

# Analysis of cytotoxic T-lymphocyte-associated antigen-4 and MMP-9 genes' methylation and their expression profiles with risk of non-alcoholic fatty liver disease

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**OBJECTIVE:** To investigate the effect of promoter methylation of cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) gene and matrix metalloproteinases (MMPs) on the risk of non-alcoholic fatty liver disease (NAFLD).

**MATERIALS AND METHODS:** CTLA-4 and MMP-9 promoter methylation were investigated using a methylation-specific polymerase chain reaction (MS-PCR) in blood samples taken from 80 NAFLD individuals and 95 healthy controls. The expression levels of CTLA-4 and MMP-9 were also assessed in 10 blood and 9 liver tissues mRNA samples from NAFLD patients. These cases were compared to the blood ( $n = 10$ ) samples of healthy controls with real-time quantitative reverse transcriptase PCR.

**RESULTS:** No significant relationship was found for methylation of CTLA-4 and MMP-9 between cases and controls. The relative expression of CTLA-4 and MMP-9 mRNA in NAFLD was not significantly different compared to healthy control samples.

**CONCLUSION:** For the first time, our outcomes indicate that the methylation status of CTLA-4 and MMP-9 genes has no significant function on the process of NAFLD.

**Key words:** Cytotoxic T-lymphocyte-associated antigen-4, expression, gene, methylation, matrix metalloproteinases-9, non-alcoholic fatty liver disease

## Introduction

Non-alcoholic fatty liver disease (NAFLD) is a common cause of chronic liver disease worldwide.<sup>[1]</sup> It also has been found to be a significant risk factor for expansion of primary liver cancer and liver-associated mortality and morbidity.<sup>[2,3]</sup> NAFLD refers to a spectrum of histological findings, ranging from simple and reversible steatosis to steatohepatitis and cirrhosis, and is diagnosed after ruling out other causes-in particular, alcoholic liver disease (ALD).<sup>[4]</sup> In addition to a higher prevalence of NAFLD in patients with obesity, metabolic syndrome, and type 2 diabetes, it also can be induced by a variety of genetic variations.<sup>[5]</sup> However, the data is sparser regarding genetic and epigenetic variations on the etiology of NAFLD. Understanding these types of alterations would have a critical effect on the clinical practice and management of disease.<sup>[6]</sup> Matrix metalloproteinases (MMPs) are a family of proteases with roles in the development and invasion of various cancers, including degrading components of the extracellular matrix, which paves the way for the transportation of tumor cells to other tissues.<sup>[7]</sup> The MMP-9 gene is placed at chromosomal location 20q13.2, and its exact expression mechanisms are unknown.<sup>[8]</sup> A few studies have evaluated the involvement of these genetic variations in development of chronic liver disease.<sup>[9]</sup>

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Cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) is a single-spanning membrane protein, the gene for which is located on chromosome 2q33.<sup>[10,11]</sup> This protein is positioned on a T-cell surface molecule which interacts computationally with the co-stimulatory molecule CD28 and plays a major role in peripheral control of the immune response.<sup>[12-14]</sup> The functional magnitude of this molecule is characterized in CTLA-4-deficient mice, which develop lethal self-reactive lymphoproliferative disease.<sup>[15]</sup> It seems likely that the decreased expression of CTLA-4 might cause autoimmune T-cell clonal proliferation.<sup>[16]</sup> Several studies have shown that the dysfunction of CTLA-4 is connected to autoimmune disorders.<sup>[15,17,18]</sup>

This study analyzed the link between the promoter hypermethylation of MMP-9 and CTLA-4 genes and their expression in blood samples of patients with NAFLD disease in a group of patients in South Eastern Iran.

## Materials and Methods

### Study subjects and specimens

This case-control study was performed on 80 patients with confirmed NAFLD and 95 healthy subjects. Samples were collected in the Ali-Ebn-Abi Taleb hospital from 2008 to 2010. Exclusion criteria were: Patients with other known causes of liver disease, including viral hepatitis B and C; hemochromatosis; Wilson disease; autoimmune liver diseases; a history of alcohol consumption of more than 100 g/week; and chronic drug use. Individuals who were overweight or (defined as a body mass index [BMI] >25 kg/m<sup>2</sup>) had type 2 diabetes or hyperlipemia and an abnormal liver function test participated in the study. Laboratory assays encompassed fasting glucose, insulin, total cholesterol, high density lipoprotein-cholesterol, low density lipoprotein-cholesterol, triglycerides, iron, TIBC, ferritin, ceruloplasmin, alanine aminotransferase, aspartate aminotransferase,  $\gamma$ -glutamyltransferase, alkaline phosphatase, bilirubin, HBS-Ag, HBc-Ab, LKM1 antibody, HCV antibody, antismooth muscle antibody and antimitochondrial antibody, and antinuclear antibody, collected after a 12-h overnight fast. Hepatic ultrasonography scanning was performed in all participants by an experienced radiologist who was

blinded to participants' details. The diagnosis of NASFLD was performed according to the clinical setting, sonographic, and laboratory findings, because the patients did not agree to undergo liver biopsy. Normal subjects were selected from the Zahedan population who participated in the metabolic syndrome project and had normal blood pressure, normal lipid profiles, normal blood glucose, normal BMIs, normal waist circumference, and no history of systematic disease. Demographic and clinical data on cases and controls are shown in Table 1. The lab work for the analysis of gene methylation was done in parallel for cases and controls.

### DNA extraction and methylation analysis

DNA was extracted from whole blood using the phenol-chloroform extraction method; then, 2  $\mu$ g of purified DNA were converted using sodium bisulfite as previously described.<sup>[19]</sup>

### Methylation-specific polymerase chain reaction

Variations in sequences of DNA after treatment by sodium bisulfate were identified by Methylation-specific PCR (MSP). The primer sequence and PCR conditions are listed in Table 2. Each MSP reaction included: 80 ng of bisulphate-converted DNA, 1  $\mu$ M of each primer, and 2U Hot Start Taq (Cat, No: #EP0602, Fermentase). Finally, PCR products were analyzed by electrophoresis on 3% agarose gel stained with ethidium bromide. Positive controls (*in vitro* methylated and bisulfite-treated human placental DNA) and negative controls (no template) were included in all reactions.

**Table 1: Demographic and clinical data on cases and controls**

Variables	Controls N=95	Cases N=80	P value
Age (year)	37.85 $\pm$ 14.77	40.82 $\pm$ 11.74	0.144
Weight (kg)	65.60 $\pm$ 15.14	82.29 $\pm$ 11.89	<0.001
Height (cm)	161.29 $\pm$ 9.39	165.79 $\pm$ 8.83	0.003
BMI (kg/m <sup>2</sup> )	25.22 $\pm$ 5.25	30.01 $\pm$ 4.21	<0.001
AST (IU/L)	20.89 $\pm$ 3.48	47.69 $\pm$ 28.61	<0.001
ALT (IU/L)	19.40 $\pm$ 2.52	71.60 $\pm$ 47.34	<0.001
FBG (mg/dL)	88.58 $\pm$ 20.06	110.24 $\pm$ 37.71	<0.001
TG (mg/dL)	167.11 $\pm$ 131.06	207.22 $\pm$ 119.80	0.051
Total chol (mg/dL)	178.50 $\pm$ 37.02	200.82 $\pm$ 38.20	<0.001
HDL-C (mg/dL)	45.02 $\pm$ 6.80	43.24 $\pm$ 8.42	0.156
LDL-C (mg/dL)	99.27 $\pm$ 30.90	108.73 $\pm$ 26.92	0.046

BMI: Body mass index, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, HDL: High density lipoprotein, LDL: Low density lipoprotein, FBG: Fast blood glucose, TG: Triglyceride, Chol: Cholesterol

**Table 2: Primers used for methylation and expression analysis**

Genes	Sequences	Annealing temperature
CTLA-4 M	F: GAGATTAGTTTGGTTAATATGGCGA R: CCAAATTAATAACAATAACGCGAT	60°C
CTLA-4 U	F: GAGATTAGTTTGGTTAATATGGTGA R: CCCAAATTAATAACAATAACACAAT	59°C
MMP-9 M	F: TGGGTAATTTAGTGTTAAAGGAATC R: AAAATTACATACGTAAACCACCGTA	63°C
MMP-9 U	F: GTGGGTAATTTAGTGTTAAAGGAATTG R: AAAATTACATACATAAACCACCATA	65°C
RNA 18s (real time-PCR)	F: GTAACCCGTTGAACCCCAT R: CCATCCAATCGGTAGTAGCG	60°C
CTLA-4 (real time-PCR)	F: CACAAGGCTCAGCTGAACCT R: AGGTGCCCGTGCAGATGGAA	60°C
MMP-9 (real time-PCR)	F: GTGCTGGGCTGCTGCTTTGCTG R: GTCGCCCTCAAAGGTTTGAAT	60°C

CTLA-4: Cytotoxic T-lymphocyte-associated antigen-4, MMP-9: Matrix metalloproteinases-9, PCR: Polymerase chain reaction

### Extraction of RNA and reverse transcription

Overall RNA from blood and tissues was extracted using the High Pure RNA Isolation Kit (Cat No: 11828665001 Qiagen, Hilden, Germany and Cat No: 04823125001, High Pure FFPE). The RNA concentration was identified spectrophotometrically, and the integrity of all samples was confirmed by electrophoresis in ethidium bromide-stained 1% agarose gel. First-strand cDNA was synthesized from 1 µg of total RNA using the First Strand cDNA Synthesis Kit (Cat No: K1611, Fermentase) according to the manufacturer's instructions.

### Quantitative real-time polymerase chain reaction with SYBR green

Real-time polymerase chain reaction (RT-PCR) was performed in order to set up a quantitative association between PCR products obtained from the target gene and a housekeeping gene (RNA 18s). Quantitative RT-PCR assays were performed with the RT-PCR System (7300, Applied Biosystems) using SYBR green fluorescence. PCR amplification was done in 20 µL of the reaction mixture containing 3 µL of cDNA, 10 µL SYBR green, 2 µL of both primers (forward and reverse), and 5 µL of H<sub>2</sub>O. The sequences of the primers used for this purpose can be found in Table 2.

### Statistical analysis

Analysis of data was based on the multivariate logistic regression analysis for estimation of methylation status in groups, and the Mann-Whitney test was used for examination of gene expression data. The level of significance was set at  $P < 0.05$ .

### Results

The outcomes of the MMP-9 and CTLA-4 genes' methylation between cases and controls are given in Tables 3 and 4. As indicated, the CTLA-4 gene was 85.0% methylated in NAFLD patients and 85.26% methylated in healthy individuals. Statistically, this variation is not significant between healthy controls and patients. A similar statistical correlation has been found for the MMP-9 gene, which was 85.26% methylated in normal individuals and 88.75% methylated in unhealthy individuals.

### CTLA-4 and MMP-9 gene expression

The differences between CTLA-4 and MMP-9 mRNA levels were assayed in 10 NAFLD (10 blood and 9 liver tissues) and 10 normal blood samples. As shown in Tables 5 and 6, there was no statistically significant assessment for relative gene expression in a comparison between cases and controls for the CTLA-4 and MMP-9 genes. Further studies utilizing larger sample sizes are necessary to achieve a conclusion regarding the effect of altered gene expression of CTLA-4 and MMP-9 genes in NAFLD.

### Discussion

NAFLD has become the leading cause of referral to hepatology clinics and is a major future research field in internal medicine. Data from interethnic differences and family studies have shown that genetic factors may have a critical function in the causation of NAFLD.<sup>[20]</sup> However,

**Table 3: Promoter methylation frequency of CTLA-4 and MMP-9 genes in patients with NAFLD and healthy controls**

Gene	Methylation status <sup>a</sup>	Controls N=95 (%)	Cases N=80 (%)	<sup>a</sup> P value
CTLA-4	M	81 (85.26)	68 (85)	0.986
	U	14 (14.74)	12 (15)	
MMP-9	M	81 (85.26)	71 (88.75)	0.573
	U	14 (14.74)	9 (11.21)	

M: Methylation, U: Unmethylation, <sup>a</sup>Variable were compared by the Fisher's exact test, NAFLD: Non-alcoholic fatty liver disease, CI: Confidence interval, OR: Odds ratio

**Table 5: Comparison of gene expression for CTLA-4 and MMP-9 between patients with NAFLD and healthy controls in the blood sample**

Gene	Samples	No.	Mean±SD	P value (Mann-Whitney test)
CTLA-4	Cases	10	0.103361±0.315171	0.44
	Controls	10	0.024059±0.041131	
MMP-9	Cases	10	0.199711±0.414876	0.15
	Controls	10	0.002419±0.005157	

CTLA-4: Cytotoxic T-lymphocyte-associated antigen-4, MMP-9: Matrix metalloproteinases-9, NAFLD: Non-alcoholic fatty liver disease

data regarding gene association studies for patients with NAFLD is sparse. Determining genetic and epigenetic risk factors that predict the level of fibrosis progression or susceptibility to viral agents may be useful for better management of patients in an individual way.<sup>[20]</sup> Evidence for the involvement of genetic factors in the development of advanced fibrosis in NAFLD has been extracted from family clustering studies.<sup>[21]</sup> Day *et al.* have reported that genetic susceptibility plays a key role in alcohol-induced liver disease.<sup>[22]</sup> In eukaryotes, gene expression is controlled by many mechanisms; DNA methylation is a general epigenetic signaling tool which cells utilize to lock genes in the "off" position.<sup>[23]</sup> The majority of DNA methyl regions were located on promoter sites, which show various patterns of expression, depending on the amount of methylated sites.<sup>[24]</sup> The finding of methylated DNA sequences in serum has been recommended as a potentially valuable biomarker for preclinical diagnosis of malignant lesions.<sup>[25]</sup> In fact, numerous studies of hepatocellular carcinoma have revealed that methylated p15, p16, and RASSF1A sequences are present in serum at the time of cancer diagnosis.<sup>[24,26,27]</sup> CTLA-4 encodes a T-cell receptor that mediates T-cell apoptosis, which has a significant effect on genetic susceptibility to autoimmune diseases, such as type 1 diabetes.<sup>[25,28]</sup> Xia Zhao *et al.*<sup>[29]</sup> have suggested that the susceptibility to variants of the CTLA-4 gene vary between the different geographic populations with Graves'

**Table 4: Risk of NAFLD based on gene promoter methylation**

Genes		Unadjusted			Adjusted <sup>b</sup>		
		OR	95%CI	P value	OR	95%CI	P value
CTLA-4	U(ref)	1.0	0.43-2.3	0.99	0.93	0.39-2.23	0.88
	M						
MMP-9	U(ref)	0.73	0.29-1.8	0.49	0.70	0.27-1.80	0.46
	M						

<sup>a</sup>Binary logistic regression analysis, <sup>b</sup>Adjusted by age and sex, OR: Odd ratio, 95%CI: 95% confidence Interval, ref: reference

**Table 6: Comparison of gene expression for CTLA-4 and MMP-9 between liver tissues of patients with NAFLD and bloods of healthy controls**

Genes	Samples	No.	Mean±SD	P value (Mann-Whitney test)
CTLA-4	Cases	9	1.995405±4.350444	0.169
	Controls	10	0.024059±0.041113	
MMP-9	Cases	9	0.200552±0.357798	0.097
	Controls	10	0.002419±0.005157	

CTLA-4: Cytotoxic T-lymphocyte-associated antigen-4, MMP-9: Matrix metalloproteinases-9, NAFLD: Non-alcoholic fatty liver disease

disease.<sup>[30]</sup> Increased levels of plasma MMP-9 have been found in a variety of malignant cancers, such as colon cancer, lung cancer, breast cancer, and hepatocellular carcinoma.<sup>[31-34]</sup> Matsumura *et al.* indicated that the T-allele in the MMP-9 promoter is associated with the invasive phenotype of gastric cancer.<sup>[35]</sup> Certain other studies suggested that plasma MMP-9 may play an important role in the progression of breast cancer.<sup>[36,37]</sup>

## Conclusion

Our study provides data that promoter methylation of CTLA-4 and MMP-9 have no significant effect on the etiology of NAFLD. Still, data from gene involvement studies in patients with NAFLD are uncertain. Most available results were performed in small cohorts of patients carrying the risk factors for the disease. The present report needs to be confirmed by a subsequent larger sample size and a number of other candidate genes that have been involved in NAFLD.

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