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Original contribution

Epigenetic silencing of Klotho expression correlates with poor prognosis of human hepatocellular carcinoma☆

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Summary Klotho is identified as a tumor suppressor in several tumors, but the expression of the Klotho gene (*KL*) and its regulative mechanism are not reported in hepatocellular carcinoma (HCC). The messenger RNA and protein levels of Klotho were measured in 64 HCC tumor tissues by real-time polymerase chain reaction and immunohistochemistry, respectively. The methylation of *KL* promoter DNA was examined by bisulfite-based polymerase chain reaction. The correlation of Klotho protein expression and methylation with survival of HCC was analyzed using Kaplan-Meier analysis. The interference of *KL* gene expression was conducted in HCC cells by DNA demethylating agent and/or histone deacetylase inhibitor. In HCC tissues, a significant loss of Klotho messenger RNA and protein expression was observed, which parallels the increased methylation in *KL* promoter DNA. Both Klotho expression and methylation correlated with the poor prognosis of HCC. Experiments with HCC cell lines showed that a combination of DNA demethylating agent and histone deacetylase inhibitor fully recovered Klotho expression and subsequently induced cell apoptosis. In conclusion, Klotho is a tumor suppressor in HCC. Both hypermethylation and acetylation are involved in the loss of Klotho expression in HCC cells. Both *KL* gene expression and its promoter DNA methylation are predictive factors for the poor prognosis of HCC. Our study also suggests that the Klotho gene could be a target for therapy of HCC.

1. Introduction

Primary liver cancer is the fifth most common cancer and the third most frequent cause of cancer-related death worldwide [1,2]. Hepatocellular carcinoma (HCC) accounts for about 85% of primary liver cancers with high prevalence in Asia and southern Africa. The incidence of HCC is increasing in the Western world [3]. HCC has poor prognosis with a 7% 5-year survival rate [4]. The mechanism driving hepatocarcinogenesis remains to be fully elucidated. Epigenetic alterations play an important role in the silencing of tumor suppressor genes [5]. However, epigenetic alterations are ill defined in HCC [6].

Klotho was discovered in 1997 as a peptide hormone with antiaging properties. A mouse model with Klotho deficiency showed multiple aging-like phenotypes and shortened life span [7,8]. Recently, Klotho has been revealed to inhibit insulin/insulin-like growth factor 1 (IGF-1) pathways. IGF is associated with cancer risk and tumor progression [8,9]. Therefore, Klotho may be involved in tumorigenesis and growth as an antitumor molecule. Importantly, the expression of Klotho was found to be down-regulated in several tumors

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[10,11]. However, the expression of Klotho in HCC has not yet been reported. Currently, the mechanism responsible for the down-regulation of Klotho gene (*KL*) expression in cancers has not been fully elucidated. Hypermethylation of the *KL* promoter is thought to be the main mechanism for the loss of Klotho gene expression in several tumors. We therefore hypothesized that methylation might also be a crucial mechanism in HCC if Klotho expression is down-regulated in HCC.

In this study, we investigated Klotho messenger RNA (mRNA) expression, Klotho protein expression, and *KL* promoter methylation in HCC and their correlation with the prognosis of patients with HCC. Using a demethylating agent and histone deacetylase inhibitor, we determined the relationship between epigenetic silencing and Klotho expression in HCC tumor cells.

2. Materials and methods

2.1. Case selection

This study was conducted ethically with preapproval from the Ethics Committee for Human Studies at Second Xiangya Hospital, Central South University. A total of 64 HCC and 30 peritumoral tissues were collected. HCC specimens were collected from patients who underwent partial hepatectomy from January 2006 to December 2007 at Second Xiangya Hospital. Diagnosis of HCC was based on morphologic criteria and immunohistochemical (IHC) staining according to the conclusions of the Barcelona–2000 European Association for the Study of the Liver conference [12]. Survival information was obtained from all patients through letters and telephone calls. Among the 64 patients with HCC, 50 patients were male and 14 were female, with ages ranging from 35 to 70 years. No patient was given chemotherapy or radiotherapy before surgery. Resected tumors and peritumoral tissues were snap frozen or paraffin embedded and kept at −80°C.

2.2. Cell culture

HepG2, BEL-7402, SMMC-7721, HL7702, HUH-7, and MHCC-97-H cells are human hepatocellular cancer cell lines, and L-02 is a normal liver cell line. All cells were obtained from the Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in either RPMI 1640 or Dulbecco's modified Eagle medium (DMEM), with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C, 5% CO_2.

2.3. IHC staining

IHC staining of Klotho protein expression was performed according to a previously published protocol [13]. Briefly, 4-μm-thick sections were cut from routinely paraffin-embedded tissues. The sections were incubated with anti-Klotho antibody overnight at 4°C and horseradish peroxidase (HRP)-conjugated second antibody for 30 minutes. The immune complex was visualized using 3,3′-diaminobenzidine. Slides were counterstained with hematoxylin. Immunostaining was evaluated by 2 "blind" investigators unaware of the study. The percentage of positive cells was calculated from 10 random fields. Cases with 10% or more positive cells were considered positive [13].

2.4. Real-time polymerase chain reaction and reverse transcriptase polymerase chain reaction of Klotho gene expression

Frozen tumor tissues, peritumoral tissues, and cultured cells were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA) using the PowerGen 125 homogenizer (Fisher Scientific, Pittsburgh, PA). Total RNA was isolated following the manufacturer's instructions. Reverse transcription was performed using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Science, Waltham, MA). Quantitative polymerase chain reaction (PCR) reactions were performed using the SYBR Premix ExTaq kit (TaKaRa, Shiga, Japan). Relative quantification (RQ) of Klotho mRNA expression was determined using the comparative cycle threshold (CT) method ($RQ = 2^{-\Delta\Delta Ct}$) and normalized to the *GAPDH* level. The *KL* gene was amplified using forward primer 5′-CACGGCAAGGGTGCGTCCAT-3′ and reverse primer 5′-TCGCGCCCACGAGATGGAGA-3′. The *GAPDH* gene was amplified using forward primer 5′-CTCATGACCACAGTCCATGC-3′ and reverse primer 5′-TTCAGCTCTGGGATGACCTT-3′.

2.5. Genomic DNA isolation, sodium bisulfite treatment, and PCR amplification

QIAamp DNA Mini Kit (Qiagen, Valencia, CA) was used to extract genomic DNA from frozen tumor tissues, peritumoral tissues, and cultured cells following the manufacturer's instructions. For bisulfite treatment, EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA) was adopted according to the manufacturer's manual. After purification, methylated genomic DNA was subjected to PCR amplification. Primers were designed according to the nonconversion (U) and CT conversion sequences (M) of the *KL* gene promoter. The methylated DNA was amplified by Klotho(M)-F: 5′-ATGAATTTGAGCGTTTACGAAAC-3′ and Klotho(M)-R 5′-ACTCCGCTAACAATAATTACC-TACG-3′, whereas the unmethylated DNA was amplified by Klotho(U)-F: 5′-ATGAATTTGAGTGTTTAT-GAAATGT-3′ and Klotho(U)-R: 5′-TCCACTAACAA-TAATTACCTACAAA-3′. The amplified fragments were 219 base pairs.