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Original Article

HPP1 gene promoter methylation in pancreatic cancer: correlation with carcinogenesis and clinical implication

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Abstract: Aberrant methylation of the promoter region in a tumor suppressor gene is a significant early event in carcinogenesis of pancreatic cancer. Methylation status of a tumor suppressor gene may be useful as a diagnostic marker for early detection of pancreatic cancer. The current study evaluated methylation status of the hyperplastic polyposis 1 (HPP1) gene transcriptional regulation region (TRR) in pancreatic cancer and normal tissue samples by bisulfite-specific PCR (BSP) cloningbased sequencing analysis and quantitative methylationspecific PCR (qMSP). Correlation of methylation with the clinicopathological characteristics of the pancreatic cancer patients was also assessed. The results demonstrated that the HPP1 promoter was significantly hypermethylated in the pancreatic cancer tissues compared with normal tissues and a significant correlation between HPP1 methylation level and tumor-node-metastasis stage was observed. Thus, our study suggests that methylation of the HPP1 gene is a potential clinically diagnostic and prognostic biomarker for pancreatic cancer.

Keywords: Pancreatic cancer, HPP1, methylation, diagnosis

Introduction

Pancreatic cancer, one of the most frequent cancers in the world, is a devastating malignant disease with a median survival of 3-6 months and a 5-year survival rate of less than 5% [1-3]. In 2010, 34,509 men and 23,226 women died from pancreatic cancer in China, a number of deaths exceeding that in the United States [4, 5]. The vast majority of patients diagnosed with pancreatic cancer have regional or distant metastasis. A multicenter nationwide study in China showed that 18.4% of pancreatic cancer patients received diagnoses at stages I or II, and 81.6% at stages III or IV [6]. Therefore, a novel diagnostic tool for early detection is urgently required for pancreatic cancer patients in China.

Increased methylation of regulatory gene sequences, so called CpG islands in the promoter region of genes, is a hallmark of tumor cells [7, 8] and can be used to discriminate normal and tumor cells [9, 10]. Aberrant methylation of CpG-rich sequences in a tumor suppressor gene leading to gene silencing is typically an early event in tumorigenesis, including pancreatic cancer. Thus, these events may be used as a diagnostic marker to detect early-stage pancreatic cancer.

HPP1, a tumor suppressive epidermal growth factor (EGF)-like ligand, mediates its effects through signal transducer and activators of transcription (STAT) activation [11]. The janus kinase (JAK)/STAT pathway is best known as an effector of signaling from immune cells, particularly from macrophages in the pancreas that secrete IL-6 and IL-6r that together activate signaling through STAT3 within tumor cells, promoting survival and proliferation [12]. HPP1 is downregulated by promoter hypermethylation in a number of tumor types [11, 13]. Detection of methylated free-circulating DNA (mfcDNA) for HPP1 in blood is correlated with a poor prognosis for patients with metastatic colorectal cancer [14]. However, the methylation status of HPP1 in pancreatic cancer patients remains unclear.

In our study, the methylation status of the HPP1 gene transcriptional regulation region was examined in primary carcinoma and paired normal tissues derived from 42 patients with pancre-

atic cancer, and association of methylation with the clinical characteristics of the patients was also assessed. Our findings suggest that HPP1 methylation status may provide a novel biomarker to improve prediction and to guide prognosis for pancreatic cancer.

Materials and methods

Cell line and culture

The pancreatic cancer BXPC-3 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were was cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO_2 atmosphere incubator.

Pancreatic tissue samples

A tissue and patient data usage protocol was approved by the Ethics Committee of our institution. Informed written consent was obtained from each patient. Slides of paraffin-embedded pancreatic cancer tissues from 42 patients, corresponding adjacent normal tissues and normal pancreatic tissues were obtained from Changhai Hospital, which is affiliated with Second Military Medical University. Sample of white blood cells (WBCs) was obtained from a healthy volunteer. Clinical characteristics, including gender, age, tumor size, differentiation, lymph node metastasis and pTNM stage were collected from the electronic medical records of the patients. The clinical stage of pancreatic cancer was classified by the American Joint Committee on Cancer (AJCC) staging system (8th edition).

DNA extraction and bisulfite modification of DNA

Genomic DNA from the tissues was extracted by phenol/chloroform method and ethanol precipitation. Genomic DNA (1 μg) was treated with the EZ DNA Methylation Kit™ (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. The bisulfite-modified DNA was then suspended in 20 μl of deionized water for immediate use or stored at -80°C until use.

Bisulfite-specific polymerase chain reaction (BSP) and DNA sequencing

The primers designed to specifically amplify bisulfite-converted DNA of HPP1 TRR were used to detect methylation of the HPP1 gene promoter. The primers custom synthesized by Shanghai Sangon (Shanghai, China) were as follows: forward, 5'-TGGTGATGGAGGAGGTTTAGTAAGT-3'; reverse, 5'-AACCTTAAAATTACACRCTCTT-3'. The bisulfite modified DNA (2 μl) from each sample was subjected to PCR analysis in a 25 μL volume containing 1 × PCR buffer, 2.0 mmol/L $MgCl_2$, 2.5 mmol/L dNTP, 1 mmol/L primer, and EX Taq DNA HS 800 U/L. The reaction mixture was preheated at 95°C for 5 min and amplified using a touch-down PCR program on a PCR system as follows: 9 cycles of 95°C for 30 s, 59°C for 30 s (next cycle touch-down 0.5°C) and 72°C for 30 s; 42 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final extension of 4 min at 72°C. The PCR products were then subjected to either direct sequencing analysis or cloning into the pMD-18-T vector (TaKaRa, Dalian, China) followed by sequencing analysis (after the cloning, 10-25 clones from each sample were randomly selected for DNA sequencing).

Quantitative methylationspecific polymerase chain reaction (qMSP)

The bisulfite-treated DNA was amplified using qMSP in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). PCR was carried with the following amplification program: Initial denaturation step of 95°C for 10 min, then 45 cycles of 95°C for 15 s, 70°C for 15 s and 60°C for 60 s. The bisulfite-treated DNA that was obtained from the BXPC-3 cells and fully methylated by SssI methylase was used as a positive control. β-actin was used as an internal control to correct for differences in quality and quantity between samples. The primers and probes designed for HPP1 and ACTB (used as a loading control) were as follows: HPP1 forward, 5'-GTTGT TTTTAGGTCGGTAAGAGC-3' and reverse, 5'-ACG TCCTACTAACGACCGACG-3'; HPP1 probe, 5'-FAM-TTAGAGAAAYGTTTTTGGTTT-MGB-3'; ACTB forward, 5'-TGGTGATGGAGGAGGTTTAGTAAGT-3' and reverse, 5'-AACCAATAAAACCTACTCCTCCC TTAA-3'; ACTB probe, 5'-FAM-TTTGTTATTGTGTG TTGGGTG-MGB-3'.

Statistical analysis

Data are reported as arithmetic means ± SE. Statistical analyses were performed using Chi-square and Student's t test with the SPSS version 17.0 software. *P* value < 0.05 was considered significant.

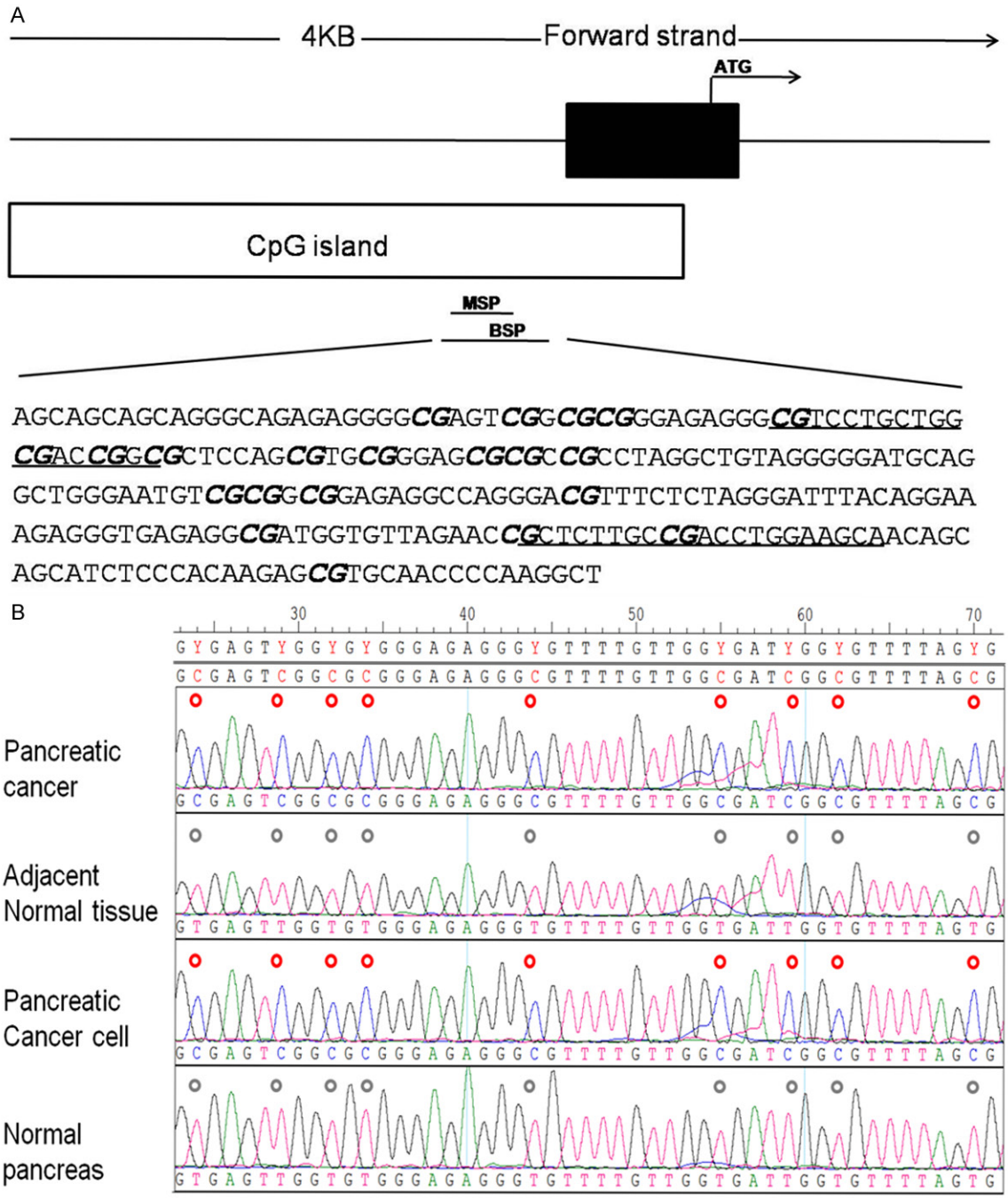

Figure 1. Detection of HPP1 gene TRR methylation. A. Structure of the HPP1 gene TRR and topology of the BSP primers. The bold italic "CG" indicates the location of 21 CpG island sites. The underlined sequence indicates the primers for BSP. B. Representative BSP-based sequencing analysis results in three different groups of pancreatic tissues and the pancreatic cancer cell line. The red dots represent methylated sites and the grey dots represent unmethylated sites.

Results

Methylation of the HPP1 gene TRR in pancreatic cancer cells and pancreatic tissues

According to the guidelines of the National Center for Biotechnology Information genome database, we analyzed the HPP1 gene TRR. **Figure 1A** demonstrated the position of the CpG island containing 21 CpG sites and the BSP primers. Then we conducted BSP-based sequencing analysis to assess the methylation status of the HPP1 gene TRR in the following four groups: pancreatic cancer cell line (BXPC-

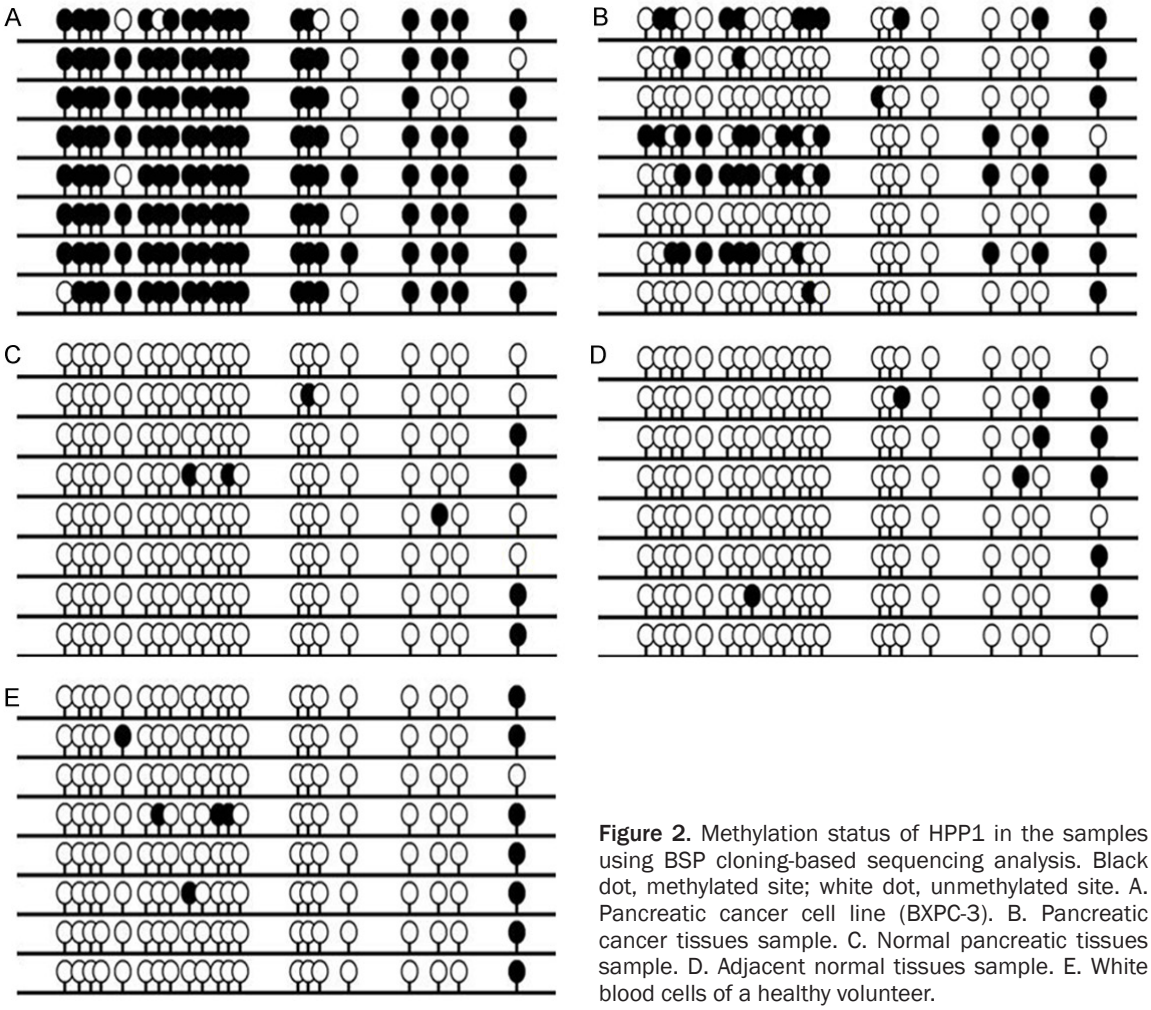

Figure 2. Methylation status of HPP1 in the samples using BSP cloning-based sequencing analysis. Black dot, methylated site; white dot, unmethylated site. A. Pancreatic cancer cell line (BXPC-3). B. Pancreatic cancer tissues sample. C. Normal pancreatic tissues sample. D. Adjacent normal tissues sample. E. White blood cells of a healthy volunteer.

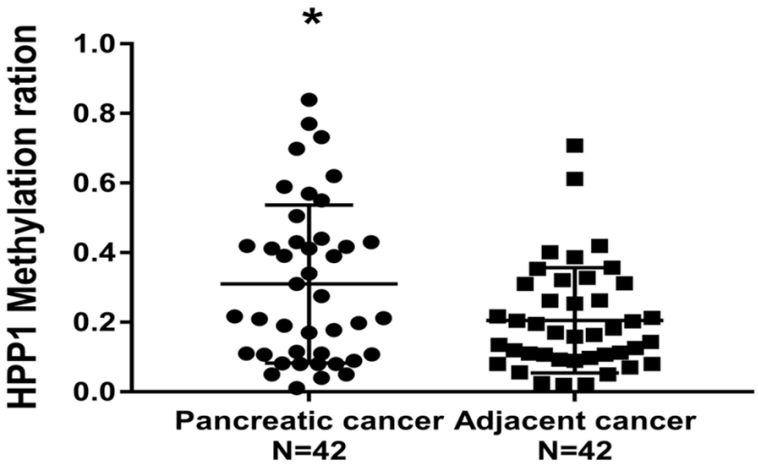

Figure 3. Quantitative analysis of the HPP1 gene promoter methylation in pancreatic tissues. The methylation level of HPP1 in 42 pancreatic cancer tissues and corresponding adjacent normal tissues was assessed by quantitative methylation-specific polymerase chain reaction (qMSP).

3), pancreatic cancer and their adjacent normal tissues, and normal pancreatic tissues. Representative results for these four different groups are shown in **Figure 1B**. Methylated sites of the HPP1 gene TRR were found in pancreatic cancer tissues and cells but not in normal pancreas and adjacent tissues.

To further confirm that hypermethylation of the HPP1 gene TRR occurs in pancreatic cancer, BSP cloning-based sequencing analysis was performed to identify the methylation patterns in the same samples above with the addition of one case of white blood cells from a healthy volunteer. The pancreatic cancer cell line and pancreatic cancer tissues (**Figure 2A** and **2B**) obviously presented more marked CpG island sites of hypermethylation

Table 1. Correlations of HPP1 methylation with clinical characteristics in pancreatic cancer

Clinicopathological characteristic	Variable	Cases	HPP1 methylation		*p*-Value
			+	-	
Gender	Male	27	15	12	0.89*
	Female	15	8	7	
Age (years)	47-80	42	60.47±5.73	58.12±6.21	0.419**
Maximal tumor size (mm)	10-100	42	4.41±1.98	4.39±1.81	0.573**
Histology	Well	30	17	13	0.695*
	Poorly	12	6	6	
Lymph node metastasis	Positive	25	13	12	0.663*
	Negative	17	10	7	
TNM stage	I+II	19	7	12	0.034*
	III+IV	23	16	7	
Total		42	23	19	

*Chi-square test; **Student's t-test; Well, well/moderately-differentiated adenocarcinoma; Poorly, poorly-differentiated adenocarcinoma.

compared with the normal and adjacent normal tissues and the WBCs from the healthy volunteer (**Figure 2C-E**). These data were consistent with the results of the BSP-based sequencing analysis.

To evaluate the difference of HPP1 methylation expression more accurately, qMSP was used to assess the methylation level of the HPP1 promoter in 42 pancreatic cancer tissues and adjacent normal tissues (**Figure 3**). The mean HPP1 expression level in the pancreatic cancer tissues was significantly higher than that of the adjacent normal tissues (cancer tissue: Mean, 0.31; 95% confidence interval, [0.24-0.38]; normal tissue: Mean, 0.21; 95% confidence interval, [0.16-0.25]; P=0.0114). Based on these data, hypermethylation was defined by a value of > 0.21. Hypermethylation of HPP1 was detected in 23 out of the 42 (54.8%) primary pancreatic cancer tissue cases, indicating that HPP1 gene TRR hypermethylation occurs frequently in pancreatic cancer.

Correlation of the HPP1 gene TRR methylation status with clinical characteristics in pancreatic cancer patients

To further investigate the relationship of HPP1 methylation with clinical characteristics of pancreatic cancer, the methylation results were assessed in 42 samples from pancreatic cancer patients. No difference was detected in HPP1 methylation status according to patient gender, age, tumor size, tumor histology, or lymph node metastasis. However, a significant correlation was observed between HPP1 methylation and tumor-node-metastasis stage (**Table 1**, P=0.034). HPP1 methylation frequently occurs in advanced pancreatic cancer, suggesting that HPP1 methylation is associated with tumor progression.

Discussion

Pancreatic cancer is a fatal disease with poor prognosis since it is difficult to detect early, and usually presents at a late stage [15]. Hypermethylation of tumor suppressor gene promoters causes DNA transcriptional inhibition, and the loss of the tumor suppressor gene is involved in carcinogenesis and tumor progression. The present study investigated the methylation status of HPP1 promoter in pancreatic cancer cell lines, pancreatic cancer, and corresponding adjacent normal tissues, and normal pancreatic tissues. Methylated sites of CpG islands were detected in pancreatic cancer tissues and cell lines, but not in the normal tissues. Further qMSP results showed that the HPP1 promoter was significantly hypermethylated in the pancreatic cancer tissues compared with the adjacent normal tissues. Moreover, according to the analyses of the relationship between HPP1 methylation and clinical characteristics of pancreatic cancer patients, a significant correlation between HPP1 methylation level and tumor-node-metastasis stage was observed, suggesting that HPP1 methylation frequently occurs in advanced pancreatic cancer. These data indicate that HPP1 promoter methylation is highly associated with pancreatic cancer. Importantly, methylation often occurs at the early stage of the tumor development and can be easily detected in a small amount of DNA [16-18], so HPP1 methylation status to be a marker for early diagnosis of pancreatic cancer. To our knowledge, this is the first report demonstrating the correlation of HPP1 promoter hypermethylation with pancreatic cancer and tumor progression.

The molecular mechanism responsible for methylation of the HPP1 gene promoter is still unknown. Previous studies have demonstrated that some environmental factors can result in methylation of certain tumor suppressor genes [19, 20], and HPP1 has been identified as a target of epigenetic inactivation in numerous tumor types, suggesting a role as a potential tumor suppressor [21]. HPP1 overexpression can upregulate JAK1 and JAK2 that are responsible for the subsequent activation of STAT1 and STAT2 [11]. STAT1 has previously been shown to have tumor suppressive activity by inhibitory modulation of tumor angiogenesis, growth, and metastasis [21, 22] and abrogation of STAT2 signaling results in partial restoration of proliferation and anchorage-independent growth [11]. Thus, HPP1 tumor suppressor activity may be mediated through activation of JAK1 and JAK2, and STAT1/STAT2. However, recent studies have also indicated that the JAK2/STAT3 signaling pathway is important for the initiation and progression of pancreatic cancer [23]. Whether HPP1 can inactivate STAT3 through interaction with upstream transcription factors in pancreatic cancer will require further study.

In conclusion, our results demonstrated that the HPP1 methylation ratio was significantly higher in pancreatic cancer samples compared with normal or adjacent normal pancreatic tissues and HPP1 hypermethylation was correlated with the progression of pancreatic cancer. The concrete cellular mechanisms are not clear and need more study, but we believe that HPP1 methylation status has the potential to be a clinically diagnostic and prognostic biomarker for pancreatic cancer and may contribute to a nationwide screening program to remedy shortcomings in China's pancreatic cancer diagnosis and treatment.

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Disclosure of conflict of interest

None.

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