# CDC20, A Hypermethylated Gene in Senescent Cells, is Associated with Hepatocellular Carcinoma Prognosis

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# Abstract

Background

Hepatocellular carcinoma (HCC) is a malignancy with poor outcome. As an anti-tumor mechanism, the methylation pattern of gene in senescent cells is different from that in malignant ones deriving from physically senescent cells. Therefore, it is possible that aberrantly methylated senescence-associated genes could predict the prognosis of HCC patients.

Methods

Differentially expressed genes with aberrant methylation were explored in two senescent cell datasets and were selected as senescence-associated genes (SAGs). And we used the proliferation, cell cycle, and senescence β-galactosidase assay to detect the effects of downregulated *CDC20,* a SAG, on cell cycle arrest and cellular senescence. Then, we investigated the relationship between the expression and methylation of *CDC20* in senescent and proliferating HCC cells which was treated by 5-Azacytidine demethylation and in HCC patients from The Cancer Genome Atlas database. Also, we combined the methylation and expression levels of *CDC20* to evaluate the prognosis of *CDC20.* Finally, pyrophosphate sequencing was performed to validate the different methylation of *CDC20*.

Results

We found one of 49 SAGs, named cell division cycle 20 (*CDC20),* to be downregulated with hypermethylation in senescent HCC cells but upregulated with hypomethylation in proliferating HCC cells. Then, restoration of *CDC20* expression was observed after demethylation treatment. Moreover, knockdown of *CDC20* was found to induce cell cycle arrest and cellular senescence. Furthermore, the expression of *CDC20* was found to be negatively correlated with its methylation, and hypomethylation of *CDC20* was found to have a significantly shorter overall survival. Finally, pyrophosphate sequencing results showed that cg16147196 in *CDC20* has a remarkably higher methylation level in senescent HCC cells than that in the proliferating HCC cells.

Conclusions

In conclusion, our study indicated that *CDC20,* a hypermethylated gene in senescent cells, could be considered as an alternative prognostic marker in HCC.


# Background

Hepatocellular carcinoma (HCC), a highly heterogeneous and invasive disease, is the fifth most common malignancy and the second leading cause of cancer-related deaths [1, 2]. The patients survive commonly for no longer than 6 months [3]. So far, liver transplantation is considered the optimal therapeutic approach for patients in the early stage. However, HCC recurrence after liver transplantation is still observed in approximately 20% of the patients [4]. Moreover, there are no effective therapeutic options for

patients in the advanced stage. Therefore, finding new effective prognostic markers is of great importance for the diagnosis and treatment of HCC.

Senescence is characterized by growth arrest when the cell is undergoing different kinds of damage stimulation. Inducing proliferating cells with the risk of neoplastic transformation into senescence is one of main anti-tumor mechanisms. Recently, it has been demonstrated that the progression of chronic liver disease results in an increase in the proportion of senescent cells in the liver. In addition, some chronic liver diseases could probably progress to HCC [5]. Comparing the expression of genes between the senescent and proliferating cells as well as between the normal and tumor tissues, we found the expression of some genes to be remarkably different. Therefore, we need to find the mechanism of differential expression of these genes, such as epigenetic regulation to seek new biomarkers in malignant tumor diagnosis and therapy.

Approximately 80% CpG sequences are found with DNA methylation in the human genome; however, they are distributed in specific regions which are enriched in either CpG sequences (CpG islands) or large repetitive sequences [6, 7]. Abnormal DNA methylation in CpG islands (CpGIs) of the promoter is a characteristic of malignant tumors. Various studies have reported that DNA hypermethylation can induce transcriptional suppression. Firstly, methylated CpGIs can recruit inhibitory proteins or stop the transcription factors from interacting with DNA sequences [8, 9]. Secondly, it was found that methyl-CpG proteins can recognize methylated CpGIs and suppress methylated DNA activity [10, 11], such as the genomic imprinting or inactivation of X-chromosome [12, 13]. Studies have demonstrated that several anti-oncogenes could alter the signal pathways of carcinogenesis after methylation-induced transcriptional silencing [14]. The results of these studies led us to speculate that the tumor-promoting DNA methylation in the malignant tumor results from cells evading senescence.

In the present study, we firstly analyzed the genome profiling data derived from senescence datasets and screened the SAGs. Next, we validated the role of the candidate gene in senescent and proliferating HCC cells, and explored the relationship between its methylation and expression level in HCC patients. Finally, we investigated its prognostic value in HCC patients. In conclusion, our study identified a hypermethylated gene in senescent HCC cells, which could act as a potential alternative prognostic marker in HCC.

# Methods

Data sources

Two datasets of senescent fibroblast cells including genomic and methylation data (GSE81798 and GSE91071) along with HCC tissue datasets containing methylation data (GSE73003) and expression data (GSE14520) were downloaded from the Gene Expression Omnibus (GEO) database. Data from GPL3921 platform in GSE14520 included 209 HCC patients and another HCC cohort data including 370 HCC patients were obtained from The Cancer Genome Atlas (TCGA) database. And the

immunohistochemical results of CDC20 in HCC and normal liver tissues were downloaded from the Human Protein Atlas database (https://www.proteinatlas.org/).

Differentially expressed gene analysis

The differentially expressed gene (DEG) analysis was conducted on two senescent cell datasets. Genes with a fold change (FC) > 1.5 and *q*-value < 0.05 were selected for further analysis. With methylation data, we removed data located on chromosome X- and Y. At the same time, we identified the differentially methylated genes with $P < 0.05$. Then, we selected the overlapped DEGs with altered methylation in the lists as SAGs in the senescent cell datasets.

Survival analysis

Log-Rank test was carried out to analyze the difference among groups and the outcomes were presented by Kaplan-Meier curves. We used the median to separate the patients into high- and low- expression or/and methylation groups.

## Cell culture and transfection

Human HCC cell lines (SMMC-7721 and Hep G2) were obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China) and cultured in 37°C incubator contained 5% $CO_2$ with Dulbecco's Modified Eagle Medium (Hyclone, Logan, UT, USA). CDC20 siRNA: 5'-GGAGCUCAUCUCAGGCCAUtt-3' (sense) and 5'- AUGGCCUGAGAUGAGCUCCtt-3' (antisense) and control were obtained from GenePharma (Shanghai, China). The siRNA (100 nM) targeting CDC20 and and control were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

## Cell treatment

To establish the senescent cell model, cells were treated with 100 μM hydrogen peroxide (Sigma Aldrich) for 24 h according to the previously described protocol [15, 16]. Then, cells were treated with 5 μM 5-Azacytidine (5-AZA), a DNA methylase inhibitor (MCE MedChem Express, Princeton, NJ, USA) for 48 h [17] and phosphate buffered saline (PBS) was added in the control group.

## RNA isolation and qRT-PCR assay

After total RNA was extracted using Trizol agent (Ambion) and quantified by absorbance at 260nm, reverse transcription was performed using the PrimeScript RT reagent Kit. The comparative-Ct method was used to calculate the relative mRNA levels. The primer sequences used were: CDC20: 5'-CTGTCTGAGTGCCGTGGATG-3' (sense), 5'-CCATGGTTGGGTACTTCCAAATAA -3' (antisense); β-actin: 5'-ATCGTGCGTGTGACATTAAGGAG-3' (sense), 5'-AGGAAGGAAGGCTGGAAGAGTG-3' (antisense). All kits and primers were obtained from Takara and all operations are performed in accordance with the manufacturer's instructions.

### Pyrophosphate sequencing

After total DNA was extracted and quantified, bisulfite treatment was performed. Then, the products were amplified using PCR and sequenced by pyrophosphate sequencing. All kits were purchased from Qiagen and the protocol followed was as per the manufacturer's instructions. The primer sequences used are given in Table 1.

### Western blot

The cells were lysed by RIPA (HAT, Xi'an, China) and the lysate was separated in 10% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Then, the membranes were incubated in the primary antibody at 4°C overnight (1:1000, proteintech, Wuhan, China) and the secondary antibodies used subsequently along with the room temperature for 2 hours (1:10000, proteintech, Wuhan, China). The visualization was performed using the ECL kit (Millipore, Bedford, MA, USA).

### CCK-8 and cell cycle analysis

The cell viability of HCC cell lines was measured using the CCK-8 Kit (Qihai, Shanghai, China), according to the manufacturer's instructions. After the cells were trypsinized, they were fixed overnight in 70% ethanol at 4°C. After centrifugation, the cells were stained using propidium iodide (PI), incubated at 37°C for 30min and then subjected to flow cytometry (Qihai, Shanghai, China) analysis**.**

### Senescence β-galactosidase (SA β-gal) assay

Cells were stained using the SA β-gal staining kit (Beyotime Inc., Nantong, China), as per the manufacturer's instructions. The cells are fixed for 15 minutes, incubated with the staining working solution overnight at 37°C and the percentage of SA-β-gal positive cells was calculated by bright-field microscopy (Zeiss 2.0, Germany).

### Statistical analysis

All experiments were repeated three times and presented as mean ± SD. The expression levels and beta-values of *CDC20* were analyzed using the *t*-test. *P* -value less than 0.05 was considered statistically significant. The statistical analysis was conducted using IBM SPSS Statistics software 24.0 (IBM Corp, NY, USA).

# Results

**Exploration of differentially expressed genes with altered DNA methylation status in cellular senescence**The overall workflow was shown in Fig. 1A. To explore the SAGs with different methylation, we firstly analyzed the DEGs with FC > 1.5 and *q*-value < 0.05, and differentially methylated genes (DMGs) with *P*-value < 0.05 in two senescent fibroblast cell datasets (GSE81798 and GSE91071), respectively. A

total of 1078 and 467 aberrantly regulated and methylated genes were screened out from the GSE81798 and GSE91071 datasets, respectively (Fig. 1B). After analyzing the above data, 49 common aberrantly methylated DEGs were selected as the SAGs (Fig. 1C).

### CDC20 was hypermethylated and downregulated in senescent HCC cells

To evaluate the clinical value of the above 49 SAGs for HCC survival prediction, we investigated the expression level and methylation status of these genes in senescent and proliferating fibroblast cells as well as in senescent and proliferating HCC cells. Intriguingly, we found that *CDC20*, a down-regulated gene with hypermethylation in senescent fibroblast and HCC cells, was significantly up-regulated and hypomethylated in proliferating fibroblast and HCC cells (Fig. 2A and B). To confirm the above results, we first detected the expression level of *CDC20* in senescent and proliferating SMMC-7721 as well as Hep G2 cells and found *CDC20* was downregulated in senescent SMMC-7721 and Hep G2 cells. Then, we explored the relationship between expression and methylation level of CDC20 in senescent SMMC-7721 and Hep G2 cells, respectively by 5-AZA treatment. Result showed that the mRNA and protein expression levels of CDC20 were rescued only in the 5-AZA-treated group, whereas, there was no significant difference in the PBS-treated group (Fig. 2C).

### Knockdown of CDC20 inhibited cell proliferation and induced cellular senescence in HCC cells

In order to explore the role of *CDC20*, we transfected siRNA of *CDC20* and control into HCC cells. After transfection, we compared the proliferative capacities between *CDC20* down-regulated and empty vector-transfected HCC cells. The results showed that the down-regulation of *CDC20* remarkably inhibited cell proliferation (Fig. 3A) and the proportion of G2/M phase was increased in *CDC20* down-regulation cells (Fig. 3B). We then investigated the effect of *CDC20* down-regulation on cellular senescence measured by SA-β-gal staining and found that the percentage of SA-β-gal positive cells was high in the *CDC20* down-regulated group compared to the control group (Fig. 3C).

### CDC20 was frequently hypomethylated in HCC tissues and was associated with poor prognosis

Based on the immunohistochemistry staining results obtained from the Human Protein Atlas database, it was found that there was a higher expression level of CDC20 in hepatocellular carcinoma as compared to that in the normal tissues. The expression and methylation levels of *CDC20* in TCGA-LIHC are presented as a heatmap in Fig. 4A. And the same results were validated in HCC methylation data (GSE73003) and expression data (GSE14520) from GEO dataset (Fig. 4B). We then evaluated the relationship between the methylation and expression levels of *CDC20* in the TCGA-LIHC cohorts and found that the expression of *CDC20* had a negative correlation with its methylation, especially in three CpG sites: cg06373377, cg05525368, and cg16147196 (Fig. 4C). For a better investigation of the prognostic value of *CDC20*, survival analysis was used to calculate the connection between the overall survival (OS) and methylation level of *CDC20* in the TCGA-LIHC cohorts. Survival time was found to be remarkably shorter in patients with *CDC20* hypomethylation (MST = 46.6 m) compared to those with *CDC20* hypermethylation (MST = 71.0 m) (log-rank *P* value = 0.046) (Fig. 4D). Interestingly, we categorized patients with *CDC20*

hypomethylation and high expression as the high-risk group, *CDC20* hypermethylation and low expression as the low-risk group, and other patients as the intermediate-risk group, and observed that the patients with a high risk had 2.94-fold higher death risk than patients with a low risk (log-rank *P* value < 0.0001) (Fig. 4D).

### Pyrophosphate sequencing confirmed the differential methylation of CDC20 in senescent and proliferating HCC cells

To confirm the abovementioned results, we performed pyrophosphate sequencing to detect the methylation level of *CDC20* at three CpG sites and promoter regions in the senescent and proliferating SMMC-7721 cells, respectively. Data showed that there were no differences in the methylation level of *CDC20* in cg06373377 and cg05525368 (Fig. 5A) between senescent and proliferating SMMC-7721 cells. However, senescent SMMC-7721 cells showed a higher methylation level of *CDC20* than that observed in the proliferating SMMC-7721 cells in cg16147196 (Fig. 5A). We did not detect any differences in the methylation levels in the promoter region of *CDC20* in the senescent and proliferating SMMC-7721 cells (Fig. 5B).

## Discussion

In this study, we firstly identified 49 SAGs with altered methylation in two senescent cell datasets and validated *CDC20*, one of SAGs, to be down-regulated with hypermethylation in senescent HCC cells, while up-regulated with hypomethylation in HCC tissues and proliferating HCC cells. Furthermore, the experiment showed that the down-regulation of *CDC20* suppressed HCC cell proliferation and induced them into senescence. Then, the methylation level of *CDC20* was shown to be negatively associated with its expression in the TCGA-LIHC cohorts. And survival analysis showed that a lower methylation level of *CDC20* resulted in a shorter OS time in TCGA dataset. Besides, the higher expression and lower methylation level of *CDC20* showed a risk with shorter OS.

The role of cellular senescence in hepatocyte malignant transformation is still unrevealed. Recently, a key finding showed that senescent cells have a boosted capacity that can promote tumor growth if they exit senescence [18, 19]. Studies have shown that the common stressor triggering senescence is chronic liver disease, which in turn could abrogate senescence and lead to aggressive HCC development [20]. For example, reducing inflammation mediated by NKT and CD4 + T-cell can prevent hepatocarcinogenesis by removing senescent hepatocytes [21]. In our data, compared to proliferating HCC cells, the senescent HCC cells showed low expression and hypermethylation of *CDC20*. And the knockdown of CDC20 led HCC cells to senescence and cell cycle arrest. It indicates that the alteration of *CDC20* methylation level participates the cellular transformation from replicative cellular senescence to tumorous proliferation and regulates the cell-cycle processes.

A malignant tumor was initially considered as a heterogeneous genetic disease, including the genome abnormally “epigenetic” diversity. Furthermore, epigenetic alternation occupies an important position in tumor progression [22], such as DNA methylation. Abnormal DNA methylation leads to cancer [23]. The

AFP regulated by methylation status of promoter had a predictive value for HCC [24]. Also, a study has shown that AFP + and AFP-HCC tumors have unique patterns of DNA methylation [25]. However, the mechanism of DNA methylation alterations in a multitude of malignant tumors is still unclear. Further, the changes of senescence-associated methylation patterns may play a role in tumorigenesis. Our result showed that CDC20 not only had differential methylation and gene expression in senescent and proliferating HCC cells, but also in normal liver and HCC tissues. And the methylation level of *CDC20* was shown to be associated with OS in TCGA-LIHC cohorts. This indicates that hypermethylation of *CDC20* results in its low expression and increases the OS time of HCC patients.

As an important cell cycle checkpoint regulator [26], *CDC20* adjusts chromosome segregation by inducing ubiquitination, and then accelerates the transition from G2/M to G1 phase [27]. Previous studies have shown that *CDC20* exhibits high expression levels in multifarious cancers [28], including HCC and over-expression of *CDC20* had a close association with the progression of HCC [29]. In addition, aberrantly methylated *CDC20* may function as a marker for clinical diagnosis and evaluation of HCC prognosis as well as in targeted therapy [30]. We found three CpG sites to be associated with the expression and differential methylated status of *CDC20*, demonstrating its potential predictive value in the prognosis and treatment of HCC patients. Furthermore, pyrophosphate sequencing validated only one CpG site to be differentially methylated between the senescent and proliferating HCC cells. All the above findings emphasized that the epigenetic alterations during tumorigenesis and senescence and the DNA methylation modality of malignant cells is different from ones undergoing senescence.

In the present study, we validated one SAG, *CDC20*, from the dataset GSE14520 with a background of cirrhosis, and validated it in the TCGA-LIHC dataset of more than half of patients with cirrhosis. And the applicability of HCC patients without underlying liver cirrhosis needs further exploration. Secondly, we validated the three CpG sites most relevant to gene expression obtained from the databaset screening. Whether there are differences in methylation levels at other sites between senescent and proliferating HCC cells needs further verification. Thus, further studies are needed to explore the mechanism of *CDC20* methylation pattern changes between cancer cells and senescenct cells.

# Conclusions

We confirmed an aberrantly methylated SAG, namely *CDC20*, between senescent and proliferating HCC cells, as well as normal and HCC tissues. The hypomethylation and hypomethylation & high expression of *CDC20* was found to be negatively associated with the OS for HCC. Our study provides novel prospective evidence on the predictive biomarkers and targeted treatment for HCC, which could help improve the clinical outcome of HCC.

# Abbreviations

HCC
Hepatocellular carcinoma

SAGs
Senescence-associated genes
CpGIs
CpG islands
GEO
Gene Expression Omnibus
TCGA
The Cancer Genome Atlas
DEG
Differentially expressed gene
FC
Fold change
PBS
Phosphate buffered saline
PI
Propidium iodide
SA β-gal
Senescence β-galactosidase
IHC
Immunohistochemistry
DMGs
Differentially methylated genes
5-AZA
5-Azacytidine

# Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

### Competing interests

None.

### Funding

This study was funded by the National Science Foundation of China (No. 81773128 and 81871998). The funders had participated in the design of the study and in writing and revising the manuscript.

### Authors' contributions

QK, LC and XXH designed the study; XXH, ZX, MXH, and MRC collected and analyzed data; QK, XXH, and FYN prepared the figures; XXH, QK, and LC drafted and revised the manuscript. All authors have read and approved the manuscript

### Acknowledgements

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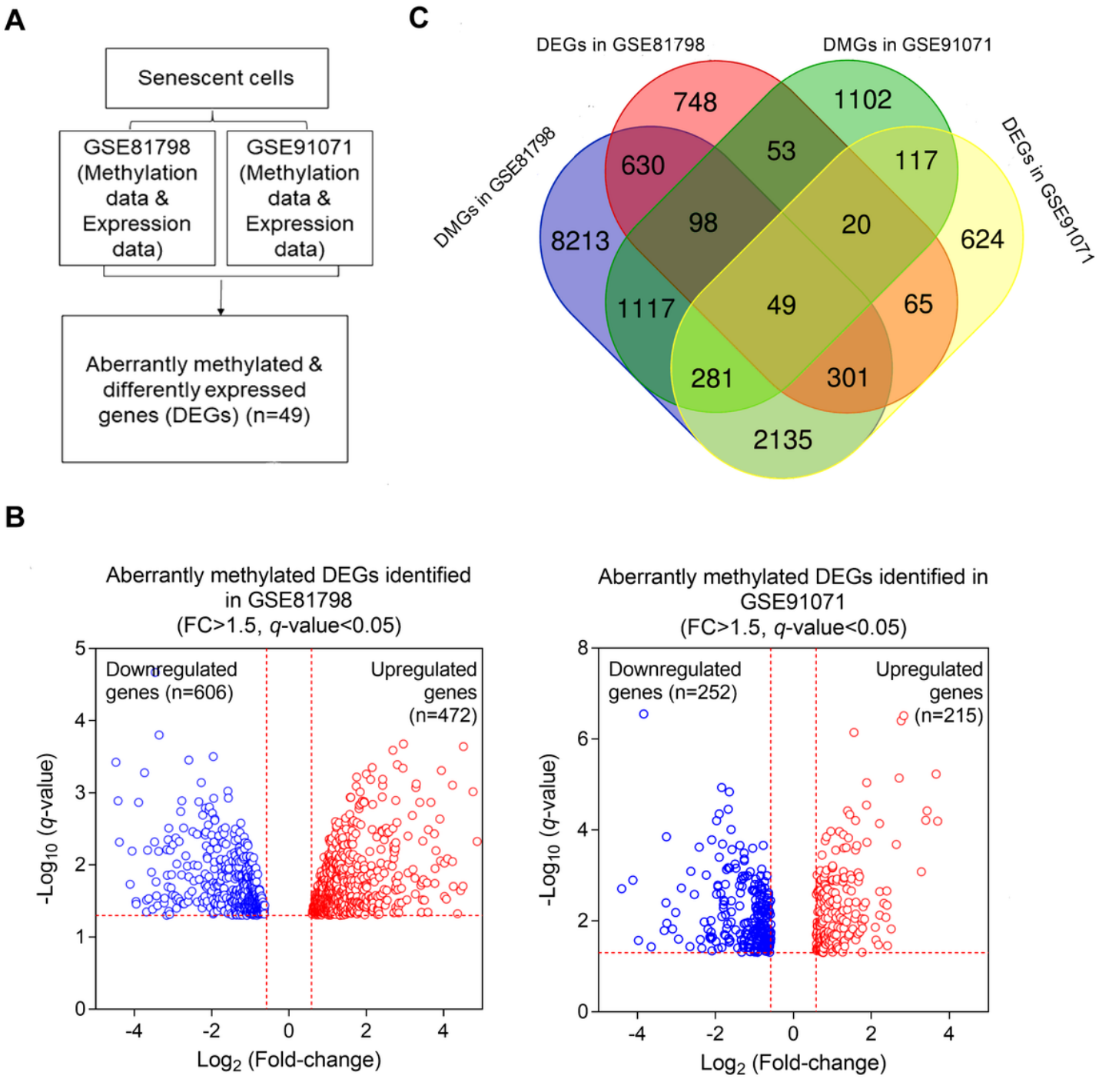
# Tables

Table 1. Primer sequences of three CpG sites of CDC20 in pyrophosphate sequencing

| CpG sites | Primer sequences (5’ - 3’) |
|---|---|
| cg06373377 | F: ATGAGGAGAGGATAAGGATGTATTTATAA |
| | R: AAAACCTACCCTTACATTCCTTCCCTAC |
| | S: AAGGATGTATTTATAATAGTTATTT |
| cg05525368 | F: GTTGTAGGATGTTATTAGAGTTTGTATT |
| | R: ACCTCTAACAAACCTAAACCTTATAAA |
| | S: AGGTATATATTGTTGTTTAGTG |
| cg16147196 | F: GAGTTTAGATGGGGTTATAGTGG |
| | R: CCATAACCTCAAAATCTCATCTACT |
| | S: GGGGTTATAGTGGTAT |
| Promoter 1 | F: TTTTTAGAAGGGGAAGGAGAG |
| | R: TTCATTCACCCTTTTCAACCTCATTTT |
| | S: AAGGGGAAGGAGAGT |
| Promoter 2 | F: GGTTTTAAAGATGTGAGGATTTTAGAGA |
| | R: TACTCCACCTCTAAACACATTCATACAATT |
| | S: AATTTATATTTTATAGATGGAGAAA |

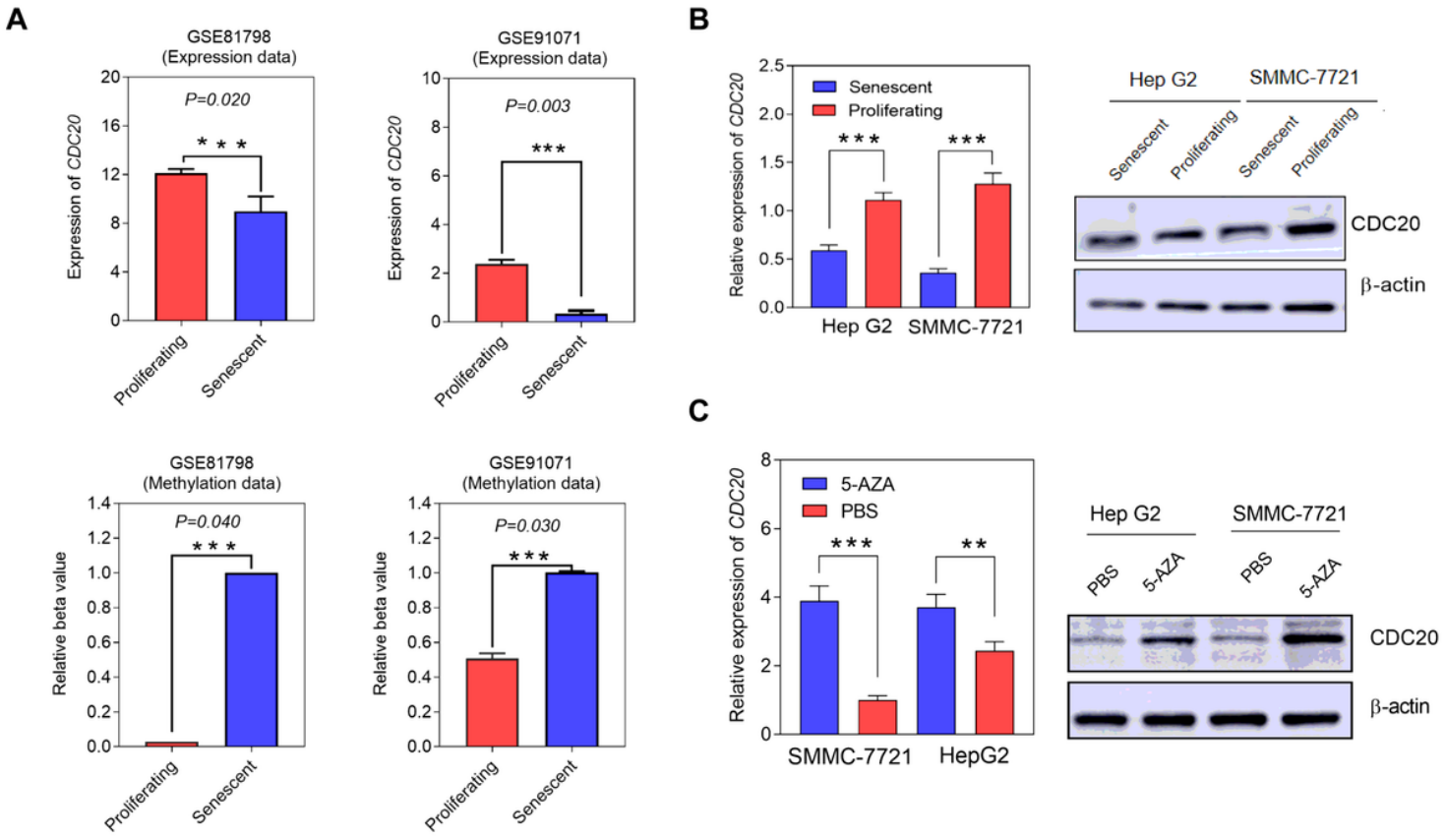
F: Forward; R: Reverse; S: sequencing

# Figures



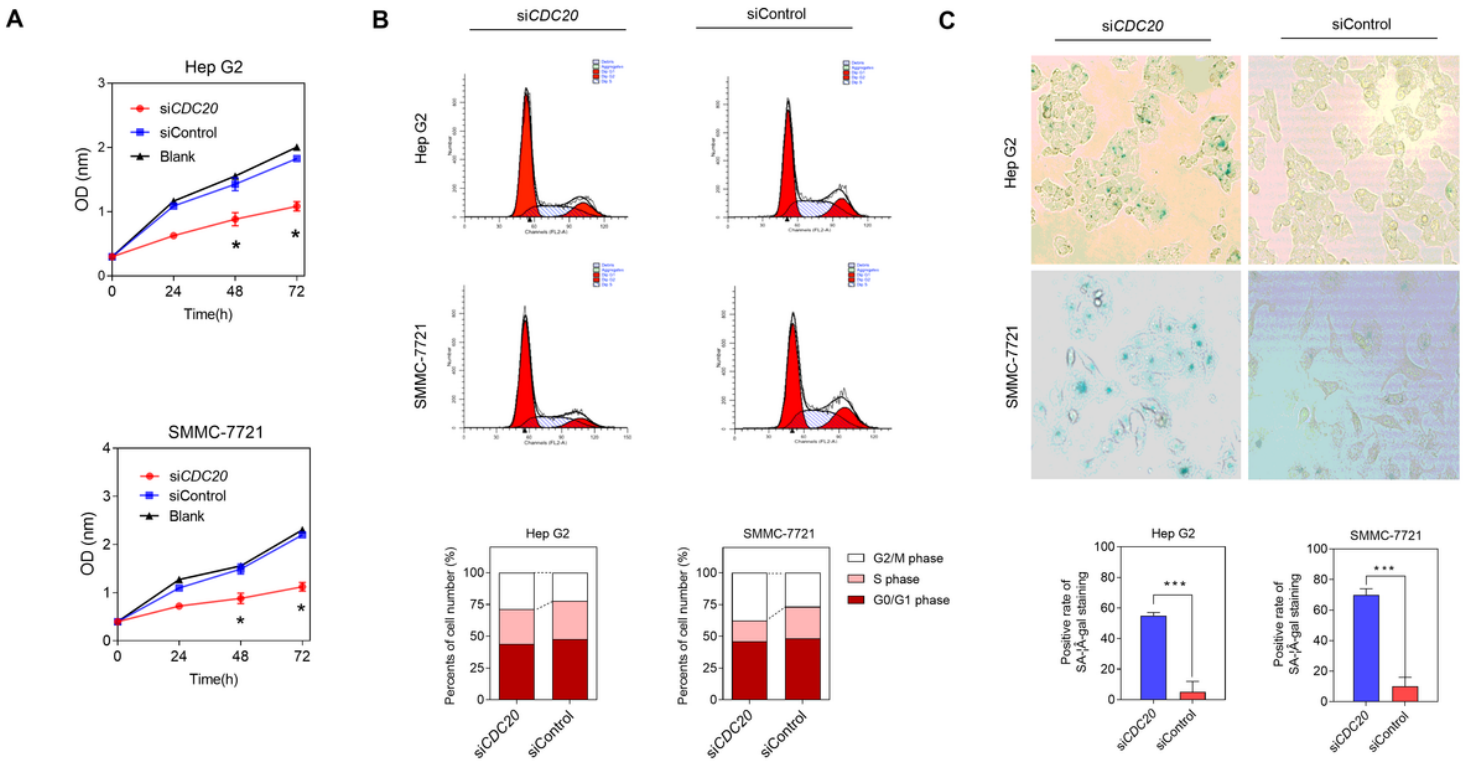
## Figure 1

Exploration of differentially expressed genes with altered DNA methylation status in cellular senescence. (A) The overall workflow of data processing. (B) The aberrantly methylated and expressed genes were identified in two senescent cell datasets. There were 1078 and 467 aberrantly regulated and methylated genes that were selected from the GSE81798 and GSE91071 datasets, respectively. (C) There were 49 common aberrantly methylated DEGs in the two lists, which were selected as senescence-associated genes (SAGs).



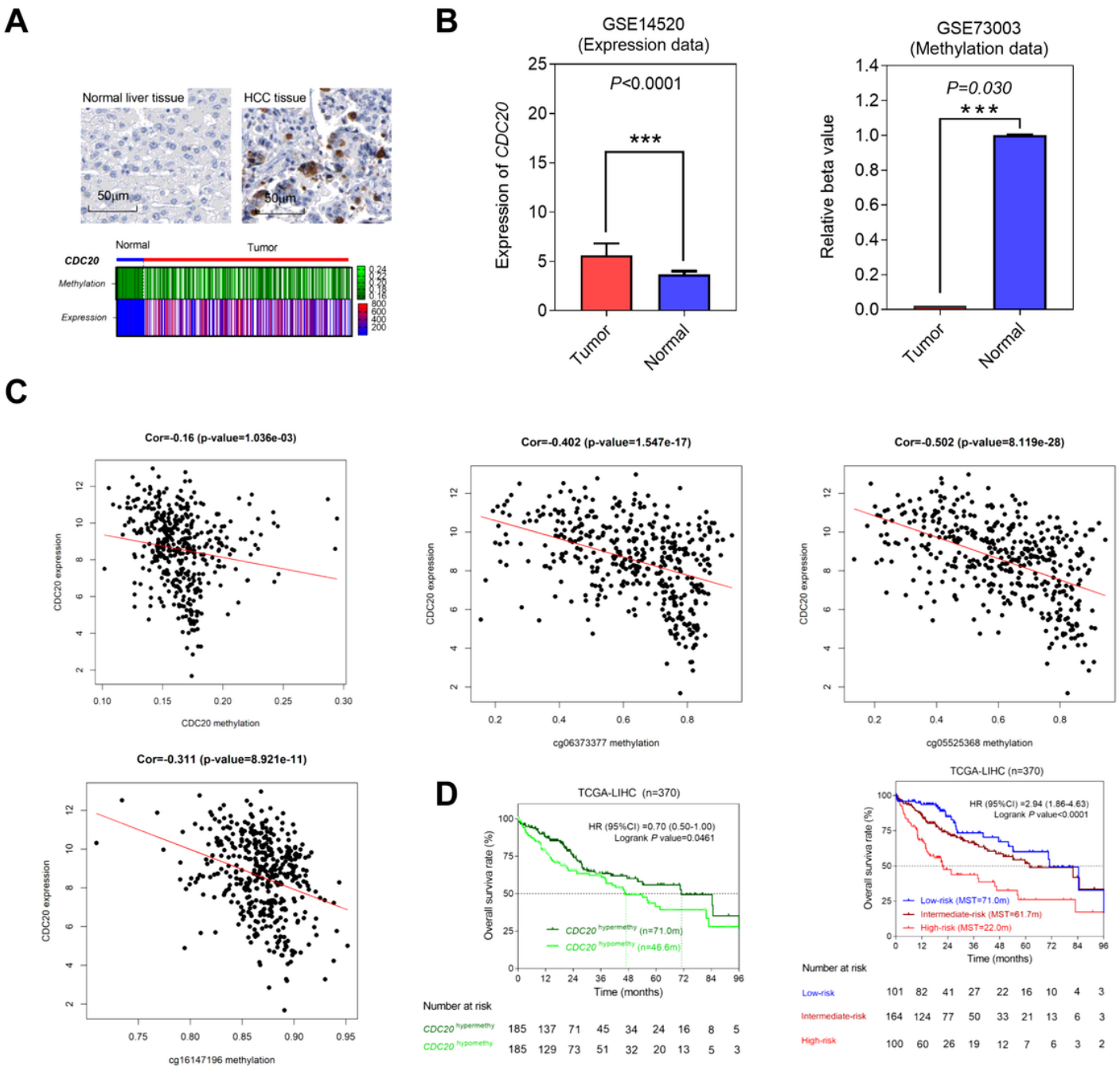

## Figure 2

CDC20 was hypermethylated and downregulated in senescent HCC cells. (A and B) CDC20 was a down-regulated gene with hypermethylation in senescent fibroblast and HCC cells, while up-regulated and hypomethylated in proliferating fibroblast and HCC cells. (C) RT-qPCR and western blot were used to examine the mRNA and protein expression levels of CDC20 after 5 μm 5-AZA treatment in senescent SMMC-7721 and Hep G2 cells (*** P < 0.001; ** P < 0.01; ).



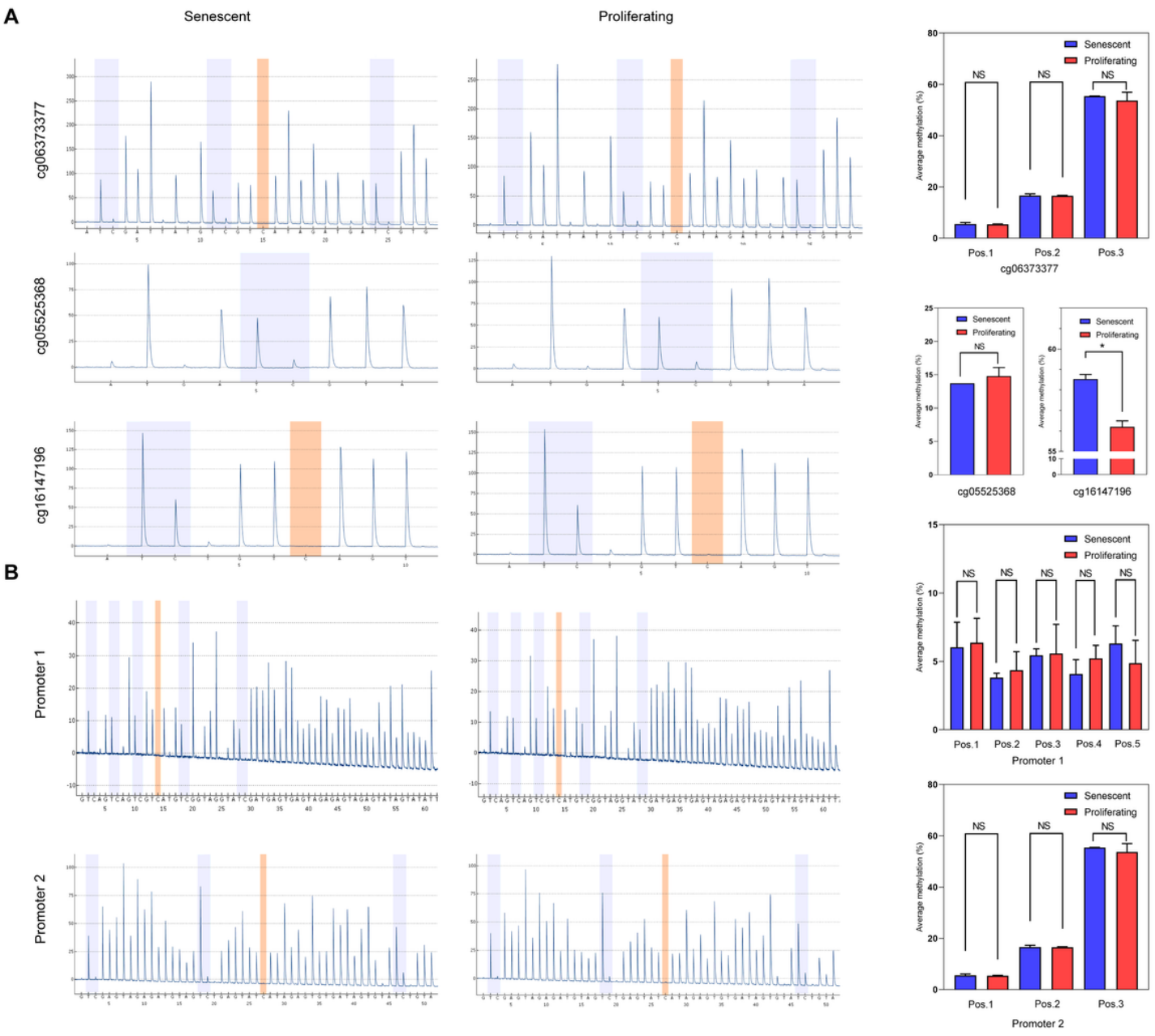

## Figure 3

Knockdown of CDC20 inhibited cell proliferation and induced cellular senescence in HCC cells. CCK-8 assay was used to detect the proliferative capacities (A) and cell cycle progression was determined by flow cytometry of propidium iodide (PI)-stained cells on a flow cytometer between siCDC20 and siControl HCC cells (B). Finally, SA-β-gal staining was conducted to validate the effects of CDC20 on cellular senescence (C) (Magnification is ×200) (*** $P < 0.001$; * $P < 0.05$).




## Figure 4

CDC20 was frequently hypomethylated in HCC tissues and was associated with poor prognosis. To assess the prognostic accuracy of the CDC20, immunohistochemistry staining showed the expression of CDC20 from The Human Protein Atlas database and the expression and methylation levels of CDC20 in TCGA-LIHC are presented as a heatmap in Figure 5A. The expression and methylation level of CDC20 was validated in HCC datasets (B). The relationship between CpG sites and expression levels of CDC20 in the TCGA-LIHC cohorts was evaluated (C). To assess the prognostic value of CDC20, the Kaplan-Meier curve was plotted to present the association between the overall survival (OS) and methylation level of CDC20 and different risk groups in TCGA-LIHC cohorts, respectively (D) (*** P < 0.001).




## Figure 5

Validation of the methylation level of CDC20 in senescent and proliferating HCC cells. The methylation levels of CDC20 were detected by pyrophosphate sequencing in cg06373377, cg05525368, cg16147196 (A) and promoter regions (B) between the senescent and proliferating SMMC-7721 cells, respectively (* $P < 0.05$; ns $P > 0.05$).