

Association of CD40 Genotyping and its Protein Expression with Airway Inflammatory Diseases

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Abstract

CD40 plays a substantial role in inflammation and has been linked to pathogenic processes of chronic inflammatory diseases such as asthma as well as chronic obstructive pulmonary disease (COPD).

Aim: The study was to investigate the association of CD40 gene (-1C/T) single nucleotide polymorphism (SNP) with the susceptibility to asthma and COPD in the Egyptian population, and its functional effect on the expression of CD40.

Methods: We analyzed -1C/T SNP of the CD40 gene in 40 patients with COPD, 50 patients with asthma, and 60 normal subjects using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). CD40 expression was measured using flow cytometry. Immunoglobulin E was also determined in asthmatics.

Results: The CT genotype was prevailing in the COPD patients and control group, while in the asthmatics it was the CC. Independent of the smoking status; being CC homozygous still conferred a 4-fold increase in the risk of asthma and a 2.5-fold increase in the risk of COPD. Carrying T allele showed a significantly lower risk for asthma. Furthermore, in both asthma and COPD, the least expression of CD40 protein was found with the TT genotype, which seems to have a protective effect. Despite the significant upregulation of total serum IgE in asthmatics it was not significantly associated with CD40 genotyping or the protein expression.

Conclusion: Our study demonstrated that CD40 - 1C/T polymorphism significantly contribute to the susceptibility to asthma and COPD in the Egyptian population. Reduced CD40 expression with the TT genotypes might imply that the - 1C/T polymorphism is linked to inflammation in addition to the initiation and development of both. The genetic predisposition to certain pathways may further help to define the development of either asthma or COPD. This may lead to stratification of patients by their genetic make-up and open new therapeutic prospects.

Keywords: Asthma; COPD; CD40 gene polymorphism; CD40 expression

Introduction

Asthma and chronic obstructive pulmonary disease (COPD) show similarities and substantial differences. It is stipulated that asthma and COPD have common genetic and environmental risk factors, which ultimately lead to clinical disease depending on the timing and type of environmental exposures. Thus, a particular group of shared genetic factors may lead to asthma when combined with specific environmental factors, whereas combination with other environmental factors, will lead toward COPD [1].

CD40 is a member of the tumor necrosis factor receptor superfamily, that plays a substantial, multi-faceted role in inflammation [2]. Previous studies have shown that interactions between CD40 and its ligand CD154 (CD40L) have been implicated in lung disorders. CD40 which is found on a variety of inflammatory cells, induces the release of inflammatory mediators and plays a role in airway inflammatory responses [3].

Chronic inflammation of the airways plays a major role in the pathogenesis of asthma as well as chronic obstructive pulmonary disease (COPD). Their pathogenesis is influenced by both environmental and genetic factors [4,5]. And since CD40 signaling has been linked to pathogenic processes of chronic inflammatory diseases, [2,3] therefore CD40 polymorphism could be associated with these two diseases.

Until now, only little information had explored the association between CD40 polymorphisms and genetic susceptibility to airway

inflammatory disease. A C/T in the 5' untranslated region of CD40 located at the -1 position within the Kozak sequence (rs1883832) has been associated with CD40 protein expression [6]. Therefore, we hypothesized that the CD40 gene (-1C/T) polymorphism may have a role in the genetic susceptibility to asthma and COPD. Therefore, we chose to study CD40 gene (-1C/T) polymorphism and investigate its functional effect on the expression of CD40 in a genetic study of asthma and COPD in the Egyptian population.

Patients and methods

Our study included 150 subjects who were divided into three groups; group 1 included 50 asthmatic patients, group 2 included 40 COPD patients, and group 3 included 60 normal subjects as control. Asthmatic patients were diagnosed according to the standard criteria, as previously described [7]. Asthmatic patients exhibited airway reversibility, as defined by an inhalant bronchodilator-induced

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improvement of forced expiratory volume in one second (FEV1) by more than 12% or 200 ml.

The diagnosis of COPD was based on the definition provided by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) [5]. As defined by a post-bronchodilator forced expiratory volume in one second (FEV1) to forced vital capacity (FVC) ratio of < 70% and a β 2-agonist reversibility of <12% or 200 mL.

Patients were excluded from the study if they had other respiratory diseases, diabetes mellitus, cardiac diseases or thyroid disease.

The control group included normal subjects who were recruited from the general population who had no respiratory symptoms, and no evidence of airflow obstruction. The majority of them were either current or ex-smokers. Individuals were excluded if they had a history of chronic lung disease or atopy, an acute pulmonary infection in the 4 weeks preceding assessment for the study, or a family history of asthma or COPD.

All the cases and controls were unrelated Egyptian people who were selected from the same population. The case groups were recruited from Alexandria main university hospital. All subjects were enrolled in the study after a written informed consent according to the protocol approved by the Ethics Committee of the Alexandria Main University Hospital. Peripheral venous blood samples of 5 ml were drawn from each individual by standard venepuncture. The blood sample was divided into two aliquots; one in a sterile tube with K2-EDTA anticoagulants for flow cytometry and genotyping, and the other one was collected in a plane tube for serum separation.

Analysis of -1C/T SNP of the CD40 gene (rs1883832)

Total genomic DNA was extracted from whole blood samples using QIAamp DNA Blood Extraction Kit (Qiagen,UK). The CD40-1C/T (ref SNP ID: rs1883832) polymorphism was determined by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. According to Hsieh et al. [8] the primer sequences were: Forward, 5'-TACACGCAAGATGCGTCC CT-3'; Reverse, 5'-AACAACTCACAGCGTCAGCAA-3'. A total of 50 ng genomic DNA was mixed with 0.25 μ l primers in a total volume of 25 μ l containing 10 mM Tris-HCL pH 8.3, 50 mM potassium chloride, 2.0 mM magnesium chloride, 3 μ l each of dNTP, and 1 U Taq DNA polymerase. The PCR amplification was performed in a programmable

thermal cycler with initial denaturation step for 5 min at 95°C, 35cycles of denaturation for 30s at 95°C, annealing for 30s at 65°C and extension for 30s at 72°C, followed by a final extension step for 10 min at 72°C.

The amplified products were digested by 5U of restriction enzyme NcoI for 8 h, according to the manufacturer's recommendation (Fermentas, Burlington, Ont., Canada). NcoI digestion cleaves the 310 bp PCR products into 2 fragments of 249 and 61bp when C allele is present. The PCR products in 0.2 ml, 12-tube strips, were transferred directly from the thermocycler into the sample tray of the QIAxcel Capillary electrophoresis from Qiagen company [9]. Separation was performed in a 12-channel gel cartridge (GCK5000) purchased from eGene Inc. (Qiagen, USA). The sizes of the alleles resolved from the subsequent separation were automatically calculated in bp and exported using the BioCalculator™ software, which provides a gel view and an electro-pherogram of the separation. QIAxcel DNA High Resolution Kit was used with Alignment Marker15-1000bp and size Marker 50-800bp.

Dual expression of CD20 and CD40 using flow cytometry: As previously described [10,11] B-cell lineage lymphocytes were stained with a phycoerythrin (PE)-conjugated anti-CD20 mouse monoclonal antibody and a fluorescein isothiocyanate (FITC)-conjugated anti-CD40 mouse monoclonal antibody (both from BD Biosciences, San Diego, CA). Gating was done on CD20 positive population of B-lymphocytes and were further analysed for staining with CD40 (Dual color). Phycoerythrin- and fluorescein isothiocyanate-conjugated mouse IgG1k antibodies (BD Biosciences) were used as isotype-matched negative controls. Dual expression of CD20 and CD40 cells was analyzed by flow cytometry : FacsCalibur from BD .

Determination of Immunoglobulin E (Ig E) concentration: Ig E concentration in human serum was detected by electrochemiluminescence on Cobas E411 from Roche (Germany) [12]. It was determined in the serum of asthmatics and controls.

Statistical analysis

Statistical analysis of data was performed using the PASW version 18. Genotype and allele frequencies of CD40-1C/T were in agreement with Hardy-Weinberg equilibrium. Genotype frequencies were compared among the studied groups using the chi square test and fisher's exact test when appropriate. Odds ratio (OR) and 95% confidence intervals (CI) were calculated to assess the relative risk conferred by a particular

	Asthmatic patients	COPD patients	Control subjects	p ₁	p ₂
Subjects (n)	50	40	60		
Age (years)	38.500±10.53	59.350 ± 10.39	52.150±16.89	<0.001*	0.414
Sex M/F (%)	25(50%)/25(50%)	30(75%)/20(25%)	45(75%)/15(25%)	0.007*	1.000
Smoking status n (%)					
Non smoker	33 (66%)	7 (17.5%)	19 (31.7%)	<0.001*	<0.001*
Current smoker	10 (20%)	19 (47.5%)	30 (60%)		
Ex smoker	7 (14%)	14 (35%)	11 (22%)		
Pack-year of smoking	15.54 ± 7.43	31.60 ± 13.81	23.93 ± 14.09	0.038*	0.024*
Post –BD FEV1 % predicted	74.78 ± 9.29	66.8 ± 12.93	-	-	-
Spirometric classification of severity	Intermittent= 7(14%) Mild= 18 (9%) Moderate= 28 (56%) Severe= 6 (12%)	Mild= 10(25%) Moderate= 21(53%) Severe= 3(8%) Very severe= 6(15%)			
Total IgE, IU/ml	221.11 ± 75.51	-	38.40 ± 12.89	<0.001*	-

n: number of subjects. Data are presented as mean ± SD. Pack-year of smoking: (packs per day) × (years smoked).
Post –BD FEV1: post bronchodilator forced expiratory volume in one second. p₁: p value for significant test between asthmatic patients and control. p₂: p value for significant test between COPD patients and control. *: Statistically significant at p ≤ 0.05.

Table 1: Characteristics of the study population.

	Control		Asthma		COPD	
	No	%	No	%	No	%
Genotype						
CC	11	18.3	26	52.0	15	37.5
CT	40	66.7	22	44.0	21	52.5
TT	9	15.0	2	4.0	4	10.0
X² (p)			14.976* (0.001)		4.642 (0.098)	
Allele frequency						
C	51	51.0	48	52.2	36	59.0
T	49	49.0	44	47.8	25	41.0
X² (p)			0.026 (0.871)		0.980 (0.322)	

χ^2 : Chi square test between control and other groups. *: Statistical significance at $p \leq 0.05$

Table 2: The genotype and allele frequencies of CD40 - 1C/T among the three studied groups.

Genotype	Control		Asthma		OR	95% CI	P
	No.	%	No.	%			
CC	11	18.3	26	52.0	4.298	1.789 – 10.321	0.001
CT®	40	66.7	22	44.0			
TT	9	15.0	2	4.0	0.404	0.080 – 2.038	FEp = 0.320
CC + TT	20	23.3	28	56.0	2.545	1.173 – 5.523	0.017*
Allele frequency							
C®	62	51.7	74	74.0			
T	58	43.3	26	26.0	0.376	0.212 – 0.666	p* = 0.001*

p#: p value for Chi square test, FEp : p value for Fisher Exact test, *: Statistical significance at $p \leq 0.05$

Table 3A: The genotype and allele frequencies of CD40 - 1C/T between asthmatic patients and controls.

Genotype	Control		COPD		OR	95% CI	P
	No.	%	No.	%			
CC	11	18.3	15	37.5	2.597	1.014 – 6.652	0.044*
CT®	40	66.7	21	52.5			
TT	9	15.0	4	10.0	0.847	0.233 – 3.078	FEp = 1.000
CC + TT	20	23.3	19	47.5	1.810	0.797 – 4.111	0.155
Allele frequency							
C®	62	51.7	51	63.8			
T	58	43.3	29	36.2	0.608	0.340 – 1.085	p* = 0.091

p#: p value for Chi square test, FEp : p value for Fisher Exact test, *: Statistical significance at $p \leq 0.05$

Table 3B: The genotype and allele frequencies of CD40 - 1C/T between COPD patients and controls.

Asthma	Genotype	Control		Cases		OR	Sig.
		No.	%	No.	%		
	CC	11	18.3	26	52.0	0.029* (0.002 – 0.356)	0.006*
	CT®	40	66.7	22	44.0		
	TT	9	15.0	2	4.0	0.000 (-)	0.999

Logistic regression analysis for the risk of asthma with the age, sex and smoking status as covariates. *: Statistical significance at $p \leq 0.05$

Table 4A: The comparison of genotype distributions between normal control subjects and subjects with asthma.

allele or genotype. Demographic and clinical data between groups were compared by the chi square test and by the Student's *t*-test. Logistic regression analysis controlling for age, sex and smoking status as covariates was done to estimate the risk in asthma. Only the smoking

status was used as a covariate in the logistic regression analysis done to estimate the risk in COPD. Mann Whitney test was done for the comparison of CD40 expression according to different genotypes in the 3 groups. Statistical significance was assumed at $P \leq 0.05$.

Results

Characteristics of the study population

The characteristics of the study population are summarized in [table 1](#). Age sex, smoking status and the total serum IgE showed a significant difference between asthmatic patients and the control group. However COPD patients showed a significant difference only in the smoking status when compared to controls.

Distribution of genotypes and alleles of CD40-1C/T polymorphism: The genotype frequencies were in agreement with the Hardy-Weinberg equilibrium in control group. Genotype distribution showed different patterns among the three studied groups ([Figure 1](#)). Where the CT genotype was prevailing in the COPD patients and control group. While in the asthmatic patients CC was the prevailing genotype considering a significant difference when compared to the control group ($p=0.001$) [Table 2](#).

CD40 -1C/T genotyping and its association with the risk for asthma and COPD: According to ([Table 3A, 3B](#)), The frequency of CC

	Genotype	Control		Cases		OR	Sig.
		No.	%	No.	%		
COPD	CC	11	18.3	15	37.5	0.357* (0.130 – 0.975)	0.045*
	CT®	40	66.7	21	52.5		
	TT	9	15.0	4	10.0	0.307 (0.067 – 1.401)	0.127

Logistic regression analysis for the risk of COPD with the smoking status as covariates. *: Statistical significance at $p \leq 0.05$

No significant association was established between genotype distributions and the smoking status nor the spirometric classification of severity in either asthmatic or COPD patients.

Table 4B: The comparison of genotype distributions between normal control subjects and subjects with COPD.

	Genotype				χ^2 (p)
		CC (n = 26)	CT (n = 22)	TT (n = 2)	
Control	CD 40				
	Range	4.0 – 11.0	3.0 – 10.0	4.0 – 8.0	
	Mean \pm SD	6.91 \pm 2.30	5.83 \pm 2.0	5.44 \pm 1.33	3.292 (0.193)
	Median	6.0	5.0	5.0	
	P ₁		0.102	0.112	
	P ₂			0.713	
Asthma	CD 40				
	Range	2.0 – 18.0	2.0 – 18.0	5.0 – 5.0	
	Mean \pm SD	9.35 \pm 4.54	11.45 \pm 4.08	5.0 \pm 0.0	7.421* (0.024)
	Median	9.0	12.0	5.0	
	P ₁		0.038	0.087	
	P ₂			0.065	
COPD	CD 40				
	Range	3.0 – 15.0	2.0 – 17.0	3.0 – 4.0	
	Mean \pm SD	7.67 \pm 3.90	9.81 \pm 4.41	3.50 \pm 0.58	9.652* (0.008)
	Median	7.0	10.0	3.50	
	P ₁		0.126	0.015*	
	P ₂			0.003*	

p: p value for Mann Whitney test between control and other groups. *: Statistical significance at $p \leq 0.05$

Table 5: The comparison of CD40 expression levels on CD20* B cells possessing different genotypes for -1C > T SNPs of CD40 in each of the studied groups.

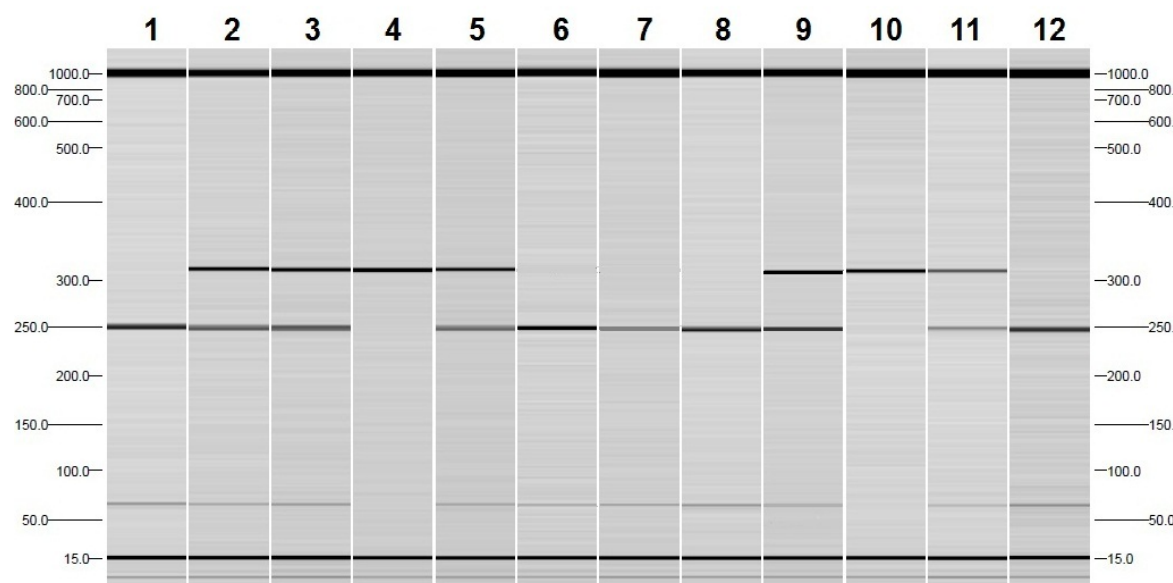


Figure 1: Gel image using QIAxcel System-genetic analyzer. Lanes 1-12 represent different samples. Lanes 1,6,7,8,12 representing the CC homozygote (two bands), lanes 4&10 representing the TT homozygote (one band) and products in lanes 2,3,5,9,11 representing the CT heterozygote (three bands). 15 base pair and 800 base pair bands are reference markers injected before the PCR products were injected.

homozygous was significantly different from CT heterozygous in both asthma and COPD patients when compared with the control group. Being CC homozygous conferred a 4-fold increase in the risk of asthma (OR= 4.30, 95% CI: 1.79-10.32, $p=0.001$) and a 2.5-fold increase in the risk of COPD (OR = 2.60, 95% CI: 1.01-6.65, $p=0.044$). The frequency of T allele was significantly different from C allele in asthmatic patients when compared to the control group ($p=0.001$), indicating that the risk of asthma was significantly lower (OR= 0.37) among individuals carrying T allele (95% CI: 0.21-0.66, $p=0.001$). However, in COPD patients no significant difference was observed between the C and T alleles.

Since there was a significant difference in the age, sex and smoking status between the asthmatics and controls (Table 1), therefore, we performed logistic regression analysis for the risk of asthma in the presence of these three covariates. Still, *CD40* polymorphism exerted a significant effect on the risk of asthma where the frequency of CC of *CD40* was significantly different from CT in the asthmatic patients (OR =0.029, 95% CI: 0.002-0.356, $p=0.006$) Table 4A.

While in COPD patients the smoking status was the only variable that differed significantly from the controls. (Table 1), therefore, we performed logistic regression analysis for the risk of COPD in the presence of this covariate. *CD40* polymorphism also exerted a significant effect on the risk of COPD where the frequency of CC of *CD40* was significantly different from CT in the COPD patients (OR =0.357, 95% CI: 0.130-0.975, $p=0.045$) Table 4B.

No significant association was established between the genotype distributions and the smoking status nor the spirometric classification of severity in either asthmatic or COPD patients.

Effect of CD40-1C/T genotyping on CD40 protein expression: The level of CD40 expression on CD20⁺ B cells was significantly higher in asthmatic and COPD patients than in controls ($p<0.001$, $p=0.015$ respectively) Figure 2, Figure 3.

When CD40 expression levels were compared according to genotypic distribution, a significant difference was observed in the asthmatic as well as the COPD group in comparison to the control group ($p=0.024$ and $p=0.008$ respectively). Our data showed a greater amount of CD40 protein in the presence of C polymorphism as it was more expressed with the CT followed by the CC genotypes and the least expression was with the TT genotype (Table 5).

The total serum IgE and its relation to CD40-1C/T genotyping and CD40 protein expression in asthmatics: The total serum IgE was significantly higher in the asthmatic patients than the control group ($p<0.001$). The associations of CD40 genotypes and the total serum IgE levels was not statistically significant neither in subjects with asthma nor the control subjects when using the multivariate general linear model type III controlling for age, sex, and smoking status as covariates. Also there was no significant correlation between the CD40 expression level and total serum IgE in both groups.

Discussion

CD40 is found on a variety of inflammatory cells and is known to influence the inflammatory state and play a role in airway inflammatory responses [3,13]. It is important for antibody class switching and is involved in the amplification and regulation of inflammatory immune responses, including regulation of T-cell-dependent B-cell responses and maturation of dendritic cells (DCs) [13] A C/T in the 5'-untranslated region of *CD40* located at the -1 position within the Kozak sequence (rs1883832) has been associated with CD40 protein expression [14,15].

These biological observations prompted us to evaluate the effect of CD40 -1C/T polymorphism on the risk for asthma and COPD. We found that this polymorphism was significantly different in the asthmatic group from the control group, independent of other confounding factors (age, sex and smoking status). Our study has demonstrated that CD40 -1C/T polymorphism significantly

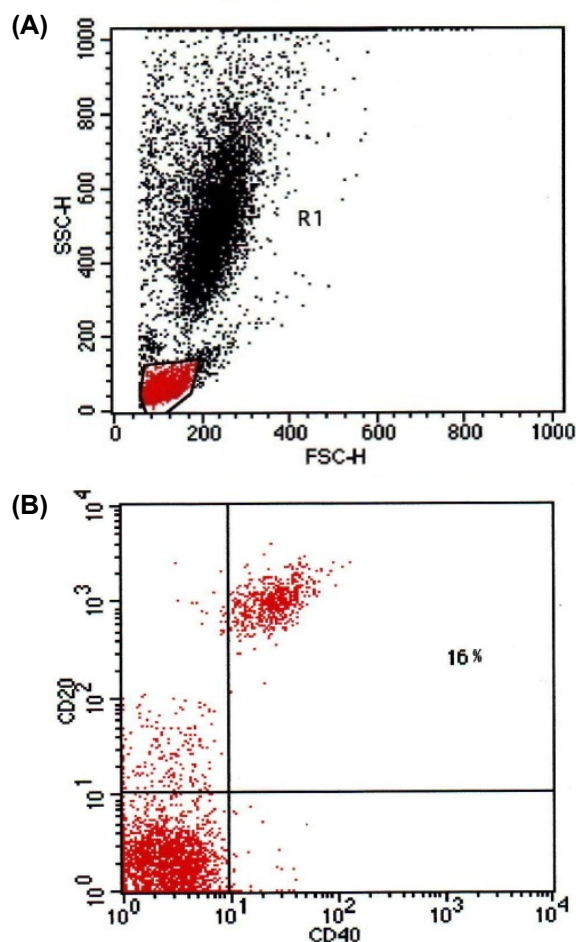


Figure 2: CD40 expression measured by flow cytometry:
A: Dot plot of forward scatter against side scatter showing gated lymphocytes.
B: Dot plot of CD20 stained by (PE) and CD40 stained by (FITC) showing CD20⁺CD40⁺ cells in a studied case.

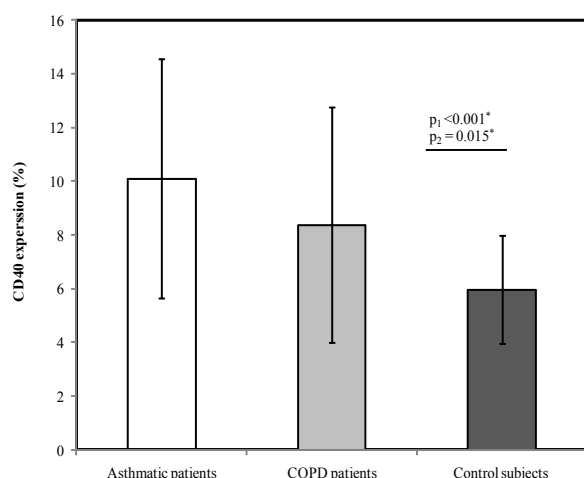


Figure 3: A comparison of CD 40 expression level among the three studied groups.
A: The data are presented as the ratio of the CD40 fluorescence intensity to that of CD20⁺ cells. *: Statistical significance at p ≤ 0.05

contribute to the susceptibility to asthma in the Egyptian population, to our knowledge, this is a novel finding. Contrary to our results Park et al who studied asthmatic patients found that CD40 -1C/T polymorphism had no effect on the development of asthma in the Korean population [16]. This could be attributed to the different studied populations.

COPD is a complex disease influenced by genetic and environmental factors. Previous studies have shown that cigarette smoking is the major environmental risk factor. However, only about a minority (10–20%) of smokers develops the clinically significant disease, [17] also there are a considerable number of people who develop COPD without having smoked cigarettes [18] indicating that susceptibility to COPD may be influenced by genetic factors. We found that (independent of the presence of the main risk factor for COPD which is the smoking status) the CD40-1C/T polymorphism significantly contributes to the susceptibility to COPD, providing further support to the influence of genetic factors. Furthermore, Liu et al. who studied COPD patients got the same finding in the Chinese population, however, the odds ratio for CD40 -1C/T polymorphism was relatively lower (1.777), and thus they suggested that other genes may contribute to the genetic susceptibility to COPD [19].

CD40 -1C/T polymorphism affects the translational efficiency of CD40 protein giving rise to inflammatory responses in the airways and lung parenchyma [20]. This fact supports our findings in asthmatic and COPD patients where the CD40 expression was significantly higher than controls. The ribosome is able to initiate translation more efficiently in the presence of the C polymorphism and results in the formation of greater amounts of CD40 protein, [6] which may imply that the -1C/T polymorphism is linked to inflammation in addition to the initiation and development of asthma and COPD. As our data have shown a greater amount of CD40 protein in the presence of C polymorphism as it was more expressed with the CT followed by the CC genotypes and the least expression was with the TT genotype, which seems to have a protective effect on the protein expression. Indeed, Jacobson et al have shown that the T allele caused a significantly reduced CD40 expression in B cells, which further supports our results [6].

Previous studies have shown that CD40 and CD40L interactions have been implicated in lung disorders, [21] giving rise to a decrease in FEV₁ and FEV₁/FVC. Interestingly, we did not find any association between the -1C/T polymorphisms and the spirometric classification of severity of asthmatic as well as COPD patients, suggesting that CD40 sequence variances have no effect on lung function decline in either of them. Hence, CD40 -1C/T polymorphism may act only as a potentiating factor in asthma and COPD, in concert with polymorphisms of other genes, as well as other environmental factors [19].

Despite the significant upregulation of total serum IgE in asthmatics it was not significantly associated with CD40 genotyping or the protein expression. Despite the fact that many studies have provided evidence that CD40 plays a role in the regulation of IgE [22]. However, our results did not show a direct link between the total serum IgE and the CD40-1C/T polymorphism. In addition, Park et al found that two CD40 SNPs, -1C/T and -580G/A, are associated with total IgE levels in individuals with asthma [16]. Further studies are needed to explore the gene–gene and gene–environment interactions involved in the development of asthma and in IgE regulation.

In conclusion, our study has demonstrated that CD40 -1C/T polymorphism significantly contribute to the susceptibility to asthma and COPD in the Egyptian population supporting the stipulation that

asthma and COPD can have common genetic risk factors. However, carrying T allele showed a significantly lower risk for asthma but not COPD. In addition, CD40 genotypes did not seem to be associated with the smoking status or clinical severity of both. Hence, CD40 – 1C/T polymorphism may act only as a potentiating factor in asthma and COPD, in concert with polymorphisms of other genes, as well as other environmental factors.

The genetic predisposition to certain pathways may further help to define the development of either asthma or COPD. In the end this may lead to stratification of patients by their genetic make-up and open new therapeutic prospects.

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