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Role of MT-1B gene polymorphism in HCC-HCV Egyptian patients

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Abstract: Background: Metallothioneins (MTs) are a group of metal-binding proteins with a high cysteine content and low molecular weight. MTs have an important role in metal metabolism and protect cells against the toxic effects of radiation, alkylating agents and oxygen free radicals. MT1B polymorphism rs964372 is located in the MT1B intron 1 (C>G) this SNP has great association with many diseases. The frequency of HCC powerfully correlates with liver inflammation like exposure to one or several risk factors including hepatitis B virus (HBV), hepatitis C virus (HCV), inherited metabolic diseases, heavy alcohol abuse, obesity, type 2 diabetes and aflatoxins. As oxidative stress drives genomic damage and genetic instability which cause mutations, and mutations have a crucial role in carcinogeneisis. **Methods:** 78 HCC patients, 53 HCV infected patients and 61 matched healthy controls were recruited. rs964372 genotypes of MT-1B were assayed with RT-PCR genotyping assays. **Results:** We found insignificant increase in CG genotype in HCC group (24.4%) compared to control group (23.5 %) and HCV group (22.2%) with higher incidence of C allele in HCV and HCC patients (85.7% and 84% respectively) in comparison to control group (82.4%). **Conclusions:** rs964372 of MT-1B gene polymorphism is neither related with HCV infection nor with HCC progression. Future studies including large sample size are recommended.
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1. Introduction:

Living organisms usually need to survive with harmful environmental circumstances such as heavy metal load, UV radiation, and oxidative stress. It is famous that the toxicity levels of metals and reactive oxygen species (ROS) vary considerably among and within species. Discrepancy between expression and function of metal binding proteins, such as metallothioneins (MTs) may be one of the reasons for this variation [1].

Metallothioneins (MTs) are a group of metal-binding proteins characterized by a high cysteine content and low molecular weight. MTs has an vital role in metal metabolism and protect cells against the toxic effects of radiation, alkylating agents and oxygen free radicals. [2]

The human MT gene is located on chromosome 16q13. The MT-1 gene has an antioxidant response element sequence and functions as an oxidative stress response gene. There are four major gene subfamilies of MTs found in humans: MT1, MT2, MT3, and MT4 [3].

MT1 and MT2 are expressed in a variety of tissues, though MT3 and MT4 are minor isoforms with limited expression in specific cells and tissues, for example the brain, reproductive organs, and stratified squamous epithelium. MT1 and MT2 are mainly expressed and extremely induced by a mixture of stimuli including metals, hormones, cytokines, growth factors, oxidants, stress, and irradiation. [4]

Hepatocellular carcinoma (HCC) is the fifth common cancer and causes more than half a million deaths every year, this makes it the third chief cause of cancer deaths in the world [5].

Chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, which induces oxidative stress and inflammation in liver are very linked to increased risk of HCC [6].

In addition, external agents such as alcohol and aflatoxin can lead to hepatocarcinogenesis by release of free radicals. [7]

Lower MT mRNA and protein expression were noticed in the cancer tissue of patients with HCC, while it was found in upper expression in normal liver tissue. This lower expression may be affected by reactive oxygen species and inflammatory cytokines, hence resulting in the cells being more subjected to DNA damage and apoptotic death [8].

2. Patients and Methods

Study population:

Our study was done in the Tropical department at Theodor Bilharz Research institute (TBRI) where 192 subjects were enrolled and divided to 3 groups; group A included 63 patients with HCV infection while group B contained 78 patients diagnosed with HCC on top of HCV. Finally; group C had 51 age and sex matched volunteers who served as a control group. We exclude those with HBV comorbidity, schistosomiasis, alcohol consumption or antiviral therapy from the study. An informed consent was taken from patients who participated in the study. Furthermore, the measures used were accepted by TBRI ethics committee in accordance with Helsinki Declaration (00010609).

Serological markers

HCV antibodies as well as HBsAg were assessed using enzyme-linked immunosorbent assay (ELISA).

Genomic DNA extraction

Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen; catalog No.: 51104). 5ml peripheral venous whole blood was taken in a sterile EDTA vacuum tube by means of standard protocol with proteinase K. Lysis of red blood cells was done 3 times using lysis buffer. Then, Sodium dodecyl sulfate (SDS) 10% and 10 ul proteinase K in the existence of guanidine HCL were added to treat the residual white cells for a short incubation time (10 minutes at 56 C) to inactivate all nucleases. Cellular nucleic acids then attach to a particular glass fibers pre-packed in high pure purification filter tube and a sequence of "wash and spin" steps were done using 500 ul Buffer AW1 and 500 ul Buffer AW2 to get rid of PCR impurities. lastly, elution buffer (200 ul Buffer AE) was added and incubation was done for 1 min at 15-25 C to release the nucleic acid from the glass fiber.

MT1 genotyping.

*MT1*gene polymorphism (rs 964372) was measured using Taq Man SNP genotyping assay. This assay consists of a single, ready to use tube that contain two sequence –specific primers for amplifying the polymorphism of interest jointly with two allele-specific Taq Man minor groove binder (MGB) probes for detecting the alleles for the specific polymorphism of interest. Each probe has a reporter dye; VIC dye is linked to the 5' end of allele C probe while FAM dye is linked to the 5' end of allele G probe. Each PCR reaction contained 2.5 ul of diluted DNA (5 ng/ul), 12.5 ul of 2× TaqMan Universal PCR Master Mix, 1.25 ul of 20× TaqMan SNP Genotyping Assay Mix and 8.75ul of Distilled water (DW). This PCR reaction was done in a thermal cycler using ABI 7500, with the next program shown in Table 1.

Table 1: PCR amplification run:

Pre-Read Run		Amplification (RQ-PCR program)			Post-Read Run	
1 Cycle		Temperature	1 Cycle	Cycles	1 Cycle	
60 °C	1 min.	95°C0	10 min	X1	60 °C	1 min
		95°C0	15 sec	X40		
		60°C	60°C			

At the end, a threshold is set at 0.1 for analysis; using ABI Prism "genetic analyzer". Then quantization of the amplified PCR product (DNA fragments) as well as determining the size of the fragments by comparing them to fragments enclosed in a size average.

Statistical analysis

The data was calculated using Microsoft Excel 2010 and statistical program for social science (SPSS version 22.0) for windows (SPSS IBM., Chicago, IL). Continuous normally distributed variables were represented as mean±SD with 95% confidence interval, as well to using the alleles and genotype frequencies and percentage for Categorical and non-parametric variables; p value of less than 0.05 was considered statistically significant. The Student's t test was performed to evaluate the means of normally distributed variables among groups. ANOVA followed by Tukey-Kramer as a post-hoc test in multi groups, $\chi 2$ test or Fisher's exact test were used to establish the distribution of categorical variables between groups.

3. Results

concerning group A "HCV patients" we set up that 34(54%) males and 29(46%) females and their age ranged from 34 to 67 years (mean± SD= 44.3 ±13.9). In group B "HCC patients" there were 50 (64%) males versus 28 (36%) females with age ranged from 48 to 60 years (Mean± SD= 46.8±15.9). furthermore, group C included 40 (78%) males and 11 (22%) females with their age ranged from 32 to 57 years (Mean±SD= 46.7±13.3).

as regards MT1 genotyping our data showed that there was an increase in CG genotype in HCC group in comparison to control group (p=0.8) and HCV group (p=0.6) while this increase were insignificant. C allele was frequent amplified "yet insignificantly" among HCV and HCC patients (85.7% and 84% respectively) compared to the healthy controls (82.4%). (Figure1 and Table 2).

Table 2: Frequency of *MT1* Allele and genotype among all the studies groups.

Mt1 (rs 964372)	CONTROL n=51	HCV n=63	HCC n=78	P. value		
				HCV Vs CONTROL	HCCVs CONTROL	HCCVs HCV
CC wild	36(70.6%)	47(74.6%)	56 (71.8 %)	0.4	0.8	0.5
CG Hetero	12 (23.5 %)	14 (22.2%)	19 (24.4 %)	0.8	0.8	0.6
GG Homo	3 (5.9 %)	2 (3.2%)	3 (3.8 %)	0.1	0.3	0.7
CG+GG	15(29.4)	16(25.4)	22(28.2)	0.4	0.8	0.5
C	84(0.824)	108(0.857)	131(0.84)	0.3	0.7	0.6
G	18(0.176)	18(0.143)	25(0.16)			

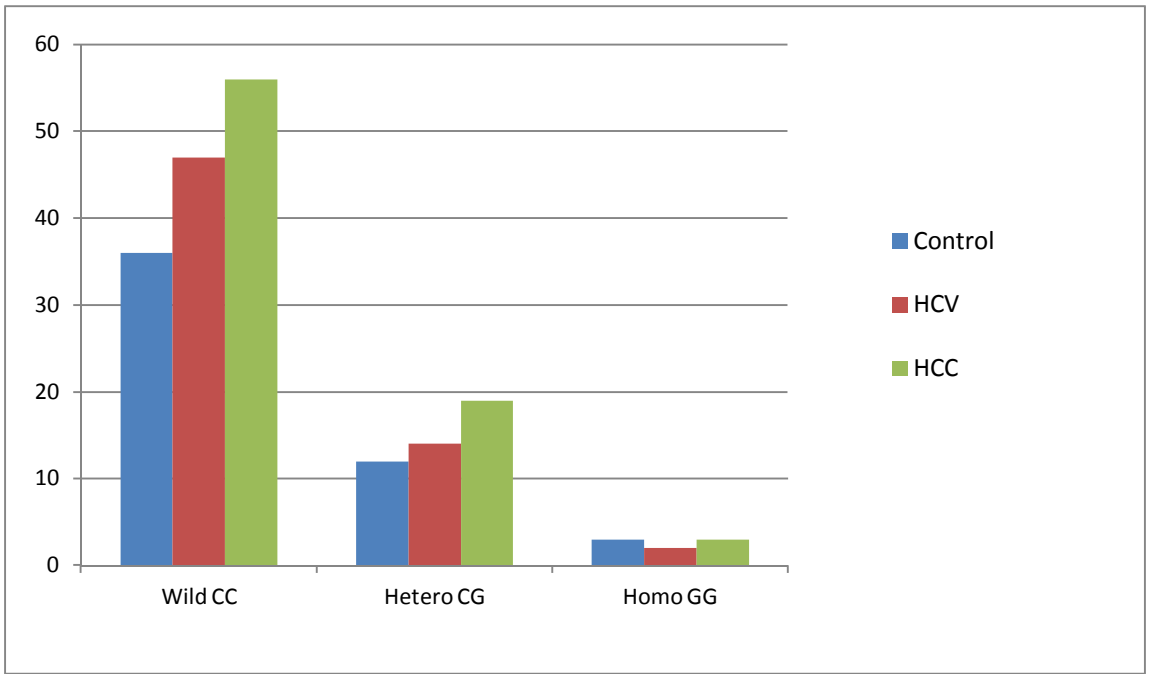

Figure 1: Frequency of *MT1* Allele and genotype among the studied group.

4. Discussion:

Metallothioneins (MTs) are a family of low molecular weight (ranging from 6 to 7 kDa), cysteine-rich proteins. [9]

MT1s are concerned with diverse functions, such as metal homeostasis, metal donation to different enzymes, angiogenesis, apoptosis, cell differentiation, radiation cell damage in addition to carcinogenesis. [10]

Given their wide regulatory properties, MT1s have also been suggested to have important roles in cell function and various pathological processes, for example neurodegeneration, osteoarthritis, metabolic disorders, and certain immune processes. [11] MTs can also attach to cadmium, mercury, platinum, or other similar heavy metals to keep cells and tissues against heavy metal toxicity. [12]

In addition, MTs play a defensive role against DNA damage and apoptosis. Great evidence indicates that MTs have important roles in carcinogenesis and cancer therapy. MTs contribute in the process of carcinogenesis and play critical roles in tumor growth, progression, metastasis, and drug resistance. [13]

In humans, MTs are encoded by a family of genes located on chromosome 16q13 and include at least 11 functional members: MT1 (MT1A, MT1B, MT1E, MT1F, MT1G, MT1H, MT1M, and MT1X; MT1C, MT1D, MT1I, MT1J, and MT1L are pseudogenes that cannot encode MT proteins), MT2 (also known as MT2A), MT3, and MT4. [14]

About 3% of the world's population has been infected with hepatitis C virus (HCV). With spontaneous clearance rates of only 20–30%, a large proportion of these individuals are at risk of becoming chronic carriers of the virus and rising long-term

disease, including cirrhosis and hepatocellular carcinoma (HCC). [15]

HCC the sixth mainly common cancer generally, is causing the second biggest number of cancer death around the world. Hepatocellular carcinoma (HCC) is a chief primary liver cancer, accounting for roughly 90% of all types of primary hepatic malignancy and triggering a major international public health trouble [16].

The pathogenesis of HCC is a multistep process caused by the progressive accumulation of gene alterations that leads to various cellular and molecular events including oxidative stress, endoplasmic reticulum stress, and abnormal cell cycle. [17]. Oxidative stress has been known to have a role in HCV infection and the development of chronic hepatitis, cirrhosis and HCC [18].

Oxidative stress causes increased production of reactive nitrogen species (RNS) or reactive oxygen species (ROS), as well as the reduced antioxidant protection which can speed up the progression of HCC. Also oxidative stress damage distributes the gene expression of cellular survival which can promote the proliferation and differentiation of normal cells and in the end lead to the reduction of cellular apoptosis or even the formation of the tumor cells [19].

There is some evidence that MT expression may promote liver cell apoptosis through NF-κB inhibition in HCV-infected livers and thus may counteract the anti-apoptotic effect of the HCV protein [20].

Therefore, HCV liver biopsies with higher MT levels may reflect an enhanced response to IFN-based therapy, as infected hepatocytes may be eliminated more easily via enhanced apoptosis [21]. Some studies have revealed that zinc, a strong MT inducer, increases the therapeutic response to IFNA in HCV infected patients [22].

55 human SNPs were identified in the MT1B gene area in accordance with the NCBI database (as of January 2013). Three of these polymorphisms (rs964372, rs8052394, and rs7191779) have a significant association with diseases. MT1B polymorphism (rs964372) This SNP is situated in the MT1B intron 1. [23]

MTs expression and their ability to attach metals can be influenced by changes at the DNA level, such as SNPs. as well changes in metal levels, some single nucleotide polymorphisms (SNPs) were found to be related to predisposition to a variety of diseases including cancer, cardiovascular diseases, and faster aging, confirming the key role of MTs in organisms against oxidative stress and toxic metals [24].

In our work we found that there was a raise in CG genotype in HCC group in comparison to control group and HCV group although this increase was statistically insignificant. There was a higher frequency of C allele among HCV and HCC patients (85.7% and 84% respectively) in comparison to the healthy persons (82.4%)," yet this finding was insignificant.

Wong., et al established increased risk of hepatocellular carcinoma was observed in rs964372 GG genotype compared with the CC genotype. At the allele level Wong., et al discovered that carrying haplotype AGT of the MT1A rs8052394 A allele, the MT1B rs964372 G allele, and the MT1B rs8052334 T allele compared to the most common ACT haplotype, particularly in those who smoke [23].

Chinese patients with type 2 diabetes mellitus with both MT1 rs8052394 GG and MT1 GA genotypes were characterized by hyperlipidemia with increased serum triglycerides and neuropathy [24]. A Taiwanese study also revealed that the MT-1 rs11076161 A, rs964372 C, and rs7191779 C alleles were protective against oral squamous cell carcinoma, whereas the rs8052394 A allele was associated with increased risk [25].

The expression of MTs is not similar in all human cancers. earlier studies have revealed that MT expression is upregulated in breast cancer, nasopharyngeal cancer, ovarian cancer, urinary bladder cancer, and melanoma [26]. Though in other cancers, such as hepatocellular carcinoma, prostate cancer, and papillary thyroid carcinoma, MT expression is down regulated [27].

Theocharis et al. also observed that between lung cancer subtypes, MT expression was prominent in squamous cell lung carcinoma and adenocarcinoma but absent in small cell lung cancer [28]. The differential expression of MTs depends on the type and differentiation status of tumors, as well as other environmental stimuli and/or gene mutations [29].

It is worth noting that this debate between our results and formerly published data may be due to different racial populations in each study, lack of clear classification of MTs genes, Most of the studies show relations between the laboratory setting only without correlation with clinical situation.

Our study had certain limitations. Referral bias might have occurred; all our patients were recruited from a single medical center. The positive rate of anti-HCV in our patients was higher than other studies.

In conclusion, the MT1B rs964372 gene polymorphism might not be a helpful determinant in predicting the outcome of HCV infection or HCC susceptibility in Egyptian population. additional prospective studies on big and different ethnic populations will be essential to confirm our result and explain the underlying molecular mechanism for the development of HCC. Also shared investigations of MT1A gene polymorphism and MT1B polymorphism

may help better understanding the functional consequences of MT1 gene polymorphism which would offer a base for future studies of this gene in the pathogenesis of HCC.

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