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Histone methyltransferase Dot1L inhibits pancreatic cancer cell apoptosis by promoting NUPR1 expression

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Abstract

Objective: To explore functions of the histone H3 lysine 79 (K79) methyltransferase Dot1L in the development of pancreatic cancer and evaluate the possibility of targeting Dot1L to inhibit pancreatic cancer progression.

Methods: Patient samples were used to detect differences in Dot1L expression between tumor and adjacent tissues and to determine correlations between Dot1L expression in patients with different stages of pancreatic cancer. Lentiviral-mediated knockdown of Dot1L expression and flow cytometry were used to detect apoptosis in pancreatic cancer lacking Dot1L expression; chromatin immunoprecipitation and quantitative PCR were used to detect downstream target genes of Dot1L.

Results: We show that Dot1L is highly expressed in pancreatic cancer, and that its expression is related to pancreatic cancer stage. Knocking down Dot1L significantly promoted apoptosis in pancreatic cancer cells, while overexpressing Dot1L inhibited apoptosis. Mechanistically, Dot1L regulated apoptosis in pancreatic cancer cells by promoting NUPR1 expression. The enriched H3K79 trimethylation in the transcription initiation region of NUPR1 promoted its expression. Overexpressing NUPR1 inhibited the pancreatic cancer cell apoptosis caused by Dot1L knockdown.

Conclusions: Dot1L inhibits pancreatic cancer cell apoptosis by targeting NUPR1; thus, Dot1L is a promising target for pancreatic cancer treatment.

Keywords

Dot1L, NUPR1, pancreatic cancer, histone methyltransferase, trimethylation, chromatin immunoprecipitation

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Introduction

Pancreatic cancer is a common malignant tumor of the digestive tract. The 5-year survival rate of pancreatic cancer is less than 10%, making it one of the deadliest tumor types.[1,2] Epigenetic modifications have been reported to regulate the etiology and pathogenesis of pancreatic cancer,[3,4] among which histone methylation plays a major role.[5,6] For example, EZH2, a member of the Polycomb repressive complex 2, has been reported to play a key role in the development of pancreatic cancer.[7] Loss of the histone H3K36 methyltransferase SETD2 also promotes the development of pancreatic cancer.[8] Disruptor of telomer silencing 1 Like (Dot1L) is a histone methyltransferase that can methylate lysine 79 of histone H3 (H3K79).[9] H3K79 methylation is primarily related to active transcription, transcription extension, and DNA repair reactions.[10,11] Dot1L was previously reported to be involved in the occurrence of prostate cancer.[12] However, its role in pancreatic cancer has not been determined. This study revealed the role of Dot1L in pancreatic cancer tumorigenesis and clarified its regulatory mechanism, providing a potential target for treating pancreatic cancer.

NUPR1 is a monomeric basic and intrinsically disordered protein (IDP) comprising 82 residues that is also known as p8 or transfer candidate (Com-1).[13,14] NUPR1 was first found in rat pancreatic acinar cells and regulates pancreatitis progression.[15] NUPR1 is a stress-induced transcription factor that is highly expressed in a variety of malignant tumors.[16,17] NUPR1 has recently attracted attention due to its role in a variety of tumor-promoting processes, including cell cycle regulation, matrix remodeling, autophagy, apoptosis, senescence, and DNA repair.[18,19] NUPR1 is always overexpressed in pancreatic cancer tissues, and previous studies have shown that inactivating NUPR1 promotes the apoptosis of pancreatic cancer cells.[20] NUPR1 has also been shown to regulate AKT signaling, and silencing NUPR1 was shown to promote apoptosis of glioblastoma cells.[21]

Here, we demonstrate that Dot1L expression is higher in human pancreatic cancer than in adjacent tissues and that it inhibits the apoptosis of pancreatic cancer cells by promoting NUPR1 expression. Knocking down of Dot1L promoted apoptosis in pancreatic cancer cells; therefore, Dot1L may be a possible target for future pancreatic cancer treatments.

Materials and methods

Tissue specimens

Pancreatic cancer tissues were collected from patients who underwent surgery at the Affiliated Hospital of Putian University between 2011 and 2015. The study protocol was approved by the ethics committee of the Affiliated Hospital of Putian University (LAEC-2021-012). None of the patients had undergone preoperative chemotherapy or radiation therapy. Written informed consent was obtained from the patient or legal guardians of the patient before any study procedures were performed.

Cell lines and cell culture

The human pancreatic cancer cell lines SW1990, BxPC-3, CFPAC-1, and Capan-1 were purchased from American Type Culture Collection (Manassas, VA, USA). SW1990 cells were cultured in L-15 medium supplemented with 10% fetal bovine serum (FBS). BxPC-3 cells were cultured in 1640 medium supplemented with 10% FBS. HPDE cells were cultured in complete keratinocyte serum-free medium supplemented with 50 μg/mL bovine pituitary extract (BPE) and 5 ng/mL epidermal growth

factor (EGF). All cell culture media contained 100 U/mL penicillin and 100 mg/mL streptomycin.

RNA extraction and quantitative PCR

We extracted total RNA using TRIzol reagent (Invitrogen, Waltham, MA, USA), and then 2.0 μg of RNA was subjected to reverse transcription using a qPCR kit (KAPA, Sigma-Aldrich, St. Louis, MO, USA) as instructed by the manufacturer. An ABI-7500 Real-Time PCR Detection System was used to perform all real-time quantitative PCR experiments. β-actin was used as a control to normalize the amplified transcript level of each indicated gene. The primers used were as follows:

Dot1LF: 5′-CAAGTTCTCGCTGCCTCACT-3′
Dot1LR: 5′-GTCCTGAGGGCTCAGCTTC-3′
β-actin F: 5′-GAAGGATTCCTATGTGGGCGA-3′
β-actin R: 5′-GATAGCACAGCCTGGATAGCAA-3′

Cell Counting Kit-8 (CCK–8) assay

We performed CCK-8 assays using a CCK-8 kit (MedChemExpress, Monmouth Junction, NJ, USA) in accordance with the manufacturer's instructions. Briefly, we seeded 3×10^4 cells into each well of a 96-well plate. Then we added 10 μL of CCK-8 solution to each well and incubated the cells in an incubator for 3 hours. We measured absorbance of the plate at 450 nm.

Immunoblotting assay

Cells were lysed in loading buffer. The Lowry protein assay was used to calculate protein concentrations. Then we used SDS-PAGE to separate 10 μg of protein lysate, which was then transferred to a PVDF membrane (Solarbio, Beijing, China). The indicated proteins were detected by immunoblotting with specific antibodies in 5% bovine serum albumin. The antibodies used were as follows: anti-Dot1L (ab72454, Abcam, Cambridge, UK), anti-NUPR1 (ab161980, Abcam), anti-GAPDH (sc-20357, Santa Cruz Biotechnology, Dallas, TX, USA), and anti-pAKT (4506, Cell Signaling Technology, Danvers, MA, USA).

Flow cytometry

We seeded 2×10^5 stable Dot1L knock-down cells into a six-well plate. After 48 hours, cells were collected and subjected to flow cytometry. Following the manufacturer's instructions, we used the Apop-Detec kit (CA1030, Solarbio) to analyze apoptosis.

Statistical analysis

Unless otherwise stated, all results are of experiments repeated at least three times. The mean ± SEM of the replicates is used to express the results. To compare differences between groups, a two-tailed paired Student's t-test was applied. Then we used the Pearson's correlation test to determine the correlation between Dot1L and NUPR1 in human tissues. When performing statistical analysis, we did not exclude any materials or data from the study.

Results

Increased Dot1L expression in human pancreatic cancer

To determine the role of Dot1L in pancreatic cancer, we first used quantitative PCR to detect its mRNA levels in pancreatic cancer patients. Follow-up of the 77 eligible cases from our institute yielded 50 cases with complete survival information. However, paired tumor and paracancerous tissues, which were used to evaluate difference in Dot1L expression in different stages

of pancreatic cancer, were only available for 30 patients. The results showed that compared with adjacent tissues, Dot1L was significantly upregulated in pancreatic cancer tissues (Figure 1a). Correlation studies also showed that Dot1L expression in pancreatic cancer patients was directly proportional to the stage of disease progression (Figure 1b). We also detected Dot1L expression in human pancreatic ductal epithelial (HPDE) cells and several pancreatic cancer cell lines. We found that Dot1L mRNA levels were higher in pancreatic cancer cell lines than in normal pancreatic ductal epithelial HPDE cells (Figure 1c). Additionally, high Dot1L expression was an independent predictor for shorter overall survival (Figure 1d). These data show that there is a causal relationship between Dot1L and the tumorigenesis of pancreatic cancer and indicate that Dot1L may be a potential biomarker of pancreatic cancer.

Dot1L inhibited apoptosis in pancreatic cancer cells

To investigate the role of Dot1L in pancreatic cancer cells, we used lentiviral infection to stably transfect two shRNAs to generate Dot1L-knockdown SW1990 and BxPC-3 cells. Detecting Dot1L RNA and protein levels by quantitative PCR and western blot, respectively, showed that Dot1L was significantly knocked down (Figure 2a).

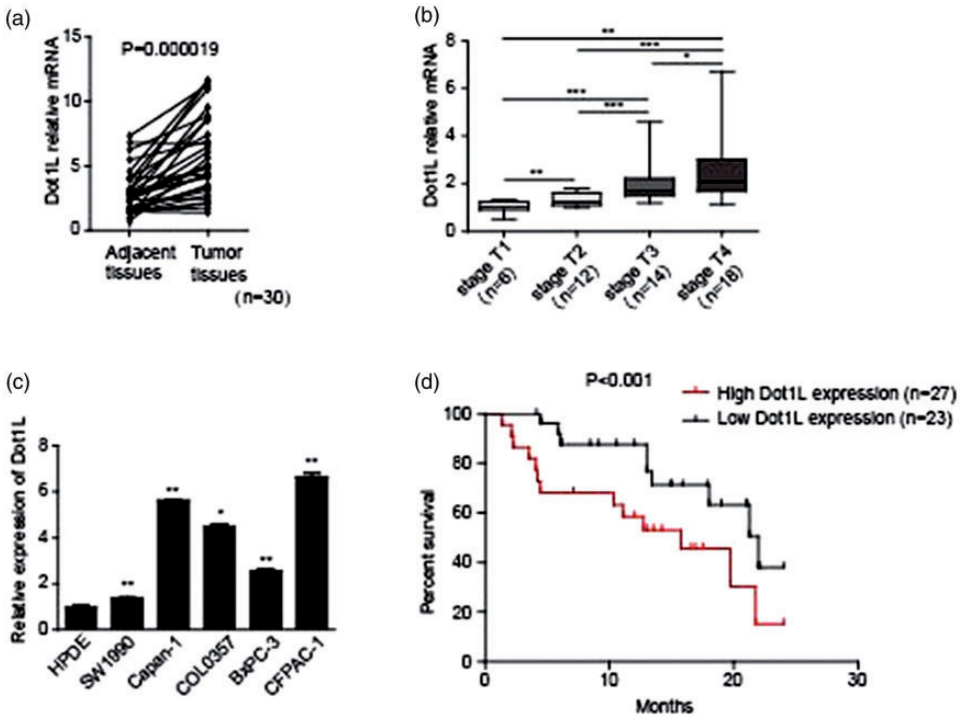

Figure 1. Dot1L expression in pancreatic cancer cell lines and clinical specimens. (a) Dot1L mRNA levels in 30 matched tumor and paracarcinoma tissues (paired t-test). (b) The box plot of Dot1L mRNA expression as assessed by quantitative PCR at different clinical stages. (c) Quantitative PCR showing the expression of Dot1L in untransformed (HPDE) or transformed pancreatic cell lines. (d) Kaplan–Meier plot of overall survival in pancreatic cancer patients on the basis of Dot1L expression. Data are expressed as mean ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (Student's t-test).

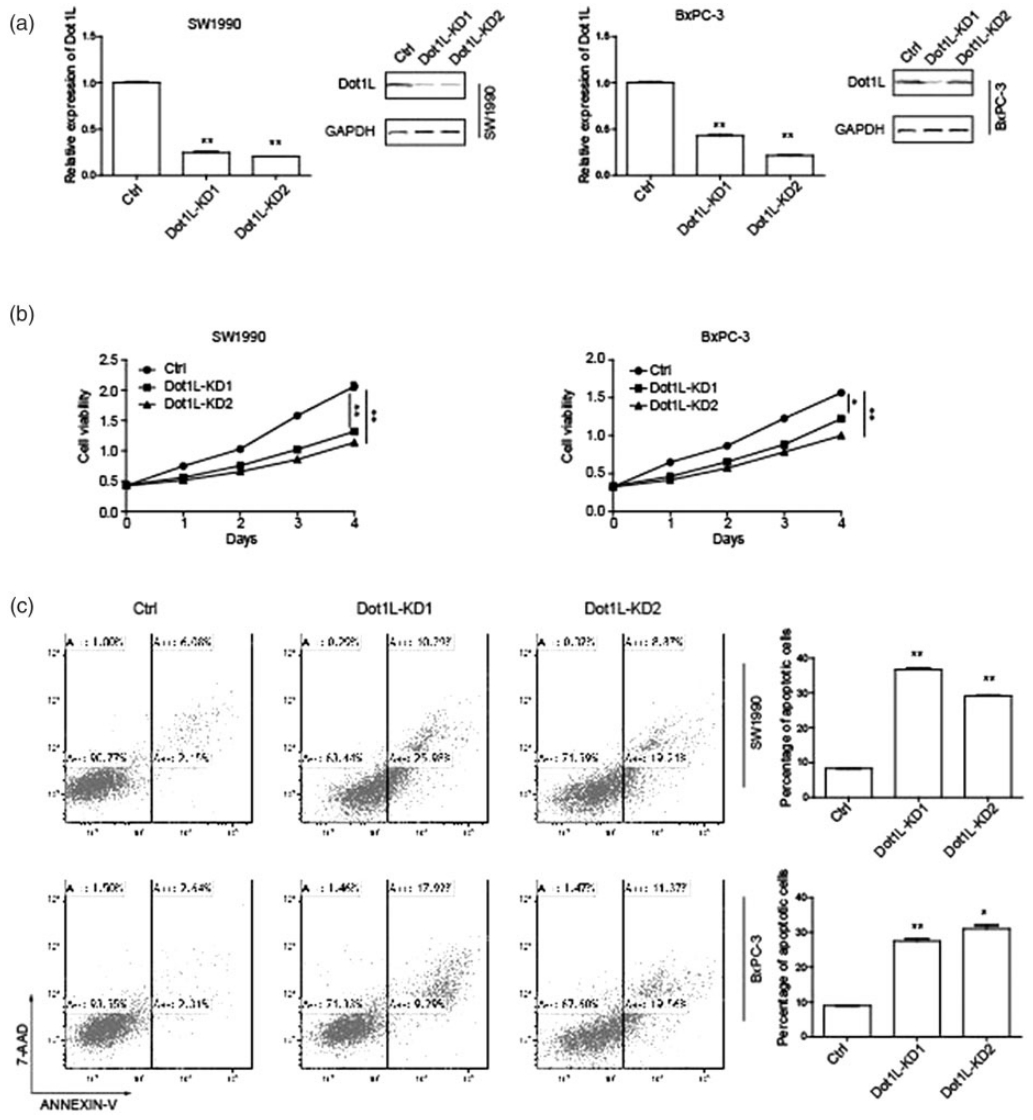

Figure 2. Knocking down Dot1L promoted apoptosis in pancreatic cancer cells. (a) Western blot and quantitative PCR analysis of the indicated proteins in control (CTRL) and Dot1L-knockdown (Dot1L-KD) SW1990 and BxPC3 cells. (b) Cell viability assays were performed in CTRL and Dot1L-KD SW1990 and BxPC3 cells. (c) SW1990 and BxPC3 cells were depleted of Dot1L, and the percentage of cells entering apoptosis was determined by flow cytometry using APC-labeled Annexin V and 7-AAD staining. All experiments were performed at least three times. Data are expressed as mean ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (Student's t-test).

Using the CCK-8 assay, we found that knocking down Dot1L suppressed the growth of pancreatic cancer cells (Figure 2b). Through a microscope, we observed a significant increase in the number of dead cells floating in the petri dish after Dot1L knockdown. To verify this finding, we measured the proportion of apoptotic

cells in pancreatic cancer cell lines 48 hours after Dot1L knockdown by flow cytometry. The results showed that knocking down Dot1L significantly increased the rate of apoptosis in SW1990 and BxPC-3 cells (Figure 2c). These data indicate that Dot1L inhibits pancreatic cancer cell apoptosis. We also overexpressed Dot1L in the pancreatic cancer cell lines SW1990 and BxPC-3 (Figure 3a). CCK-8 assays

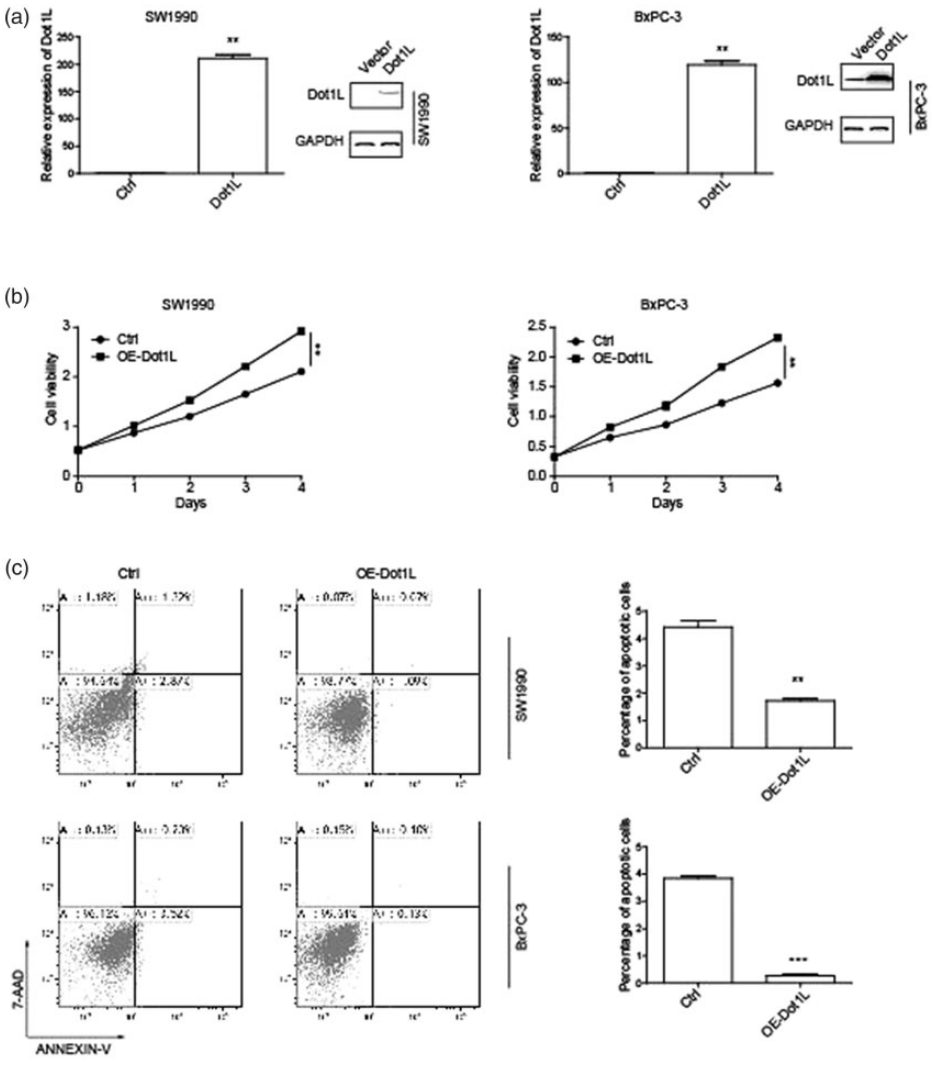

Figure 3. Overexpressing Dot1L inhibited apoptosis in pancreatic cancer cells. (a) Western blot and quantitative PCR analysis of the indicated proteins in control (CTRL) and Dot1L-overexpressing (Dot1L) SW1990 and BxPC3 cells. (b) Cell viability assays were performed in CTRL and Dot1L SW1990 cells and BxPC3 cells. (c) SW1990 and BxPC3 cells overexpressing Dot1L were sorted by flow cytometry to investigate the percentage of cells entering apoptosis using APC-labeled Annexin V and 7-AAD staining. All experiments were performed at least three times. Data are expressed as mean ± SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student's t-test).

showed that Dot1L overexpression increased the number of pancreatic cancer cells (Figure 3b). Flow cytometry showed that overexpressing Dot1L significantly inhibited apoptosis in pancreatic cancer cells (Figure 3c).

Dot1L inhibited apoptosis by promoting NUPR1 expression

According to previous reports, NUPR1 promotes the occurrence and development of a variety of tumor types.[22] Recently, it was discovered that NUPR1 promotes cell proliferation in glioblastoma by activating AKT.[23] Because NUPR1 is a potential factor in inhibiting pancreatic cancer apoptosis, we speculated that Dot1L may upregulate NUPR1 and activate AKT signaling, thereby inhibiting pancreatic cancer cell apoptosis. To assess the correlation between Dot1L and NUPR1, we used quantitative PCR to simultaneously measure the mRNA levels of Dot1L and NUPR1in 50 pancreatic cancer tissues. This analysis revealed a significant positive correlation ($p < 0.01$) between the expression of Dot1L and NUPR1 (Figure 4a). We also performed chromatin immunoprecipitation (ChIP) assays. The distribution of H3K79me3 was primarily found at sites 1 kb upstream of the NUPR1 transcription

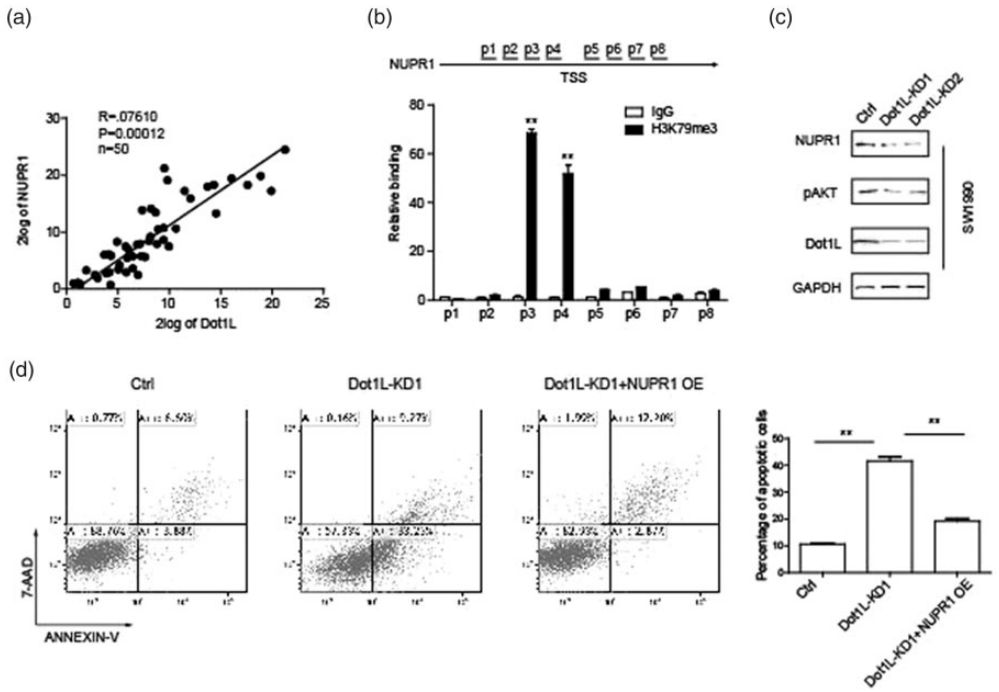

Figure 4. Dot1L regulates apoptosis by promoting NUPR1 expression. (a) Correlation between Dot1L and NUPR1 expression in pancreatic cancer patients ($n = 50$); each point represents one independent sample, and the correlation coefficient (R) and P-value is presented. (b) Chromatin immunoprecipitation and quantitative PCR analysis of H3K79me3 binding to the NUPR1 promoter in SW1990 cells. (c) Western blot analysis of Dot1L, NUPR1, and pAKT in SW1990 and BxPC3 cells. (d) SW1990 cells depleted of Dot1L and overexpressing NUPR1. The percentage of cells entering apoptosis was determined by flow cytometry using APC-labeled Annexin V and 7-AAD staining. All experiments were performed at least three times. Data are expressed as mean ± SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student's t-test).

start site (TSS) (p3) and 500 bp upstream of the NUPR1 TSS (p4) (Figure 4b). We used western blotting to confirm the protein expression levels of NUPR1 and AKT phosphorylation in Dot1L-knockdown and control pancreatic cancer cell lines (Figure 4c). We found that compared with control cells, NUPR1 expression in Dot1L-knockdown cells was decreased, as was AKT phosphorylation. These data further confirmed that NUPR1 is regulated by Dot1L in pancreatic cancer. Finally, we overexpressed NUPR1 after knocking down Dot1L, which rescued the apoptosis caused by Dot1L knockdown (Figure 4d). These results show that Dot1L inhibits pancreatic cancer cell apoptosis by promoting NUPR1 expression.

Discussion

NUPR1 is upregulated in many different tumor types and is considered an oncogene.[17] NUPR1 expression is activated in a variety of tumors and inhibits tumor cell apoptosis.[22,23] Specifically targeting NUPR1 to promote apoptosis could be used to produce therapeutic benefits. In this study, we found that Dot1L is highly expressed in pancreatic cancer cells, and that its expression level is positively correlated with the stage of pancreatic cancer progression. Knocking down Dot1L promoted apoptosis of pancreatic cancer cells by downregulating NUPR1 expression and AKT phosphorylation. Therefore, Dot1L could become a new diagnostic biomarker and a potential therapeutic target for pancreatic cancer.

To date, there have been few reports on the role of Dot1L in cancer, but a few related studies have shown that Dot1L is an oncogene.[12,24] However, whether Dot1L is also involved in the occurrence and development of pancreatic cancer through genes other than NUPR1 remains unclear and will need to be further determined by RNA-sequencing. Through chromatin immunoprecipitation and quantitative PCR, we found that the promoter region of NUPR1 was enriched for the H3K79 methylation modification, but whether Dot1L is involved in regulating pancreatic cancer cell apoptosis by modifying other genes through H3K79 also needs to be further determined.

It has also been reported that NUPR1 inhibits ferroptosis in pancreatic cancer,[25] so whether Dot1L regulates ferroptosis in pancreatic cancer cells by regulating NUPR1 is also worth exploring. The Dot1L inhibitor EPZ-5676 is currently under clinical investigation in a phase I study for mixed lineage leukemia. The pharmaco-physiological mechanism of Dot1L inhibition using EPZ-5676 in mixed lineage leukemia is not fully understood.[26] We found that Dot1L promotes the development of pancreatic cancer by inhibiting apoptosis of pancreatic cancer cells, so the role of this inhibitor in pancreatic cancer is also worth studying. EPZ-5676 is also believed to inhibit the stemness of cancer breast cancer stem cells.[27] So whether Dot1L also affects pancreatic cancer stem cells to regulate the occurrence and development of pancreatic cancer should be investigated.

In summary, we have shown that Dot1L inhibits pancreatic cancer cell apoptosis by targeting NUPR1; thus, Dot1L is a promising target for future pancreatic cancer treatments.

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Author contributions

LS conceived the project; LS and CH designed the research, analyzed the data, prepared the figures, and wrote the manuscript; LS, CH, ZJ, and CQ performed most of the experiments.

Declaration of conflicting interest

The authors declare no potential conflicts of interest.

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