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IL-6 −572G/C and −174G/C polymorphisms association with hepatitis C virus-induced hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer worldwide and the second primary aetiology of death due to cancer. Hepatitis viruses are responsible for around 70% to 80% cases of HCC [1]. HCV contributes to oncogenic transformation through induction of chronic inflammation which plays an important role in tumour initiation, promotion, and progression [2]. Liver inflammation is an important factor in HCC progression and the balance between pro-inflammatory and anti-inflammatory cytokines is an important key for controlling the disease progression [3].

Interleukin-6 (*IL-6*) is a pleiotropic and proinflammatory cytokine produced by lymphocytes, macrophages, and fibroblasts which plays an integral role in stimulation of the immune response through the initiation of acute phase reactants and lymphocyte proliferation. *IL-6* has two signal pathways: the classic pathway moderates the immune response, while the trans-signalling pathway is associated with liver regeneration and several chronic inflammatory diseases [4]. Several studies have found high *IL-6* serum levels in patients with HCC, the finding suggests a relation between *IL-6* and this disease [5,6].

IL-6 is situated on the short arm of chromosome 7 spanning a 5 kilobase length, and consists of five exons and four introns. *IL-6* single nucleotide polymorphisms (SNPs) that lie in its promoter region (−174G/C, and −572G/C) have a significant impact on transcription, and therefore, the amount of the cytokine produced [7]. Many studies have investigated links between *IL-6* SNPs with HCC risk of HCC, but a consensus has not been reached. Therefore, we tested the hypothesis of a link between the roles of the −174G/C and −572G/C *IL-6* SNPs in HCC patients with HCV infection.

We tested our hypothesis in a case–control study of 100 patients diagnosed as HCC with HCV, enrolled from internal medicine department, Mansoura University hospital, and 100 healthy controls. HCC patients were positive for HCV antibodies (Access BIO – RAD Co., France), confirmed by quantitative viral RNA detection using Taq-Man HCV quantitative test version 2.0 (Roche Molecular Diagnostic, Branchburg, NJ, USA). Exclusion criteria were HBV and other cancers. All patients were diagnosed by clinical, laboratory and radiological investigations including abdominal ultrasound and computed tomography.

The research was approved by Mansoura University ethical committee (Code: R/19.04.476) and informed consent was obtained from each participant.

Ten mL whole blood samples were obtained in EDTA and no anticoagulation for genomic DNA and serum. Sera were tested for routine laboratory measurement of alpha-fetoprotein (AFP) and *IL-6*. Genomic DNA was isolated from whole blood by DNA extraction kit (Qiagen-Germany). The −572G/C SNP of *IL-6* was determined by two sets of primers: F; (5′GGAGACGCCTTGAAGTAACTGC3′) and R; (5′GAGTTTCCTCTGACTCCATCGCAG3′). The PCR program was initial denaturation at 94°C for 5 min, then 35 cycles at 94°C for 60 s, 55°C for 60 s, 72°C for 60 s, then at 72°C for 5 min as a final extension step. Each PCR product was electrophoresed using 2% agarose gel. The PCR product was 163 bp fragment. PCR products were digested with BsrBI restriction enzymes. G allele gave 102 and 61 bp fragments while C allele remains uncut. (Figure 1). The −174G/C SNP was investigated by the use of two sets of primers: F; (5′TGACTTCAGCTTTACTCTTTGT3′) and R; (5′CTGATTGGAAACCTTATTAAG3′). PCR program was initial denaturation at 94°C for 5 min, then 35 cycles at 94°C for 60 s, 53°C for 60 s, 72°C for 60 s, then at 72°C for 5 min as a final extension. Each PCR product was electrophoresed using 2% agarose gel. The PCR product was 198 bp fragment. PCR products were

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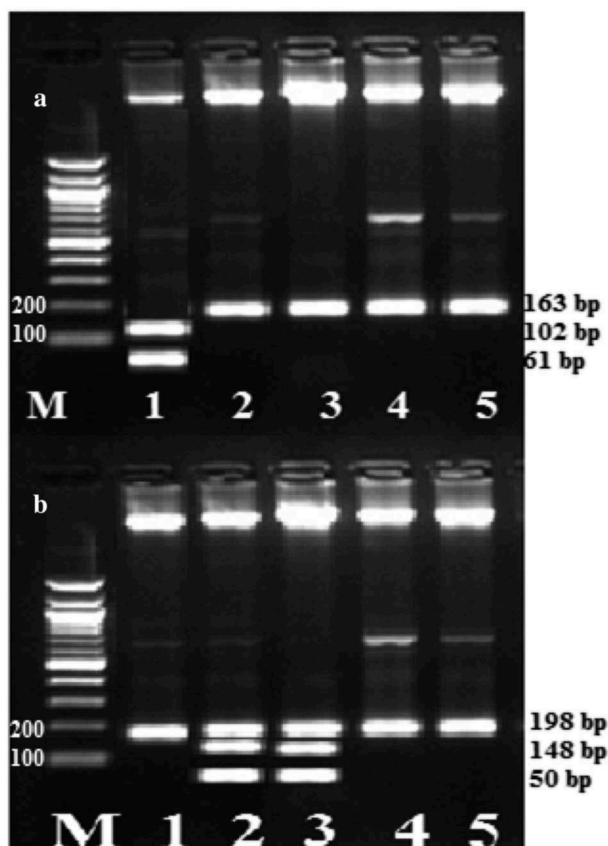

Figure 1. Agarose gel electrophoresis showing the −572G/C of *IL-6* (A) using 100-bp DNA size ladder. The C allele (163 bp band), and G allele (102 and 61 bp bands). For −714 G/C polymorphism (B), The C allele (198 p band), and G allele (148 and 50 bp bands).

digested with SfaNI restriction enzymes. G allele gave 148 and 50 bp fragments while C allele remains uncut (Figure 1). Serum *IL-6* and alpha-fetoprotein (AFP) were measured by commercial ELISA kits (R&S Systems, USA, and ELISA-DRG International Inc., USA). Results were analysed using SPSS v22. Continuously variable data were analyzed by one-way ANOVA and T-tests. Categorical data were analyzed by χ^2 tests. Odds ratios (95% confidence interval) were calculated to detect risk ratio. The frequencies of genotypes and alleles were examined for Hardy–Weinberg Equilibrium. Receiver Operating Characteristic (ROC) curve analysis test was used to discriminate diseased cases from non-diseased cases. The Spearman's correlation is used to determine the correlation between two non-normally distributed continuous variables. $p < 0.05$ was considered significant.

The patients were 77 males and 23 females, the controls 55 males and 45 females ($p = 0.001$). Mean [SD] of the age of the patients was 50.6 [5.9] years versus 49.5 [5.1] in the controls ($p = 0.07$). As expected, serum *IL-6* was increased in HCC/HCV patients as compared to healthy group, 16.0 [2.1] pg/mL and 2.1 [0.6] pg/mL, respectively ($p < 0.001$). Also, as expected, the median (IQR) of AFP was higher in HCC/HCV as compared to the healthy group (24.7 (8.1–1806) ng/ml and 8 (6–9) ng/ml, respectively) ($p < 0.001$). Receiver operating characteristic (ROC) curve of the efficiency of serum AFP and *IL-6* in differentiating HCC/HCV from the controls showed that *IL-6* was more sensitive and specific than AFP, the area under the receiver operating characteristic (AUROC) with 95% (CI) for *IL-6* was 1.00 (1.0–1.0), with 100% sensitivity, a specificity of 99%, and an optimal cutoff value of 3.25 pg/mL. The AUROC for AFP with 95% (CI) was 0.80 (0.72–0.86), with 73% sensitivity and 94% specificity, and a cut-off of 9.4 ng/mL ($p < 0.001$). Correlations between IL-6 and AFP in HCC and control groups was $r = 0.63$, $p = 0.53$ and $r = 0.1$, $p = 0.32$, respectively.

The distribution of genotypes and alleles of −572G/C and −174G/C SNP of *IL-6* are shown in Table 1. The frequency of the *IL-6* −572G/C GG genotype SNP was increased in HCC/HCV patients as compared with controls. The frequency of G allele was higher in patients compared to the controls while C allele was more frequent in the controls versus the patients. The frequency of the *IL-6* −174G/C GG genotype SNP was higher HCC/HCV patients compared to the controls. The frequency of G allele was more frequent in the patients than the controls, with increased frequency of C allele in the controls than the patients. There was a mean [SD] increase in serum *IL-6* in patients with −572 G allele as compared to those with −572 C allele, 16.3 [3.1] pg/mL and 13.8 [2.05] pg/mL, respectively ($p < 0.001$). However, there was no difference in the serum *IL-6* level between

Table 1. Distribution of genotypes and alleles of *IL-6* SNPs.

	HCC Patients (n = 100)	Controls (n = 100)	OR (95% CI)	*p*-value
−572G/C SNP				
Codominant model				
GG	76	61	Ref.	–
GC	18	31	0.4 (0.2–0.9)	0.02
CC	6	8	0.6 (0.19–1.8)	0.37
Dominant Model				
GG	76	61	Ref.	
GC+CC	24	39	0.5(0.26–0.9)	0.02
Recessive Model				
GG+GC	94	92	Ref.	
CC	6	8	0.73 (0.2–2.1)	0.6
Alleles				
G	170	153	Ref.	0.03
C	30	47	0.57 (0.3–0.9)	
−174G/C SNP				
Codominant model				
GG	75	48	Ref.	–
GC	24	50	0.3 (0.16–0.5)	<0.001
CC	1	2	0.3 (0.02–3.6)	0.35
Dominant Model				
GG	75	48	Ref.	
GC+CC	25	52	0.3 (0.16–0.56)	<0.001
Recessive Model				
GG+GC	99	98	Ref.	
CC	1	2	0.49 (0.04–5.5)	0.56
Alleles				
G	174	146	Ref.	
C	26	54	0.4 (0.2–0.6)	<0.001

OR (95% CI); Odds ratio 95% confidence interval. Ref. = reference.

G and C allele of–174G/C SNP 16 [2.1] and15.9 [2.1] pg/mL, respectively (p = 0.5).

The objective of the present study was to test the hypothesis of a link between −572G/C and −174G/C SNPs in *IL-6* in HCC patients with HCV genotype 4 infection. Most HCC cases were men as HCC is common in males than females which might be explained by the protective effect of estrogen against HCC [8]. The frequency of −572 GG genotype was higher in HCC patients as compared with controls. In addition, the frequency of −572 G allele was more prevalent in patients compared to the controls. Other reports found an association between the *IL-6* −572G/C SNP with the development of HCC and pathogenesis of hepatitis B virus infection [9], in contrast to a meta-analysis study by Liu et al. [10] who showed that there was no relation between the *IL-6* −572G/C SNP and HCC. The study of the SNP in the −174G/C of the *IL-6* promoter region revealed higher frequency of GG genotype in HCC patients as compared with controls. The frequency of G allele was more prevalent in HCC patients compared to the controls. The meta-analysis of Liu et al. [10] reported that carriers of the −174G allele (GG+CG) were at a higher risk of developing HCC. This finding may contributed to the view that the G allele of the −174G/C SNP is related to its *in vivo* production and that the G carriers have higher levels of soluble *IL-6*, while, the change from G to C allele decreased the transcription and production of *IL-6* [11].

IL-6 is a multifunctional cytokine which has a contradictory role in cancer as it has both immunostimulation and cancer-induced immuno-suppression [5]. Previous studies showed that serum *IL-6* was increased in HCC patients and could be used as a tumour marker for HCC; however, the carcinogenesis effect of *IL-6* in HCC remains unclear [12]. We found that the serum level of *IL-6* was significantly higher in HCC patients when compared to the controls and is considered more specific marker than AFP in HCC diagnosis. This finding with is in agreement of Lippitz and Harris [5], and meta-analysis of Shakiba et al. [13] found that *IL-6* was increased in HCC and could be used as a diagnostic marker especially in cases with low AFP level. There is evidence that the inflammatory regulatory role of *IL-6* in the pathophysiology and development of liver cancers indicating the crucial role of this cytokine in chronic inflammation that leads to HCC and experimental models of HCC in mice have suggested that chronic exposure to high *IL-6* level is associated with increased liver injury and HCC [14]. We failed to find a significant correlation between AFP and *IL-6* levels in both studied groups, as did Othman et al. [15] although Porta et al. [12] found a significant positive correlation between AFP and *IL-6* in HCC patients.

The cytokines levels in the blood could be affected by the SNPs in the interleukin genes promoter region. We found that G allele of–572G/C of *IL-6* was associated with a significant increase in serum *IL-6*. This result is in agreement with Jerrard-Dunnec [16] but in contrast to Mattos et al. [17] who found no association between *IL-6* concentration and *IL-6* SNPs. In addition, we found no association between *IL-6* level and −174G/C SNP, a result in agreement with Mattos et al. [17] and in contrast to Bennermo et al. [11] who found that −174G allele was associated with high *IL-6* serum.

We acknowledge weaknesses in our study – that the groups are unmatched for sex (although there is no evidence that this influences measured indices), small numbers, and that we cannot speculate if the effect we have observed are specific for HCV infection or for HCC. Nevertheless, this work represents an advance in biomedical science because it shows that the GG genotype and G allele of *IL-6* −572G/C and *IL-6* −174G/C is associated with HCC in patients with HCV infection when compared to the control subjects, and that the G allele of −572G/C of *IL-6* was associated with a significant increase in serum levels of *IL-6*.

Disclosure statement

No potential conflict of interest was reported by the authors.

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