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Association between the organic cation transporter 3 methylation and hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) is one of the deadliest cancers in the world and has a high death rate in the world. This research while examining the expression of OCT3 at the mRNA level has also studied gene methylation profile in patients with HCC in comparison with people without HCC. The volunteers were: patients with HCC (n=81) and a healthy control group (n=90). The expression of OCT3was studied using the qRT-PCR method. The methylation profile was evaluated by genomic DNA using methylation specific PCR (MSP) method. The expression level of OCT3 marker mRNA in patients has decreased significantly compared to healthy individuals (0.58 ± 0.311 *vs* 1.20 ± 0.355, P <0.001). No significant statistical relationship was found between demographic data and OCT3 expression in participants (P >0.05). The amount of methylation (UM + MM) in cancer patients has raised *vs* controls (P <0.001) and has increased the risk of cancer (OR=0.379, 95% CI=1.171-2.839, P <0.001, and OR=2.727, 95% CI=1.251-5.945, P <0.001, respectively).Changes in OCT3 levels appear to be associated with HCC. Also, changing the methylation pattern of this gene can reveal HCC pathology.

Keywords: Hepatocellular carcinoma, Methylation, Organic cation transporters 3, Quantitative real-time PCR

Approximately 85% of liver cancers occur in less developed and developing countries, especially in East and Southeast Asia and Central and West Africa[1,2]. The precise prevalence of liver cancer in Iran is still unknown. Iran is located in a region with a relatively high risk of developing HCC and less than 5 people per 1,000,000 people die of this cancer annually[3,4].

Although in some patients with HCC, surgery can lead to long-term control in the early stages after diagnosis, the recurrence rate is still high (about 50% in 3 years)[5]. In addition, due to the asymptomatic nature of HCC in the early stages, lack of awareness, and poor screening strategies, about 80% of these patients usually encounter the advanced or irreversible disease. Therefore, the detection and validation of specific biomarkers for HCC are essential, and it should be noted that these markers should greatly reduce false-negative results and thus prevent misdiagnosis[6,7].

DNA methylation is the most common change in epigenetics, including changes in histones, acetylation, and deacetylation[8,9]. DNA methylation occurs during an enzymatic reaction after DNA replication. If the rhythm and rate of methylation change, many diseases will spread, including cancers[10,11]. Methylation inhibits gene expression by two separate mechanisms: in the first case, transcription of the methylated chromosome is inhibited, and in the second case, two types of proteins, including methylation-dependent proteins and histone deacetylases, act on the chromosome surface[10]. Recent studies suggest that an abnormal pattern of methylation is effective in the tumorigenesis of cells[12].

Organic cation transporters (OCTs) are effective factors in the cell membranethat play a vital role in the metabolism and elimination of toxins. The members of this family include OCT1 to 3, each of them is sensitive to specific substrates and has a different expression pattern in tissues.In humans, compared to OCT1 and OCT2,OCT3 has been detected in many tissues (placenta, heart, liver, and skeletal muscle). Actually, OCTs are present in many metabolic pathways and enhance each other's effects[13,14].

Many cancer cell lines and tumors express OCT.In this regard, Heise and Lautem[15,16] in two separate studies showed that the expression of OCT1 and OCT3 is reduced in patients with liver failure which is

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significantly associated with cancer and patient life expectancy. The expression process of OCTs is regulated by complex mechanisms[17] and although several studies have been performed to identify these mechanisms, the exact pathway to control the function of OCTs has not yet been identified. It seems that OCTs alone can be a vital cause of cancer. Given that OCT3 is an effective membrane protein, it can be said that genetic and epigenetic changes will affect the expression and function of OCT3. Therefore, considering the importance of HCC in the world and the mortality rate of this cancer and due to the lack of screening programs and early diagnosis of HCC, increased incidence of this cancer, and low survival of patients, we intend to investigate the possible effects of OCT3 expression and methylation pattern changes in liver cancer and consider its role in cancer diagnosis and prognosis.

Materials and Methods

HCC people who had a tumor smaller than 5 cm and did not undergo vascular invasion were selected as the patient group and compared with healthy people (they were selected from referral hospitals in Iran between September 2015 to September 2021). Exclusion criteria: smokers, pregnant women, patients with diabetes (type 1 or 2), heart and kidney failures, infectious diseases, patients with any autoimmune disease, and cancerous lesions. People who were completely healthy and volunteered to donate liver tissue were selected as the control group. In all patients, the presence of HCC was assessed by: histopathological evaluations, biochemical tests related to liver damage, hepatic synthesis index, cholestasis index, liver ultrasound, and nuclear magnetic resonance imaging (NMR), in accordance with WHO criteria. Clinical and diagnostic informations of all study participants were gathered from their medical files. Written consent was obtained from all participants according to the ethics committee of the ZAUMS (IR.ZAUMS.REC. 1399.240).

Total RNA extraction mini kit, Favorgen Biotech was used to extract total RNA according to the manufacturer's instructions. In summary, 1 mL of red blood cells lysis buffer was added to 200 µL of anticoagulant-preserved fresh whole blood and incubated on ice for 10 min. After Centrifuge, 350 µL of FARB Buffer and 3.5 µL of β-Mercaptoethanol were added to the cell pellet and the mixture was centrifuged at 18000 × g for 2 min. 1 volume of 70% RNase-free ethanol was added to the supernatant and transferred to a FARB Mini Column and centrifuged at 18000 × g for 1 min. DNase I was added to the digestion genomic DNA contamination and washed the column with Wash Buffer 1 and 2. The column was centrifuged at full speed for 3 min to dry the column and added 50 µL of RNase-free ddH2O finally, column was centrifuged at full speed for 1 min to elute RNA. USA - Perkin Elmer xenon flash Tube Model 550 - s was used to measuring the quality of extracted RNA. The light absorption ratio was measured at 260.280 and the samples that had the best purity (260.280 OD> 1.8) were kept at –80°C.

OCT3 mRNA expression was assessed with the qRT-PCR method. 2-Steps RT-PCR Kit, RTPL 12, Vivantis was used for cDNA synthesis. In the first step, a solution containing RNA, Oligo d(T)18, and DNTP was prepared and incubated for 5 min at 65°C and for 2 min at –20°C. After that, a solution containing B.M-MulV and M-MulV RT was prepared and added to the first and incubated for 60 min at 42°C and then 5 min at 85°C.

OCT3 RNA expression was assessed by LightCycler ABI 7500 system (Applied Biosystems Inc., Foster City, CA) using a solution containing cDNA, DNTP, primers, and Taq. Specific primers were designed by real-time method using Oligo software, F5′- GCTGTACAATCTG AGGACTTG -3′ and R5′- AATCTGTGGCTCTAGG TGTC-′ for OCT3 and F5′- CATGAGAAGTAT GACAACAGCC -3′ and R5′- GGGGTGCTA AGCAGTTGGTG -3′ for Glyceraldehyde 3-phosphate dehydrogenase as the internal control. The PCR reaction was repeated twice for each sample. Relative copy number was determined using the 2-ΔΔCT method[18] and the gene expression compared to the control.

Genomic DNA extraction was performed using the 500 µL of peripheral blood leukocytes and the manual method, according to what the authors have given in previous studies[19-21]. Methylation specific PCR (MSP) method was used to study methylation and Sodium bisulfite modification was done manually in accordance with our previous studies[22]. The PCR conditions were as follows: initial denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 40 sec, annealing at 56°C for methylated OCT3 (MOCT3), 58°C for unmethylated OCT3 (UOCT3) for 40 sec and extension at 72°C for 40 sec, final extension step at 72°C for 10 min. Specific primers were designed by MSP using Oligo software, F5′-CGGGTAT AGGTATCGGATTC -3′ and R5′- CGCAACCTCCG ACCTAACG-3′ for MOCT3 (208bp) and F5′-

TGGGTATAGGTATTGGATTTGG -3′ and R5′-ACACAACCTCCAACCTAACAC -3′ for UOCT3 (209 bp).

The Chi-square test was used to compare the frequency of methylation and unmethylation gene. T-test was used to compare quantitative variables. To prove the data adherence to the normal distribution, the Kolmogorov-Smirnov one sample test was performed. The Tukey's posthoc test was used to compare the means and differences of the experimental groups. To compare and analyze dependent variables that had more than two modes, the Multinominal Logistic Regression model was used. SPSS software version 20 was used to analyze all data and perform statistical tests. Differences were assumed to be significant when $P \leq 0.05$.

Results

In this case-control study, 81 HCC patients (Male=68, Female=22) and 90 healthy individuals (Male=72, Female=18) were included. The mean ages of patients and healthy individuals were 54.78 ± 8.211 (Age range: 30-72) and 53.21 ± 5.611 (Age range: 37-69), respectively. There was no statistically significant difference in terms of age and gender between the two groups. The clinical characteristics of people with cancer are summarized in (Table 1). Total bilirubin, ALT and AFP concentrations were different between the two groups and there were significant differences (P <0.001), (Table 1).

OCT3 expression was assessed by real-time technique (Fig. 1).The mean OCT3 expression in patients with HCC was 0.58 ± 0.311(Median=0.50,

Table 1 — Demographic and clinical data of control (C) and hepatocellular carcinoma (HCC) groups

Parameters	C, N (%)	HCC, N (%)	P-value
Age (years)	Mean age 53.21 ± 5.611 Age range 37-69 Median 53 years	Mean age 54.78 ± 8.211 Age range 30-72 Median 56 years	P=0.121 F=1.522
Sex			P=0.621
Male	72(80.0)	68(75.5)	F=0.503
Female	18(20.0)	22(24.5)	
Hepatocellular Carcinoma:	-		-
Well or moderately differentiated		80(88.8)	
Poorly differentiated		10(11.1)	
HCC grading:	-		-
Early		83(95.1)	
G1		5(2.4)	
G2-G3		2(2.4)	
Total bilirubin (μ mol/l)	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

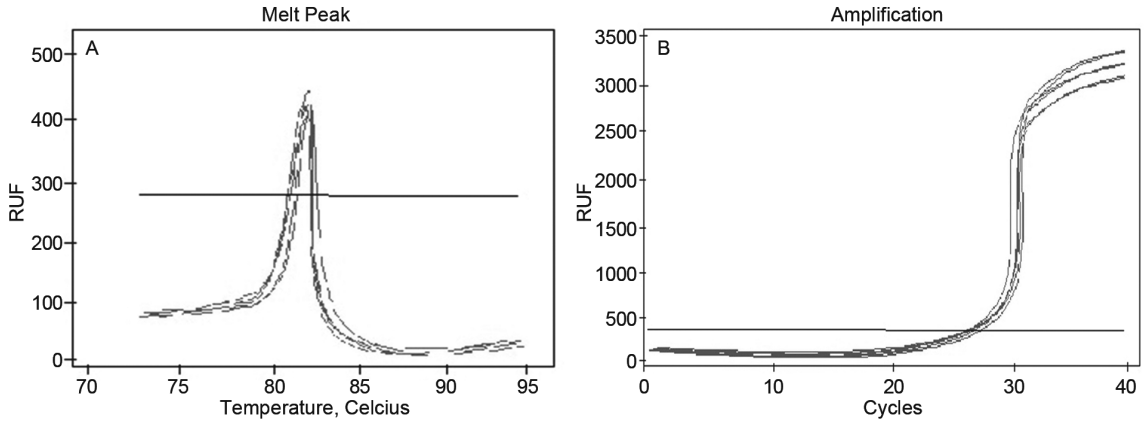

Fig. 1 — Melting curve (A) and amplification curve; and (B) from a qRT-PCR assay of OCT3. qRT-PCR, quantitative reverse transcription-polymerase chain reaction

Table 2 — Promoter DNA Methylation of the OCT3 Gene in HCC and Controls

Methylation status	HCC n (%)	Controls n (%)	OR (95%CI)	P
UU	11 (13.6%)	27 (30%)	1	
UM	57 (70.4%)	53 (58.9%)	0.379 (1.171-2.839)	*P* <0.001
MM	13 (16%)	10 (11.1%)	0.313 (0.106-1.925)	P=0.683
UM+ MM	70 (86.4%)	63 (70%)	2.727 (1.251-5.945)	*P* <0.001

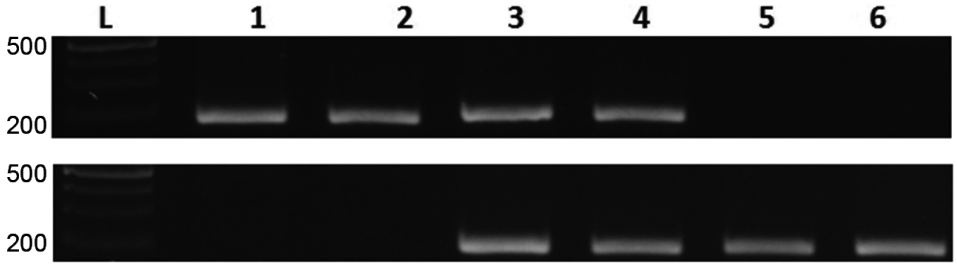

Fig. 2 — Different methylation patterns of the OCT3 in cases and controls. Lanes 1, 2, methylated/methylated status; Lanes 3 and 4, methylated/unmethylated status; Lanes 5, 6, unmethylated/unmethylated status and L, Ladder

0.20-1.30) and in healthy individuals was 1.20 ± 0.355 (Median=1.23, 0.60-2.10) and there was a statistically significant difference (*P* <0.001). No significant relationship was observed between OCT3 expression level and clinical and demographic characteristics (*P* >0.05).

The frequency of different states of OCT3 gene methylation (complete methylation=MM, relative methylation=UM, unmethylation=UU) are shown in (Table 2 and Fig. 2). The frequency distribution of different methylation patterns between the two groups was statistically significant (X^2 =6.819, P=0.033). The relative methylation, as well ascomplete methylation (UM+ MM), significantly increased the risk of HCC in comparison with unmethylation (OR=0.379, 95% CI=1.171-2.839**,** *P* <0.001, and OR=2.727, 95% CI=1.251-5.945, *P* <0.001, respectively). There was not any significant relationship between the groups concerning the frequency of different states of OCT3 gene methylation and HCC differentiation and grading (*P* >0.05).

Discussion

In this study, it was found that OCT3 mRNA expression in HCC patients is reduced compared to healthy people which is similar to Li *et al*.[23], Vollmar *et al*[24], Heise and Lautem *et al*[25,15] studies. Our findings showed that the frequency of methylation in patients with HCC was lower than in healthy people which is consistent with the Chen *et al* study[26].

Abnormal patterns in DNA methylation are commonly seen in many diseases[7]. When abnormal methylation occurs in DNA, four conditions are likely to occur: inactivation of mismatch repair in nucleotides, chromosome instability, hypomethylation of cancer genes, and hypermethylation of tumor-inhibiting genes. CpG islets are usually located in the promoter regions, and if the methylation pattern is normal, transcription factors are easily attached to the transcription starting site and gene expression occurs naturally. With increasing methylation in these areas, the expression of the desired gene decreases and the gene may even be completely silenced. Reducing methylation in the regulatory regions of oncogenes increases their expression and thus leads cells to the tumor. This mechanism is involved in the spread of cancer cells by activating oncogenes involved in cell growth and survival, apoptosis, and cell cycle[27].

Studies have shown that OCT1 activity is associated with the action of tyrosine kinase (TK1) inhibitors and leads to increased TK1 sensitivity in patients with chronic leukemia cancer[28]. The expression of SLC22A1 may also affect how it responds to therapies containing sorafenib[29]. Grimm *et al*. showed that in patients receiving a sorafenib diet, the expression of OCT1 mRNA increases, and this phenomenon can be used as a positive and significant prognostic factor in patients[30]. Although these studies show the role of OCT1 in HCC, the role of OCT3 is not well understood. In these studies, the effective function of OCT1 in HCC disease has been confirmed.

SLC22A3 is a gene encoding the OCT3 membrane protein weighing 62 kDa, and recent

studies have shown that SLC22A3 plays a vital role in the pathogenesis of heart disease[25]. To assess the expression of OCT3 in hepatocarcinogenesis, Vollmar *et al*. conducted an *in vivo* study on Oct3-knockout mice and they found that deletion of OCT3 could lead to the development of malignant lesions and hepatocarcinogenesis[24]. Heise and Lautem in two separate studies showed that the expression of OCT1 and 3 is reduced in patients with liver failures, and this decrease in expression is significantly associated with tumor progression and patient survival[25,15]. Studies show that Oct3 - / - mice are highly susceptible to the development and progression of liver tumors, and confirm that decreased expression of OCT proteins in people with HCC may be associated with the progression of the lesion to a malignant tumor[15]. By studying the HepG2 liver cancer cell line, it was found that the expression of SLC22A3 is directly related to the carcinogenicity of polycyclic and aromatic hydrocarbon compounds (PHA)[31]. In a study conducted in Asia, genetic mutations in the SLC22A3 gene have been reported as a risk factor for distal colon cancer[32]. Chen[33] studies indicate destructive changes in the metabolism of cells that lack OCT1, and Fu[34] has shown that the OCT3 complex affects the mechanisms involved in the development of esophageal cancer metastasis. Nies[33] reports that the OCT3 protein is predominantly expressed in the basement membrane of hepatocytes, and in patients with hepatic cholestasis, its expression is reduced at the mRNA level.In a study conducted in America, Chen found that genetic and epigenetic changes in the promoter region could affect OCT3 expression. The abundance of specific haplotypes in African-American ethnic groups was associated with increased expression of this protein. They showed that OCT3 mRNA expression was higher in the liver tissue of Asians and Caucasians with the homozygous g.-2A/A allele than in other ethnic groups. Methylation of the promoter region of this gene was also closely related to the expression of OCT3 protein and tumorigenicity in various prostate cancer cell lines[26].

Our findings, like some other reports[24,23], showed a significant relationship between OCT3expression and HCC susceptibility. The OCT3 methylation profile showed that methylation frequency is probably associated with the HCC riskand people with at least one methyl allele are more likely to develop cancer.One of the limitations of this research is the small sample size, which seems that if a larger sample size is used, the obtained results will be more reliable.

Conclusion

Given the role of genetic and epigenetic changes in gene and protein function, the role of changes such as methylation of the OCT3 gene and expression of this gene, which has been identified as a membrane transporter in many tissues, cannot be ignored. Comparing the allele frequencies of OCT3 methylation variant in our findings and other studies confirm that thisepigenetic variant is a highly effective change in cancer progression. Moreover, OCT3 can also be improved as a reliable marker in early detection of HCC.

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Conflicts of interest

All authors declare no conflicts of interest.

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