous systems development (Montgomery et al., 2007; Sher et al., 2008; Thiel et al., 2011). Recent studies provide evidence that high expression of EZH2 is associated with aggressive clinical behavior in several human malignancies, such as breast cancer, hepatocellular carcinoma and prostate cancer (Kleer et al., 2003; Sudo et al., 2005; Bryant et al., 2007). Wagener et al. (2010) also documented that EZH2 could be considered an independent prognostic factor in renal cell carcinoma (RCC). Furthermore, they advanced pointed out that EZH2 knockdown mediated by RNA interference (RNAi) in several RCC cell lines could reduce tumor cell proliferation and increase apoptosis (Wagener et al., 2008). But, there are few articles referring to the internal mechanism of EZH2 in promoting oncogenesis of RCC.

The Wnt signaling pathways, activated by at least nineteen Wnt isoforms, are considered to play very important roles in many cellular processes, such as proliferation, migration, differentiation and apoptosis (Smalley et al., 1999; Clevers, 2006). Additionally, the Wnt pathway also regulates critical aspects during embryonic development (Smalley et al., 1999). The Wnt signaling pathways include four branches: the Wnt/β-catenin (canonical) pathway, the planar cell polarity (PCP) pathway, the WNT/Ca^{2+} pathway and the protein kinase A pathway (Smalley et al., 1999; Chen et al., 2005; Clevers, 2006). Among these, Wnt/β-catenin signaling pathway has been most thoroughly studied. This signaling pathway is activated after the Wnt proteins (including Wnt3a) binding to the Frizzleds (Fzs)/low-density lipoprotein receptor-related protein 5/6 (LRP5/6) complex on the cell membrane. Then the activation of the pathway leads to the stabilization and accumulation of β-catenin in the cytoplasm, which will translocate into the nucleus and stimulate the transcription of downstream target genes. On the other hand, glycogen synthase kinase-3β (GSK-3β), adenomatous polyposis coli (APC), casein kinase 1/2 (CK1/2) and Axin form a complex, which mediates the degradation of β-catenin in the cytoplasm and inhibits the transduction of canonical Wnt signals. Several studies

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documented that the dysregulation of Wnt/β-catenin signaling pathway played a crucial role in many human cancers including RCC (Banumathy et al., 2010; Choi et al., 2010; Wan et al., 2012). But, to the best of our knowledge, whether EZH2 regulates the RCC by Wnt/β-catenin signaling pathway has not been reported so far. For this reason, in the present study, we used short interfering RNA (siRNA) to knock down the expression of EZH2 for evaluating the regulatory effects of EZH2 on Wnt/β-catenin signaling pathway activation in human RCC cell line ACHN. Meanwhile, the proliferation and invasion abilities of ACHN cells were also assayed. At 72h post-transfection, the expression of three key molecules in Wnt/β-catenin signaling pathway (Wnt3a, β-catenin and GSK-3β) were detected by real-time PCR and Western blot, respectively.

Materials and Methods

Reagents and Suppliers

LipofectamineTM 2000 and Trizol Reagent were obtained from Invitrogen (Carlsbad, CA, USA). DMSO and MTT solution were purchased from Sigma (St. Louis, MO, USA). Antibodies of mouse anti-EZH2, anti-Wnt3a, anti-β-catenin and anti-GSK-3β were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Mouse anti-GAPDH antibody and horseradish peroxidase-conjugated anti-mouse secondary antibody were purchased from ProMab (Richmond, CA, USA) and Amersham Biosciences (Piscataway, NJ, USA), respectively.

Cell Culture

The human RCC ACHN cells, which stably express the EZH2, were purchased from the American Type Culture Collection (Manassas, VA, USA). ACHN cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) in a humidified 5% CO_2 incubator at 37°C.

siRNA Preparation

As Bracken et al. described (Bracken et al. 2003), the shRNA oligonucleotides were designed for EZH2 (sence, 5'-GATCCCGAAGACTCTGAATGCAGTTGCTTT CA AGAGAAGCAACTGCATTCAGA GTCTTTTTT TTC CAAA-3'; antisense, 5'-AAGCTTTTGGAA AAAAAA GACTCTGAATGCAGTTGCTTCTCTTGAA AGCAA CTGCATTCAGAGTCTTCGG-3') The expression plasmid pGPU6/GFP/Neo-EZH2 shRNA and negative control plasmid pGPU6/GFP/Neo without shRNA were synthesized by GenePharma Company (Shanghai, China).

Cell Transfection

All the ACHN cells, used in the following experiments, were divided into three groups: the ACHN group, the NC group and the siRNA group. When the cultured ACHN cells in the three groups reached 70% confluency, they were transfected respectively with the pGPU6/GFP/Neo-EZH2 shRNA (the siRNA group), the empty pGPU6/GFP/Neo without EZH2 shRNA (the NC group) or no plasmid (the ACHN group) by LipofectamineTM 2000. At 6h post-transfection, the medium was replaced by RPMI-1640 with 10% FBS, in which the cells were cultured up to 72 hours after transfection. Then we used the fluorescent microscopy to observe the transfection efficiency of pGPU6/GFP/Neo (in NC group) and pGPU6/GFP/Neo-EZH2 shRNA (in siRNA group) plasmids into ACHN cells, which expressed enhanced green fluorescent protein (EGFP). Both of them were more than 82%, and they could be used for the further treatments as described below.

Semiquantitative RT-PCR Analysis of Wnt3a, β-catenin and GSK-3β mRNA

Total mRNAs were extracted from ACHN cells in these three groups using Trizol Reagent according to the manufacturer's instructions. And reverted first strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD, USA) was used to obtain cDNA. The human GAPDH gene was used as the endogenous control to evaluate the semi-quantification of mRNA expressions of Wnt3a, β-catenin and GSK-3β in ACHN cells. DNA primer sequences were designed as follow: for human Wnt3a gene, forward 5'-GTAGCGAGGACATCGAGTT-3' and reverse 5'-CGTACTTGTCCTTGAGGAAG-3'; for human β-catenin gene, forward 5'-GAGGAGATGTACATTCA GCAG-3' and reverse 5'-GTCTCCGACCT GGAAAAC -3'; for human GSK-3β gene, forward 5'-TCACCACTCA AGAACTGTCA-3' and reverse 5'-AAGCAGCATTATT GGTCTGT-3'; for human GAPDH gene, forward 5'-ACCACAGTCCATGCCATCAC-3' and reverse 5'-TCCACCACCCTGTTGCTGTA -3'. All the primers were synthesized by Sangon Biotech Co. Ltd (Shanghai, China). And the PCR cycle conditions were as follow: initially 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 59°C for 30 s and 72°C for 45 s and then finally 72°C for 5 min. RT-PCR procedures were repeated three times and the images were obtained by using ultraviolet light.

Western Blot Analysis of EZH2, Wnt3a, β-catenin and GSK-3β Proteins

At 72h post-transfection, ACHN cells in three groups were collected simultaneouly and washed with cold PBS three times separately. Then the cells were lysed in cold RIPA lysis buffer (100 mM NaCl, 20 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0, 0.5% Triton X-100, 0.5% Nonidet P-40) for 20 min and total proteins were extracted after sonicating and centrifuging. Equal amounts (40 ug) of proteins were separated in 12% SDS polyacrylamide gels and then were electrotransferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Membranes were blocked in 5% non-fat milk in TBS and probed with primary antibodies of mouse anti-EZH2 (1:300), anti- Wnt3a (1:300), anti-β-catenin (1:300), anti-GSK-3β (1:300) or anti-GAPDH (1:1000) overnight at 4°C respectively. After being washed with TBS for three times, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse secondary antibodies (1:1000). All bands were detected by an enhanced chemiluminescent substrate kit (Pierce, Rockford, IL, USA) and exposed to films. These procedures were repeated on three occasions.

Cell Proliferation Assay

The tetrazolium-based assay (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, MTT assay) was used to assess the effects of the EZH2 on the ACHN cells proliferation in vitro. The stably transfected ACHN cells in the three groups were placed into 96-well plates at an approximate density of 1×104 cells/well. Fifty μl of MTT solution (5 mg/ml) was added into each well at 24h, 48h, 72h and 96h post-transfection, and all plates were incubated at 37 °C for 4h until the formazan crystal formed. Then, the 150μl DMSO was used to replace the MTT-containing medium and to dissolve the formed crystal. The absorbance values were measured at the wavelength of 570 nm. And the growth curve was made according to the measured absorbance values of the three groups at the four different time points.

Cell Invasion Assay

In our study, the ACHN cells invasion capabilities in vitro were measured in transwell chambers (6.5 mm, 8 μm pores, Corning, NY, USA) according to the manufacturer's instructions. 80μl diluted (3.9 μg/μl) extracellular matrix gel was put into upper transwell chambers. The inserts were incubated at 37°C for 30 min to allow the gel aggregate formation. Then, the stably transfected ACHN cells in the three groups in 2 ml FBS-free DMEM (cell density: 5×10^4/ml) were seeded into the upper chambers of the transwells respectively. Meanwhile, 1 ml DMEM containing 10% FBS were added into the lower chambers. After being incubated at 37°C for 24h, the cells in the upper chambers were removed by a cotton swab. The invading cells were mixed for 20 min and then stained with toluidine blue for 5 min. The numbers of invading cells of the three groups in five random fields were counted under the microscope at 1000× magnification. These procedures were repeated on three occasions.

Statistical Analysis

Quantitative values were expressed as mean ± standard deviation (SD). One-way ANOVA followed by the Student-Newman-Keuls (SNK) test, was used to compare the statistical differences among the three groups underwent various treatments. Statistical analyses were performed applying the statistical software package SPSS13.0 (SPSS Inc, Chicago, IL). It was considered statistically significant when the P value was <0.05.

Results

Effects of EZH2 siRNA on EZH2 Expression and Wnt/β-catenin Signaling Pathway in ACHN Cells

To identify the inhibiting effects of EZH2 siRNA on EZH2, the Western blot assay was performed to detect the protein EZH2 among the three groups. Because the transfection efficiency in ACHN cells of the NC group and the siRNA group was over 82% at 72h post-transfection, the cells were harvested at that time point. The results (Figure 1a) showed EZH2 protein level was reduced significantly in siRNA group than both ACHN and NC groups (P <0.001). But, there was no statistical difference between the ACHN group and NC group (P =0.065).

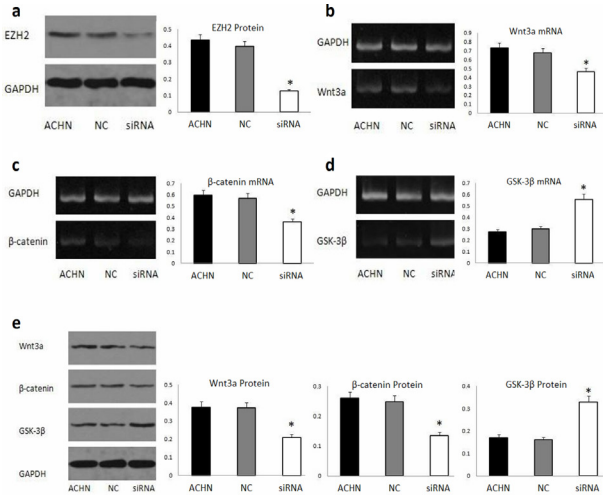

Figure 1. The Expressions of EZH2 and Three Key Molecules in Wnt/β-catenin Signaling Pathway (Wnt3a, β-catenin and GSK-3β) after EZH2 Knockdown in ACHN Cells. Western blot analysis confirmed that EZH2 was suppressed in the siRNA group (a). Semiquantitative RT-PCR analysis was performed to detect the mRNA levels of Wnt3a, β-catenin and GSK-3β at 72h post-transfection (b-d). The expression profiles of Wnt3a, β-catenin and GSK-3β protein were measured after EZH2 knockdown in the three groups (e). GAPDH was used as the endogenous control to evaluate the expressions among groups. The Y-axis represents the relative mRNA/protein level. Values are expressed as the mean ± SD. *P <0.05, significant difference compared with both NC and ACHN groups

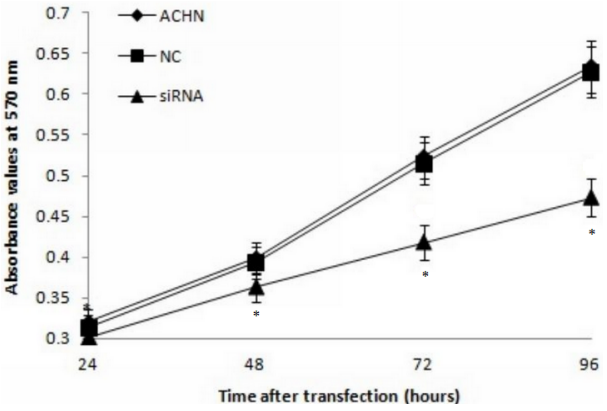

Figure 2. Effects of EZH2 Knockdown on ACHN Cell Proliferation. Cell proliferation was examined using MTT at 24, 48, 72 and 96h; the absorbance values were measured at 570 nm to compare cell viability among the three groups. *P <0.001, significant difference compared with both NC and ACHN groups after 48h post-transfection

Semiquantitative RT-PCR analysis was performed to detect the mRNA expression of three key molecules in Wnt/β-catenin signaling pathway (Wnt3a, β-catenin and GSK-3β) at 72h post-transfection. As compared with ACHN and NC group, Wnt3a and β-catenin mRNA expression were reduced, while GSK-3β mRNA expression was increased in siRNA group (P <0.05). All three mRNA expressions had no significant differences between ACHN and NC group (P >0.05) (Figure 1b, c and d).

Western blot assay was used to detect the protein expression of Wnt3a, β-catenin and GSK-3β. Wnt3a

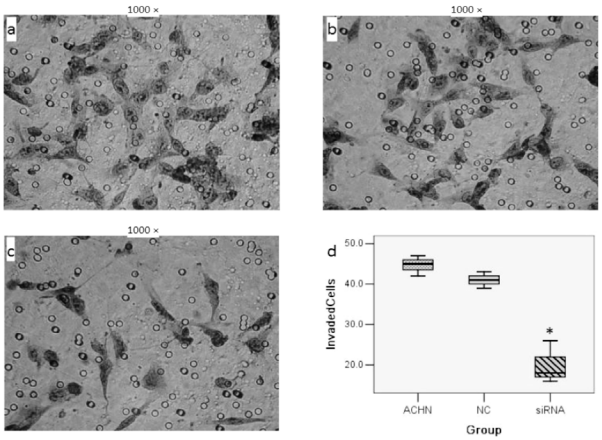

Figure 3. Effects of EZH2 Knockdown on ACHN Cell Invasion. ACHN cell invasion abilities in the ACHN group (a), NC group (b) and siRNA group (c) were measured by transwell invasion assay at 72h post-transfection, respectively. The Y-axis represents invading cells (d). Quantitative values were expressed as mean ± SD. *P <0.01, significant difference compared with both NC and ACHN groups

and β-catenin protein expression were decreased, while GSK-3β protein expression was elevated in siRNA group as compared with ACHN and NC group (P <0.05). Meanwhile, there was no significant differences of all three protein expressions between ACHN and NC group (P >0.05) (Figure 1e). And the results of Western blot assay were consistent with the real-time PCR findings.

The Inhibiting Effects of EZH2 siRNA on Proliferation Ability of ACHN Cells

The 570 nm absorbance values directly represented the number of living cells of the three groups at different time points after transfection. As showed in Figure 2, there was no statistical difference among groups at the 24h time point post-transfectionally (P =0.113). But, the absorbance value was significant reduced in the siRNA group as compared with both ACHN and NC groups after 48h post-transfection (P <0.001). Meanwhile, the empty pGPU6/GFP/Neo plasmid showed no inhibitory effects on proliferaion ability of ACHN cells (P =0.073, at 48h; P =0.118, at 72h; P =0.243, at 96h).

The Inhibiting Effects of EZH2 siRNA on Invasion Ability of ACHN Cells

The numbers of invading cells of the three groups in the lower transwell chambers after experiment were 44.7 ± 2.5 (ACHN group), 41.0 ± 2.0 (NC group) and 20.0 ± 5.3 (siRNA group), respectively (Figure 3). Comparing to the other two groups, the number of invading ACHN cells was significantly reduced in siRNA group (P <0.01). While, there was no statistical difference of ACHN cell invasion ability between the ACHN and NC groups (P =0.266).

Discussion

The human EZH2 gene, which comprised 20 exons of length 41-323bp, located at chromosome 7q35 (Cardoso et al., 2000). Several studies have reported that EZH2 overexpressed in many human carcinomas, including RCC. Wagener et al. (2008) reported that EZH2 was overexpessed in many RCC cell lines, including the ACHN cell. They further demonstrated that siRNA-mediated inhibition of EZH2 expression could conspicuously decrease the growth rate and increase apoptosis in RCC cell lines of 786-O and CaKi-1. But, as far as we know, there are no studies in the literatures, regarding the effect of EZH2 siRNA on ACHN cell line. In this paper, we chose the ACHN cell line as our research object, and specifically knocked down the EZH2 expression by shRNA. To study the inhibitory effect of EZH2 siRNA on ACHN, we compared the ACHN cells transfected by pGPU6/GFP/Neo-EZH2 shRNA (siRNA group as described) with the cells in ACHN and NC group. In our study, the EZH2 expression level detected by Western bolt assay was significantly reduced in siRNA group compared with the other two groups (P <0.001). Furthermore, we also certificated that the ACHN cells in siRNA group had lower proliferation and invasion ability than in both ACHN and NC group (P <0.01). Meanwhile, we demonstrated that the empty pGPU6/GFP/Neo plasmid without EZH2 siRNA did not play any independent role in both EZH2 expression in ACHN cells and the proliferation/invasion capabilities of ACHN cells (P >0.05).

Wnt/β-catenin signaling pathway, as a conserved pathway in metazoan animals, has been reported to be associated with various kinds of human diseases, such as human carcinomas and congenital defects (Andersson et al., 2010; Li et al., 2010). Wnt3a and β-catenin are considered functioning as positive regulators of this pathway mediated by promoting transcription of downstream target genes (Wang et al., 2012). And some target genes are involved in oncogenesis, such as MMP-7 regulating cancer invasion (Miyata et al., 2006), Myc and CyclinD1 regulating cancer proliferation (Herold et al., 2009; Stocker et al., 2009). Meanwhile, GSK-3β is considered playing a negative role in this pathway by degradation of β-catenin (Liu et al., 2002). Several studies reported that EZH2 contributed to constitutive activation of Wnt/β-catenin signaling pathway and induced tumorigenesis of breast cancer and hepatocellular carcinomas (Shi et al., 2007; Li et al., 2009; Cheng et al., 2011). To the best of our knowledge, there is no article referring the relationship between EZH2 and Wnt/β-catenin pathway in the oncogenesis of RCC. So we chose and concentrated on the expression of three key component elements of Wnt/β-catenin pathway (Wnt3a, β-catenin and GSK-3β) in ACHN cells post-transfected with EZH2-siRNA to search whether EZH2 regulates the RCC cell line via this signaling pathway. In our study, comparing to the other two groups, EZH2 siRNA in the siRNA group inhibited the expression of WNT3α and β-catenin (P <0.05), and it markedly promoted the expression of GSK-3β (P <0.01). Meanwhile, we also confirmed that the empty pGPU6/GFP/Neo plasmid without EZH2 siRNA did not affect the expressions of all three studied genes at both mRNA and protein levels (P> 0.05).

Taking all the data together, our results demonstrate that the elimination of EZH2 protein by RNAi can inhibit invasion and proliferation capability of RCC cell line

ACHN. Moreover, our preliminary findings support the hypothesis that EZH2 promotes the tumourigenicity of RCC via WNT/β-catenin signaling pathway. And it seems reasonable for us to speculate that EZH2-mediated excessive activation of Wnt/β-catenin signaling pathway plays an important role in the progression of human RCC.

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