RESEARCH ARTICLE

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The Genetic Influence of Programmed Death Ligand-1 (*PD-L1*) Single Nucleotide Polymorphisms on the Incidence of B-Cell Non-Hodgkin Lymphoma in a Cohort of Egyptians

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Abstract

Objective: This study was conducted to investigate the relationship between rs4143815 (C>G) and rs2890658 (A>C) of the *PD-L1* (Programmed Death-Ligand 1) gene and the risk of developing non-Hodgkin's lymphoma (B-NHL) in a cohort of Egyptian population. **Methods:** In this case-control study, which included one hundred patients (males and females) diagnosed with B-NHL from Kasr El Aini hematology clinic, their age ranged between 18 and 71 years and 100 age and sex matched healthy controls from outpatient clinic. Three ml venous blood was withdrawn from all subjects and collected in EDTA vacutainer® and kept frozen at -20˚C till time of DNA extraction. DNA samples were extracted from blood samples using GeneJETTM Genomic DNA Purification Kit from Thermo Fisher. Genotyping was determined using Custom TaqMan® SNP Genotyping Assays from Applied biosystems by real-time PCR, and subsequently extensive statistical analysis was performed to investigate the clinical value of these polymorphisms. Patients' clinical information was obtained from patient's medical records. **Results:** The obtained results of the current study demonstrated GG genotype of *PD-L1* rs4143815 and the CC genotype of *PD-L1* rs2890658 were observed to be more prevalent among NHL patients compared to those reported for the healthy controls (62%, 80% vs 58%, 77%), respectively, however these results revealed no significant association between the studied SNPs of PDL1 gene and risk of NHL (p=0.837, *). Regarding survival analysis, results of 36 and 60 months DFS for the common genotype (GG) of rs4143815 versus the combined genotype (GC & CC) were (67%, 44.6% vs 63.8%, 63.8%), respectively (p=0.249) as well as results of 36, 60 months DFS for the common genotype (CC) of rs2890658 versus the combined genotype (AA & AC) revealed (61.5%, 46.1% vs 83.3%, 83.3%), respectively which was statistically insignificant (p=0.599), therefore PDL1 rs4143815 & rs2890658 polymorphisms have no significant impact on patients' DFS. **Conclusions:** Our findings provided the first evidence that *PD-L1* rs4143815 (C>G) and rs2890658 (A>C) are not molecular susceptibility markers for B-NHL in Egyptians, at least in the studied population.

Keywords: B-NHL- PDL1- rs4143815- rs2890658- SNP- Real-Time PCR

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Introduction

Cancer is considered a highly significant global health problem and worldwide leading cause of death. Thus, the etiology and pathogenesis of malignancy have not been completely elucidated and their understanding is still decisive. During the past decades, several investigators focused their research on the functional role of the immune system in the process of carcinogenesis [1]. The results point out that mutations in immune-related genes may alter cancer predisposition and susceptibility [2].

Human cancers, including hematological malignancies, have developed multiple effective strategies to evade the host immune surveillance. Mechanisms used by tumors for immune evasion have been extensively studied in the last decade [1,3], and a better understanding of these decisive mechanisms has facilitated the development of novel therapies aimed at stopping tumor immune evasion. Tumor cells are capable of evading immune surveillance by over-expressing the ligands of checkpoint receptors, bringing T lymphocytes to a state of anergy or exhaustion [4].

Cancer cells can work on a variety of immune checkpoint signaling pathways such as programmed cell death protein 1 (*PD-1*) and cytotoxic T lymphocyte antigen 4 (CTLA4) to elicit immunosuppressive functions. The *PD-1*/*PD-L1* signaling pathway has an important role in immune tolerance and prevention of occurrence of autoimmune disorders [5].

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Although many studies on chemical therapy for cancer have generated promising results, a major breakthrough has been made by using immunotherapeutic agents that unleash our ability to recognize and overcome cancer [6]. During the last decade we have witnessed a remarkable development of immunotherapy targeting numerous checkpoint molecules of the immune system [7].

Under normal physiological circumstances, the *PD-1*/*PD-L1* checkpoint pathway plays a vital role in maintaining immune homeostasis. *PD-L1* is a ligand bound to *PD-1* receptor and their interaction down-regulates T cell response for optimal control of the immune system in normal cells. In cancer, tumor cells use *PD-L1* to escape from immune surveillance where they overexpress this ligand at a high level to downregulate tumor-specific T lymphocytes and inhibit their proliferation [8].

Many SNPs that might alter the genetic predisposition of cancer have been reported in *PD-L1* genes recently. These polymorphisms might affect tumor growth, behavior, and response to therapy [9]. Since a limited number of studies have investigated the association between *PD-L1* polymorphisms and the risk of lymphoma occurrence, it is necessary to examine the effect of these polymorphisms on the tumor behavior, prognosis, and response to treatment. The Aim of this study was to investigate the possible relation between polymorphisms of *PD-L1* rs4143815 and rs2890658 with the susceptibility of B-NHL in a Cohort of Egyptians.

Materials and Methods

Study Population

The study included 100 adult patients (54 males and 46 females) diagnosed with NHL based on histological confirmation with age range of 18-71 years. The patients were recruited from the Department of Hematology and Medical Oncology at Kasr Al-Ainy Hospital, Cairo University. In addition, 100 unrelated healthy controls of the same ethnicity were enrolled. The study was carried out in the interval between June 2020 and December 2021. Patients with double malignancy or with any other hematological disorders are excluded from the study.

Ethical statement

The research protocol was approved by the Research Ethics Committee (REC) of Kasr Al Ainy Faculty of Medicine, Cairo University. Written informed consent was obtained from all the participants before involvement in the study.

Sample collection and DNA extraction

Three ml venous blood were withdrawn from all subjects and collected in EDTA tubes and kept frozen at -20˚C till time of DNA extraction. Genomic DNA was isolated using GeneJET Whole Blood Genomic DNA Purification Mini Kit (Cat No: #K0781, Life Technologies Inc., 5781 Van Allen Way, Carlsbad, California) applied by Thermo Fisher according to the manufacturer's instructions.

Genotyping of PDL1 rs4143815 (C/G) and rs2890658 (A/C)

PD-L1 allelic discrimination was assessed using Sequence-specific primers and Custom allele-specific TaqMan® SNP Genotyping Assays provided from Applied biosystems then consequent analysis was performed on DNA-Technology StepOne ™ Real-Time PCR system. Also, each allele-specific TaqMan probe has a reporter dye at its 5′end (FAM dye is linked to the 5′end of the Allele 1 probe, and VIC dye is linked to the 5′ end of the Allele 2 probe), which allows the allele-specific fixation of each probe.

The context sequence for rs4143815 of PDL1 was

TTGCCTCCACTCAATGCCTCAATTT[C/G]TTTTCTGCATGACTGAGAGTCTCAG

Variant: G>C, transition substitution,

while the context sequence for PDL1 rs2890658 was

CAAGAGGAAGTGAAATAATCAAGGC[A/C]GCCATTTAATAGTGAGCAGCCACTC

Variant: C<A, transition substitution.

Amplification of DNA was done in a real-time thermocycler. After PCR amplification, an endpoint plate read was performed using an DNA-Technology Real-Time PCR System, The DTMaster Software uses the fluorescence signal measurements which were made during the plate reading to plot fluorescence values based on the signals from each well.

Statistical Analysis

With the help of the statistical programme SPSS version 22, data were coded and examined. Analysis of variance (ANOVA) with multiple comparisons post hoc testing was used to compare the groups. An analysis using the Chi square (2) test was done to compare categorical data. It was determined the odds ratio (OR) and its 95% confidence interval. Statistics were considered significant for P-values under 0.05. The Fisher exact test, the Pearson chi-square test, or the Kruskal-Wallis test were all used to analyze univariate association. The Kaplan-Meier method was used to produce the survival curves, and the log-rank test was used to compare them.

Results

Demographic, clinical and laboratory data

They were 54 males and 46 females with a mean age of 52.7 years for the patients and 52 male and 48 female (with a mean age of 52.5 years for the controls. Clinicopathological data of B-NHL patients at diagnosis are presented in Table 1.

Analysis of the relation between incidence of NHL and the selected SNPs

According to the distributions of different genotypes and alleles frequency, our findings revealed that genotype distribution of *PD-L1* rs4143815 GG, GC, and CC genotypes were 62%, 33%, and 5% among B-NHL patients, compared to 58%, 36%, and 6% in the control

Table 1. Clinicopathological Data of B-cell non-Hodgkin Lymphoma Patients at Presentation

Item / Patient group (n=100)	Number (%)
Sex (male/female)	(54/46)
Age (years)	
≤ 60	77 (77)
< 60	23 (23)
B- symptoms	
Fever, night sweats, weight loss	55 (55)
Lymphadenopathy	91 (91)
Extra-nodal involvement	
< 2	81(81)
≥ 2	19 (19)
Bulky disease	29 (29)
Splenomegaly	58 (58)
Hepatomegaly	43 (43)
Bone marrow infiltration	34 (34)
HCV positivity	25(25)
Clinical stage	
Early (I&II)	24 (24)
Late (III&IV)	76 (76)
P.S	
Score <2	70 (70)
Score ≥2	30 (30)
IPI risk group	
Low & Low-intermediate	62 (62)
High & High-intermediate	38 (38)
IPI risk groups for DLBC subtype (n=84)	
Low/ Intermediate low	57 (67.8)
Intermediate high/ High	27(32.1)

P.S, Performance status; HCV, Hepatitis C virus; IPI, International prognostic index.

group, respectively (p=0.837). Furthermore, the frequency of G and C alleles was 78.5%, 21.5% among patients compared to 76%, 24% in the controls, respectively with no statistical significance (p=0.551). In *PD-L1* rs2890658 polymorphism, the distribution of CC, AC and AA genotypes were 80%, 18% and 2% among B-NHL patients, compared to 77%, 22% and 1% in the control group, respectively. Furthermore, the frequency of C and A alleles was 89% and 11% among patient group compared to 88% and 12% in the control group respectively with no statistical significance (p=0.787), and the dominant alleles are similarly represented in both groups of our study. As a net result, data revealed from analysis of genotype distribution and allele frequencies of *PDL-1* rs4143815 (C<G) and rs2890658 (A>C) SNPs, did not show any association with the susceptibility of B-NHL Table 2.

Analysis of the relation between different PD-L1 Genetic variants and clinicopathological features

Statistical comparison between two studied groups of B-NHL regarding allele frequency of *PD-L1* rs4143815 revealed that the presence of mediastinal lymphadenopathy, hepatomegaly, extra-nodal involvement ≥2 sites and bone marrow infiltration were more prominent in those harboring the G allele and results showed statistical significance (P=0.001, p=0.009, p=0.013, <0.001), respectively. On multivariate analysis, hepatomegaly retains statistical significance (OR= 2.426, P value=0.035) Table 3.

Concerning *PD-L1* rs2890658 analysis, the presence of nodal involvement, advanced clinical stage III & IV, presence of anemia, presence of bone marrow infiltration and increased level of LDH were more prominent in those harboring the C allele and results showed statistical significance (P=0.017, p=0.049, p=0.012, p=0.009, 0.002),

Table 2. Genotype Distribution and Allele Frequencies of *PD-L1* rs4143815 and rs2890658 in B-NHL Patients and Control Groups

Genotype	Group		P-value
	Patients (n=100) %	Controls (n=100) n(%)	
	PD-L1 rs4143815 (C > G)		
GG	62 (62)	58 (58)	0.837
CG	33 (33)	36 (36)	
CC	5 (5)	6 (6)	
G allele	157/200 (78.5)	152 /200 (76)	0.551
C allele	43/200 (21.5)	48/200 (24)	
GG vs GC+CC	62 (62) vs 38 (38)	58 (58) vs 42 (42)	0.564
CC vs GG+GC	5 (5) vs 95 (95)	6 (6) vs 94 (94)	0.756
	PD-L1 rs2890658 (A < C)		
CC	80 (80)	77 (77)	
AC	18 (18)	22 (22)	*
AA	2 (2)	1 (1)	
C allele	178/200 (89)	176/200 (88)	0.787
A allele	22/200 (11)	24/200 (12)	
CC vs AC/AA	80(80) vs 20(20)	77 (77) vs 23(23)	0.606
AA vs AC/CC	2(2) vs 98 (98)	1(1) vs 99(99)	*

P value <0.05, Is significant; *PD-L1*, Programmed death ligand-1; *, p value cannot be calculated because of small number within strata.

Table 3. Comparison between Allelic Frequency of *PD-L1* rs4143815 in B-NHL Patients and Their Clinicopathological Features at Diagnosis.

Item		*PD-L1* rs4143815		P-value
		C allele 43/200 (21.5%)	G allele 157/200 (78.5%)	
Gender				
Female		20/43 (46.5)	86/157 (54.8)	0.336
Male		23/43 (53.5)	71/157 (45.2)	
Age				0.65
≤ 60		32/43 (74.4)	122/157 (77.7)	
> 60		11/43 (25.6)	35/157 (22.3)	
B-symptoms (present)		19/43 (44.2)	91/124 (58.0)	0.108
Lymphadenopathy		38/43 (88.4)	144/157 (91.7)	0.497
Groups of lymph nodes involved	Cervical	23/43 (53.5)	89/157 (56.7)	0.708
	Inguinal	16/43 (37.2)	70/157 (44.6)	0.387
	Mediastinal	0/43 (0)	32/157 (20.4)	0.001
	Mesenteric	7/43 (17.9)	15/157 (12.0)	0.341
	Para-aortic	17/43 (39.5)	57/157 (36.3)	0.698
Splenomegaly		21/43 (48.8)	95/157 (60.5)	0.169
Hepatomegaly		11/43 (25.6)	75/157 (47.8)	0.009
Extra-nodal involvement				
No		19/43 (44.2)	39/157 (24.8)	0.013
Yes		24/43 (55.8)	118/157 (75.2)	
An Arbor stage				
Stage I&II		13/43 (30.2)	35/157 (22.3)	0.28
Stage III&IV		30/43 (69.8)	122/157 (77.7)	
P.S				
Score <2		33/43 (76.7)	107/157 (68.2)	0.276
Score ≥2		10/43 (23.3)	50/157 (31.8)	
IPI risk				
Low/Intermediate low		30/43 (69.8)	94/157 (59.9)	0.236
Intermediate high/ High		13/43 (30.2)	63/157 (40.1)	
Treatment Outcome				
CR		38/43 (88.4)	128/157 (81.5)	0.29
Non-CR		5/43 (11.6)	29/157 (18.5)	
Rs2890658				
A allele		16/43 (37.2)	6/157 (3.8)	<0.001
C allele		27/43 (62.8)	151/157 (96.2)	

P-value < 0.05, Significant; P.S, Performance status; IPI, International prognostic index; CR, Complete remission

respectively. By multivariate analysis of the variables, nodal involvement (OR=4.3, P=0.037), BM involvement (OR=6.2, P=0.017) and high LDH (OR=4.0, P=0.004) show statistical significance. Moreover, co-inheritance of the common alleles of both SNPs of PDL1 show very high statistical significance by both univariate and multivariate analysis (OR=14.086, p=<0.001) Table 4.

Regarding the potential role of these SNPs as molecular prognostic markers

The 12 months, 36 months, and 60 months DFS rates and PDL1 rs4143815 & rs2890658 genetic variants were assessed for their impact on patients' DFS. The 36 months DFS` rate for the combined genotype (GC & CC) of *PD-L1* rs4143815 (C>G) was 63.8% versus 67% for the common genotype (GG), while the 60 months DFS rate for the combined genotype (GC & CC) was 63.8% versus 44.6% for the common genotype (GG) with no statistically significant difference (p=0.249). While the 36 months` DFS rate for the combined genotype (AA & AC) of rs2890658 (A>C) was 83.3% versus 61.5% for the common genotype (CC), and the 60 months DFS rate for the combined genotype (AA & AC) was 83.3% versus 46.1% for the common genotype (CC), which was statistically insignificant (p=0.599), therefore PDL1 rs4143815 & rs2890658 polymorphism have no impact on patients' DFS Table 5.

By univariate analysis between clinicopathological

Table 4. Comparison between Allelic Frequency of *PD-L1* rs2890658 in B-NHL Patients and Their Clinical Data

Item		PD-L1 rs2890658		P-value
		A allele 22/200 (11%)	C allele 178/200 (89%)	
Gender				0.13
Female		15/22 (68.2)	91/178 (51.1)	
Male		7/22 (31.8)	87/178 (48.9)	
Age				0.974
≤ 60		17/22 (77.3)	137/178 (77)	
> 60		5/22 (22.7)	41/178 (23)	
B-symptoms (present)		11/22 (50.0)	99/178 (55.6)	0.617
Lymphadenopathy		17/22 (77.3)	165/178 (92.7)	0.017
Groups of lymph nodes involved	Cervical	11/22 (50)	101/178 (56.7)	0.548
	Inguinal	4/22 (18.2)	62/178 (34.8)	0.117
	Mediastinal	1/22(4.5)	31/178 (17.4)	0.12
	Mesenteric	2/22 (10.0)	20/178 (13.9)	0.633
	Para-aortic	6/22 (27.3)	68/178 (38.2)	0.316
Splenomegaly		11/22 (50)	105/178 (59.0)	0.42
Hepatomegaly		6/22 (27.3)	80/178 (44.9)	0.114
Extra-nodal involvement				0.42
No		8/22 (36.4)	50/178 (28.1)	
Yes		14/22 (63.6)	128/178 (71.9)	
Clinical stage				0.049
I&II		9/22 (40.9)	39/178 (21.9)	
III&IV		13/22 (59.1)	139/178 (78.1)	
P.S				0.844
Score <2		15/22 (68.2)	125/178 (70.2)	
Score ≥2		7/22 (31.8)	53/178 (29.8)	
IPI risk				0.766
Low/Intermediate low		13/22 (59.1)	111/178 (62.4)	
Intermediate high/ High		9/22 (40.9)	67/178 (37.6)	
Rs4143815				<0.001
G allele		6/22 (27.3)	151/178 (84.8)	
C allele		16/22 (72.7)	27/178 (15.2)	
Treatment Outcome				0.099
CR		21/22 (95.5)	145/178 (81.5)	
Non-CR		1/22 (4.5)	33/178 (18.5)	

P-value < 0.05, significant; P.S, Performance status; IPI, International prognostic index; CR, Complete remission.

factors and DFS; patients with B symptoms, PS ≥2, advanced An Arbor stage, presence of anemia and high TLC count were found to have a statistically significant relation with poorer DFS (p=0.016, p=0.039, p=0.023, p=0.038, p=0.019), respectively. It was found that An Arbor stage (HR=7.53, P=0.053) is an independent predictor of DFS by multivariate analysis. Other potential prognostic factors, such as the patients' age at diagnosis, sex, International Prognostic Index score, extra-nodal involvement, and LDH level did not affect the DFS of our patients.

Discussion

Several SNPs in *PD-L1* genes are heavily studied for their contribution to the susceptibility of cancer occurrence, tumor growth, behavior, and response to therapy [10]. However, the relationship between the *PDL-1* genetic variations and B-NHL remains ambiguous [11].

So far, this is considered the first study to address the association between *PD-L1* SNPs (rs4143815 and rs2890658) and the susceptibility of NHL in Egypt. Results of *PD-L1* rs4143815 demonstrated similar genotype distribution between both patient and control groups with no statistical significance revealed (p=0.551). These results were in agreement with recent study in 2020, that revealed no significant association between *PD-L1* rs4143815 and the risk of NHL incidence [11], also in agreement with Du (2017)' study that revealed

Table 5. Impact of *PDL1* rs4143815 & rs2890658 Polymorphisms on the DFS of B-NHL Patients

Item	No.	No. of	Cumulative survival at 12 months (%)	Cumulative survival at 36 months (%)	Cumulative survival at 60 months (%)	p-value
rs4143815						0.249
GG	51	9	84.0	67	44.6	
GC & CC	32	5	100.0	63.8	63.8	
rs4143815						*
CC	4	0	NR	NR	NR	
GG & GC	79	14	90.5	64.8	54.0	
rs2890658						0.599
CC	65	11	93.0	61.5	46.1	
CA & AA	18	3	83.3	83.3	83.3	
rs2890658						*
AA	2	0	NR	NR	NR	
CC & CA	81	14	90.5	63.8	53.1	

NR, Not reached; p-value significant < 0.05; *, p value cannot be calculated because of small number within strata.

no significant association between *PD-L1* rs4143815 polymorphism and occurrence of Lung cancer [12].

Discordant results from a meta-analysis by Zou (2019) in Asian population revealed that rs4143815 (C>G) is associated with increased cancer susceptibilities, including gastric, bladder cancer and HCC, indicating that this SNP might be used as a genetic biomarker tool to predict the cancer susceptibility [13]. Moreover, GG genotype in rs4143185 (C>G) SNP was found to be related to increased risk of HCC in another study (p< 0.001) [14].

Hashemi and colleagues, revealed that *PDL-1* rs4143815 polymorphism is associated with decreased overall risk of tumour occurrence giving it a protective value against cancer, while no significant association found between rs2890658 SNP and the overall risk of cancer in parallel to our results [15]. Moreover, the pooled analysis from Zou (2019) study did not support an association between *PD-L1* rs2890658 polymorphism and the cancer susceptibilities [13].

On the other side, data reported by Hoseini (2020) demonstrated significant association of *PD-L1* rs2890685 (A>C) SNP with increased risk of NHL (p<0.0001), where rs2890685 AA genotype distribution was significantly prevalent in patients with NHL in comparison to controls [11]. In addition to, Zhou (2016) who reported that *PD-L1* rs2890658 SNP is associated with increased risk of oesophageal carcinoma in group of smokers although these results show conflict with our observation [16].

Since prognostic markers are important clinical tools used in assessing the newly diagnosed patient to help predict the patient's clinical outlook. For B-NHL patients, many prognostic markers such as age< 60 years, advanced clinical stage (III&VI), performance status≤ 2 on ECOG scale, high LDH level, extra-nodal sites >1, presence of BM involvement, anemia and B symptoms have been used to predict future clinical outcome of patients. Here, we studied the relationship between the allele frequencies of the *PD-L1* polymorphisms and adverse clinicopathological factors of the patients which revealed some positive results.

Our findings revealed, the presence of mediastinal nodal involvement, hepatomegaly, extra-nodal involvement and BM infiltration were more prominent in patients with G allele of rs4143815 and results showed a statistical significance (P=0.001, p=0.009, p=0.013, <0.001), respectively. These findings are consistent with Wu (2006)' study which found significant relation between rs4143815 SNP and clinicopathological features of gastric cancer including tumour size, differentiation grade, depth of tumour infiltration, lymph node metastasis, and TNM stage, and considered this variant might be a potential risk factor for cancer predisposition [17].

Another study done by Du et al., was in agreement with our findings, which reported that rs4143815 SNP had significant associations with clinicopathological features, including depth of tumour infiltration, distant metastasis, lymph node metastasis and TNM stage in lung cancer; therefore, it might be possible future prognostic tool [12].

So, we can conclude that our results provided the first evidence that *PD-L1* rs4143815 and rs2890658 genetic variants are not considered genetic predisposing factors in susceptibility to B-NHL in a cohort of Egyptian population, at least in the studied population. Although the polymorphism at *PD-L1* gene has no impact on patients' survival; rather, there was a significant association between the dominant alleles of *PD-L1* rs4143815 and rs2890658 SNPs and the adverse clinico-pathological features of B-NHL patients. Therefore, these polymorphic sites could be candidates for predicting the adverse clinicopathological features of B-NHL and might be valuable for prognostic values in Egyptian population. Although, validation by a larger prospective study from diverse ethnic population is warranted to confirm our findings.

Study Limitations

The relatively small sample size of this study is a limitation of the present work. Larger sample size is recommended to validate our results regarding the role of the studied SNPs as molecular risk factors for B-NHL and to clarify their impact on therapeutic response and disease course. Furthermore, *PD-L1* expression or serum *PD-L1*

level should been examined to conclude the association between functional mutations and B-NHL.

Author Contribution Statement

All the authors contributed to data collection and wrote the manuscript, All the authors read and approved the final manuscript. Conception: Manal W El-Masry, Interpretation or analysis of data: Marwa T Hassan, Heba M Gouda, Manal W El-Masry, Preparation of the initial manuscript: Marwa T Hassan, Heba M Gouda, Revision for important intellectual content: Heba M Gouda, Mervat M Matter, Supervision throughout the work: Manal W El-Masry, Mervat M Matter.

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If it was approved by any scientific Body/ if it is part of an approved student thesis

This study is a part of MD thesis and approved by the scientific committee that judged the thesis.

How the ethical issue was handled (name the ethical committee that approved the research)

The current study was approved by the Research Ethics Committee (REC) of Faculty of medicine in Cairo University, Egypt.

Availability of data (if apply to your research)

All the data generated or analysed during this study were included in this published article and available on request.

Informed consent

Written informed consent for publication was obtained from all the participants.

Conflict of Interest

The authors declare that they have no conflicts of interest concerning the research, relationships authorship, affiliations, and/or publication of this article.

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