mRNA expression and epigenetic-based role of chromodomain helicase DNA-binding 5 in hepatocellular carcinoma

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Abstract

Objective: Chromodomain helicase DNA-binding 5 (CHD5) acts as a tumor suppressor gene in some cancers. CHD5 expression levels may affect an individual’s susceptibility to hepatocellular carcinoma (HCC). This study aimed to evaluate the methylation pattern of the CHD5 promoter region and the gene’s corresponding mRNA expression in HCC patients compared with healthy individuals.

Methods: In this case–control study, CHD5 mRNA gene expression levels and DNA methylation patterns were analyzed in 81 HCC patients and 90 healthy individuals by quantitative reverse transcription polymerase chain reaction and methylation-specific polymerase chain reaction, respectively.

Results: The CHD5 gene was hypermethylated in 61.8% of the HCC patients and 54.4% of the controls, and this difference was statistically significant. The CHD5 mRNA expression levels were significantly lower in the HCC patient group.

Conclusions: Hypermethylation of the CHD5 promoter region may significantly lower the expression of this gene, affecting the incidence and severity of HCC. The methylation status of CHD5 can also be further studied as a prognostic factor in HCC.

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Introduction
Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world and the third leading cause of cancer-related death. The prevalence of this disease varies in different parts of the world, but is highly prevalent in parts of Asia and Africa because of weak health systems. However, in developed countries, HCC cases are becoming increasingly more frequent from hepatitis B (HBV) and C virus infections, alcohol use, and the spread of liver cirrhosis.1

With advanced understanding of the biology of tumors, the importance of cancer molecular biomarkers are becoming increasingly appreciated. Cancer is a multi-stage process involving alterations to many genes, such as oncogenes and tumor suppressor genes, and changes in mechanisms that control cell growth, cell proliferation, and the DNA repair process. It is necessary to study the epigenetic mechanisms that can regulate cancer-related gene expression and the effects of certain drugs on these processes.2 Switching between heterochromatin and euchromatin, DNA methylation, and histone modifications are proposed as epigenetic mechanisms that can regulate transcription factor access to target genes. Additionally, the interactions of non-coding RNAs, such as microRNAs (miRNAs), with target genes can impact cell growth, differentiation, and death rates. Thus, epigenetic factors can directly or indirectly alter the expression of genes in cells. Interfering with these mechanisms can lead to the activation or inhibition of various signaling pathways and the development of diseases such as cancer.3

Chromodomain helicase DNA-binding 5 (CHD5) is a member of a family of chromatin-transforming proteins called SWItch/Sucrose Non-Fermentable (Swi/Snf). These proteins are localized in the nucleus of eukaryotic cells and can control DNA packaging pathways and affect gene expression.4

Bagchi et al.5 performed mouse model experiments and found that CHD5 had specific tumor suppressor functions. Even a slight defect in the function of CHD5 could disrupt the P53 pathway, activating mechanisms that enhance the progression and spread of cancer. As a tumor suppressor, CHD5 possibly plays crucial roles in the regulation of gene expression and protein function.6

Studies on neuroblastoma have shown that deletion of the CHD5 locus on human chromosome 1p36.3 is commonly observed in this disease, suggesting its loss can contribute to the development of cancer.7 CHD5 expression levels are significantly reduced in cancer cell lines and primary neuroblastoma samples, although methylation of this gene may have a direct effect on its expression.8,9

Evidence supporting the role of CHD5 in human cancers has been obtained from genome-wide association studies (GWAS) on breast and colon cancer samples and DNA sequencing analysis.10 Destructive heterozygous mutations were observed in two of 24 patients with primary breast cancer and one of 11 tuberculosis samples.
This phenomenon is very common in breast and ovarian cancers.\textsuperscript{11,12} Therefore, these previous results provide a rationale for studying the role of CHD5 in a variety of cancers.\textsuperscript{13}

The methylation pattern of the CHD5 gene promoter region, CHD5 mRNA expression levels in HCC, and effect of this gene on HCC susceptibility are all not well understood. Therefore, the aim of the present study was to investigate these points by comparing HCC patients with healthy individuals.

**Materials and methods**

In this case–control study, 81 HCC patients were included and 90 healthy individuals were used as a control group. Patients referred to Namazi Hospital in Shiraz, Iran and Shaheed Labbafinezhad Hospital in Tehran, Iran from September 2015 to September 2021 were included. They were selected based on clinical findings and histopathological evaluations of liver tissue and liver ultrasounds, in accordance with WHO criteria. To participate in the study, healthy individuals were required to be in the same age range, have no family relationship with any patient, not smoke, and have no inflammatory disease, chronic disease, autoimmune disorder, or cancer. Written informed consent was obtained from all participants. The Institutional Ethics Committee of the Zahedan University of Medical Sciences (IR. ZAUMS.REC. 1399.240) approved this study. All procedures were in accordance with the ethical standards of the research committee and the Declaration of Helsinki. The required demographic information, such as age, tumor size, tumor grade status, and stage of the disease, was obtained from the patient’s medical records. Demographic characteristics of the patients and normal control group are summarized in Table 1. Each participant provided 5 mL of peripheral blood, which

| Parameter | C, N (%) | HCC, N (%) | P-value |
|-----------|----------|------------|---------|
| Age (years) | Mean age | Mean age |  \( P = 0.121 \) |
| | 53.21 ± 5.61 | 54.78 ± 8.21 | \( F = 1.522 \) |
| Age range | 37–69 | 30–72 |  \( P = 0.121 \) |
| Median | 53 | 56 | \( P = 0.121 \) |
| Sex | Male | 72 (80.0) | 68 (75.5) |  \( P = 0.621 \) |
| | Female | 18 (20.0) | 22 (24.5) | \( F = 0.503 \) |
| HCC differentiation: | – | – | \( P = 0.621 \) |
| | Well or moderately differentiated | 80 (88.8) | – | \( P = 0.621 \) |
| | Poorly differentiated | 10 (11.1) | – | \( P = 0.621 \) |
| HCC grading: | – | – | \( P = 0.621 \) |
| | Early | 83 (95.1) | – | \( P = 0.621 \) |
| | G1 | 5 (2.4) | – | \( P = 0.621 \) |
| | G2–G3 | 2 (2.4) | – | \( P = 0.621 \) |
| Total bilirubin (\( \mu \text{M} \)) | 16.54 ± 5.86 | 30.77 ± 10.30 | \( P < 0.001 \) |
| ALT (U/I) | 27.64 ± 9.14 | 103.00 ± 23.12 | \( P < 0.001 \) |
| AFP (ng/mL) | 2.87 ± 1.26 | 477.87 ± 93.54 | \( P < 0.001 \) |

ALT, alanine transaminase; AFP, alpha-fetoprotein.
was divided into two parts. A portion of the blood was transferred to a tube containing ethylenediaminetetraacetic acid (EDTA) used for DNA extraction. The other part of the blood was immediately transferred to a −80°C freezer for later RNA extraction. All molecular experiments were performed in the Infectious Diseases and Tropical Medicine Research Center, Zahedan, Iran.

**RNA extraction, cDNA synthesis, and quantitative real-time PCR analysis (qPCR)**

RNA was extracted from the peripheral blood samples using a Total RNA Extraction Mini Kit (Favorgen Biotech, Ping-Tung, Taiwan) according to the manufacturer’s instructions. The concentration and quality of RNA were respectively determined by using a Nanodrop Lightwave II spectrophotometer (Biochrom, Cambridge, UK) to measure the 260/260 absorbance ratio and by electrophoresis of the RNA product on a 0.8% agarose gel and evaluating the 18s and 28s ribosomal bands.

cDNA synthesis was performed according to the optimized instructions using a 2-Steps RT-PCR Kit (Vivantis, Shah Alam, Malaysia). The LightCycler ABI 7500 system (Applied Biosystems Inc., Foster City, CA, USA) was used to perform qPCR. *CHD5* gene-specific primers were designed using Oligo software (Molecular Biology Insights, Inc., Colorado Springs, CO, USA). The primers for the *CHD5* gene are Forward: 5′-TACAGGTTGTGG TGCATCAG-3′ and Reverse: 5′-CGCTG CTTGAGGAGTCAG-3′. *GAPDH* was used as an internal gene control; Forward: 5′-CATGAGAAGTATGACAACAGCC-3′ and Reverse: 5′-GGGGTGCTAAGCTTGTTGGTG-3′. A 20-μL solution containing cDNA, dNTPs, primers, and Taq polymerase was used to perform PCR. The cycling conditions were: initial denaturation step at 95°C for 5 minutes, then 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. All assays included negative control samples (reactions containing water in place of cDNA) to ensure that the solution was not contaminated by genomic DNA. *CHD5* gene expression data were normalized to *GAPDH* data. Melting curve analysis was used to determine the specificity of the PCR products. In addition, PCR products were loaded on 1.5% agarose gels to ensure the products were of the correct size and specificity. Primer specificity was investigated using standard curves generated using expression assays with different known concentrations of cDNA. Then, the 2-ΔΔCT method was used to determine the relative *CHD5* expression levels.

**DNA extraction, bisulfite treatment, and CHD5 gene methylation**

Genomic DNA was extracted from the peripheral blood samples of patients and healthy individuals. For this purpose, the salting-out method was used in accordance with our previous studies. The quantity and quality of the extracted DNA were examined using a spectrophotometer. Then, DNA samples with sufficient quality were bisulfite-treated. Treatment of target DNA with sodium bisulfite results in the conversion of unmethylated cytosines to uracil, while methylated cytosines remain unchanged. This change allows for different patterns of methylated and unmethylated forms to be studied. Briefly, 4μL of 2 M sodium hydroxide was added to the microtube containing 2μg of DNA and incubated at 50°C for 15 minutes. Then, 2% agarose was added and incubated for 15 minutes at 50°C. Cold mineral oil was added to produce the agarose granules. Fresh 5 M sodium bisulfite and
hydroquinone solutions were added to the agarose and incubated for 18 hours at 50°C. Finally, the agarose grains were washed with TE buffer, 0.2 M sodium hydroxide, and then TE again. The modified DNA was purified using an AccuPrep® Gel Purification Kit (Bioneer Corporation, Daejeon, Republic of Korea). For methylation-specific PCR (MS-PCR) primer design, the sequence of the genomic DNA treated with sodium bisulfite was considered as the target sequence.

After treatment with sodium bisulfite, all cytosines except CpG cytosines that are methylated are converted to uracil and finally to thymine. Relevant primer sequences: methylated CHD5 forward: 5'-GTTCGGGGTTAGGTTGTTTC-3' and reverse: 5'-GAAAACTTACGAACCCGAACG-3', with a 108 bp expected amplicon size; non-methylated CHD5 forward: 5'-GGTTTGGGGTTAGGTTTGGTGG-3' and reverse: 5'-GAAAACTTACAAACCCGAACCG-3', with a 110-bp expected amplicon size. These CHD5-specific methylated and non-methylated primer sets and bisulfite-treated DNA were used in MS-PCRs. These reactions were performed at a final volume of 20 μL and contained 1 μL of bisulfite-modified DNA, 0.5 μL of each primer (10 mM), 10 μL of 2X Prime Taq Premix (Genet Bio, Daejeon, Republic of Korea), and 8 μL ddH2O. The cycling conditions were: initial denaturation step at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 40 s, annealing for 40 s (at 58°C for methylated CHD5 (MCHD5) and 61°C for unmethylated CHD5 (UCHD5)), and extension at 72°C for 40 s, followed by a final extension step at 72°C for 10 minutes. After cycling was complete, the products were electrophoresed on a 1.5% agarose gel. Separate PCRs were performed for each pair of methylated and non-methylated primers. We uploaded all supporting data to our Github page (https://github.com/bitamoudi/bitamoudi.git).

The reporting of this study conforms to STROBE guidelines.19,20

**Statistical analysis**

Statistical analysis of the data was performed using SPSS software version 20 (IBM Corp., Armonk, NY, USA). P-values <0.05 were accepted as statistically significant. The Smirnov–Kolmogrov test was used to assess the normal or abnormal distribution of data. The chi-square test was used for statistical analysis of data related to CHD5 gene methylation status in experimental and control groups. To compare and analyze dependent variables that have more than two modes, Multinomial Logistic Regression was used.

**Results**

Demographic and clinical information are summarized in Table 1. Our study involved 90 HCC patients (68 men, 22 women; age: 54.78 ± 8.211 years) and 90 healthy individuals (72 men, 18 women; age: 53.21 ± 5.611 years). No significant differences concerning age and sex were observed between the groups (P > 0.05).

The promoter methylation status of the CHD5 gene was assessed by MS-PCR. As shown in Table 2, the distribution of methylation patterns was different in the two groups. There was a statistically significant hypermethylation of the CHD5 gene in 61.8% of the HCC patients and 54.4% of the controls. We computed the odds ratio (OR) and 95% confidence interval (CI) from logistic regression analysis to obtain the association between methylation status and HCC. The results suggest that hypermethylation of the CHD5 gene is significantly associated with HCC risk (OR = 0.418, 95% CI = 1.219–2.799, P = 0.008). Clinical features (age and sex) and HCC state (differentiation, grading, total bilirubin, alanine transaminase (ALT), and alpha-fetoprotein...
(AFP) were not correlated with the methylation status of the CHD5 gene ($P > 0.05$). Considering the impact of different covariates in the current study, we found no associations between CHD5 methylation status and patients’ age ($P = 0.342$), sex ($P = 0.641$), differentiation ($P = 0.279$), grading ($P = 0.511$), total bilirubin ($P = 0.087$), ALT ($P = 0.354$), or AFP ($P = 0.277$).

The CHD5 mRNA expression levels were evaluated by qPCR. CHD5 mRNA expression levels were significantly lower ($P < 0.001$) in HCC patients ($1.82 \pm 0.671$, Median = 1.60, 0.80–3.80 for control group; $0.83 \pm 0.405$, Median = 0.80, 0.20–1.80 for HCC patients). Clinical features (age and sex) and HCC state (differentiation, grading, total bilirubin, ALT, and AFP) were not correlated with CHD5 expression ($P > 0.05$). Considering the impact of different covariates in the current study, we found no associations between CHD5 expression and patients’ age ($P = 0.420$), sex ($P = 0.514$), differentiation ($P = 0.394$), grading ($P = 0.451$), total bilirubin ($P = 0.076$), ALT ($P = 0.452$), or AFP ($P = 0.741$).

### Discussion

In the present study, the methylation status of the CHD5 promoter region and its corresponding mRNA expression levels were evaluated in blood samples of HCC patients and compared with healthy individuals in an Iranian population. Using MS-PCR analysis, the methylation frequency of the CHD5 gene was found to be 61.8% in HCC patient samples and 54.4% in healthy samples. Individuals with CHD5 gene methylation were at higher risk of HCC than healthy people ($P = 0.008$). In these patients, CHD5 mRNA expression levels were significantly decreased relative to the control group.

Deletions in the 1p36 chromosomal region have been observed in many malignant tumors,7,21,22 and the tumor suppressor genes located in this region appear to be effectively lost or inactivated during cancer development and progression.5 The effects of deletions of the 8p, 13q, and 17p sequences in HCC have been investigated and their role in cancer incidence has been established. However, chromosomal defects in the 1p36 region are rare in liver cancer.23,24 This suggests that other mechanisms, such as epigenetic changes, are responsible for the reduced CHD5 gene expression in HCC patients. Hypermethylation of the CHD5 promoter region can lead to silencing of its expression, and this phenomenon has been identified in various cancers such as colorectal, breast, lung, and gastric cancers.25–28

Cancer cells are characterized by a large reduction in methylation, with up to a 5% to 6% reduction in total 5-methyl cytosine. However, the simultaneous acquisition of specific patterns of CpG island hypermethylation of specific gene promoters is widely observed. In general, hypermethylation mainly occurs in repetitive DNA sequences and promotes chromosomal instability and alterations.29 Hypomethylation of specific promoters can activate the expression of ectopic oncogenes. When this occurs in tumor cells, expression of the oncogene is increased and tumor growth is supported.30
Unlike overall hypomethylation, hypermethylation occurs in specific CpG islands. Transcriptional inactivation from promoter hypermethylation can affect genes involved in major cellular cancer-related pathways, including DNA repair, vitamin response, signaling, cell cycle control, and apoptosis pathways.31

Hypermethylated promoters can be considered as new biomarkers in cancer diagnosis and prognosis. Although the majority of CpG island studies focus on promoter regions, recent findings suggest that most ectopic methylation events in cancer occur in the margins of the CpG islands.32 Notably, most of the changes in the CpG island margins (45% to 65%) are related to areas that become hypermethylated during natural tissue differentiation.33

Human tumors are also characterized by decreased expression levels of certain miRNAs, which is often a result of hypermethylation of the miRNA promoter.34 Interestingly, the suppression of miRNA expression by hypermethylation is associated not only with cancer development, but also with its metastasis.35

Hypermethylation patterns are specific to the type of tumor, and it is not yet clear why certain areas become hypermethylated while other areas remain hypomethylated. One possibility is that inactivation of certain genes leads to an increased growth capability for that cell and ultimately a clonal selection. Another hypothesis is that aberrant methylation of CpG islands could be from the invocation of DNA methyltransferases to specific target genes by fusion proteins.36 Furthermore, it is possible that methylation is extended from the hypermethylated regions to the surrounding areas. Epigenetic extinction from DNA methylation has reportedly been able to cover regions up to 1 mb on a chromosome and imitate a state similar to heterozygosity loss, which is often seen in human tumors.37 Overall disruption of the DNA methylation pattern can also be from an impaired regulation of DNA methyltransferase expression. The expression levels of these enzymes are often increased in many tumor types. In addition, DNA methyltransferase expression can be regulated by miRNAs.38

Unlike genetic mutations, most epigenetic changes can be reversible or preventable. Therefore, restoring abnormal epigenetic events in neoplastic cells is a developing strategy for the treatment or prevention of cancer. Currently, it is possible to target the DNA methylation and histone acetylation and methylation processes. Pharmacological inhibition of DNA methylation works by targeting enzymes such as DNA methyltransferases, which leads to restoration of expression of genes that were silenced by ectopic hypermethylation. This simultaneously inhibits clonal proliferation and growth of tumor cells and induces their differentiation and death.39 How enzyme inhibitors specifically act on tumor cells is not well understood, but if these factors are associated with newly amplified DNA, then they will only target rapidly proliferating cells like tumor cells. Extensive clinical studies have shown that enzyme inhibitors have short-term, controllable side effects at appropriate doses.39,40

Appreciation of the important role of epigenetic dysregulation in the incidence and progression of cancer has increased in recent years. Disrupting certain epigenetic mechanisms can promote tumor growth by affecting and cooperating with carcinogenic mutations. Therefore, managing these abnormal epigenetic events to target the formation or progression of cancer has become a novel treatment strategy.41 The effectiveness of epigenetic therapies to treat various syndromes indicates the precedence of epigenetic disorders over the onset of cancer. Epigenetic therapy is therefore a promising way to prevent and treat malignancies. Interestingly, epigenetic therapy
can enhance the effectiveness of other anti-cancer therapies. Understanding the relationship between epigenetics and cancer has practical applications for evaluating prognosis and treatment plans. Many aspects of epigenetics remain unknown and numerous studies are underway to investigate other epigenetic mechanisms, their relationships with each other, and how they affect the occurrence and progression of various diseases such as cancer.

The present study has some limitations. The study population was relatively small, and future evaluation with more samples will help confirm our results. In this study, the methylation status of only one CpG island was investigated. In addition, increased methylation of the CHD5 promoter region was associated with decreased gene expression. Therefore, in future studies, we will examine CHD5 expression along with its methylation status in patients with different grades of cancer. In general, the results of this study indicate that methylation of the CHD5 promoter region may play a role in the expression of this gene and the incidence and severity of HCC. Methylation of this gene can also be a prognostic factor in HCC. In future studies, the number and variety of liver cancer tumor samples will be increased in terms of patient location, pathological features, and age. Examination of tumor tissue samples is also recommended. Additionally, the clinical response of patients with CHD5 hypermethylation to drug therapies should be evaluated and compared with the control population.

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Author contributions

BM and AAR collected the data, performed the genetic assays and statistical analyses, interpreted the results, and drafted the manuscript. ZH and HMS co-designed the study and supervised the experimental design. All authors read, modified, and approved the final version of the manuscript.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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