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RESEARCH ARTICLE

CYTOTOXIC T-LYMPHOCYTE ANTIGEN 4 (CTLA-4) GENE POLYMORPHISMS IN BRONCHIAL ASTHMA.

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Abstract

Asthma is a polygenic, multifactorial disorder, these factors are both genetic and environmental. CTLA-4 gene is located on chromosome 2q33, a key inhibitor of T cell activation, expressed only on activated T helper cells and plays a negative regulatory role in T cell response. CTLA-4 gene polymorphisms have been linked to susceptibility to autoimmune disease as rheumatoid arthritis, celiac disease, type I diabetes, myasthenia gravis, and bronchial asthma. The aim of the current study was to analyze the relation between CTLA-4 gene SNPs (A/G polymorphism at position 49 in Exon 1 and C/T-60 polymorphism in the 3' untranslated region) and serum IgE in bronchial asthma. This study was carried out on 62 patients with bronchial asthma attending inpatients and outpatients clinics of Thoracic Medicine Department, Mansoura University Hospital. Also 38 apparently healthy volunteers were taken as a control group. Ten ml blood was withdrawn from each individual and divided in two tubes. One for measurement of serum IgE by ELIZA and the other for polymorphism genotyping of CTLA4 gene by PCR-RFLP. The results of the current study showed that there was no statistically significant difference between patients and control groups as regard A/G polymorphism at position 49 in Exon 1 of the CTLA4 gene. On the other hand there was a statistically significant difference between patients and control groups as regard C/T-60 Polymorphism in the 3' untranslated region of the CTLA-4 gene. CC genotype was significantly higher in asthmatic than control group. It can be concluded that CTLA-4 gene C/T 60 polymorphisms could confer susceptibility to bronchial asthma and could confirm the usefulness of the CTLA-4 genotyping in predicting bronchial asthma.

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Introduction:-

The pathogenesis and etiology of asthma are very complex and not fully understood, although an interaction of multiple genetic loci and a variety of environmental factors have been suggested as important determinants⁽¹⁾. An increasing number of chromosomal regions have shown evidence of linkage to atopic traits. One such region is on chromosome 2q32-33, harboring the candidate gene-cytotoxic T-lymphocyte antigen 4 (CTLA-4)⁽²⁾. CTLA-4

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molecule is a member of the immunoglobulin superfamily⁽³⁾. It has a central role as a negative costimulator in T-cell regulation as it binds to CD28 (CD28 costimulation promotes T-cell activation) after binding CTLA-4 is weakened this positive costimulation by competing with CD28 for extra cellular ligands on the antigen presenting cell, and hindering the T-cell receptor/CD28 complex during an immune response⁽⁴⁾.

CTLA-4 is expressed on activated T-cells; it binds to B7 molecules on antigen-presenting cells. CTLA-4-B7 binding delivers negative signals to the T-cell, affecting T-cell proliferation, cytokine production and immune responses. Breakdown in the B7-CD28/CTLA-4 pathway may alter T-cell response leading to immune-mediated diseases⁽¹⁾.

Several studies had reported associations between single nucleotide polymorphisms (SNPs) within and around the CTLA-4 region and rheumatoid arthritis⁽⁵⁾, type I diabetes⁽⁶⁾, myasthenia gravis⁽⁷⁾ and autoimmune pancreatitis⁽⁸⁾. Moreover, CTLA-4 gene had also been implicated in other autoimmune disorders like Hashimoto's Thyroiditis⁽⁹⁾, Addison's disease⁽¹⁰⁾ and Multiple sclerosis⁽¹¹⁾.

Howard et al.,⁽¹²⁾ had identified single nucleotide polymorphisms in CTLA4 gene associated with asthma and allergic phenotypes. Additionally, combinations of polymorphisms within CTLA4 increase the risk for increased total serum IgE levels.

The present study aimed to explore the relation between different CTLA-4 gene polymorphisms and serum IgE in bronchial asthma.

Materials and Methods:-

Subjects:-

This study was conducted at Chest Medicine and Medical Biochemistry Departments Mansoura University. This study was carried out on 62 patients with bronchial asthma; they were diagnosed to have bronchial asthma by history, clinical examination and confirmed by pulmonary function tests. Also 38 apparently healthy volunteers were taken as a control group. The subjects included in this study were classified into the following groups:

Group A: included 62 patients with bronchial asthma (23 males and 39 females) with a mean age of 26.27 ± 12.54

Group B: included 38 age and sex matched healthy subjects have been considered as control group (21 males and 17 females) with a mean age of 25.47 ± 9.55 .

Patients receiving immunotherapy or receiving anti allergic drugs during the previous week of taking blood sample were excluded from the study. Also, smokers and patients suffering from chronic chest diseases as COPD, bilateral bronchiectasis and TB, Heart diseases as heart failure or any endocrinal disorders as thyrotoxicosis were excluded from the study.

Ethical approval had been obtained from Medical Research Ethics Committee of Faculty of Medicine (code no: MS/353), Mansoura University. Patients signed their written consents after detailed explanation of the study protocol.

All patients were subjected to the following:

1. Clinical evaluation: Thorough medical history with stress on cough, hemoptysis, chest pain and dyspnea.
2. Radiological investigations: X-ray chest postero-anterior view.
3. Pulmonary function tests: Spirometry was performed at the start of the study, such as forced expiratory volume in the first second (FEV1%) of predicted, FVC and FEV1/FVC ratio were measured by spirometry and reversibility test was done (using Smart PFT CO, Medical Equipment Europe GmbH, Germany).

The diagnostic criteria of bronchial asthma:-

History of cough, chest tightness, wheeze and shortness of breath. Expiratory wheezing on auscultation. Air flow limitation in pulmonary function tests (Reduced FEV1/FVC ratio and positive reversibility test (increase Forced Expiratory Volume1 more than 12% post bronchodilator)⁽¹³⁾.

Ten ml blood was withdrawn from each individual of the study and divided in two tubes.

- a) 5 ml on EDTA used for DNA extraction, the samples are preserved at temperature -30°C until used.
- b) 5 ml allowed for clotting (15 minutes) then centrifuged (7000 rpm for 10 minutes) for serum separation and

preserved at -30°C until used for measurement of Ig E.

- c) Measurement of serum IgE levels by ELISA kit supplied by (International Immuno-diagnostics USA) Catalog Number: 343E

CTLA4 polymorphism genotyping:-

Two SNPs in CTLA4 gene A/G polymorphism at position 49 in Exon 1 and C/T-60 polymorphism in the 3' untranslated region were genotyped by PCR-based restriction fragment length polymorphism (PCR-RFLP) technique.

DNA Extraction: DNA isolation was done using G-spin™ Total DNA Extraction Kit, Reference number 17045, Intron Biotechnology, Korea.

Genotyping of A/G polymorphism at position 49 in Exon 1 of the CTLA4 gene⁽⁶⁾:-

DNA amplification was done for each sample using the following primer sequences:

1. Primer F (Biolegio BV): 5` GCTCTACTTCCTGAAGACCT 3`.
2. Primer R (Biolegio BV):5` AGTCTCACTCACCTTTGCAG 3`.

PCR was performed using thermal cycler (TECHANE TC-321, model FTC3102D, UK) with the following program: Initial denaturation 94°C for 5 minutes (One cycle), denaturation 94°C for 30 seconds (30 cycles), Annealing 55°C for 30 seconds (30 cycles), Extension 70°C for 30 seconds (30 cycles), Final extension 70°C 5 minutes (One cycle).

DNA-PCR products were fractionated on 2% agarose gel using 50 bp DNA ladder markers (*Fermentas life science, Canada*). A positive sharp bands of 162 base pairs were identified. The restriction enzyme *BbvI* was used to cut the 162 bp PCR product only if the G allele is present at this site resulting in fragments of 88 and 74 bp.

Wild type (A/A), 1 band: 162 bp. **Heterozygous mutant (A/G)**, 3 bands: 162, 88 and 74 bp. **Homozygous mutant (G/G)**, 2 bands: 88 and 74 bp (figure 1).

Genotyping of C/T-60 SNP in the 3 UTR of the CTLA4 gene⁽⁷⁾:-

DNA amplification was done for each sample using the following primer sequences:

1. Primer F (Biolegio BV): 5` GAGGTGAAGAACCTGTGTAAA 3`.
2. Primer R (Biolegio BV):5` ATAATGCTTCATGAGTCAGCTT 3`.

PCR was performed using thermal cycler (TECHANE TC-321, model FTC3102D, UK) with the following program: Initial denaturation 94°C for 5 minutes (One cycle), denaturation 94°C for 30 seconds (30 cycles), Annealing 55°C for 30 seconds (30 cycles), Extension 72°C for 30 seconds (30 cycles), Final extension 72°C 5 minutes (One cycle).

DNA-PCR products were fractionated on 2% agarose gel using 50 bp DNA ladder markers (*Fermentas life science, Canada*). A positive sharp bands of 178 base pairs were identified. The restriction enzyme *HPYCH4IV* was used to cut the 178 bp PCR product only if the C allele is present at this site resulting in fragments of 107 and 71 bp.

Wild type (C/C), AGE allows visualization of 2bands: 107 bp, and 71 bp.

Heterozygous mutant (C/T), AGE allows visualization of 3bands: 107, 71 &178 bp.**Homozygous mutant (T/T)**, AGE allows visualization of 1 band: 178 bp (figure2).

Statistical analysis:-

Data were analyzed using the computer program SPSS (Statistical package for social science) version 17.0.

Mann-whitney U test: Used to compare between two groups of numerical (non-parametric) data. Polymorphisms and genotype frequencies were evaluated by gene counts. When the observed genotype frequencies fit to Hardy-Weinberg equilibrium, X² tests (2-by-2 tables) were performed to calculate significantly different genotype distributions between patients and controls and also odd's ratio (OR) and confidence interval 95% (CI 95%) were calculated to detect risk ratio. *P* value <0.05 was considered statistically significant in all analyses.

Results:-**Table 1:-** Comparison of serum IgE between asthmatic group and normal control group.

		Group A Asthmatic (n=62)	Group B Control (n=38)
IgE	Median	215.000*	59.600
	Minimum	57.470*	35.790
	Maximum	411.000*	165.900

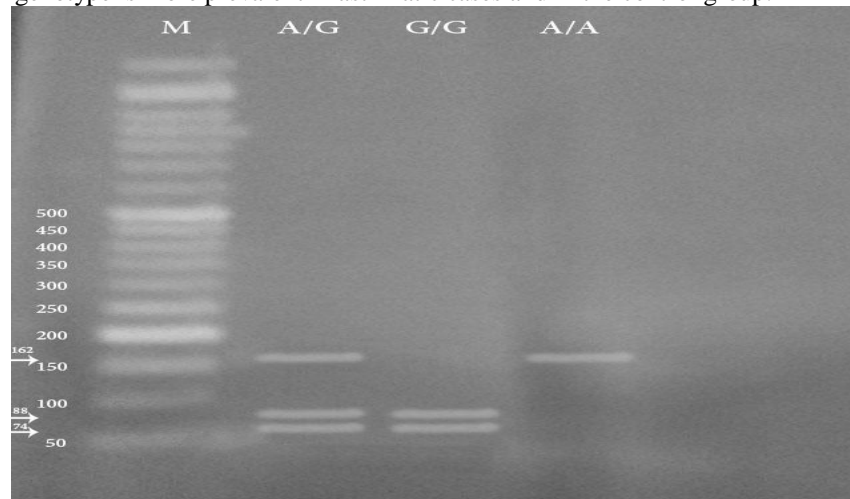
*p< 0.05 Test used: Mann-Whitney test

Table 2:- Comparison between asthmatic group and normal control group as regard the genotyping of CTLA-4 (A/G polymorphism at position 49 in Exon 1):

		Group A Asthmatic (n=62) No (%)	Group B Control (n=38) No (%)	OR (CI 95%)
Genotypes	AA	47 (75.8%)	28 (73.7%)	1(Ref)
	AG	14 (22.6%)	9 (23.7%)	0.9(0.36-2.4)
	GG	1 (1.6%)	1(2.6%)	0.6(0.04-9.9)
	AG+GG	15 (24.2%)	10 (26.3%)	0.89(0.35-2.25)
Allels	A	108 (87.0%)	65(86.0%)	1(Ref)
	G	16 (13.0%)	11 (14.0%)	0.87(0.38-2.002)

OR: odd's ratio CI: confidence interval

It reveals that AA genotype is more prevalent in asthmatic cases and in the control group.

**Figure 1:-** AGE analysis of the +49A/G SNP in CTLA-4 gene after digestion by BbvI. The AA genotype produced only one 162 bp. band (lane 4), the GG genotype produced two bands at 88 and 74 bp. (lane 3) and AG genotype produced three bands: 162, 88 and 74 bp. (lane 2). Lane (1) represents the marker.**Table 3:-** Comparison between asthmatic group and normal control group as regard the genotyping of CTLA-4 (C/T-60 polymorphism in the 3'Untranslated Region):

		Group A Asthmatic (n=62) No (%)	Group B Control (n=38) No (%)	OR (CI 95%)
Genotypes	TT	13 (21.0%)	16 (42.1%)	1(Ref)
	CT	36 (58.0%)	20 (52.6%)	2.2(0.89-5.5)
	CC	13 (21.0%)*	2(5.3%)	8.0(1.5-42.03)
	CT+CC	49 (79.0%)*	22 (57.9%)	2.7(1.1-6.7)
Allels	T	62 (50.0%)	52(68.0%)	1(Ref)
	C	62(50.0%)*	24 (32.0%)	2.16(1.19-3.9)

*p< 0.05OR: odd's ratio CI: confidence interval

The CC genotype is significantly higher in asthmatic than control group.

It also represents that in asthmatic cases T allele (50.0%) is equal to C allele (50.0%), in control group T allele (68.0%) is more than C allele (32.0%) and odd ratio for C/T is 2.16 (95% CI 1.19-3.9; p=0.01).

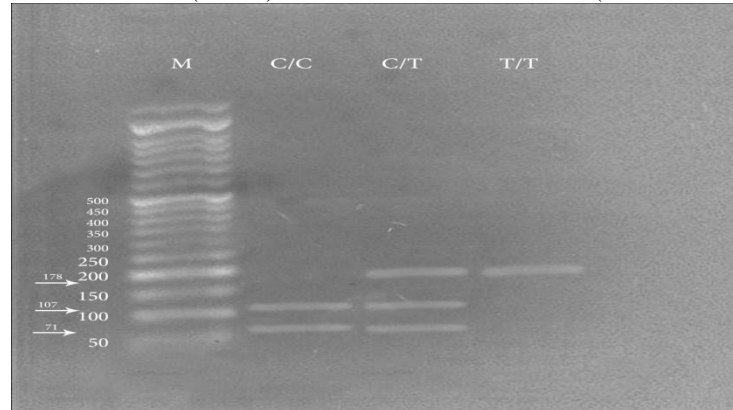


Figure 2:- AGE analysis of the CT60 SNP in CTLA-4 gene after digestion by HpyCH4IV. The TT genotype is presented by a single band at 178 bp (lane 4), the CC genotype is presented by 2 bands at 107 and 71 bp (lane 2) and the CT genotype is presented by 3 bands: 178, 107 and 71 bp (lane 3). Lane (1) represents the marker.

Table 4:-Comparison between asthmatic group and normal control group as regard the haplotyping of CTLA-4 (A/G polymorphism at position 49 in Exon 1) and (C/T-60 polymorphism in the 3'Untranslated Region):

	Group A Asthmatic (n=62) No (%)	Group B Control No (%) (n=38)	OR (CI 95%)
AATT	13 (21.0%)	12 (31.6%)	1(Ref)
AACT	24 (38.7%)	14 (36.8%)	1.58(0.57-4.4)
AACC	10(16.1%)	2(5.3%)	4.6(0.83-25.5)
AGTT	0 (0%)	4(10.5%)	-
AGCT	12 (19.4%)	5 (13.2%)	2.2(0.6-8.17)
AGCC	2 (3.2%)	0 (0%)	-
GGTT	0 (0%)	0 (0%)	-
GGCT	0 (0%)	1 (2.6%)	-
GGCC	1 (1.6%)	0 (0%)	-

OR: odd's ratio CI: confidence interval

It reveals that haplotype AACT is the most prevalent in asthmatic group as well as in the control group

Table 5:-Comparison of serum Ig E between patients and control groups according to genotyping of CTLA-4 (A/G polymorphism at position 49 in Exon 1) and (C/T-60 polymorphism in the 3'Untranslated Region):

IgE			A/G polymorphism at position 49 in Exon 1			C/T-60 polymorphism in the 3'Untranslated Region		
			AA	AG	GG	TT	CT	CC
Groups	Group A Asthmatic (n=62)	Median	215.000	310.000	317.470	108.000	215.000	312.000
		Minimum	57.470	198.000	317.470	57.470	91.360	211.000
		Maximum	401.000	414.000	317.470	215.000	366.000	411.000
	Group B Control (n=38)	Median	59.600	59.600	91.360	54.580	63.150	123.000
		Minimum	35.790	35.790	91.360	35.790	35.790	112.000
		Maximum	165.900	122.000	91.360	84.660	165.900	134.000
	P	*	*	-	*	*	*	

*p< 0.05(Test used: Mann-Whitney U test)

As regard the the comparison of serum Ig E according to genotyping of CTLA-4 (A/G polymorphism at position 49 in Exon 1) between patients and control groups, there is a statistically significant increase of Ig E in patients than control in AA and AG genotypes.

As regard the comparison of serum Ig E according to genotyping of CTLA-4 (C/T-60 polymorphism in the 3' Untranslated Region) between patients and control groups, there is a statistically significant increase of Ig E in patients than control in TT, CT and CC genotypes

Discussion:-

Asthma is a polygenic, multifactorial disease, many factors are involved in its development. These factors are both genetic and environmental⁽¹⁴⁾. CTLA-4 plays an important role in the downregulation of T-cell response⁽³⁾. **Howard et al.**⁽¹²⁾ had reported that there were significant associations between CTLA4 polymorphisms and serum IgE levels, allergy, allergic asthma, asthma and reduced lung function, suggesting a major contribution of the polymorphisms to atopy.

In the present study the comparison between asthmatic group and normal control group as regard the genotyping of CTLA-4 (A/G polymorphism at position 49 in Exon 1) revealed that there was no statistically significant difference between patients and control groups as regard A/G polymorphism at position 49 in Exon 1 of the CTLA4 gene.

This is in accordance with **Munthe-Kaas et al.**⁽¹⁵⁾ who also did not find statistically significant difference between patients and control groups as regard A/G polymorphism at position 49 in Exon 1 of the CTLA4 gene.

Also **Heinzmann et al.**⁽¹⁶⁾ had reported that SNPs in the CTLA-4 gene are not associated with asthma or atopy, and **Nakao et al.**⁽¹⁷⁾ found no association between CTLA-4 gene polymorphisms and atopic asthma in a Japanese population.

However this result disagrees with the results of **Lee et al.**⁽¹⁸⁾ who demonstrated that the CTLA-4 exon 1 +49 A/G polymorphism may have a disease-modifying effect in asthmatic airways. Also **Sohn et al.**⁽¹⁾ had found significant associations between +49 A/G polymorphisms in CTLA-4 and atopic asthma in Korean children.

A meta-analysis performed by **Yao et al.**⁽¹⁹⁾ suggested that CTLA-4 exon 1 +49A/G polymorphism might be a risk factor for asthma susceptibility.

In the current study the comparison between asthmatic group and normal control group as regard the genotyping of CTLA-4 (C/T-60 Polymorphism in the 3' Untranslated Region) revealed that there was a statistically significant difference between patients and control groups as regard C/T-60 Polymorphism in the 3' Untranslated Region of the CTLA-4 gene. CC genotype was significantly higher in asthmatic than control group. This indicates that this SNP is associated with susceptibility to asthma.

These results in harmony with the results of **Munthe-Kaas et al.**⁽¹⁵⁾ who suggested that C/T 60 was involved in asthma pathogenesis. In contrast, **Heinzmann et al.**⁽¹⁶⁾ had reported that SNPs in the CTLA-4 gene are not associated with asthma.

This discrepancy of the results may be due to influence of environmental exposures and different genetic backgrounds (genetic heterogeneity exists in different populations). The same polymorphism plays a different role in different ethnic populations or across different studies⁽¹⁹⁾.

As regard serum level of IgE, this study demonstrated that there was a statistically significant increase in IgE level patient than control.

This finding is in agreement with **Hendeles and Sorkness**⁽²⁰⁾ who found that serum immunoglobulin E levels were high in asthmatics as compared to normal subjects and the IgE levels increased as the severity of asthma increased.

Also this is in harmony with previously published reports of **Agha et al**⁽²¹⁾, **Deo et al**⁽²²⁾, **Dávila et al**⁽²³⁾ who had reported that serum levels of IgE were higher in asthmatics than normal subjects.

As regard genotyping of CTLA-4 (A/G polymorphism at position 49 in Exon 1) this study demonstrated a statistically significant increase of IgE in patients than control in AA and AG genotypes. As regard genotyping of CTLA-4 (C/T-60 polymorphism in the 3' Untranslated Region) this study demonstrated a statistically significant increase of IgE in patients than control in TT, CT and CC genotypes.

In accordance with these results, *Munthe-Kaas et al.*,⁽¹⁵⁾ found a strong association between CTLA-4 polymorphisms and serum IgE level. Also *Howard et al.*,⁽¹²⁾ and *Sohn et al.*,⁽⁴⁾ had demonstrated a significant association between CTLA-4 polymorphisms and serum IgE level.

However, this result is in contrast to *Heinzmann et al.*,⁽¹⁶⁾ who had reported that SNPs in the CTLA-4 gene are not associated with asthma nor with total serum IgE levels.

Conclusion:-

CTLA-4 gene C/T 60 polymorphisms could confer susceptibility to bronchial asthma and could confirm the usefulness of the CTLA-4 genotyping in predicting bronchial asthma. There were associations between CTLA4 polymorphisms and increase serum IgE levels suggesting a major contribution of the polymorphisms to atopy. Further large scale studies about CTLA-4 gene polymorphisms in asthmatic patients will help to identify people at risk of developing asthma.

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