CLDN14 is epigenetically silenced by EZH2-mediated H3K27ME3 and is a novel prognostic biomarker in hepatocellular carcinoma

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Abstract

Trimethylation of lysine 27 on histone H3 (H3K27ME3) is a transcription-suppressive histone mark mediated by enhancer of zeste homolog 2 (EZH2). We have previously suggested that EZH2-mediated H3K27ME3 plays a critical oncogenic role in human hepatocellular carcinoma (HCC) aggressiveness. However, the direct downstream targets of EZH2-H3K27ME3 and the molecular mechanisms by which regulates HCC pathogenesis remain unclear. In this study, we used chromatin immunoprecipitation together with high-throughput sequencing (ChIP-seq) and gene expression profiling by microarray analysis to assess genome-wide chromatin occupancy of H3K27ME3 in HCC cells. We identified that claudin14 (CLDN14) is a potentially direct target for EZH2-mediated H3K27ME3 in HCC. In a large cohort of clinical HCC tissues, we found that low expression of CLDN14 was significantly associated with advanced tumor stage and determined to be an independent predictor of shortened survival of HCC patients. Next, functional experiment demonstrated that depletion of CLDN14 substantially restored EZH2-silenced HCC cells motility and invasive capacities and supported cell epithelial–mesenchymal transition (EMT). Furthermore, downregulation of CLDN14 dramatically re-enhanced the wnt/β-catenin signaling activity in EZH2-silenced HCC cells by increasing the levels of active β-catenin and promoting the nuclear localization of β-catenin. These results, collectively, uncover that CLDN14 is a novel direct target of EZH2-mediated H3K27ME3, and provide an explanation for the aggressive nature of HCC with downregulation of CLDN14 and the underling mechanism that links the tumor suppressor CLDN14 to the wnt/β-catenin signaling pathway.

Introduction

Hepatocellular carcinoma (HCC) is a major lethal malignancy with a high prevalence worldwide. However, the prognosis of HCC patients remains unsatisfactory, largely due to the frequent intrahepatic spread and extrahepatic metastasis of HCC (1,2). Therefore, a better understanding of the biology of HCC metastasis is crucial for the improvement of treatment.

Enhancer of zeste homologue 2 (EZH2), performs its role as a transcriptional regulator by methylating lysine 27 of histone 3 (H3K27ME3). This modification leads to chromatin condensation and transcriptional silencing of downstream target genes (3–6). Our group’s previous work suggests that overexpression of EZH2 and H3K27ME3 was positively associated with advanced clinical stage of HCC and vascular invasion, suggesting a potential oncogenic role of EZH2-H3K27ME3 in HCC metastasis (7,8). However, the downstream target genes and the molecular mechanisms by
which EZH2-H3K27ME3 mediates HCC metastasis have not been clearly elucidated.

Chromatin immunoprecipitation followed by sequencing (Chip-seq) is a highly sensitive and specific set of methods for identifying protein-binding sites on DNA (9, 10). Using anti-H3K27ME3 Chip-seq together with gene expression profiling in a highly invasive human hepatoma cell line sk-hep-1 and sk-hep-1-shEZH2, we identified that claudin14 (CLDN14) is a novel direct target gene of EZH2-mediated H3K27ME3 in HCC cells. This study was undertaken to determine the role of CLDN14 in EZH2-H3K27ME3-mediated HCC aggressiveness.

Materials and methods

HCC cell lines and cell cultures

Six HCC cell lines (7721, sk-hep-1, MHCC-97L, 7402, HepG2 and Huh7) and one normal hepatic cell line (Lo2) were obtained from the American Type Culture Collection (Manassas, VA), where the cell lines were authenticated by STR profiling before distribution. These cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (HyClone Laboratories) at 37°C with 5% CO2 in this study.

Construction of the recombinant retroviral and lentiviral vectors

The EZH2 expression construct was generated by subcloning the PCR-amplified human EZH2 coding sequence into the pEZ-Lv201 (GeneCopoeia, Rockville, MD) retroviral vector. EZH2 was knocked down using one of our previously constructed specific lenti-short hairpin RNAs (shRNAs), which can efficiently silence endogenous EZH2 in cancer cells (11).

Western blotting analysis

Equal amounts of whole cell and tissue lysates were harvested in ice-cold lysis buffer at 4°C. Lysates were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and then electrotransferred onto a polyvinylidene difluoride membrane (Millipore). The membranes were then blocked with milk and incubated in primary antibodies against EZH2, H3K27ME3, CLDN14, tubulin, GAPDH, Histone H1 (Santa Cruz Biotechnology, Santa Cruz, CA), fibronectin, E-cadherin, α-catenin, β-catenin, Vimentin, (Becton Dickinson Transduction Laboratories, Franklin Lakes, NJ), respectively. The immunoreactive signals of horseradish peroxidase-conjugated secondary antibodies were detected by ECL detection system (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions.

Wound-healing and matrigel invasion assays

Approximately 4 × 10^5 cells were plated into six-well culture plates and a line was scraped in the central area of the confluent cells 24 h later. Wound healing in confluent monolayer was observed and photographed at indicated time points using phase contrast microscopy. To evaluate the effect of EZH2 and CLDN14 on cells, the Matrigel invasion assays were carried out using the 24-well transwell units (Matrigel-coated membrane, BD Biosciences) with polycarbonate filters (pore size 8 μm). Cells were seeded into the upper chamber containing serum-free medium while the lower chamber contained with medium with 10% fetal bovine serum as a chemoattractant. After 24 h, cells invaded to other side of the membrane were counted and imaged under a microscope after fixing with 2% methanol and staining with 1% crystal violet solution. The experiments were repeated three times independently.

Chromatin immunoprecipitation (ChIP) and high-throughput sequencing

ChIP was performed according to the protocol described previously (12). Detailed in Supplementary information, available at Carcinogenesis Online. The ChIP-seq results described in this study have been deposited in the GEO database (GSE68758).

Microarray analysis

Detailed in Supplementary information, available at Carcinogenesis Online. The microarray results described in this study have been deposited in the GEO database (GSE68863).

KEGG pathway analysis

Pathway enrichment analysis of the candidate target sets was completed using the database DAVID v6.7 for visualization, annotation and integrated discovery.

Quantitative real-time PCR (qPCR)

Detailed in Supplementary information, available at Carcinogenesis Online.

Patients and tissue microarray (TMA)

In this study, formalin-fixed, paraffin-embedded pathological specimens from 212 patients who underwent initial surgical resection between March 2003 and August 2006 were obtained from the archives of the Department of Pathology of the First Affiliated Hospital, Sun Yat-sen University (Guangzhou, China). In parallel, another randomly collected, independent validation cohort of 126 HCCs between July 2005 and May 2008 were assessed. These patients underwent initial surgical treatment at Sun Yat-sen University Cancer Center (Guangzhou, China). Patients with HCC were selected for both the testing and validation cohorts only if they had been given a distinctive pathological diagnosis, were undergoing primary and curative resection, and had not received preoperative anticancer treatment. Availability of patients’ resection tissues and follow-up data were also criteria for selection. TMA was constructed as described previously (13). Tumor stage was defined according to the tumor-node metastasis classification system from the American Joint Committee on Cancer/International Union Against Cancer. This study has been approved by the Institute Research Medical Ethics Committee of Sun Yat-sen University.

Immunohistochemistry (IHC) and selection of cut-point score

Detailed in Supplementary information, available at Carcinogenesis Online.

RNA interference

Lipofectamine 2000 reagents (Invitrogen, Carlsbad, CA) were used for siRNA transfection according to the manufacturer’s instructions. siRNAs specifically targeting CLDN14 and the corresponding control scrambled siRNA were purchased from Ambion (Austin, TX). The effect of gene silencing on protein level was measured by western blotting at 48 h post-transfection.

Luciferase reporter assays

Luciferase reporter assays for the wnt/β-catenin pathway were performed as described previously (14). FLuc and RLuc activities were assessed using the Dual-Luciferase Assay System (Promega).

Subcellular fractionation

Approximately 10^6 cells were harvested into 10 ml isonicotinic fractionation buffer (0.5 mM EDTA, 20 mM Hepes, 250 mM sucrose and 500 μM Na3VO4 at pH 7.2) supplemented with protease inhibitor cocktail complete (Roche Molecular Biochemicals, Mannheim, Germany) and centrifuged at 900 g for 5 min. The nuclear protein and cytoskeleton fraction was extracted as described previously (15).

Immunofluorescence staining

Cells were grown on glass coverslips up to 80% confluence and washed twice with phosphate-buffered saline, fixed in 4% paraformaldehyde and...
then processed for immunofluorescence staining. For immunofluorescence staining, cells were first incubated with primary mouse anti-β-catenin and CLDN14 antibodies (1:100 dilutions) overnight at 4°C. After thorough washing, cells underwent 1 h incubation with fluorescence-conjugated secondary antibody. Finally, cells were washed and mounted with mounting medium containing DAPI (Vector Laboratories, Burlingame, CA) before imaging by a fluorescence microscope (Olympus, Lake Success, NY).

Statistical analysis

For survival analysis, the optimal cut-point for CLDN14 expression was obtained using X-tile software version 3.6.1 (Yale University School of Medicine, New Haven, CT). The correlation between CLDN14 expression and clinicopathological features of HCC patients was analyzed using the χ² test or Fisher’s exact test. For univariate survival analysis, survival curves were obtained using the Kaplan–Meier method. The Cox proportional hazards regression model was performed for multivariate survival analysis. The independent Student’s t test was performed to analyze the statistical significance between two preselected groups. P values of less than 0.05 were considered statistically significant.

Results

Expression levels of EZH2 and H3K27ME3 influence the invasive capacity of HCC cell lines

We first examined the protein levels of EZH2 by Western blotting in six HCC cell lines (7721, 7402, sk-hep-1, MHCC-97L, HepG2 and Huh7) and one normal hepatic cell line (Lo2). We found that the levels of EZH2 in the six HCC cell lines were higher than that in the normal liver cell line Lo2. Of the HCC cell lines, the highly invasive human hepatoma cell line, sk-hep-1 (16), exhibited high levels of EZH2, while MHCC-97L cells showed the lowest levels of EZH2 (Figure 1A). To determine the relationship between EZH2 and H3K27ME3 expression in HCC cells, EZH2 was knocked down in sk-hep-1 cells to generate sk-hep-1-shEZH2 cells and overexpressed in MHCC-97L cells to generate MHCC-97L-EZH2 cells. Consistent with the role of EZH2 in mediating H3K27ME3, we found that H3K27ME3 levels were substantially decreased in sk-hep-1-shEZH2 cells as compared to that in control sk-hep-1 cells. In contrast, H3K27ME3 level was dramatically increased in MHCC-97L-EZH2 compared to that in MHCC-97L cells (Figure 1B). In addition, using wound healing assays and matrigel invasion assays, decreased migration and invasive capacities were observed in sk-hep-1-shEZH2 cells compared to those in sk-hep-1 cells. On the other hand, increased migration and invasion were observed in MHCC-97L-EZH2 cells compared to those in MHCC-97L cells (Figure 1C and D). Collectively, these results indicate that H3K27ME3 is regulated by EZH2 and that expression levels of EZH2 and H3K27ME3 influence the invasive capacity of HCC cells.

Combinational analysis of chromatin binding and gene expression data identified genes regulated by EZH2-mediated H3K27ME3

As sk-hep-1 cell line has been demonstrated to be highly invasive and had great metastatic capacity (16), we next choose this cell line to investigate the potential mechanisms of HCC cell metastasis regulated by EZH2-mediated H3K27ME3. Then the anti-H3K27ME3 ChIP-seq assay and microarray analysis were carried out in sk-hep-1 and sk-hep-1-shEZH2. We compared the anti-H3K27ME3 ChIP-seq dataset against the transcriptional profiling data of sk-hep-1 versus sk-hep-1-shEZH2 cells. Among the 2980 genes identified in the Chip-seq assay, 243 genes were upregulated and 51 genes were downregulated in sk-hep-1-shEZH2 cells (Figure 2A). Since modification of EZH2-mediated H3K27ME3 represses expression of its target genes, we considered the 243 genes exhibiting increased expression in sk-hep-1-shEZH2 cells to be direct and functionally relevant EZH2-H3K27ME3 target genes (Supplementary Table 1, available at Carcinogenesis Online). Kyoto Encyclopedia Genes and Genomes (KEGG) pathway analysis of these genes revealed significant alterations in the biological pathways of actin cytoskeleton regulation and tight junctions (Figure 2B).

CLDN14 is negatively regulated by EZH2-H3K27ME3

CLDN14 was the gene with largest change in expression among EZH2-H3K27ME3 target genes in sk-hep-1-shEZH2 compared with sk-hep-1 (Figure 3A; Supplementary Table 1, available at Carcinogenesis Online). In order to determine the reproducibility of this finding, we further applied anti-H3K27ME3 ChIP-on-chip data (GSE52301) analysis in paired HCC specimens. In these HCC specimens, EZH2 and H3K27ME3 were overexpressed in cancer tissues (17). We found that the H3K27ME3 modification level on CLDN14 gene was significantly higher in HCC tissues compared with adjacent liver tissues (Supplementary Table 9, available at Carcinogenesis Online). Further ChIP-PCR validated that CLDN14 is modified by EZH2-mediated H3K27ME3 in sk-hep-1 cells (Figure 3B) and a series of HCC cell lines (Supplementary Figure 1A, available at Carcinogenesis Online) and clinical samples (Supplementary Figure 1D, available at Carcinogenesis Online). However, EZH2 and H3K27ME3 are overexpressed in HCC cell line and tissues (7,8). This phenomenon indicated a possible negative correlation of EZH2-H3K27ME3 and CLDN14 in HCC. Western blotting and qPCR assays demonstrated that CLDN14 expression was increased in EZH2 knocked down HCC cell lines and decreased in MHCC-97L-EZH2 cells compared with that in MHCC-97L cell (Figure 3C, Supplementary Figures 1E and 1F, available at Carcinogenesis Online). Correlation analysis performed on normalized EZH2 and CLDN14 expression values for each sample of clinical paired specimen and HCC cell lines from Gene expression profile data (GSE56140 and GSE36133) also suggested a significant negative correlation in HCC paired specimen (Figure 3D) and HCC cell lines (Supplementary Figure 1G, available at Carcinogenesis Online). In fresh paired clinical samples, EZH2 and H3K27ME3 were overexpressed in cancer tissue while CLDN14 expression was decreased in cancer tissues compared with adjacent non-tumor tissue using (Figure 3E). All of these results suggested that EZH2-H3K27ME3 negatively regulates the expression of CLDN14 in HCC.

Expression of CLDN14 in liver tissues and its correlation with HCC patients’ clinicopathological features and survival

Next, the expression of CLDN14 was examined by IHC in a large cohort of clinical HCC tissues. This cohort of HCCs has recently been studied and increased expression of both EZH2 and H3K27ME3 was associated closely with patients poor survival (7,8). In this study, we found that low expression of CLDN14 was observed in 129/212 (60.8%) of primary HCC tissues (Figure 4A). Based on an X-tile analysis, the cut-point for high expression of CLDN14 was defined when H scores were above 150. Correlation analysis demonstrated that low expression of CLDN14 was positively associated with tumor size and more advanced clinical stage of HCC (P < 0.05, Supplementary Table 2, available at Carcinogenesis Online). Kaplan–Meier
analysis showed that the mean disease-free survival time for patients with low CLDN14-expressing HCCs was 32.8 months compared with a survival time of 50.1 months for patients with high CLDN14-expressing HCCs (P < 0.0001, log-rank test, Figure 4B, Supplementary Table 3, available at Carcinogenesis Online). Further multivariate Cox regression analysis also indicated that CLDN14 expression is an independent prognostic factor for poor survival of HCC patients (Supplementary Table 4, available at Carcinogenesis Online). To determine the reproducibility of these findings, the expression dynamics of CLDN14 in HCC were next evaluated in our validation cohort. In this cohort, decreased expression of CLDN14 was observed in 72/126 (57.1%) and associated with a more aggressive phenotype of the tumor (Supplementary Table 6, available at Carcinogenesis Online). Kaplan–Meier analysis established that, in the validation cohort, patients with HCC who negatively expressed CLDN14 also exhibited a shorter survival time than patients with HCC who positively expressed CLDN14 (Supplementary Table 7, available at Carcinogenesis Online). Consistently with our results in testing cohort, CLDN14 expression is an independent prognostic factor.

Figure 1. EZH2-H3K27ME3 overexpression enhances HCC cells migration and invasion. (A) EZH2 expression was examined in Lo2, Huh7, 7721, 7402, HepG2, sk-hep-1 and MHCC-97L cells by western blot. (B) Western blotting was used to examine the levels of EZH2-H3K27ME3 in sk-hep-1, sk-hep-1-shEZH2, MHCC-97L and MHCC-97L-EZH2 cells. (C) Representative results of wound-healing assays of sk-hep-1, sk-hep-1-shEZH2, MHCC-97L and MHCC-97L-EZH2 cells were examined using a matrigel invasion assay. (D) The invasive abilities of sk-hep-1, sk-hep-1-shEZH2, MHCC-97L and MHCC-97L-EZH2 cells were examined using a matrigel invasion assay.

Figure 2. Combinational analysis of Chip-seq data and gene expression profile data. (A) Venn diagram shows the number of H3K27ME3-enriched genes that are downregulated (51 genes) and upregulated (243 genes) in sk-hep-1-shEZH2 cells. (B) Functionally relevant EZH2-H3K27ME3 target genes were subjected to ingenuity KEGG pathway analysis.
for poor survival of HCC patients (Supplementary Table 8, available at Carcinogenesis Online). Furthermore, we evaluated the potential correlations between expression of CLDN14 and that of EZH2 and H3K27ME3 examined by IHC in our previous studies, in which the same cohort of HCC TMA tissues was used (7,8). We found that expression levels of CLDN14 were significantly inversely correlated that of either EZH2 (P = 0.013) or H3K27ME3 (P = 0.006, Chi-square test, Supplementary Table 5, available at Carcinogenesis Online).

CLDN14 is involved in EZH2-H3K27ME3-mediated invasiveness of HCC cell lines

To understand the role of CLDN14 in EZH2-H3K27ME3-mediated invasiveness of HCC cells, a series of experiments were performed. CLDN14 expression is stably silenced in Huh7-shEZH2 and sk-hep-1-shEZH2 cells and that siRNA2 had a better suppressing effect (Figure 5A). Therefore, siRNA2 was used to knock down CLDN14 in subsequent experiments. Wound healing assays and matrigel invasion assays showed that after siCLDN14 treatment in sk-hep-1-shEZH2 and Huh7-shEZH2 cells, the decreased invasiveness of sk-hep-1-shEZH2 and Huh7-shEZH2 cells reached a level approaching that of control sk-hep-1 and Huh7 vector cells (Figure 5B and C). Our previous work has demonstrated that decreased expression of EZH2 resulted in increased EMT (20). When CLDN14 was silenced in sk-hep-1-shEZH2 and Huh7-shEZH2 cells, the decreased EMT was also restored (Figure 5D). These results provide evidence that CLDN14 is an important target for EZH2-H3K27ME3-mediated aggressiveness of HCC cells.

Decreased expression of CLDN14 in HCC cells results in an increased wnt/β-catenin signaling activity

A previous study in HCC cells demonstrated that CLDN3, a member of the claudin family, mediates inhibition of HCC EMT via wnt/β-catenin signaling (21). This prompted us to investigate whether the levels of CLDN14 influence wnt/β-catenin signaling pathway in HCC. Gene set enrichment analysis (GSEA) of
CLDN14 mRNA expression levels and wnt/β-catenin regulated gene signatures from two collections of published HCC patient profiles (GSE25097 and GSE54238) revealed that CLDN14 expression negatively correlated with wnt/β-catenin signaling gene signatures (Figure 6A and Supplementary Figure 2A, available at Carcinogenesis Online). In HCC cell lines, wnt/β-catenin signaling activity was analyzed using the TCF/LEF luciferase reporter assay. TCF/LEF reporter expression levels were significantly decreased in sk-hep-1-shEZH2, Huh7-shEZH2 cells and substantially restored when CLDN14 was further obligated (Figure 6B). In addition, the levels of nuclear β-catenin, as well as the pool of activated β-catenin (i.e. dephosphorylated β-catenin on Ser37 and Ser41), were decreased in EZH2-silenced cells, and restored when CLDN14 was further knocked down in these cells (Figure 6C). Further immunofluorescent (IF) staining also showed a clearly decreased distribution of nuclear β-catenin in EZH2-silenced cells and subsequently increased expression of β-catenin in the nuclear (Figure 6D and Supplementary Figure 2B, available at Carcinogenesis Online). Taken together, these results provided evidence that decreased expression of CLDN14 in HCC cells results in an increased wnt/β-catenin signaling activity.

Discussion

Our previous study revealed that upregulation of EZH2 and H3K27ME3 is associated with advanced clinical stage and/or poor prognosis in nasopharyngeal carcinoma patients, and that upregulation of EZH2-H3K27ME3-mediated transcriptional repression of E-cadherin was sufficient to promote cell invasiveness and/or metastasis (11). Previously, we also found that upregulation of EZH2 and H3K27ME3 is associated with a later clinical stage and/or vascular invasion in HCC patients (7,8). However, the direct downstream genes epigenetically silenced by EZH2-mediated H3K27ME3 in HCC remained unclear.

To identify direct and functionally relevant target genes of EZH2-mediated H3K27ME3, we initially analyzed an anti-H3K27ME3 ChIP-seq dataset against transcriptional profiling data between EZH2-H3K27ME3 and control HCC cell lines. A KEGG pathway enrichment analysis of identified target genes indicated that the biological pathways of actin cytoskeleton regulation and tight junctions were altered. The deregulation of both pathways has previously been shown to be important in cancer metastasis (22,23). CLDN14 was identified as the
target gene of EZH2-mediated H3K27ME3 with the most significant transcriptional repression and chosen for subsequent investigation.

CLDN14 is a member of the claudins family, which is the major integral membrane protein family forming the backbone of tight junctions (24). Deregulation of tight junction

Figure 5. Knockdown of CLDN14 increases the migration and invasion capacities of EZH2-silenced HCC cells. (A) Western blotting reveals that CLDN14 was efficiently knocked down in indicated cells. (B) Wound-healing assay shows that siCLDN14 treatment increased the migration of Huh7-shEZH2 cells and sk-hep-1-shEZH2 cells. (C) Cell invasion was evaluated using matrigel invasion assay. Data are the mean ± SE of three independent experiments. *P < 0.05, **P < 0.01 by Student’s t test. (D) Western blotting reveals the expression of epithelial makers (E-cadherin and α-catenin) and mesenchymal markers (fibronectin and vimentin) in the indicated HCC cells.
may eventually lead to the formation of cancer metastasis (25–28). Recent studies have demonstrated that the expression of several claudin family members is altered in human cancers (29). However, little is known about the role and underling molecular mechanisms of CLDN14 deregulation in HCC pathogenesis.

In our study, the expression dynamics of CLDN14 were thus examined using IHC in a HCC TMA together with complete patient follow-up data. The results demonstrated that expression of CLDN14 was frequently decreased in HCC tissues and was significantly correlated with tumor advanced clinical stage. Decreased expression of CLDN14 in HCC may facilitate an
aggressive phenotype. Importantly, we also found that decreased expression of CLDN14 in HCC was a strong and independent predictor of short cancer-specific survival. Examination of low CLDN14 expression by IHC could thus be used to identify HCC patients at increased risk of tumor invasion and/or metastasis. In HCC cell lines and fresh tissues, we found that the expression of CLDN14 is negatively correlated with EZH2. Furthermore, using Chip-PCR assay, we did verify the epigenetic silencing of CLDN14 by EZH2-mediated H3K27ME3. These findings suggest a potentially important role of epigenetic silencing of CLDN14 by EZH2-mediated H3K27ME3 as an underlying biological mechanism in the development and/or progression of HCC.

In HCC cell lines, significant decreased invasiveness was observed in EZH2 repressed HCC cell lines with upregulated expression of CLDN14. However, when EZH2 depleted HCC cells were transfected with siCLDN14, the migratory/invasive abilities and EMT were successfully restored. These results indicated that the decreased expression level of CLDN14 is essential for EZH2-mediated HCC cell invasiveness, as well as EMT. EMT is the key process by which epithelial cancer cells acquire increased invasive capacity and eventually migrate to adjacent tissues or distal parts of the body (30). Our recent study provided evidence that ectopic expression of EZH2 in HCC cells resulted in enhancement of EMT (20). These data, taken together, support our emerging view that decreased expression of CLDN14 increases HCC cell aggressiveness. However, to date, the precise molecular mechanisms by which CLDN14 regulates HCC cell malignant phenotype remains unclear.

It is known that Wnt/β-catenin signaling is an important pathway for the promotion of EMT (31). Recently, Jiang et al. (21) reported that CLDN3, another member of claudins family, was frequently downregulated through promoter methylation in HCC tissues and cells, and decreased expression of CLDN3 was important in the acquisition of an aggressive and/or poor prognostic phenotype of the HCC patients. Further studies showed that deceased expression of CLDN3 in HCC could substantially activate the wnt/β-catenin signaling which leads to the EMT of HCC cells. In this study, GSEA analysis revealed a significantly negative correlation between CLDN14 expression and wnt/β-catenin signaling in a large cohort of HCC specimens, suggesting that CLDN14 might also involve the wnt/β-catenin signaling pathway. Next, in our HCC cells, TCF/LEF luciferase reporter assay provided evidence that decreased wnt/β-catenin signaling activity during EZH2 knockdown was eliminated with siCLDN14 treatment. In addition, the levels of nuclear β-catenin and active β-catenin were all decreased in EZH2-silenced HCC cells and substantially rescued when CLDN14 expression was further knocked down. These results suggested that the epigenetic silencing of CLDN14 by EZH2-H3K27ME3 in HCC cells may promote EMT and/or aggressiveness by activation of the wnt/β-catenin pathway. Finally, as a result of our collective present data, herein we propose a new downstream direct target, CLDN14, for EZH2-mediated H3K27ME3 and its underlying mechanisms in promoting HCC cell aggressiveness. An illustration of the major molecular mechanism of CLDN14, in HCC cells aggressiveness, is provided in Figure 6E.

In summary, our report describes, for the first time, that CLDN14 is a direct target gene for EZH2-mediated H3K27ME3 and that low expression of CLDN14 in HCC is important in the acquisition of an aggressive and/or poor prognostic phenotype. Furthermore, our functional and mechanistic studies revealed that CLDN14 is critical for EZH2-H3K27ME3-mediated live cancer cell aggressiveness through its modulation of wnt/β-catenin signaling. Our findings, collectively, suggest that CLDN14 could be employed as a prognostic marker and/or as an effective therapeutic target for HCC.

Supplementary material
Supplementary Tables 1–9 and Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

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