

Aberrant KLK4 gene promoter hypomethylation in pediatric hepatoblastomas

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Abstract. DNA methylation has a crucial role in cancer biology and has been recognized as an activator of oncogenes and inactivator of tumor suppressor genes, both of which are mechanisms for tumorigenesis. Kallikrein-related peptidase 4 (KLK4), has been suggested to be an oncogene in various types of cancer. The aim of the present study was to assess the DNA methylation patterns of the KLK4 gene in cancerous samples harvested from patients with hepatoblastoma (HB). KLK4 mRNA expression levels were detected using reverse transcription-quantitative polymerase chain reaction and assessed its DNA methylation patterns using high-throughput mass spectrometry on a matrix-assisted laser desorption/ionization time-of-flight mass array. A total of 10 HB and 10 normal liver tissue samples were obtained from patients with HB. The results of the present study showed that a significantly higher level of KLK4 mRNA expression levels were detected in HB tissues, as compared with the matched controls. Furthermore, the KLK4 gene promoter region was distinctively less methylated in the HB samples compared with the controls and negatively correlated with KLK4 mRNA expression levels. These findings indicate that aberrant methylation of KLK4 may contribute to its upregulated mRNA expression in HB.

Introduction

Hepatoblastoma (HB) is the most common type of malignant liver tumor in infants and children. Although it accounts for just 0.5-1.5 cases per million children per year, the mortality rate is 35-50% in high-risk patients (1). Previous studies have suggested an association with familial adenomatous polyposis (FAP) (2), both low and high birth weights (3), and constitutional trisomy 18 (4); however its etiology remains unknown.

Currently, alphafetoprotein levels, histological analysis, tumor resectability and tumor metastasis are the only prognosis factors for HB. Therefore, novel targets for early detection and improved therapies and prognosis are required.

Epigenetic reprogramming has a crucial role in tumorigenesis; one of the key mechanisms is DNA methylation. DNA methylation, maintained by DNA methyltransferases, refers to the addition of a methyl group to the carbon 5 of the cytosine ring in CpG dinucleotides of gene promoter regions (5). DNA methylation is the only genetically programmed DNA modification process in mammals that is involved in the regulation of various biological processes, including gene transcription, X-chromosome inactivation, genomic imprinting and chromatin modification (6-8). It is generally recognized that DNA methylation is a form of gene closure; the higher the level of methylation, the more silent a gene is. Therefore, the hypermethylation of suppressor genes or hypomethylation of oncogenes may contribute to tumorigenesis. This hypothesis has been investigated and supported in various studies on tumors (9), including HB (10).

Kallikrein-related peptidase 4 (KLK4), which is a member of the KLK family, is considered to be an oncogene associated with various types of cancer, including colorectal adenocarcinoma and prostate cancer (11,12). In a pilot study, we detected aberrant expression of KLK4 in HB and normal tissues, and theorized if alterations in methylation status affect its expression. Therefore, the present study aimed to investigate KLK4 methylation in 10 HB samples in order to profile genes that are differentially methylated in this disease.

Materials and methods

Patients and sample collection. The present study utilized 10 HB and adjacent non-tumor tissues from patients who

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Table I. Clinical and pathological characteristics of the study subjects.

Subject	Age (months) ^a	Gender	Diagnosis type	Alpha-fetoprotein (ng/ml)
1	7	Male	Mixed embryonal/fetal subtype	68490
2	23	Male	Mixed embryonal/fetal subtype	>121,000
3	11	Female	Mixed embryonal/fetal subtype	>121,000
4	10	Female	Mixed embryonal/fetal subtype	>121,000
5	20	Female	Mixed embryonal/fetal subtype	>121,000
6	7	Male	Mixed embryonal/fetal subtype	>121,000
7	30	Male	Epithelial type	>121,000
8	14	Male	Epithelial type	>121,000
9	19	Male	Epithelial type	>121,000
10	7	Female	Epithelial type	>121,000

^aOn the date of serum sample collection.

ATCACGAACTATTTTTGGAACCTTGGGCAAGTGACTGCACCCCTGTGAGCCTGTTTTCCAAC TG
 TAAAACTGGAGTGACCATTATGCCCCATTTCCCAAGTGGATTCAAAGAGGTAATTCAGGGAAA
 GGCACACAGTGCCAGGCACATTGTAGGCTCTCGGTGAGCGGGAGAACAAAAAGTCTTCGCTCA
 CpG_1 CpG_2 CpG_3
 GTGAAAAGCAGCTGAGTGCCTACCACACTCGAGCTCTCGGCAGGTGCTGGGGCCACAGGAG
 CpG_4 CpG_5
 GAACCAGCAAAGTCCTCGTCACTGGGGATCGCGTCTACCGGGGCACAGACCATAAAGGAA
 CpG_9
 AACTGCAGGGACCAGCATATAAGGTGGTGGTGGGGAGACTCAGAGAAGGGGAACATTTAAG
 CpG_10
 CTGAGATCCCAGGGAAGAGGAGGAGGAGGGTCCGCGCAGCAGGGGCGGTTCGGGGTGCAGG
 CpG_11.12 CpG_13 CpG_14
 GGAGAACTTGCTCCAGCCAGAGGAGGGCAGGGCGGGTAGGAGGGCAGGGCGGGTAGGAGCG
 CAGACCGGGAACCGGGCGGGCCTGCAGGAGTTTACGTACCAGGATTAAGGAGCTTGGGTTTTA
 CTTAAGTGCAATGGATTTTAAGCA

Figure 1. DNA sequence of the kallikrein-related peptidase 4 promoter. Primers are underlined.

underwent partial hepatectomy at the Children's Hospital of Fudan University (Shanghai, China). The patients ranged in age from 7 to 30 months. Informed consent was obtained retrospectively from clinical files and the diagnoses were confirmed by the Department of Pathology following the presence of >80% viable tumor cells (13). Clinical and pathological data are listed in Table I. The Ethics Committee of the Children's Hospital of Fudan University approved the use of these human samples. Genomic DNA was extracted according to standard procedures from 10 matched HB tumors and non-tumor tissue sections that were stored at -80°C immediately after surgery.

DNA/RNA extraction and reverse transcription quantitative-polymerase chain reaction (RT-qPCR). DNA was extracted from 10 HB primary tumors and adjacent non-tumor tissues. DNA samples were stored at -80°C until subsequent use for mass spectrometry analysis. Total RNA was extracted from 10 HB tumor and non-tumor pairs using TRIzol reagent (Invitrogen; Thermo Fisher Scientific,

Inc., Waltham, MA, USA), and, reverse-transcribed into cDNA using a PrimeScript RT reagent kit (Perfect Real Time) with gDNA Eraser (Takara Biotechnology Co., Ltd., Dalian, China) following the manufacturer's protocol. This was then subjected to qPCR analysis to assess the levels of KLK4 mRNA expression using a SYBRGreen PCR kit (Takara Biotechnology Co., Ltd.). The KLK4 primers used for qPCR were: Forward, 5'-GGACTCCTGCAACGGTGA CTCT-3' and reverse, 5'-TAGACACCTGGCAGCCAACT T-3'. The PCR cycling conditions were as follows: 95°C for 30 sec, followed by 95°C for 5 sec and 60°C for 34 sec, for 45 cycles. For quantitative results, KLK4 expression was represented as fold-change by the $2^{-\Delta\Delta C_q}$ method and statistically analyzed (14).

Primer design and PCR tagging for EpiTYPER assay. Primers for the KLK4 gene were designed to cover the regions with the most CpG sites (Fig. 1). Selected amplicons were 500 bp upstream of the promoter of the KLK4 gene. Genomic DNA was bisulfite-treated, and its reverse primer

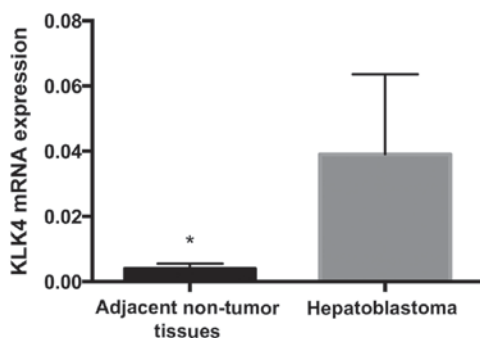


Figure 2. KLK4 mRNA expression levels were significantly greater in the HB samples, as compared to adjacent non-tumor tissues (* $P<0.01$). KLK4, kallikrein-related peptidase 4.

was supplemented with a T7-promoter tag for subsequent PCR amplification.

Mass spectrometry. Following treatment with shrimp alkaline phosphatase (SAP) *in vitro* transcription and uracil-specific cleavage, the DNA samples were robotically dispensed onto silicon matrix preloaded chips (SpectroCHIP; Sequenom, San Diego, CA, USA). Mass spectra were collected using a MassARRAY Compact matrix-assisted laser desorption/ionization time-of-flight system (Sequenom), and the methylation ratios of the spectra were generated using EpiTYPER software v1.0 (Sequenom).

Statistical methods. Statistical analyses and graphical depiction of data were generated using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Results are presented as the mean \pm standard error of the mean and were evaluated using a two-tailed Student's t-test unless otherwise specified, in which instance unpaired t-test or Pearson's correlation analysis were used. Statistical analyses were also performed using SPSS 19.0 software (IBM SPSS, Armonk, NY, USA) for Windows. $P<0.05$ was considered statistically significant to indicate a statistically significant difference.

Results

KLK4 mRNA expression levels are elevated in HB. Expression levels of KLK4 mRNA were analyzed in the 10 pairs of tumor ($n=10$) and adjacent normal liver ($n=10$) tissues using RT-qPCR. Expression levels of KLK4 in HB tissues were significantly increased, as compared with matched non-tumor liver tissues (0.039 ± 0.0077 vs. 0.004 ± 0.0005 , respectively; $P<0.01$; Fig. 2).

DNA methylation of KLK4 is reduced in HB. Methylation patterns of KLK4 were verified in the 10 HB samples and match non-tumor controls. Enriched methylation positions located at 500 bp upstream of the transcription initiation site were analyzed; 11/22 sites in the amplicons were amenable to analysis. Using two-way hierarchical cluster analysis, two sites were found to be at a significantly lower degree of methylation in the HB tissues, as compared with their non-tumor counterparts (Fig. 3). Furthermore, these sites exhibited a significant difference in the mean methylation levels of the

KLK4 gene between HB and adjacent non-tumor tissues (0.3755 ± 0.03218 vs. 0.4885 ± 0.01176 , respectively; $P<0.01$; Fig. 4).

Correlation between hypomethylation with KLK4 and expression of mRNA. Using linear Pearson's R correlation, the correlations between KLK4 mRNA expression and DNA methylation status of CpG4 and CpG9 sites harvested from the 10 samples were analyzed. This analysis demonstrated that the expression of KLK4 is negatively correlated with its methylation status ($r=-0.47$; $P=0.037$; Fig. 5).

Discussion

Pediatric solid tumors develop after relatively short latency periods, and thus are becoming one of the most common reasons for child mortality (15,16). HB is the most common liver tumor found in children, and its incidence is increasing in North America and Europe (17). Although its pathogenesis and progression have been extensively studied for the last two decades, its etiology remains to be fully elucidated. Through a previous study that involved sequencing of the HB exome, we recently detected a novel oncogene (caprin family member 2) and three tumor suppressors (speckle-type POZ protein, olfactory receptor family 5 subfamily I member 1 and cell division cycle 20B) that influence HB cell growth (18). The present group has also detected an association between long non-coding RNA (lncRNA) and HB in a previous study (19).

Hypermethylation or hypomethylation of gene promoter regions is recognized as one of the mechanisms that can silence or activate the oncogenes (20). It has recently been indicated that aberrant promoter methylation of the RAS association domain family protein 1 may contribute to the pathogenesis of HB and is considered to be a significant prognostic indicator in HB (1,21).

KLK4 is a member of the KLK family. It has previously been reported that KLK4 is overexpressed in endometrial carcinoma (22), colorectal adenocarcinoma, and prostate cancer; however, the mechanism remains unclear. Detection of KLK4 mRNA expression levels in HB and normal liver tissues by qPCR in the present study demonstrated a significant difference between tissue types. KLK4 mRNA expression levels in HB tissues were significantly higher than in normal liver tissues. In an independent cohort of 10 adjacent HB-non-tumor tissues pairs, we theorized whether there was a correlation between KLK4 mRNA expression and DNA methylation. To further understand the association between HB and KLK4 methylation, the methylation status of KLK4 genes were analyzed in the 10 paired samples. Following methylation quantification of the KLK4 gene using MALDI-TOF MS, three CpG sites were detected per sample. Hypermethylation degrees of two CpG sites in the promoter regions of the KLK4 gene for normal liver tissues were significantly higher than those of HB samples. Furthermore, correlation analysis indicated that expression of KLK4 mRNA is significantly negatively correlated with its methylation status. This finding supports previously published data, which suggested that KLK4 may be an oncogene (23-26).

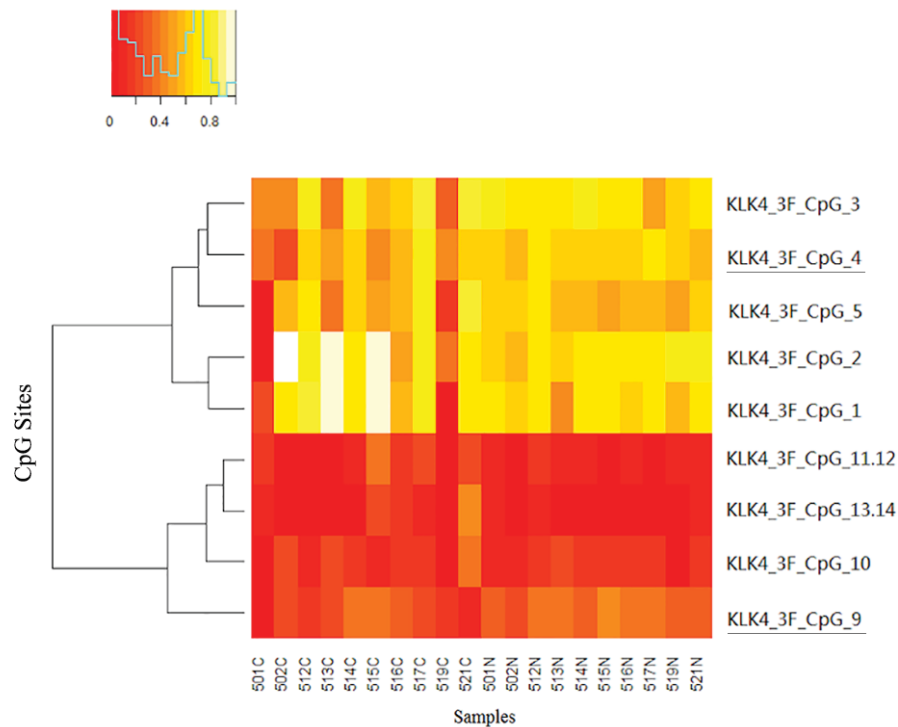


Figure 3. Two-way hierarchical cluster analysis of methylation patterns between 10 hepatoblastoma and adjacent non-tumor tissues. The two aberrant methylation sites are underlined. Red clusters indicate 0% methylated, whereas yellow clusters indicate 100% methylated. The color gradient between red and yellow indicates methylation ranging from 0 to 100, and white clusters indicate CpG sites that were not analyzed. KLK4, kallikrein-related peptidase 4.

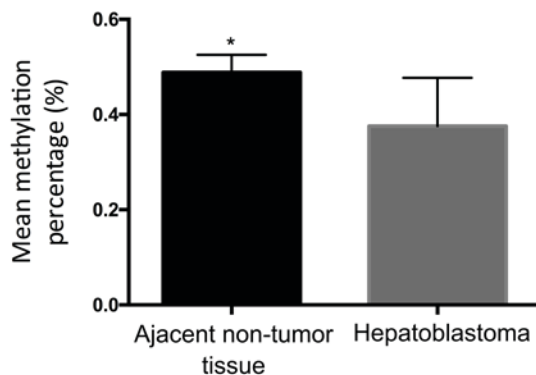


Figure 4. Methylation levels of KLK4 were significantly reduced in the hepatoblastoma samples ($n=10$), as compared with adjacent non-tumor tissues ($P<0.01$). KLK4, kallikrein-related peptidase 4.

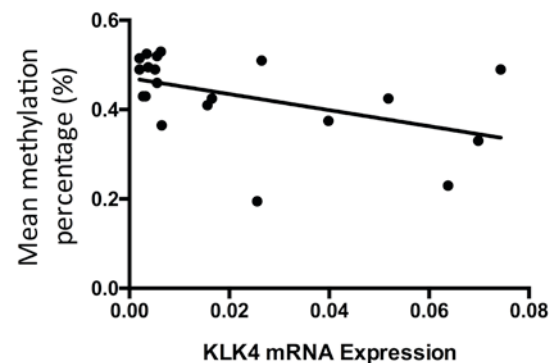


Figure 5. Correlation analysis of KLK4 mRNA expression and the percentage of DNA methylation in 10 hepatoblastoma primary tissues (two-tailed Pearson Correlation: $r=-0.47$; $P=0.037$). KLK4, kallikrein-related peptidase 4.

To the best of our knowledge, the present study is the first to examine the methylation of KLK4 genes in HB, and an association between the level of KLK4 mRNA and methylation was successfully detected. This finding is consistent with the results of previous studies, demonstrating that hypomethylation of promoter regions of crucial genes is able to activate relevant gene expression and may contribute to tumorigenesis (27-29). A limitation of the present study is that only 10 pairs of patient samples were used. Additional samples are required for investigation in order to more accurately represent the population.

In conclusion, the present findings indicate that aberrant promoter methylation of KLK4 may contribute to the tumorigenesis of HB, and that KLK4 may be a potential biomarker for HB. However, since HB is an uncommon disease, its

etiology, pathophysiology, and molecular mechanisms remain unknown. Further studies are required in order to fully understand and treat this disease.

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