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## Study the association between glutathione peroxidase-1 gene in patients with hepatocellular carcinoma in Egypt

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### Abstract

cancer in the world. The main risk factors associated with HCC are hepatitis B and hepatitis C viral infections and other factors that play a role in HCC development. Oxidative stress is an imbalance between production and elimination of reactive metabolites of oxygen and nitrogen, in favor of their production leading to potential damage. During oxidative stress, biologically important molecules and cells can be damaged, and this can be significant in the pathogenesis of many diseases. Reactive oxygen species (ROS) is known to activate the apoptosis of some hepatocytes and therefore contribute to inflammation, regeneration, fibrogenesis, and carcinogenesis. The enzyme generally considered to be the frontline defense against ROS is glutathione peroxidase (GPX). The present study aims to investigate the association of progression of HCC with GPX1 (Pro198Leu) gene polymorphisms in HCC Egyptian patients. One hundred HCC cases and matched 100 controls were recruited from National Cancer Institute and Kasr El-Aini respectively. The detection of the genetic polymorphisms of GPX1 (Pro198Leu) were determined by single nucleotide polymorphisms (SNPs) using real time PCR technique. The results showed that there is a significant association between the GPX1 polymorphisms and the progression of HCC, the distribution of different GPX1 polymorphisms in HCC patients infected with HCV was (47.5% CC, 47.0% CT and 81.3% TT) respectively and in controls was (52.5% CC, 53.0% CT and 18.8% TT) respectively ( $P=0.033$ ). Our findings suggested that the genetic polymorphisms in GPX1 play a role in the etiology of hepatocellular

carcinoma. **Conclusion:** AFP is highly significant in relation to HCC cases and in also with GPX1 gene mutation. Our findings suggested that the genetic polymorphisms in GPX1 play a role in the etiology of hepatocellular carcinoma.

**Keywords:** Hepatocellular carcinoma, glutathione peroxidase gene, gene polymorphisms, Egyptian patients

### 1 Introduction

Hepatocellular carcinoma (HCC) is a major source of cancer burden worldwide, and is the third leading cause of cancer-related mortality (Zhou *et al.*, 2013). Incidence varies widely between geographical areas, probably because of variations in the exposure to hepatitis virus and other environmental pathogens (El-Serag, 2001). In Egypt, HCC is the second most common cancers in men and the 6<sup>th</sup> most common cancers in women (GLOBOCAN 2008 database). As with many cancers, variants of genes involved in multistage carcinogenesis may determine an individual's susceptibility to develop HCC. Single nucleotide polymorphisms (SNPs) are the most common type of genomic sequence variation and are thought to be associated with population diversity, susceptibility to disease, and individual response to drug treatment (Shastri, 2002).

The clinical risk factors include cirrhosis, Chronic Hepatitis C Virus (HCV) Infection, Chronic Hepatitis B Virus (HBV) Infection, Co- Infection of HBV and HCV Virus, Schistosomiasis, aflatoxin B1 (AFB1),

and alcoholism. The environmental risk factors include the pesticides and Cigarette Smoking (Anwar *et al.*, 2008).

HCV infection causes liver inflammation and variable grade of damage to the organ that over decades can lead to cirrhosis (Ikeda *et al.*, 1993; Tsukuma *et al.*, 1993; Lauer and Walker, 2001). Egypt has one of the highest prevalence rates of HCV infection. Disease progression is influenced by additional factors such as duration of infection, age at infection, gender, co-infection with HBV, and the level of HCV viraemia and its genotype (Anwar *et al.*, 2008).

Free radicals that contain the element oxygen are the most common type of free radicals produced in living tissue. Another name for them is "Reactive Oxygen Species" or "ROS". Antioxidants are chemicals that interact with and neutralize free radicals, thus preventing them from causing damage. Antioxidants are also known as "free radical scavengers" (Diplock *et al.*, 1998; Bouayed and Bohn, 2010; Poli *et al.*, 2004). Glutathione peroxidase (GPX1) is an antioxidant enzyme that protects the organisms from oxidative damage (Chandrasena *et al.*, 2006).

Glutathione Peroxidase (GPX) catalyzes the reduction of H<sub>2</sub>O<sub>2</sub> or organic hydroperoxides to water or the corresponding alcohols using reduced glutathione. The mammalian GPX family is divided into six clades according to their amino-acid sequence, substrate specificity and sub-cellular localization: classical or cytosolic (GPX1), the first mammalian GPX to be identified; gastrointestinal (GPX2); plasma (GPX3); phospholipid hydroperoxide (PHGPX or GPX4); epididymal (GPX5); olfactory epithelium (GPX6) (Mills, 1957; Flohe *et al.*, 1973; Rotruck *et al.*, 1973).

GPX-1 Mills first described GPX activity in 1957 (Mills, 1957), and its function was hypothesized to be protection of red blood cells against hemolysis by oxidation (Bhabak and Mugesh, 2010). Activity levels of the antioxidant enzyme GPX1 is likely affected by functional polymorphisms in the genes encoding them. A polymorphism in the GPX1 gene (Pro198Leu), encoding the isoenzyme GPX1, was reported to have a relation to HCC development (Sutton *et al.* 2006).

The genetic polymorphism of glutathione peroxidase-1 may have a significant effect on the enzyme activity. In particular, polymorphism in GPX1 Pro198Leu (C→T) located in the second exon of gene GPX1 has a high level of heterozygosity; it induces a proline (CCC)–leucine (CTC) substitution. Moreover, it may have an effect on the catalytic enzyme activity, its affinity to the substrate, specificity, structure stability, etc (Zheikova, 2012). With the use of cell lines, it was shown that Pro198Leu enzyme had lower activity compared to wild type protein. Catalytic gene activity was found to be 5% lower in each additional T copy in patients with this allele (Ravn\_Haren *et al.*, 2006).

Genetic variations in the antioxidant gene coding for the GPX1 enzyme may cause decreased or impaired regulation of their enzymatic activity and alter ROS

detoxification. Therefore, genetic variations among these enzymes that protect the cell against ROS may lead to disease (Forsberg *et al.*, 2001). Due to the high interaction potentiality of ROS with genetic material, polymorphisms in genes coding for antioxidant enzymes may play an important role for inter-individual differences in maintaining the human genome's integrity. Genetic polymorphisms in GPX1 have been implicated in proneness to cancer and other diseases (Tang *et al.*, 2010; Cebrian *et al.*, 2006). The present study aims to investigate the association of progression of HCC with GPX1 (Pro198Leu) gene polymorphisms in HCC Egyptian patients.

## 2 Materials and methods

### Sample Collection:

This study is a case-control study. It was conducted on a total number of 200 individuals divided into two groups, Group I included 100 HCC cases and group II included 100 healthy subjects recruited from National Cancer Institute and Kasr Al-Aini hospital respectively. Ten ml of blood withdrawn from cases and controls in EDTA tubes under complete aseptic condition. All samples were centrifuged, plasma and Buffy coat separated, aliquoted and stored at -80°C. Buffy coat used for detection of polymorphism of GPX1 gene and plasma used for HCV PCR testing and determination of AFP.

### Methods:

All HCC cases and controls included in this study were subjected to the Alphafeto Protein (AFP) using AxSYM AFP (Abbott Laboratories, USA, Diagnostics Division), HCV PCR by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and detection of GPX1 gene polymorphism by real time PCR.

### HCV RT-PCR

HCV RNA RT-PCR was performed in a total volume of 100µl containing 1X Taq buffer with 1.5mM MgCl<sub>2</sub> (Roche Molecular Biochemicals, Mannheim, Germany), 0.2mM dNTPs (Promega Madison, WI, USA), 20 pmole of each primers (P1 and P2), 20 units of Human Placental Ribonuclease Inhibitor (HPRI) (RNasin) (Promega Madison, WI, USA), 10 units of AMV Reverse Transcriptase (RT) (Promega Madison, WI, USA), and 2.5 units Taq DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany). A 90µl of master mix was added to each sample and the mixture was incubated at 42°C for 30 min for RT (one cycle), 95°C for 4 min (one cycle), followed immediately by 35 cycles of the following conditions: 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute. A final cycle of 72°C for 10 minutes at the GeneAmp PCR Systems 9600 (Applied Biosystems). Nested PCR was performed by the transfer of 10µl of the first PCR product to 90 µl of the second master mix containing 1X Taq buffer (Roche Diagnostics), 0.2mM dNTP's (Promega), 20 pmole of each nested primer (P3 and P4), 2.5 units Taq polymerase (Roche Diagnostics). The samples were incubated for 35 cycles as in step 2 without the initial RT step. Finally,

PCR results were visualized by electrophoresis on ethidium bromide stained 3% agarose gel in 0.5X TBE buffer (GIBCO-BRL, Life Technologies, Gaithersburg, MD, USA). The primers were derived from the highly conserved 5'-untranslated region (5'UTR) of HCV genome (Abdel-Hamid et al., 1997).

#### Detection of GPX1 gene polymorphism by Real time PCR technique:

DNA was extracted from Buffy Coat samples by using QIAamp DNA Mini Kit (Qiagen, Santa Clarita, USA). The polymorphism for GPX1 (rs1050450) was genotyped by real-time PCR fluorescence detection on an ABI PRISM® 7500 Real Time PCR System (Applied Biosystems, USA) using fluorescent labeled probes. Each 15 µl PCR reaction contained 20 pmol of forward primer 5'-CATCGAAGCCCTGCTGTCT-3', 20 pmol of reverse primer 5'-CACTGCAACTGCCAAGCA-3', 5 pmol of fluorescent oligonucleotide probes 5'-(FAM)-ACAGCTGAGCCCTT-(MGB)-3', 5 pmol of fluorescent oligonucleotide probe 5'-(VIC)-ACAGCTGGGCCCTT-(MGB)-3', 7.5 µl of 2X TaqMan Universal PCR Master Mix (Applied Biosystems) and 2 ng DNA. The PCR cycling conditions consisted of: 2 min cycle at 50°C and 10 min cycle at 95°C, followed by 49 cycles of 95°C for 30 s and 60°C for 1 min. Distilled water was used as a negative PCR control in each amplification.

### 3 Results

Table 1 shows that the HCC GPX1 cases had AFP ranged 354.58±449.6 for CC genotype, 733.77±463.9 for CT genotype and 872.16±385.6 for TT genotype which is a highly significant ( $P<0.0001$ ) and the controls for GPX1 had AFP ranged 3.37±2.4 for CC genotype, 3.03±2.27 for CT genotype and 2.22±.26 for TT genotype which is not significant ( $P=0.606$ ).

Table 2 shows that Among HCC cases; the HCV antibodies (HCV Ab) were positive in 43(89.6%) of GPX1 (CC genotype) and negative in 5(10.4%) of CC genotype. HCV Ab were positive in 34(87.2%) of CT genotype and negative in 5(12.8%) of CT genotype. HCV Ab were positive in 12(92.3%) of TT genotype and negative in 1(7.7%) of TT genotype, which is not significant ( $p=0.863$ ).

Among HCC cases; the HCV PCR were positive in 39(81.3%) of GPX1 (CC genotype) and negative in

9(18.8%) of CC genotype. HCV PCR were positive in 32(82.1%) of CT genotype and negative in 7(17.9%) of CT genotype, while HCV PCR were positive in 11(84.6%) of TT genotype and negative in 2(15.4%) of

TT genotype, which is not significant ( $p=0.961$ ). Among controls; HCV antibodies (HCV Ab) were positive in 22(41.5%) of GPX1 (CC genotype) and negative in 31(58.5%) of CC genotype. HCV Ab were positive in 18(40.9%) of CT genotype and negative in 26(59.1%) of CT genotype. HCV Ab were positive in 1(33.3%) of TT genotype and negative in 2(66.7%) of TT genotype, which is not significant ( $P=0.961$ ).

Among controls; HCV PCR were positive in 14(26.4%) of GPX1 (CC genotype) and negative in 39(73.6%) of CC genotype. HCV PCR were positive in 11(25.0%) of CT genotype and negative in 33(75.0%) of CT genotype, while HCV PCR were negative in all TT genotype 3(100%), which is not significant ( $P=0.590$ ).

Table 3 shows that regarding to the HCC cases; GPX1 (CC polymorphism) was found in 48(48%) of the HCC cases, while it was found in 53(53%) of the controls; CT polymorphism was found in 39(39%) of HCC cases while it was found in 44(44%) of the controls; TT polymorphism was found in 13(13%) of HCC cases while it was found in 3(3%) of the controls, which is significant ( $P=0.033$ ).

Regarding to the HCC cases; the functioning GPX1 (CC+CT polymorphisms) were found in 87(87%) of HCC cases while they were found in 97(97%) in the controls. The Malfunction GPX1 (TT polymorphism) was found in 13(13%) of HCC cases while it was found in 3(3%) in the controls, which is significant ( $P=0.009$ ).

### 4 Discussion

Our work was conducted to detect the association between glutathione peroxidase 1 (GPX1) gene in patients with Hepatocellular Carcinoma in Egypt.

Glutathione peroxidase-1, a selenium dependent enzyme, and the proline-leucine substitution makes it less sensitive to stimulation by the addition of selenium (Hu and Diamond, 2003). Several research groups have revealed the association between the polymorphism and various diseases caused by the oxidative stress (breast cancer, lung cancer, leukemia, metabolic syndrome, CAD) (Tang *et al.*, 2008, Ravn\_Haren *et al.*, 2006, Cox *et al.*, 2006).

In this study, we found a significant association between the GPX1 polymorphism and the progression of HCC, the distribution of different GPX1 polymorphisms in HCC patients infected with HCV was (48.0% CC, 39.0% CT and 13.0% TT) respectively and in controls was (53.0% CC, 44.0% CT and 3.0% TT) respectively ( $P=0.033$ ).

Table.1 The mean and SD of AFP in HCC GPX1 patients and controls

	HCC GPX1	N/Total (%)	Mean	SD	P value	Controls	N/Total (%)	Mean	SD	P value
AFP	CC	48(48%)	354.58	449.6	<0.0001	CC	53(53%)	3.37	2.4	0.606
	CT	39(39%)	733.77	463.9		CT	44(44%)	3.03	2.27	
	TT	13(13%)	872.16	385.6		TT	3.0(3%)	2.22	26	

Table.2 HCV Ab and HCV PCR in HCC GPX1 patients and controls

	HCC GPX1	N/Total (%)	Positive	Negative	P value	Controls	N/Total (%)	Positive	Negative	P value
HCV Ab	CC	48(48%)	43(89.6%)	5(10.4%)	0.863	CC	53(53%)	22(41.5%)	31(58.5%)	0.961
	CT	39(39%)	34(87.2%)	5(12.8%)		CT	44(44%)	18(40.9%)	26(59.1%)	
	TT	13(13%)	12(92.3%)	1(7.7%)		TT	3.0(3%)	1(33.3%)	2(66.7%)	
HCV PCR	CC	48(48%)	39(81.3%)	9(18.8%)	0.961	CC	53(53%)	14(26.4%)	39(73.6%)	0.590
	CT	39(39%)	32(82.1%)	7(17.9%)		CT	44(44%)	11(25.0%)	33(75.0%)	
	TT	13(13%)	11(84.6%)	2(15.4%)		TT	3.0(3%)	0	3(100%)	

Table.3 Polymorphism and functioning GPX1

	HCC	N/Total (%)	Controls	N/Total (%)	P value	GPX1	HCC	Controls	P value
GPX1	CC	48(48%)	CC	53(53%)	0.033	Functioning (CC+CT)	87(87%)	97(97%)	0.009
	CT	39(39%)	CT	44(44%)		Malfunction (TT)	13(13.0%)	3(3.0%)	
	TT	13(13%)	TT	3.0(3%)					

In agreement to our study, Ravn-Haren *et al.*, 2006 found that erythrocyte GPX1 activity was lower in individuals carrying the Leu allele than in individuals carrying the Pro allele. Genetic variant in GPX1 gene polymorphism that increases cancer risk may also cause alteration in the activity of the enzyme and hence alteration in the oxidant-antioxidant balances. Thus, the less active Leu-GPX1 variant may increase the risk of bladder cancer due to decreased protection against ROS-induced oxidative damage.

Another study done by Abd El-Ghaffar *et al.*, (2015) who found that GPX1 gene polymorphism individuals bearing Leu allele had a 4.915-fold when comparing the HCC group to the control subjects ( $P=0.001$ ). These results suggested that Pro/Leu genotype might be risky for the development of the inflammation

resulting from HCV infection and passes through liver cirrhosis to the development of HCC. Although none of their participants had Leu/Leu genotype, our allelic results suggest that this genotype might possess the highest risk in this process.

Another study with Nahon and co-outers 2012 (Nahon *et al.*, 2012) Nahon *et al.*, 2012 who carried out study on HCV-induced cirrhosis patients and followed them up for HCC development, it was found that patients with HCC had a frequency of Pro/Pro genotype (29.4%), Pro/Leu genotype (38.7%), and Leu/Leu genotype (45%). They stated that patients bearing two Leu-GPX alleles had higher rates of death.

Our results were supported by the results of Sutton *et al.*, 2006 who found that Leu GPX allele bearing patients had a higher incidence of HCC during follow-up of

alcohol-induced cirrhotic patients. They explained that by the slow detoxification of hydrogen peroxide by the low activity-associated Leu-GPX variant.

However, in a study was done by Ezzikouri *et al.*, 2010 and found no statistical difference in GPX1 genotypic frequency between HCC and control groups ( $P=0.685$ ).

With regard to GPX1 (Pro 198 Leu) gene, our study showed a statistically significant difference on comparing AFP between GPX1 genotypes (Pro/Pro, Pro/Leu, Leu/Leu) among HCC patients. The median of AFP were (354.58±449.6), (733.77±463.9) and (872.16±385.6) respectively ( $P<0.0001$ ).

In accordance with our results, a study by Abd El-Ghaffar *et al.*, 2015 found a statistically significant difference on comparing AFP between GPX1 genotypes (Pro/Pro, Pro/Leu) among HCC patients ( $P=0.004$ ).

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