Background: Hepatitis C virus (HCV) infection is a major risk factor for developing HCC. Cytokines are involved in various cancers. This work aimed to investigate the relationship between the gene polymorphisms of interleukin-1 beta (IL-1β), IL-1 receptor antagonist (IL-1RN) and tumor necrosis factor alpha (TNF-α) and the development HCV-related HCC.

Methods: The study included 100 HCV patients (50 with HCC and 50 without HCC) and 70 apparently healthy individuals. Gene polymorphisms of IL-1β–31 C/T, IL-1β–511 C/T, IL-1RN variable number of tandem repeats (VNTR), and TNF-α–308 G/A were investigated by PCR based assay.

Results: There were significant increase in the frequency of IL-1β–31 TT, IL-1RN A2A2 and TNF-α–308 AA genotypes in HCC group when compared to controls. There were, in addition, significant increase in the frequency of IL-1β–31 TT, IL-1RN A2 and TNF-α–308 A alleles in HCC group when compared to controls. However, there was no significant difference in IL-1β–511 gene polymorphism between different groups.

Conclusion: HCV patients’ carriers for IL-1β–31 T, IL-1RN A2 or TNF-α–308 A alleles could be at risk of developing HCC.

Introduction
Hepatitis C virus (HCV) infection is a global health problem, being the second most common chronic viral infection in the world with a global prevalence of about 3% (about 180 million people) (Craxi et al., 2008). Egypt is an endemic area of hepatitis C. It was found that about one person in seven of Egyptian population tested positive for antibodies against HCV. However, nearly one person in ten carries its viral RNA and is therefore chronically infected (Yehia et al., 2011). Patients infected with HCV have different clinical outcomes, ranging from acute resolving hepatitis to chronic liver disease including liver cirrhosis or hepatocellular carcinoma (HCC) (Zaltron et al., 2012). HCC is the fifth most common tumor and the third most common cause of cancer-related deaths worldwide (Okamoto et al., 2010). Its incidence has increased sharply over recent decades and has been partially attributed to chronic HCV infection (Bosch et al., 2005). In Egypt, the growing incidence of HCC is nearly doubled over the last decade (Freedman et al., 2006). This is parallel with the Egyptian patients with chronic HCV infection (HCC and non-HCC) and healthy individuals to assess whether these genes are involved in chronic HCV susceptibility and/or HCC development.

Cytokines, as the products of host response to inflammation, play an important role in the defense against viral infections. However, in HCV infection they may play a prominent role in liver damage (Koziel, 1999). Interleukin-1 beta (IL-1β) and tumor necrosis factor-α (TNF-β) are key cytokines in the inflammatory response (Dinarello, 1996). Interleukin 1 beta (IL-1β) is one of the potent pro-inflammatory cytokines and has a wide array of biological functions, including cell survival and proliferation (Roshak et al., 1996). Some single nucleotide polymorphisms (SNPs) have been reported in IL-1β, from which cytosine (C)/ thymidine (T) base transitions at –31 bp from the transcriptional start site. It has been shown that the –31T allele enhances IL-1β transcriptional activity (El-Omar et al., 2000) and a number of studies reported that –511C/–31T is a risk haplotype for the development of cancer (Chang et al., 2005). Another cytokine that has an important influence on IL-1β levels is the IL-1 receptor antagonist (IL-1RN); the gene encoding this cytokine; IL-1RN, is also known to be polymorphic (Santtila et al., 1998).

Circulating TNF-α levels increase during HCV infection and hepatitis viral infection induces TNF-α production in human hepatocytes (Cua et al., 2007). An elevated TNF-α level correlates with the severity of hepatic inflammation, fibrosis, and tissue injury (Akpolat et al., 2005). Persistent immune mediated hepatic injury can initiate the process of fibrosis, cirrhosis, and, eventually HCC (Leverero, 2006). Polymorphism in the human TNF-α promoter at –308 (Wilson et al., 1992) involves the substitution of adenosine for guanine in the uncommon alleles. A variety of infectious diseases and inflammatory disorders are associated with TNF-α–308 alleles (Bdwell et al., 2001). The role of polymorphisms in TNF-α in the pathogenesis of HCV infection has been investigated, but some results are contradictory (Abbas et al., 2005). The aim of the present study was to determine the genotype and allele frequencies of IL-1β, IL-1RN and TNF-α polymorphisms among the Egyptian patients with chronic HCV infection (HCC and non-HCC) and healthy individuals to assess whether these genes are involved in chronic HCV susceptibility and/or HCC development.

Subjects and Methods
Patient group: The study included 170 subjects; 100 patients attending Oncology Center - Mansoura University HCV (50 patients with HCC and 50 patients without HCC) and 70 healthy individuals with no liver affection (mean age/years ± SD: 55.24±5.72). The patients were subjected to full history taking and thorough physical examination. General physical examination included examination of metabolic, endocrine, cardiovascular, respiratory, gastro-intestinal and neurological systems to exclude presence of abnormalities. —
The HCC was diagnosed by ultrasonography and computer tomography (CT) as well as liver histopathological examinations. Other features including gender, age, tumor size, liver function tests, serum tumor marker alpha fetoprotein (AFP) level were done. The patients of HCC on top of viral hepatitis infection included in the study were positive for serum HCV RNA. Exclusion criteria were: diabetes mellitus, chronic renal failure, coronary artery disease, end stage liver disease, positive serum antinuclear antibody (ANA). Patients and controls had no history of other malignancy. All subjects were informed of the purpose of the study and informed consent was obtained. The study was approved by the ethical committee of Mansoura University.

Biochemical investigation:
Fasting blood samples (one ml) were collected on ethylenediamine tetraacetic acid (EDTA) and stored at −20°C for DNA extraction.

DNA extraction and investigation of IL-1β and TNF-α polymorphisms:
Genomic DNA was extracted from peripheral blood using a Gentra genomic DNA purification kit (Qiagen; Hilden, Germany). The region containing SNP –31C/T within the IL-1β gene was detected by restriction fragment length polymorphism (RFLP) (Hwang et al., 2002). The forward primer used was: F: 5’-AGGATTCTGCA CCAATACT-3’. The reverse primer used was: R: 5’-GAAGCTTCCACCAATACT-3’. Each PCR included 300 ng DNA, 200 mM dNTP, 500 nM primer, and 2.5 U Taq DNA polymerase (Ampli Taq Gold, Perkin-Elmer, Norwalk, CT, USA). DNA was initially denatured for one minute at 95°C, and then PCR amplification was performed in a PTC-200 thermal cycler (MJ Research, Essex, UK) using the following temperature program: 36 cycles of denaturation at 94°C for 45 seconds, annealing 54°C for 50 seconds, and extension at 72°C for 60 seconds. The PCR amplification was completed by a final extension at 72°C for 7 min. The amplification yielded a product of 448 bp. Upon cleavage with 5 U Alu I (Life Technologies Corporation. Invitrogen. Cat. no. 45200-029, China) overnight at 37°C, the DNA fragments were detected using 3% agarose gel stained with ethidium bromide and visualized under UV light. C allele: 344, 79, 20 and 5 bp, while T allele: 247, 97, 79, 20 and 5 bp (figure 1).

The region containing SNP IL-1β –511 C/T was detected by RFLP (Mansfield et al., 1994). The forward primer used was: F: 5’-TGGCATTTGATCTGGTTCATC-3’. The reverse primer used was: R: 5’-GAAGCTTCCACCAATACT-3’. Each PCR included 300 ng DNA, 200 mM dNTP, 500 nM primer, and 2.5 U Taq DNA polymerase (Ampli Taq Gold, Perkin-Elmer, Norwalk, CT, USA). DNA was initially denatured for one minute at 95°C, and then PCR amplification was performed in a PTC-200 thermal cycler (MJ Research, Essex, UK) using the following temperature program: 36 cycles of denaturation at 94°C for 45 seconds, 55°C for 80 seconds, and 72°C for 2 minutes. The amplification was completed by a final extension at 72°C for 7 min. The amplification yielded a product of 836 bp. Upon cleavage with 5 U Ava I (Gibco BRL, Life Technologies Corporation) overnight –308. 5’-ATAGGTTTTGAGGGGCATCG-3’ specific for G or 5’-ATAGGT TTTGAGGGGCATCA-3’ specific for A. DNA was initially denatured at 96°C for 3 minutes, then 30 cycles of 96°C for 45 seconds, 55°C for 80 seconds, and 72°C for 2 minutes followed by 3 minutes extension at 72°C (figure 4).

Amplification of 410 bp for allele A1, 240 bp for allele 2 within intron 2 of IL-1RN VNTR using primers: Forward: 5’- TCACGCCCTGCTTTTG-3’. Reverse: 5’-CTAAGCACA ACCTC-CTAT-3’. Initial one minutes denaturation at 96°C followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60 for 1 min, extension at 70°C for 60 seconds and a final extension for 7 minutes at 70°C (Wilkinson et al., 1999) (figure 3).

Amplification of an 836 base pair (bp) fragment of the TNF-α promoter region at position –308 (G-A) was amplified by amplification refractory mutation system (ARMS) (Saarig et al., 2000) using primers: a common forward primer 5’-CTG- CATCCCCGTCTTTTCTCC-3’ and one of two reverse primers with a 3’ mismatch corresponding to a G or and A at posi-

Figure (1): Ethidium bromide stained 2% agarose gel shows enzymatic digestion of – 31C/T polymorphism of IL-1β gene for different groups studied; Lane M: 50 bp DNA size marker, lane 1 (C/C genotype), lane 2 (C/T genotype) and lane 3 (T/T genotype).

Figure (2): Ethidium bromide stained 2% agarose gel shows enzymatic digestion of -511C/T polymorphism of IL-1β gene for different groups studied; Lane M: 50 bp DNA size marker, lane 1 (C/C genotype), lane 2 (C/T genotype) and lane 3 (T/T genotype).

Figure (3): PCR amplification for TNF-α at position –308 (G-A) polymorphisms using SSP for G allele (above) and A allele (below) show: lane M, DNA size marker; lanes 1, 2, 4, 6 (+ve for G and A alleles giving G/A genotype), lane 5 (–ve for G allele and +ve for A allele giving A/A genotype), lane 7 (+ve for G allele and –ve for A allele giving G/G genotype) and lane 3 –ve control. Band size: 836 bp.
Table (1): Descriptive and biochemical data in Control and HCV groups:

| Parameter               | Control (N=70) | HCV-patients with HCC (N=50) | HCV-patients without HCC (N=50) |
|-------------------------|----------------|-----------------------------|---------------------------------|
| Age (year) Mean ± SD    | 56.02±5.62     | 57.55±5.33                  | 56.45±4.56                      |
| Sex No (%) Male Female  | 35 (50%) 35 (50%) | 31 (62%) 19 (38%)           | 32 (64%) 18 (36%)               |
| Tumor size (cm) Mean ± SD| NA            | NA                          | 2.04±0.41                       |
| INR                     | 1.11 ± 0.02    | 1.35 ±0.03                  | 1.38 ±0.04                      |
| ALT (U/L)               | 19.36 ± 0.56   | 44.54 ± 2.89                | 57.35 ±3.58                     |
| AST(U/L)                | 18.36 ± 1.11   | 78.95 ± 5.44                | 87.08 ±5.33                     |
| Albumin (mg%)           | 4.22 ±0.09     | 2.86 ±0.16                  | 2.66 ±0.11                      |
| Total bilirubin (mg%)   | 0.84 ±0.06     | 1.80 ±0.17                  | 1.84 ± 0.18                     |
| HCV-Ab                  | Negative       | Positive                    | Positive                        |
| HCV-PCR                 | Negative       | Positive                    | Positive                        |
| AFP µg/ml               | 9.45±5.7       | 256± 126.9                  | 1828±1426.4                     |

Abbreviations: ALT, alanine aminotransferase; AST, Aspartate transaminase; AFP, alpha-fetoprotein; INR, international normalized ratio; Ab, antibody; PCR, polymerase chain reaction, NA: not applicable.

Table (2): IL-1β−31, IL-1β−511, TNF−α−308 and IL-1RN VNTR genotype frequencies among cases with hepatitis C virus compared to controls with their statistical significance

| Genotypes            | Control group (N=70) | HCV-patients with HCC (N=50) | HCV-patients without HCC (N=50) |
|----------------------|----------------------|-----------------------------|---------------------------------|
| IL-1β−31             |                      |                             |                                 |
| TT                   | 19 (27.1%)           | 21 (42%) a                  | 16 (32%)                        |
| TC                   | 22 (31.4%)           | 18 (36%)                    | 20 (40%)                        |
| CC                   | 29 (41.5%)           | 11 (22%) a                  | 14 (28%)                        |
| IL-1β−511            |                      |                             |                                 |
| TT                   | 29 (41.4%)           | 20 (40%)                    | 18 (36%)                        |
| TC                   | 25 (35.7%)           | 22 (44%)                    | 24 (48%)                        |
| CC                   | 16 (22.9%)           | 8 (16%)                     | 8 (16%)                         |
| TNF−α−308            |                      |                             |                                 |
| GG                   | 56(80%) a            | 25 (50%) a, b               | 35 (70%)                        |
| GA                   | 8 (11.4%) a          | 15 (30%) a, b               | 8 (16%)                         |
| AA                   | 6 (8.6%) a           | 10 (20%) a                  | 7 (14%)                         |
| IL-1RN               |                      |                             |                                 |
| A1A1                 | 41 (59%)             | 6 (12%) a                   | 6 (12%) a                       |
| A1A2                 | 26 (37%)             | 32 (64%) a, b               | 35 (70%) a                      |
| A2A2                 | 3 (4%) a             | 12 (24%) a, b               | 9 (18%) a                       |

a: There is statistically significant difference when compared to control group
b: There is statistically significant difference when compared to HCV-patient group without HCC.
Hepatitis C virus (HCV) is a major cause of chronic liver disease (Shepard et al., 2005). Approximately 80% of infected patients fail to clear the virus and progress to chronic hepatitis. Some of those with chronic HCV infection may progress to liver cirrhosis and eventually hepatocellular carcinoma (HCC) (Lauer and Walker, 2001). Because risk for HCC increases with the severity of hepatic inflammation and chronic inflammation developing through the action of various inflammatory mediators is known as a cofactor of carcinogenesis (Coussens and Werb, 2002).

In this study, we examined the association of IL-1β−31 and −511 gene polymorphisms with development of HCC in patients with hepatitis C virus. It was found that TT genotype and T allele of IL-1β−31 but not IL-1β−511 were more frequent in HCC on top of HCV compared to control group. Those findings are in accordance with Wang et al. (2003) who stated that the proportion of IL-1β−31 TT genotype was higher than T/C and C/C genotypes in patients with HCC and concluded that IL-1β−31 genotype T/T is associated with presence of HCC in Japanese patients with chronic HCV infection. On the other hand, Okamoto et al. (2010) reported that the IL-1β−31 genotype has no relationship with HCC diagnosis, but the higher transcriptional genotype, the −31T homozygote, has a poorer prognosis than C allele carriers among HCC patients and that gene polymorphism might be potential marker for predicting the prognosis of HCC patients.

The IL-1β−31 polymorphism, which involves TATA box, is reported to affect lipopolysaccharide induced IL1β production by modifying DNA protein interactions. It is hypothesized that the IL-1β−31 T allele enhances IL-1β production in the liver and induces hepatocyte damage that may finally lead to HCC development (El-Omar et al., 2001). The IL-1β−31 and −511 were in near complete linkage disequilibrium as was observed in previous reports (Takamatsu et al., 2000) indicating that one or both of these loci may functionally affect HCC development in patients with chronic HCV infection.

Our results found that A2A2 genotype and A allele of IL-1RN gene were more frequent in HCC on top of hepatitis C virus rather than control group. The IL-1RN gene has a pentan-allelic 86-bp tandem repeat polymorphism (variable number tandem repeat; VNTR) in intron 2, of which the less common allele 2 (IL-1RN*2) is associated with a wide range of chronic inflammatory and autoimmune conditions. IL-1RN*2 is associated with enhanced IL-1β production in vitro (Santtila et al., 1998) and in vivo (Hwang et al., 2002).

In the current study, we found that AA genotype and A allele of TNF-α−308 gene were more frequent in HCC on top of hepatitis C virus rather than control group. In considering the relationship between −308 polymorphisms and the severity of HCV infection, Barrett et al. (2000) and Hohler et al. (1998) reported an association between these factors in patients with chronic HCV infection; Romero-Gomez et al. (2004), found no association between −308 polymorphisms and the severity of fibrosis in HCC. The −308A promoter variant has been associated with enhanced transcription of TNF-α (Wilson et al., 1997), but other investigations have failed to confirm this finding (Brinkman et al., 1996). Yee et al. (2000) found that −308As was associated with increased cirrhosis in HCV infected patients. SNPs at −308 showed inconsistent associations with various HCV outcomes (Constantini et al., 2002). These discrepancies may be due to ethnic differences in the examined populations, leading to a differential distribution of cytokine gene polymorphisms.

**Conclusion:**

IL-1β, IL-1RN VNTR, and TNF-α genes polymorphism could be risk factors for the development of HCC in patients with chronic HCV in Egyptian population, and to confirm this results, large number of cases and other risk factors should be studied in the future.

**Discussion:**

Hepatitis C virus (HCV) is a major cause of chronic liver disease (Shepard et al., 2005). Approximately 80% of infected patients fail to clear the virus and progress to chronic hepatitis. Some of those with chronic HCV infection may progress to liver cirrhosis and eventually hepatocellular carcinoma (HCC) (Lauer and Walker, 2001). Because risk for HCC increases with the severity of hepatic inflammation and chronic inflammation developing through the action of various inflammatory mediators is known as a cofactor of carcinogenesis (Coussens and Werb, 2002).

In this study, we examined the association of IL-1β−31, IL-1β−511, TNF-α−308 and IL-1RN VNTR allele frequencies among cases with hepatitis C virus compared to controls with their statistical significance.
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