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Interleukin-27 levels and its Polymorphism in Hepatocellular carcinoma associated with Hepatitis C

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Abstract

The prevalence of Hepatocellular carcinoma (HCC) in Egypt represents about 11% of the gastrointestinal tract malignancies. The development of HCC usually is preceded by chronic infections with Hepatitis C virus (HCV). Interleukin-27 (IL27) is implicated in the antiviral immunity. Little is known about the use of IL27 as a biomarker for the development of HCC associated with HCV. Moreover, the role of IL-27 rs153109 gene polymorphism in the development of HCC associated with HCV has not been well studied yet. A case control study included three groups; Group I, 60 healthy control patients, Group II, 60 patients with cirrhosis associated with HCV infection, and Group III, included 60 patients with HCC associated with HCV. ELISA technique was used to measure serum IL27, while restriction fragment length polymorphism (RFLP) was used to detect single-nucleotide polymorphisms (SNP). Results showed that the serum levels of IL27 is significantly higher in both cirrhotic and HCC groups. G allele is significantly associated with HCC and cirrhotic cases, compared with the healthy control group. Current results encourage the use of IL 27 as a diagnostic marker for both cirrhotic and HCC diseases. The aims of current work were to study the serum levels of IL27 in HCV positive hepatic patients, to evaluate its role as a biomarker for early detection of HCC, and to find any association of rs153109 with the hepatic diseases.

Keywords: IL27, PCR, RFLP, ELISA, HCC, HCV

1. Introduction

Hepatocellular carcinoma (HCC) is defined as a common malignancy that spreads worldwide. Dai *et al*., (2014) reported that it represents the sixth most prevalent cancer and the third common cause of deaths due to malignancy. In Egypt, the prevalence of HCC is around 11 % of the gastrointestinal tract malignancies, and represents about 2 % of all malignancies (Mokhtar *et al*., 2007) The development of HCC is usually proceeded by chronic infections with hepatitis C HCV) or/and hepatitis B viruses (HBV) (Gomaa *et al*., 2014).

Human viral infections are controlled by the immune system, especially cytokines. Cytokines production is subjected to genetic variations among the affected individuals. Variations in a single nucleotide due to single-nucleotide polymorphisms (SNPs) in the cytokine genes affect cytokine productions/ functions, leading to the disturbance of the immune response to the viruses infections, and may contribute to HCC development (Dondeti *et al*., 2016).

Hui and Lau, (2005); Ikeguchi and Hirooka, (2005) studied the various cytokines polymorphism and their relations to the development of HCC including; IL-1β, TNF-α, IFN-γ, IL-6, IL-10, IL-15, IL-18, TNF-α, IFN-α, and TGF-β1.

Interleukin-27 (IL27) together with other cytokines, are implicated in the antiviral immune response. IL-27 is a comprising of p40-related protein, EBI3, and IL-12 p35-related protein, p28. In a previous study, Owaki *et al*., (2006) reported that IL27 is secreted from the activated antigen presenting cells after viral infections, and mediates antiviral action through activation of the T helper 1 lymphocytes, and later leads to an anti-inflammatory reaction by secretion of IL10.

Moreover, Raphael *et al*., (2015) added that IL-27 has broad stimulatory as well as inhibitory effects on Th1, Th2, Th17, Treg, and Tr1 subsets of T cells, NK cells and B cells. When IL-27 becomes organized with other IL-12 family cytokines, it coordinates inflammatory processes and stands immune polarization. Recent reports by Zicca *et al*., (2014); Zhang *et al*., (2015) hypothesized that IL-27 has antitumor activity through impairment of angiogenesis, by induction of IFN-γ inducible protein-10 (IP-10) and enhancement of lymphocytes cytotoxic activity.

The IL-27gene is located on chromosome 16p11, and contains five exons. Several reports propose a role of IL-27 polymorphisms in cancer threat such as; esophageal cancer (Tao *et al*., 2012), ovarian cancer (Zhang *et al*., 2014), nasopharyngeal carcinoma (Wei *et al*., 2009), hepatocellular carcinoma (Peng *et al*., 2013) and glioma (Zhao *et al*., 2009). Jankowski *et al*., (2010) pointed that the expression of IL-27 is increased in several diseases including; tuberculosis, Cohn's disease, multiple sclerosis, uveitis, rheumatoid arthritis, psoriatic arthritis, and systemic sclerosis.

Activation of the NK cells by small quantity of IL-27 produces IFN-δ, which in turn activates the antigen presenting cells (APCs) in the presence of a microbial agent. This induces the production of much larger amounts of IL-27, which have both local and general effects on APCs and lymphocytes in the ensuing adaptive phase of the immune response (Ziblat *et al*., 2015).

To the best of our knowledge, this is the first study that investigates the influence of IL27 rs153109 on IL27 gene expression/ function, susceptibility to and severity of cirrhosis and HCC in Egyptians HCV patients. The objectives of the present study were; to compare the serum levels of IL27 in patients with cirrhosis and HCC associated with hepatitis C, to evaluate IL27 role as a biomarker for early detection of HCC, and to study the genetic polymorphism of IL27 rs153109 associated with cirrhosis and HCC compared to the healthy control.

2. Material and methods

2.1. Materials

The study is a case-control conducted at Mansoura University hospital, Egypt. Samples were collected from out patient's clinics from January 2016 - January 2017. Moreover, healthy control participants were included in the study. The study protocol conformed to the 1975 Declaration of Helsinki, and was approved by the institutional review boards of Mansoura Faculty of Medicine ethical committee IRB R/17.12.54, and the informed consent was obtained from each participant.

The study included three groups; Group I, thirty health control participants; Group II, were of thirty patients with cirrhosis associated with HCV infection; and Group III, included thirty patients with HCC

associated with HCV. Staging was performed according to the clinical and laboratory data, radiological investigations including ultrasound and computed topography (CT) were indicated and reported according to the Barcelona Clinic Liver Cancer (BCLC) staging system. Inclusions criteria for the patients were patients >18 years with cirrhosis, and HCC associated with HCV. This was in addition to patients with hepatitis B virus, patients with liver diseases due to causes other than HCV, patients with HIV and patients with other cancer types.

2.2. Blood samples

A sample of ten milliliter of blood was obtained under complete sterile conditions from each participant. This sample was divided into three aliquots; one aliquot of 2 ml over EDTA for the genomic DNA separation from the mononuclear cells for IL27 gene polymorphism. The second aliquot of the blood was subjected to sera separation for routine laboratory study of; complete liver functions by auto-analyzer (Dialab 450 system, Austria), determination of circulatory anti-HCC by Elecys system (Roche-diagnostic), and alpha fetoprotein (AFP) measurement by enzyme linked immunosorbant assay (ELISA - DRG International Inc., USA.). The third aliquot of blood sample was subjected to sera separation, and then the sera was kept frozen at -20°C for further measurement of IL27 by ELISA using invitrogen kit (Thermofisher-scientific).

2.3. IL27 p28 964A/G rs153109 Gene Polymorphism

The DNA was extracted from the peripheral blood mononuclear cell (PBMCs) using a DNA Purification Kit (Qiagen-Germany) CAT no 51304, according to the manual of the manufacturers. Amplification of the required region of the gene was performed by polymerase chain reaction (PCR) using the primers; Forward: 5- GGCTGTGCTGGAAGGGAGAC-/3, and Reverse: 5-ATATCTGGGACCAGGGTTAGG-/3, having amplicon size of 468 bp. The amplification volume was 25 microns with the use of 10 pmol/ l of each primer, and 0.1 µg DNA. The amplification mixtures were supplied by Qiagen.

The PCR cycling was performed as follows; 1 cycle of 94°C for 5 min., followed by 35 cycles of 94°C for 1 min., 56°C for 45 sec, 72°C for 1 min., and a final extension step at 72°C for 10 min. per cycle. PCR reaction was performed in a Biometra thermal cycler (Biometra GmbH, Germany). The 468 bp PCR products were visualized on 2 % agarose gel. About 15 µl of the PCR product was subjected to digestion with XhoI restriction enzyme (Fermentas, Vilnius).

2.4. Statistical analysis

Data were coded and entered using the statistical package SPSS (Statistical Package for the Social Sciences) version 23. Data was summarized using median, minimum and maximum in quantitative data, using frequency (count) and relative frequency (percentage) for categorical data. Comparisons between quantitative variables were done using the non-parametric Kruskal Wallis and Mann-Whitney tests. For comparing the categorical data, test was performed. Exact test was used instead when the χChi square expected frequency is less than 5.

Correlations between quantitative variables were done using Spearman correlation coefficient. Genotype and allele frequencies were compared between each two groups by chi-square tests. Odds ratio (OR) with 95 % confidence intervals was calculated. ROC curve was constructed with area under curve analysis performed to detect best cutoff value of IL27 for detection of HCC. Multivariate logistic regression analysis was done to detect the independent predictors of HCC. Statistically significant *p*-values were less than 0.05.

3. Results

The present study comprised of 60 patients with liver cirrhosis (LC) and 60 HCC cases, in addition to 60 healthy control participants of matched age and gender. These samples of individuals were selected randomly from population in Dakahleya Governorate

in lower Delta, Egypt. Applying Hardy Weinberg equation, revealed that the studied genotypes in each group were in HW equilibrium value >0.05. All the HCC and cirrhotic patients were HCV-Ab positive, HBV-Ag and HIV-Ab negative, and all groups were of matched age and gender.

The studied biochemical markers in cirrhotic patients showed significantly higher aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities, total bilirubin, alpha fetoprotein (AFP) and IL27 levels. On the other hand, they demonstrated significantly lower WBC and platelets accounts, Hb concentration and albumin level, in comparison with the control group. However, the HCC cases presented significantly higher activities of AST, ALT, AFP and IL27 levels. On contrast, they expressed significantly lower WBC and platelets accounts and albumin level, when compared to the control group. On comparing the cirrhotic cases to the HCC group, they exhibited significantly higher total and direct bilirubin levels, significantly lower platelets accounts, albumin, AFP and IL27 levels, as clear in Table (1).

The median serum level of IL27 in the cirrhotic patients is 6.6 pg/ ml (range: 3.8-11), it increased several folds in the HHC group as its median reached 122.5 (range: 110.5-225) pg/ ml, and has a high statistical difference ($p < 0.001$). The AFP median level in the cirrhotic patient is 11 ng/ ml (range: 0.7-69), it increased several folds in the HCC group as its median is 87.5 ng/ ml (range: 2.3- 447), and also showed significant statistical difference ($p < 0.001$). However, we realized an overlap in the range of serum levels with the normal levels, which we are unable to depend on AFP alone in diagnosis of HCC as shown in Table (1).

The DNA fragments of different genotype bands were, one band of 468 bp for A/A, two band for GG at 347/121 bp, and three bands at 468/347/121 bp for A/G.

Considering AA genotype, A allele as references; AG, GG, AG+GG genotypes, G allele showed significantly higher frequency in cases of the liver cirrhosis group, and in the HCC group, compared to the control group, $p < 0.05$ (Table 2). The stratified analysis of the combined genotype AG, GG, AG+GG versus AA in HCC, according to the; sex, age, tumor site, size, grade and BCLC showed no significant differences. However, the cirrhotic patients AG+GG genotypes have appreciable difference in relation to the gender i.e.: is more common in females, child pugh classes, child C class, lower RBG and AFP, in comparison with AA genotypes as shown in Table (2).

The ROC curve was used to assess the sensitivity of both AFP and IL27, for discrimination between the cirrhotic and HCC patients. They were measured and the area under the curve AUC is 0.893 and 1, respectively. The AFP cut off value is >55.4ng/ ml with a sensitivity of 56.7 % and specificity of 96.7 %. With regard to IL 27, it has a cut off value of > 60.75 ng/ ml, the sensitivity is 100 % and the specificity is 100 % ($p < 0.001$), when comparing both parameters in the cirrhotic and HCC patients (Table 3). The IL27 levels are higher in the HCC group compared to cirrhotic group, and this is statistically significant, but the genotypes and alleles associated with IL 27 showed no-significance (Fig. 1).

Regression analysis was conducted for prediction of LC and HCC within control participants using age, gender, smoking, AST, ALT, AFP, IL27, and genotypes as covariates. Higher AST, ALT, AFP, IL27, AG+GG genotypes are significantly associated with prediction of LC within the control group in univariable analysis. Taking those variables with significant association in univariable analysis into multivariable analysis, revealed that AFP and IL27 are independent prognostic factors for prediction of LC within the healthy control group. However, only AFP and IL27 are independent prognostic factors for prediction of HCV and HCC within the healthy control group.

Table (1): Comparison of the laboratory data including CBC liver kidney function tests, and IL27 with AFP between all the studied groups

	Median Control	**Median LC**	**Median HCC**	***P1***	***P2***	***P3***	***P4***
WBC	7.2 (4.2-13.5)	4.3 (1.5-8.2)	5.4 (2.52-27.2)	< 0.001	< 0.001	0.002	0.061
HGB	12.4 (11- 17.1)	12.1 (8-16.2)	13.1(8.8- 17.5)	0.015	0.007	0.413	0.073
PLT	223 (125-354)	75 (32-168)	146 (35.5- 366)	< 0.001	< 0.001	<0.001	< 0.001
INR	1.2 (1-14)	1.5 (1-4.3)	1.1 (1-2.3)	0.056	0.162	0.276	0.063
Sodium		138 (133-143)	139.5 (130-144)	< 0.001			0.432
Potassium		4 (3.5-4.7)	4.3 (3.3- 5.5)	< 0.001			0.387
RBG		114 (65-221)	99.5 (23-189)	0.029			0.129
Creatinine	0.9 (0.8-1.3)	0.8 (0.5-1.7)	0.8 (0.6- 1.8)	0.182	0.263	0.078	0.858
Albumin	3.8 (3.2-5)	3.1 (2.2-4.2)	3.7 (2.7-4.8)	< 0.001	< 0.001	0.013	0.062
AST	27 (21-36)	64 (20-93)	65 (21- 596)	< 0.001	< 0.001	< 0.001	0.150
ALT	28 (20-37)	38.5 (20-81)	48 (20- 685)	< 0.001	< 0.001	< 0.001	0.006
Tbilq	0.8 (0.7-1)	2.90 (0.7-14.3)	0.9 (0.5-1.8)	< 0.001	< 0.001	0.238	< 0.001

DBil		1.6 (0.5-9)	0.4 (0.2-0.9)	< 0.001			< 0.001
AFP	6 (3-9)	11(0.7-69)	87.5 (2.3- 447)	< 0.001	0.002	< 0.001	< 0.001
Il27	1.8 (1-3.4)	6.6 (3.8-11)	122.5 (110.5-225)	< 0.001	<0.001	< 0.001	< 0.001

Where; P1: comparison between 3 groups; P2: comparison between control and LC; P3: comparison between control and HCC; P4: comparison between LC and HCC. The LC cases showed significantly higher AST, ALT, total bilirubin, AFP, IL27, significantly lower WBC, hb, plt, albumin, when compared to the control group. The HCC cases expressed appreciable higher AST, ALT, AFP, IL27, and lower WBC, plt, albumin, in comparison with the control group. The LC cases exhibited higher total and direct bilirubin, significantly lower plt, albumin, AFP, IL27, compared to the HCC group

Table 2: IL27 polymorphic alleles between LC and HCC in relation to the control groups

	Control		**LC**		**P**	**OR**	**95% CI**	
	N=60		**N=60**					
AA	31	51.7	16	26.7		1		
AG	21	35.0	30	50.0	0.014	1.886	1.136	3.131
GG	8	13.3	14	23.3	0.022	2.138	1.116	4.099
AG+GG	29	48.3	44	73.3	0.005	1.958	1.223	3.134
A	83	69.2	62	51.7		1		
G	37	30.8	58	48.3	0.006	2.099	1.238	3.556
	Control		HCC		p	OR	95 % CI	
	N= 60		N= 60					
AA	31	51.7	18	30.0		1		
AG	21	35	28	46.7	0.043	1.680	1.016	2.778
GG	8	13.3	14	23.3	0.036	1.989	1.044	3.788
AG+GG	29	48.3	42	70.0	0.016	1.769	1.113	2.813
A	83	69.2	64	53.3		1		
G	37	30.8	56	46.7	0.012	1.963	1.158	3.328

Where; OR: odds ratio, CI: confidence interval

Table 3: AUC and performance characteristics of the AFP and IL27 concentrations, for discrimination between the different studied groups

	Control and LC		**Control and HCC**		**LC and HCC**	
	AFP (ng/ml)	IL27	AFP (ng/ml)	IL27	AFP (ng/ml)	IL27
AUC	0.666	1	0.893	1	0.777	1
P	0.002	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
95% CI	0.560-0.773	1-1	0.825-0.962	1-1	0.693-0.861	1-1
Cut off value	10	3.6	19.4	56.95	55.4	60.75
Sensitivity (%)	53.3	100	83.3	100	56.7	100
Specificity (%)	100	100	100	100	96.7	100
PPV (%)	100.0	100	100	100	94.4	100
NPV (%)	68.2	100	85.7	100	69.0	100
Accuracy (%)	76.7	100	91.7	100	76.7	100

Where; AUC: area under ROC curve, PPV: positive predictive value, NPV: negative predictive value

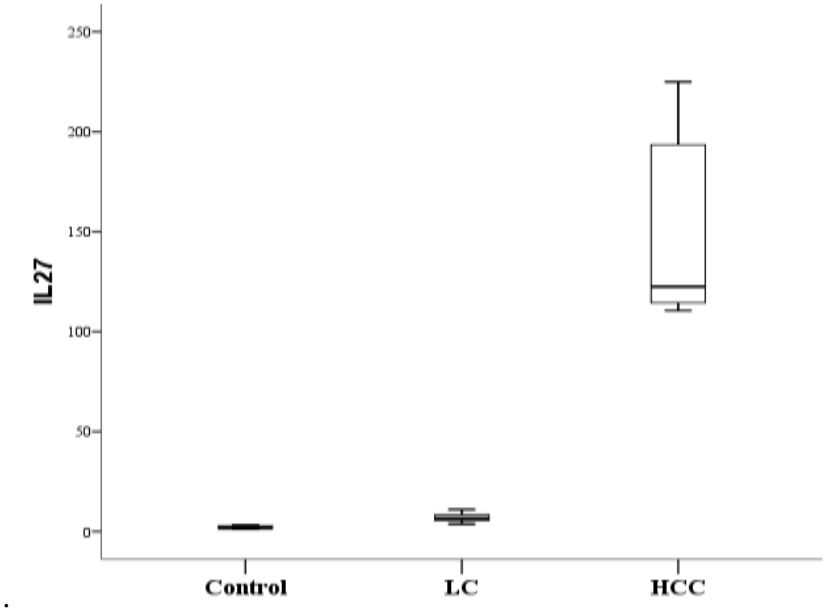

Fig. 1. IL27 concentration in all the studied groups

For prediction of HCC within the LC cases, same regression analysis was conducted and revealed that a higher AST, ALT, AFP, IL27, AG+GG genotypes are significantly associated with prediction of LC within the HCC group in univariable analysis. Taking those variables with significant association in univariable analysis into multivariable analysis, revealed that AFP and IL27 are independent prognostic factors for prediction of HCC within LC group as obvious in Table (4).

Table 4: Regression analysis for predicting HCC within the LC cases

	Univariable				Multivariable			
	p	**OR**	**95 % CI**		***p***	**OR**	**95 % CI**	
Age	0.645	0.987	0.935	1.043				
Gender	0.142	0.550	0.247	1.221				
Smoking	0.366	1.418	0.665	3.022				
AST	0.012	1.013	1.003	1.024	0.072	1.514	0.865	1.967
ALT	0.003	1.027	1.009	1.045	0.973	0.999	0.927	1.076
AFP	0.002	1.027	1.010	1.044	0.049	1.320	1.046	1.965
Il27	< 0.001	1.026	1.014	1.038	0.008	1.442	1.101	1.889
Gene	0.685	0.902	0.548	1.484				

4. Discussion

The most collective sources of human genetic variants are the single nucleotide polymorphisms, and this is due to the individual predisposition to HCC. IL27 has an anti-proliferative and anti-angiogenic effects by direct action on the tumor cells (Lyu *et al*., 2015). The main function of IL27 is to induce the proliferation of naïve T- lymphocytes, and synergize with IL12 to produce IFN-δ by activated naïve cells and natural killer cell (NK). According to Hisada *et al*., (2004), the principle function of IL27 is the promotion of T cells helper 1 function toward tumor cells, and this function was supported by experimental animal's studies.

Thus, the polymorphism of IL27 promoter region may have an impact on the role of IL27 on patients having HCC associated with HCV infection. In their review, Dondeti *et al*., (2016) found only few studies on the IL-27 gene polymorphism, and the hepatitis-related HCC risk. Previously, Ali et al., (2014) studied the IL27 gene polymorphism and the hepatitis- related HCC risk, and reported that IL27 gene polymorphism is not contributing to HBV infection. Similarly, this was supported by Peng *et al*., (2013) who studied the

same association in Chinese HBV- related HCC patients. In a similar study of Zicca *et al*., (2014) as they used direct sequencing on the amplified DNA to detect rs153109 genotypic distribution in only 15 patients with chronic hepatitis C. They were 5 patients A/A, 9 patients A/G and only 1 patient GG genotype, and they stratified the patients in relation to their viral response into responder, relapser and non-responder after treatment with peginterferon-a (PegIFN- a) combined with ribavirin (RBV). The analysis of allele distributions of the SNPs shows a prevalence of allele A in all genotypes in spite of allele G.

Moreover the study of Fawzy *et al*., (2016) included 111 patients with chronic HCV infection, and assessed their genotypes of IL27rs153109 by PCR-RFLP. They reported similar genotypic distribution to the current results, and a higher IL27-AA frequency followed by AG, and GG. They concluded that HCV infected patients with the GG genotype were more expected to clear their HCV infection than those with the AA genotype.

Our results with regard to IL27 serum level comes in agreement with those recorded by Houssen *et al*., (2015) who stated that the mean serum levels of IL-27 in Egyptian patients with HCC were significantly elevated, compared to either HCV patients or healthy control group. In addition, the serum IL27 levels were positively correlated with serum AST activity and serum viral load as assessed by quantitative PCR titer. They concluded the possibility of using serum IL27 as a novel marker for HCC in HCV patients. This comes in agreement with our results and also with those by Zhu *et al*., (2009) who demonstrated a higher level of IL 27 in both cirrhotic and HCC studied groups.

Results of the biochemical markers in our studied groups of LC reported a significantly higher total and direct bilirubin levels, noticeable lower platelets accounts, albumin and AFP levels when compared to HCC group. Recently, Bai *et al*., (2017) stated that AFP despite being the most popular diagnostic marker of HCC with a concentration of more than 400 ng/ ml, but both its accuracy and use for HCC surveillance programs was debated, thus it was removed from following the international strategies for HCC. In order to have a more accurate test, many suggestions were advised by Arrieta *et al*., (2007); El-Serag and Kanwal, (2013) for the justification of using AFP by the mean of serial measurement. This is because many causes of increased AFP levels were reported such as; cirrhosis, lung cancer, biliary cancer, gastric cancer, and pancreatic cancer. So we encourage using IL27 as a marker in diagnosis of both of cirrhotic and HCC patients, as current results showed no overlap between IL27 serum level in both patients and the control. This comes in agreement with Houssen *et al*., (2015) who advised further research to ensure the safety of using IL27 as immunotherapy in HCC. Moreover, similar results were reported by recent metadata analysis that showed significant association between IL27-964A/G rs153109 polymorphism and cancer risk in colorectal cancer (Xu *et al*., 2017). However, large-scale and well-designed studies are still needed to confirm the results of this meta-analysis. The most interesting findings of the present study were the significant association of AG and G alleles with HCC, and cirrhosis with HCV. This may reflect the depressed innate immune response mediated by IL27 on chronic HCV, leading to progression of the infection to HCC.

Conclusion

We concluded that IL27 is a perfect marker in detecting HCC, as we did not find significant difference when comparing its level between different genotypes of IL27 p28.rs153109. In addition, we recorded that G allele is a risky allele as it increased in HCC and cirrhotic groups. These findings may modulate the treatment strategy of HCV positive patients; however, further follow up of patients is needed to plain their treatment. The conflict between the current study and the previous global studies might be attributed to the modifications in disease etiology, the important molecular mechanisms, and environmental involvements in the different inhabitants.

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Conflict of interests

The authors declare that they have no conflict of interests.

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Ethical approval

The study protocol conformed to the 1975 Declaration of Helsinki, and was approved by the institutional review boards of Mansoura Faculty of Medicine ethical committee IRB R/17.12.54, and the informed consent was obtained from each participant.

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