IL-2 polymorphisms and IL-2 serum levels association with susceptibility to HBV-related Hepatocellular Carcinoma

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Abstract: Polymorphisms in cytokine genes responsible for inflammatory and immune responses are associated with risk of hepatocellular carcinoma (HCC) in high-risk population. Interleukin-2 (IL-2) is an immuno-regulatory cytokine produced by T cells and plays an important role in anti-tumor immunity. Variations in the DNA sequence of the IL-2 gene may lead to altered cytokine production and/or activity, and thus modulate an individual's susceptibility to hepatitis B virus-related hepato-cellular carcinoma (HBV-related HCC). **Aim:** This study aimed to investigate whether IL-2 gene polymorphisms and its serum levels are associated with HBV-related HCC. **Patients and Methods:** The -384 T/G polymorphisms in the IL-2 gene were examined in 25 cases of chronic hepatitis B (CHB), 25 cases of HBV-related liver cirrhosis (LC), 25 cases of HBV-related HCC, and 25 healthy controls by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) . The serum IL-2 levels were measured by enzyme-linked immunosorbent assay (ELISA). **Results:** The results showed that there were highly significant difference between HBV-related HCC patients and healthy controls regarding the genotype and allele frequencies of the IL-2 polymorphisms respectively. The TG (OR = 4.81, 95% CI, 1.14 – 20.25, $P = 0.03$) and GG (OR = 11.67, 95% CI, 2.13 – 64.04, $P = 0.003$) genotypes were correlated with a significant increased HCC risk as compared with the TT genotype and the G allele was correlated with a significant increased HCC risk when compared with the T allele (OR = 4.2, 95% CI, 1.81 – 9.73, $P = 0.001$). There was significant decrease in serum IL-2 levels between HBV-related HCC patients (177.78±71.7) and healthy controls (256.9±33.2). The genotypes of the IL-2 gene polymorphism were observed significantly correlated with serum IL-2 levels in HBV-related HCC patients with highly significant decrease in serum IL-2 levels in individuals with homozygous GG genotypes (107.7±.8 ng/L) or heterozygous TG genotypes (194.8±12.5 ng/L) than homozygous TT genotypes (306.3±29.8 ng/L) **Conclusion:** The results suggested that the IL-2 -384 T/G polymorphism might contribute to an increased risk of developing HBV-related HCC by affecting the serum IL-2 secretion.
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Key words: IL-2 polymorphisms, IL-2 serum levels, HBV-related Hepatocellular Carcinoma

1. Introduction:

Primary liver cancer is the fifth and the eighth most frequent cancer worldwide in men and women, respectively, accounting for 4% of all newly diagnosed cancers in both sexes and the dominant form of primary liver cancer is hepatocellular carcinoma (HCC), which is the third most common cause of cancer-related death in the world (1). In Egypt, HCC reports to account for about 4.7% of chronic liver disease patients (2).

Most HCC in high-risk areas can be attributed to hepatitis B virus (HBV) infections and aflatoxin exposure (3).

It is believed that immune system-mediated chronic inflammation of the liver can lead to HCC development because the former induces continuous cell death, resulting in cell proliferation and increased frequency of genetic alterations (4). Thus, ineffective immune response can be a principal oncogenic factor in a chronic HBV- or HCV-infected individual. If the T-cell response in an infected individual is strong enough, HBV or HCV can be eliminated from the liver; if not, a procarcinogenic effect can be induced by permanently triggering necroinflammatory disease without resulting in final eradication of HBV or HCV from the liver. The inflammatory response is mediated by cytokines (5).

Interleukin-2 (IL-2), secreted by Th1 cells, plays a central role in the activation of T cell-mediated immune responses (6). IL-2 is a cytokine involved in the regulation of proliferation and functional activities of T-cell and NK-cell. It functions as a T cell growth factor that can augment NK cell cytolytic activity and promote immunoglobulin production by B cells (7). In addition, IL-2 contributes to the regulatory T cells development and regulates the expansion and apoptosis among activated T cells (8). With respect to its relation to cancer, IL-2 has been reported to play an essential role in antitumor immunity (9). Clinical studies have shown that the IL-2 gene transfected into tumor cells can enhance both specific and nonspecific antitumor

immune responses (10). In addition, recombinant IL-2 has been shown to be a promising agent for the activation of immune response against tumors (11).

These observations suggested that the IL-2 gene might be a candidate for cancer gene therapy or treatment. The gene encoding IL-2 is located on chromosome 4q26–q27 in humans. The IL-2 gene consists of five exons and five introns. Genetic polymorphisms in the IL-2 gene may affect IL-2 production or protein expressions thus modulate cancer risk. Recently, genetic polymorphisms of the IL-2 gene have been associated with the susceptibility to a range of cancers, including nasopharyngeal carcinoma (12), head and neck cancer (13), and bladder cancer (14).

Many studies have investigated the association between IL-*2* polymorphism and cancer risk; however, the results remain controversial (15).

2. Patients and methods

1- Study population:

In brief, this was a hospital-based case-control study performed in a total of 100 subjects involving 75 patients and 25 healthy controls with matched age and sex, their ages ranged between 40 – 72 years. They were randomly selected from Menoufia University Hospitals in the duration between May 2014 and November 2015, based on the following inclusion criteria:

1. Chronic hepatitis B patients confirmed by consistent detection of HBV-DNA (6 months), positive HBsAg, Total anti-HBC ,HBeAg by third generation enzyme immunoassay ,elevated liver enzyme ALT & AST > 40 IU/ L and histological examination performed by an experienced pathologist.

2. Negative for hepatitis C Antibodies.

3. Negative for antibodies to human immunodeficiency virus type 1 and type 2. Exclusion criteria were: Patients with chronic liver diseases of different origin than hepatitis B viral etiology, past and present substance abuse.

The study was approved by the ethics committee of our medical faculty and written informed consent was obtained from all subjects before study entry.

The studied patient's were divided into 4 groups:

Group I: included 25 chronic HBV infected patients (15 males and 10 females), their ages ranged between 45 – 72 years.

Group II: included 25 patients with liver cirrhosis on top of chronic HBV (12 males and 13 females), their ages ranged between 49 – 62 years.

Group III: included 25 patients with HCC on top of HBV (14 males and 11 females), their ages ranged between 48 – 66 years with AFP ≥ 400 ng/ml.

Group IV: included 25 apparently healthy age and gender matched subjects as a control group (12 males and 13 females), their ages ranged between 41- 67 years.

2- Methods and techniques: For all subjects, the followings were done:

- 8 ml blood sample were collected from each individual after 12 hours fasting, under aseptic condition by clean veinupuncture without venous stasis. It was divided into two parts:

a - 4 ml were added to an EDTA-contained sterile tube for the determination of IL-2 polymorphisms. The sample were stored in the refrigerator for not more than a week till extraction of DNA or stored as a cell pellet on lysate for longer period at -80º C till extraction.

b - 4 ml were added to a sterile plain tube for immediate assessment of serum ALT, AST ,alpha fetoprotein and hepatitis B viral markers . The blood was left to clot at 37ºC and rapidly centrifuged at 4000rpm for 10 min.

Biochemical tests for detection of serum ALT and AST were done on AU- 480 autoanalyser using kit supplied by Beckman using the IFCC reference methods.

1- Serum HBsAg, Total **anti-HBC** , **HBeAg** & **AFP** were done by mini VIDAS systems (bioMérieux, Marcy l'Etoile, France) which is an automated enzyme-linked fluorescent immunoassay (ELFA) based on a one-step immunoassay sandwich method and a final fluorescent detection step for the quantitative measurements.

Two ready-to-use reagents; The SPR® Solid Phase Receptacle serves as a solid phase and pipetting device, coated with mouse monoclonal anti-human immunoglobulins and the reagent strip contains pre-dispensed reagents.

2- Serum IL-2 was determined by the Enzyme-Linked Immunosorbent Assay (ELISA) using IL-2 ELISA kit (IBL international, Germany). The assay uses a quantitative sandwich enzyme immunoassay technique that measures human IL-2. Standards and samples were sandwiched by the immobilized antibody and biotinylated polyclonal antibody specifc for IL-2, which was recognized by a streptavidin–peroxidase conjugate. All unbound material was then washed away and a peroxidase enzyme substrate was added. The color development was stopped and the intensity of the color was measured.

3- Genotyping: Determination of IL-2 - 384T/G gene polymorphism by PCR- RFLP

Blood for genotyping was drawn into EDTA-containing receptacles. Genomic DNA was prepared from peripheral blood leukocytes according to the standard procedure (the standard phenol–chloroform method), dissolved in 300 uL of Tris–HCl buffer (10 mmol/L, pH8.0) containing 1 mmol/L ethylenediaminetetraacetic acid (EDTA) and stored at -20 C until use. To analyze polymorphic sites, we used

separate PCR analyses followed by subsequent restriction fragment length polymorphism (RFLP) analysis. The primer sets used were: Forward primer: 5-ATTCACATGTTCAGTGTAGTTCT-3 and Reverse primer: 5- GTGATAGCTCTAATTCATGC-3. Briefly, 300 ng of DNA was added to a PCR mixture (final volume of 50 ul) containing 1.5 mM MgCl2, 50 pmol of each primer, 200M dNTPs, and 1 U of Taq polymerase (Promega, Madison WI, USA) in a reaction buffer recommended by Promega. The PCR conditions for the IL-2 - 384T/G RFLPs were one cycle at 95 C for 5 min followed by 35 cycles of 30 s at 94 C; 45 s at 56 C, and 50 s of elongation at 72 C, followed by a final elongation step of 72 C for 7min. Negative controls (no DNA added) were included in every PCR run to check for contamination. The PCR amplified products were digested for over night with 5-10 U of the appropriate Bfa I restriction enzyme at 37 C, and the fragments were analyzed using electrophoresis on 2.0% agarose gel. The alleles are named according to the presence or absence of the restriction site (FigI).

4- Statistical analysis:

The SPSS statistical software package version 13.0 was used for all of the statistical analyses.

The Chi square test and Fisher's exact test when appropriate were used or comparing qualitative data. Odds ratio (OR) and 95% confidence intervals (CIs) were calculated by binary logistic regression to assess the odds ratio conferred by a particular allele and genotype. One-way ANOVA test was used to test the difference between normally distributed quantitative data among the studied groups. Kruskal Wallis test was used to test the difference between not normally distributed quantitative data among the studied groups.

All tests were two-tailed, statistical significance was assumed at the $P < 0.05$ level.

3. Result:

There was no significant difference in age, gender distribution between the cases and controls, suggesting that subject matching based on these variables was adequate as shown in table 1.

Gene polymorphism and allele among the studied groups:

The results showed that there were no significant differences between CHB patients and HBV-related liver cirrhosis patients when compared with healthy controls regarding the genotype and allele frequencies of the IL-2 -384 polymorphisms respectively .However, there were significant difference and highly significant d T/G difference between HBV-related HCC patients and healthy controls the genotype and allele frequencies of the IL-2 -384 T/G polymorphisms respectively. The TG (OR = 4.81, 95% CI, 1.14 – 20.25, $P = 0.03$) and GG (OR = 11.67, 95% CI, 2.13 – 64.04, $P = 0.003$) genotypes were correlated with a significant increased HCC risk as compared with the TT genotype and the G allele was correlated with a significant increased HCC risk when compared with the T allele (OR = 4.2, 95% CI, 1.81 – 9.73, $P = 0.001$) as shown in table 2.

Serum IL-2 levels:

The results showed that there were no significant differences between CHB patients and HBV-related liver cirrhosis patients when compared with healthy controls regarding Serum IL-2 levels. Also, the results showed that there were no significant differences between Serum IL-2 levels between CHB patients and HBV-related liver cirrhosis patients. However, there was significant decrease in serum IL-2 levels between HBV-related HCC patients (177.78±71.7) and healthy controls (256.9±33.2) as shown in table 3 and Fig II.

Association of IL-2 gene polymorphisms and serum IL-2 levels

There was no significant association between the IL-2-384 T/gene polymorphisms and serum IL-2 levels in healthy controls, CHB and HBV related liver cirrhosis. However, the genotypes of the IL-2 gene polymorphism were observed significantly correlated with serum IL-2 levels in HBV-related HCC patients with highly significant decrease in **serum IL-2 levels** in individuals with homozygous GG genotypes (107.7±.8 ng/L) or heterozygous TG genotypes (194.8±12.5 ng/L) than homozygous TT genotypes (306.3±29.8 ng/L) as shown in table 4 and Fig III.

Table 1: age and sex distribution among the studied groups

	Control N =25	Chronic hepatitis B patients N = 25	Liver cirrhosis on top of HBV N = 25	HCC on top of HBV N = 25	Test	*P* value
Age Mean±SD Range	54.88±7.10 40 – 67	55.6±6.61 45 – 72	55.388±4.29 48 – 62	58.68±5.25 48 – 66	ANOVA 1.98	0.12
Sex Male Female	12 (48.0) 13 (52.0)	14 (56.0) 11 (44.0)	12 (48.0) 13 (52.0)	14 (56.0) 11 (44.0)	X^2 0.64	0.89

SD = standard deviation, ANOVA = one way ANOVA test, X^2 = chi square test

Table 2: gene polymorphism and allele among the studied groups

	Control N =25	Chronic hepatitis B patients			Liver cirrhosis on top of HBV N = 25			HCC on top of HBV		
		N = 25 (%)	OR (95% CI)	*P*	N = 25 (%)	OR (95%CI)	*P*	N = 25 (%)	OR (95% CI)	*P*
Gene Polymorphism			REF (1) 0.51 (0.14 – 1.93)			REF (1) 0.66 (0.18 – 2.36			REF (1) 4.81 (1.14 – 20.25)	
TT	14(56)	17(68)			16 (64)			4 (16)		
TG	8 (32)	5 (20)	0.82 (0.14 – 4.74)	0.32	6 (24)	0.88 (0.15 – 5.05)	0.52	11 (44)	11.67(2.13 – 64.04)	0.03
GG	3 (12)	3 (12)		1.0	3 (12)		0.88	10 (40)		0.003
Allele	N=50	N=50	REF (1)		N=50	REF (1)		N=50	REF (1)	
T	36 (72)	39 (78)	0.73 (0.29 – 1.80)	0.49	38 (76)	0.81 (0.33 – 1.99)	0.65	19 (38)	4.2 (1.81 – 9.73)	0.001
G	14 (28)	11 (22)			12 (24)			31 (62)		

X^2 = chi square test

Table 3: interleukin 2 level among the studied groups

	Control N =25	**Chronic hepatitis B patients N = 25**	**Liver cirrhosis on top of HBV N = 25**	**HCC on top of HBV N = 25**	U test	*P* value
IL 2 level					0.26	0.79[1]
Mean±SD	256.9±33.2	254.4±36.3	250.4±38.7	177.8±71.7	0.79	0.43[2]
Range	190 – 305	194 – 300	183 – 330	95 – 350	4.16	<0.001[3]
					0.65	0.52[4]
					4.01	<0.001[5]
					3.70	<0.001[6]

U = Mann Whitney U test
1 = comparing Chronic hepatitis B patients with control
2 = comparing Liver cirrhosis on top of HBV patients with control
3 = comparing HCC on top of HBV patients with control
4 = comparing Chronic hepatitis B patients with Liver cirrhosis on top of HBV patients
5 = comparing Chronic hepatitis B patients with HCC on top of HBV patients
6 = comparing Liver cirrhosis on top of HBV patients with HCC on top of HBV patients

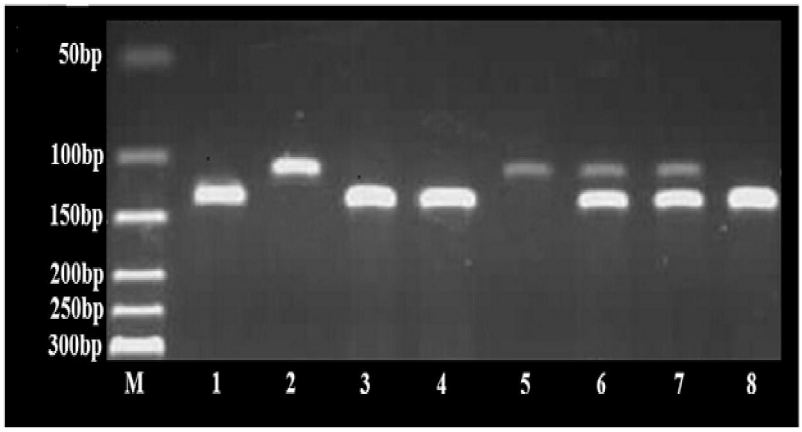

Fig. 1. Polymerase chain reaction–restriction fragment length polymorphism assay for analyzing IL-2 _384T/G polymorphism. Lane 1: negative control; lanes 3, 4, and 8 show _384 TT genotypes; lanes 6, and 7 show _384 TG genotypes; lanes 2, and 5 show _384 GG genotypes.

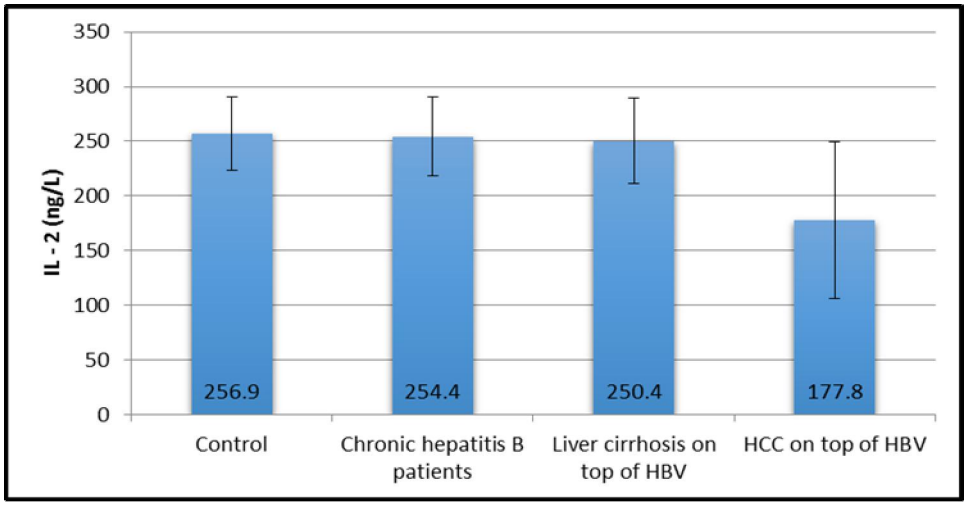

Fig II **: interleukin 2 level among the studied groups**

Table 4 **:Interleukin 2 level** in relation to gene polymorphism in different studied groups

	Interleukin 2 level			
	Control N =25	**Chronic hepatitis B patients N = 25**	**Liver cirrhosis on top of HBV N = 25**	**HCC on top of HBV N = 25**
Gene Polymorphism				
TT	267.9±25.2	254.6±35.7	257.7±31.5	306.3±29.8
TG	244.9±38.5	244.8±34.1	234.0±53.0	194.8±12.5
GG	238.0±44.2	269.0±52.0	244.7±47.2	107.7±.8
K test	3.18	2.16	1.12	20.38
***P* value**	0.20	0.34	0.57	<0.001

K = Kruskal Wallis test

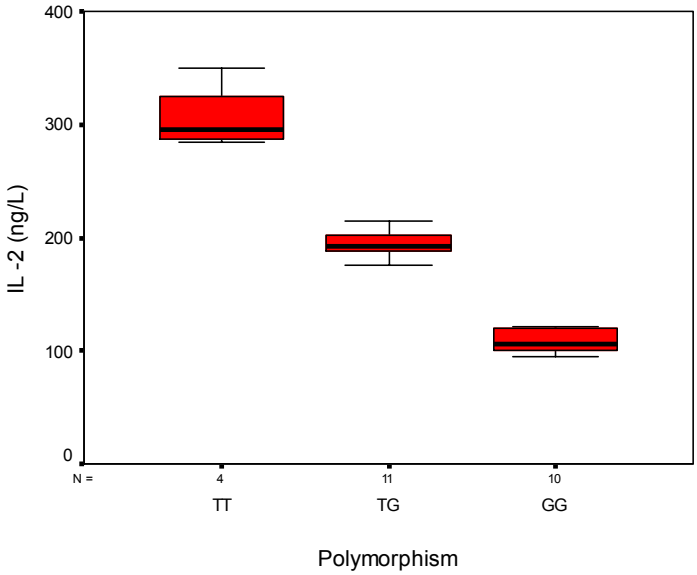

Fig III :Association between IL- 2 -384 T/G polymorphism and IL2 level among HCC patients group

4. Discussion

The effect of inflammation and inflammatory cells on the process of tumor development and progression is increasingly recognized (16).

It is now evident that a substantial proportion of cancer cases worldwide arise from infection and chronic inflammation (17). Both inflammatory and tumor cells produce an assorted array of cytokines and chemokines, which mediate all aspects of inflammation

and profoundly affect the development and progression of cancer (18).

IL-2 is a proinflammatory and immunoregulatory cytokine which plays a central role in the interaction between innate immunity and adaptive immunity by inducing IFN-c production and generating T helper cell 1 (Th1) responses and CTL. In several comparative studies, IL-2 has been revealed to be the most effective cytokine that could induce eradication of experimental tumors, prevent development of metastases, and elicit long-term anti tumor immunity (19**).**

Due to the important role of IL-2 in anti-tumour effect, polymorphisms in the IL-2 gene and susceptibility to different types of cancers have been extensively studied (20)**.**

In the present study we found that there was non-significant difference among CHB patients, HBV-related liver cirrhosis patients versus healthy controls as regarding the genotype and allele frequencies of the IL-2 -384 T/G polymorphisms. On the other hand we found significant difference and highly significant difference between HBV-related HCC patients and healthy controls as regards the genotype and allele frequencies of the IL-2 -384 T/G polymorphism respectively. The TG and GG genotypes were correlated with a significant increased HCC risk as compared with the TT genotype and the G allele was correlated with a significant increased HCC risk when compared with the T allele.

This is in agreement with ***Peng et al (2014)*** who found that there were no significant differences between the genotype polymorphisms and allele frequencies of the IL-2 gene, and CHB risk and HBV-related liver cirrhosis risk compared with control group. T, but on the other hand they found that there were significant differences in the genotype and allele frequencies of the IL-2 -384 T/G polymorphism between HBV-related HCC patients and healthy controls and that; TG and GG genotypes were correlated with a significant increased HCC risk as compared with the TT genotype (OR = 1.988, 95% CI, 1.034–3.480, P = 0.009 for TG genotype, and OR = 1.975, 95% CI, 1.012–3.341, P = 0.013 for GG genotype, respectively) and the G allele was correlated with a significant increased HCC risk when compared with the T allele.

In another study done by ***Wei et al (2008)*** they found that IL-2 gene T/G polymorphism is associated with chronic hepatitis B and G allele is an important genetic susceptibility gene for chronic hepatitis B. In IL-2 gene G allele carriers the risk of increasing HBV-DNA copies may be related to the pathogenesis of chronic hepatitis B.

Some studies have investigated the association of *IL-2* T/G polymorphism with cancer risk, but the previous results were conflicting and had relatively low statistical power. Thus, a study done by ***Zhao and Wang (2015)*** involving a total of ten studies including 3,060 cases and 3,435 controls were involved in this meta-analysis. The results indicated that *IL-2* -384 T/G polymorphism was significantly associated with cancer risk([OR =2.03, 95% CI =1.40–2.95] for GG vs. TT ; [OR =1.37, 95% CI =1.11–1.69] for GT vs. TT; [OR =1.46, 95% CI =1.18–1.81] for [GG + GT] vs. TT; [OR=1.66, 95% CI =1.24–2.23] for GG vs. [GT + TT] and [OR =1.35, 95% CI =1.16-1.57]for G vs. T.

This is in contrast to a study done by ***Gao et al (2010)*** who found that IL-2 T/T was associated with an increased risk, but IL-2 G/G was associated with reduced risk of persistent HBV and/or HCV infection, and with the development of mild CH, moderated/severe CH, and cirrhosis. Moreover, a study done by ***Yafeng et al (2015)*** showed that *IL-2* rs2069762 T>G polymorphism may not be correlated with cancer risk. In this study analogous results were found after stratified by cancer type and ethnicity and this was the first meta-analysis including a total of 3095 cases and 4480 controls from 10 case-control studies examining the association of *IL-2* rs2069762 T>G polymorphism with cancer risk. With the increase of gene association studies, it is even more encouraged to synthesize available data to solve persistent difficulties in obtaining replicable and robust results. Most common gene defects usually make small-to-moderate contributions to malignancy risk. So far, many studies had concentrated on the association between *IL-2* rs2069762 T>G polymorphism and cancer risk, but the results were still vague. Several individual studies have provided positive signals of *IL-2* gene rs2069762 T>G polymorphism with cancer, such as hepatocellular carcinoma, contrastingly, as illustrated in this meta-analysis among 3095 cases and 4480 controls, there was null association, even in different ethnicity and cancer type. In consideration of a modest sum of studies included in this meta-analysis and most of small sample size, there is a suggestion of possible beneficial, reinforcing additional large studies to overthrow or confirm these findings. It is also possible that the latent role of this polymorphism is masked or diluted by other gene-environment or gene-gene interactions. Carcinogenesis is a multifactorial process involving interaction between genetic and environmental factors.

In the subgroup analysis of ethnicity ***Yafeng et al (2015)*** found no obvious association was found among Caucasians and Asians, which was consistent with the overall data. Variations on various genes may alter cancer susceptibility among different ethnicities. Thus, Variations of *IL-2* rs2069762 T>G polymorphism may exert different influences on cancer risk among different races. Nevertheless, the results of this study suggested that the polymorphism variations may exert

little influence on cancer susceptibility. In this meta-analysis, only two studies about Caucasians were obtained. The insufficient number may give rise to limited statistical power to assess race effect of gene. Therefore, the results should be interpreted carefully.

The present study showed that there were no significant differences between CHB patients and HBV-related liver cirrhosis patients when compared with healthy controls regarding serum IL-2 levels. Also, the results showed that there was no significant difference between CHB patients and HBV-related liver cirrhosis patients regarding serum IL-2 levels. this is not in agreement with a study done by ***Antonia et al (2014)*** who found that, IL-2 levels in HBV patients were higher than in healthy controls and this difference was statistically significant ($p < 0.05$) and high positive correlation was observed between IL-2 levels and AST, ALT and GGT in HBV patients, suggesting that IL-2 expression correlates with the degree of necroinflammation. Also, in a study by ***Bozkaya et al (2000)*** all HBV patients groups had higher IL-2 levels compared to controls suggesting that IL-2 production is increased when liver disease becomes active in HBeAg-positive phase of HBV infection and that circulating cytokine profile in chronic hepatitis B is related with the HBeAg status, replication level of the virus and the activity of liver disease a result that coincides with that of ***Debnath et al (2005)*** which supported the observation that IL-2 level can be used as a marker of activity in CHB patients as IL-2 level was significantly detected in the CHB patients with raised Alanine aminotrasferase (ALT) (> 80 iu/L).

In the present study there was significant decrease in serum IL-2 levels between HBV-related HCC patients (177.78±71.7) and healthy controls (256.9±33.2).

This is in agreement with ***Peng et al (2014*)** who found that the serum IL-2 levels were significantly lower in HBV-related HCC patients compared with the healthy controls suggesting that a depressed IL-2 response might play a role in HBV-related HCC etiology by reducing Th1 cytotoxic response to the delayed acquisition of common anti-tumor activities.

The present study shows that there was no significant association between the IL-2 gene polymorphisms and serum IL-2 levels in healthy controls, CHB and HBV related liver cirrhosis. However, the genotypes of the IL-2 gene polymorphism were observed significantly correlated with serum IL-2 levels in HBV-related HCC patients with highly significant decrease in **serum IL-2 levels** in individuals with homozygous GG genotypes (107.7±.8 ng/L) or heterozygous TG genotypes (194.8±12.5 ng/L) than homozygous TT genotypes (306.3±29.8 ng/L). This agrees with a study done by ***Peng et al (2014)*** who demonstrated that the serum IL-2 levels were significantly reduced in heterozygous TG genotypes or homozygous GG genotypes than in individuals with homozygous TT genotypes. And they suggested that the IL-2 gene polymorphism may contribute to an increased HBV-related HCC risk through regulating the expression of serum IL-2 levels. Thus, genotypes carrying the IL-2 G variant allele had a decreased ability to produce IL-2, which may contribute to HCC susceptibility.

In conclusion

we found that the IL-2 T/G polymorphism might contribute to an increased risk of developing HBV-related HCC by affecting the serum IL-2 secretion. Additional studies with larger sample sizes will be necessary to confirm our findings. Because genetic polymorphisms often vary between different ethnic groups, further studies in diverse ethnic populations are needed to clarify the association of the IL-2polymorphisms with HBV-related HCC risk.

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