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Study of SNPs in the promoter region of IL-6 gene and other inflammatory markers as predictors of acute appendicitis in Iraqi patients

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Abstract
This study was done to find out if the SNPs (-174G/C) in the regulatory (promoter) region of IL-6 gene can be considered as helpful marker and contribute in the prediction of acute appendicitis combined with other inflammatory marker like WBC count and serum CRP levels in 86 Iraqi patients admitted to emergency department of Alsader Teaching Hospital in Al-Najaf province with clinical and physical diagnosis of acute appendicitis, and 40 healthy individuals without any inflammatory disorders or clinical manifestation of any disease as control group. The distribution of patients according to gender and age showed higher incidence rates in male and in the second age group (16-22years) respectively, but without significant differences ($p > 0.05$). This study also showed that the patients had significantly ($p<0.05$) higher mean WBC counts and CRP when compared to controls. Finally, allelic distributions of SNPs (-174G/C) in the promoter region of IL-6 gene showed no significant difference ($p > 0.05$) between acute appendicitis patients and controls. That means polymorphism plays no role in acute appendicitis prediction so this SNPs cannot use to confirm the clinical and physical diagnosis of acute appendicitis, while other inflammatory markers (WBC count and serum CRP levels) were useful in the prediction of acute appendicitis in Iraqi population.
Key word: SNPs, IL-6 gene, acute appendicitis, Iraq, polymorphism.

Introduction

Appendicitis is the inflammation and the infection of the appendix. It is the most common cause of acute abdominal pains that requiring surgery (appendectomy).

The etiology of acute appendicitis is not certain, may be any mechanical obstruction of appendiceal lumen can caused it (6.8% and 10.5% of appendicitis specimens had mechanical obstruction), like in fecaliths [1,2], it also related with other many other medical complications (lymphoid hyperplasia, viral, bacterial and fungal infections, foreign bodies, Crohn's disease, tumors, and tuberculosis) [3,4,5]. Other study reported that in some cases of appendicitis lumen obstruction was result from spasms of neuromusculature that caused by imbalance of an autonomic nervous [6].

Fast diagnosis of appendicitis is very important because it's out come like peritonitis or appendiceal rupture can cause death [7].

The diagnosis of acute appendicitis was depending on many factors like clinical manifestations (right lower abdominal pain, fever, and anorexia) and physical examination (tenderness and muscular rigidity in the abdomen) [8]. In spite of that 10-30% of appendectomy was reported as negative because the diagnosis (even by experienced surgeons) was a challenging [9, 10].

The Radiological diagnosis also can used to investigate the acute appendicitis. The ultrasonography and computed tomography are used for scanning of acute appendicitis but that must be after the clinical diagnosis [11,12,13].

Ultrasonography is depended on the operator and required careful examination. However it's hard to recognize the normal appendix by Ultrasonography. Furthermore computed

tomography is exposed the patients (in adults and particularly in children) to ionizing radiation and then increased the risk of cancer [14,15].

Another study found that, in spite of the use of the Radiological diagnosis but there was no reduction in the rate of negative appendectomy incidence may be because its low sensitivity [16]. So, other approaches are necessary to investigate the acute appendicitis.

Blood tests, especially the inflammatory response agents, give evidence to insure the diagnosis of appendicitis. The white blood cell count and CRP are used in the diagnosis of appendicitis. The WBC count commonly elevated and may be reached to 12,000/mm3. However, the WBC may be decreased rather than increased, so this test is neither sensitive nor specific enough to detect this disease [17].

Many studies have used the CRP values to diagnose acute appendicitis [18, 19, 20, 21]. In spite of this rise in serum CRP levels in appendicitis, the increase is not necessarily associated with the severity of inflammation .Like other acute phase proteins, CRP increase in response to tissue damage. It also rises in response to bacterial or viral infections and in other (noninfectious) conditions like cancer, myocardial infarction, and arthritis [22].

So to confirm the reason for CRP rise is acute appendicitis we need a combination of tests to achieve diagnostic value. The addition of some inflammatory markers considered as useful method to insure the diagnosis of acute appendicitis, like IL-6 which represents one of the important cytokines of inflammatory response [23].

SNPs (single nucleotide polymorphisms) in the regulatory (promoter) region of IL-6 gene had been found to be related to the biological functions of inflammatory cytokines pathways. One of these SNPs is -174G/C which changes the rate of gene transcription and then the amount of IL-6 produced [24, 25].

This study was done to find out if the SNPs (-174G/C) in the regulatory (promoter) region of IL-6 gene can be considered as helpful marker and contribute in the prediction of acute appendicitis combined with other inflammatory marker like WBC count and serum CRP levels.

Materials and Methods

This study was carried out in Alsader Teaching Hospital in Al-Najaf province and laboratory of molecular biology in the Department of Biology in the Faculty of Science – Kufa University, during the period from September 2014 through May 2016.

1. Study and Control Subjects

a) Study group: All 86 patients in this study were admitted to Alsader Teaching Hospital/ emergency department with clinical and physical diagnosis of acute appendicitis.

b) Control group: It consists from 40 healthy individuals; all were without any inflammatory disorders or clinical manifestation of any disease.

2. Collection of Blood Samples

Four ml of venous blood was collected from clinically suspected patients and control.

a) One ml was allowed to clot at room temperature then centrifuged at 3000 rpm for 5 minutes the serum was used freshly for the serological test of serum CRP.

b) One ml was collected in EDTA tubes and used freshly for WBC count.

c) Two ml was collected in EDTA tubes and store at $^{-}20\,^{o}C$ until used for PCR test.

3. WBC and CRP

a) The WBC count was performed by Sysmex automated hematology analyzer KX-21N (produced by Sysmex Corporation, Wakinohama–Kaigandori, Chuo–Ku, Kobe, Japan).

b) Serum CRP was assayed using a rapid latex agglutination test kit for the detection of the CRP in human serum, provided by AVITEX Inc. (CRP test, *Omega***,** UK).

4. DNA isolation and Polymerase Chain Reaction (PCR)

Genomic DNA was isolated using protocol from Genomic DNA Mini Kit (Geneaid Biotech. Ltd., Taiwan Company, Cat. No. GB100, LOT. No. TJ21207), which designed specifically for purifying DNA from frozen blood.

A sequence of single nucleotide Polymorphic variants in the region of promoter in IL-6 gene (−174 G/C) was amplified using the primer-pairs listed in table 1.

Amplification refractory mutation system (ARMS-PCR) was performed in two tubes to amplify the both alleles, G and C, of IL-6 in a single PCR reaction; one corresponding to the WT sequence and the other corresponding to the mutation. This enabled homo/heterozygosity to be easily determined which mean the genotyping of IL-6.

The primers gene (synthesized by AccuOligo® Bioneer Corporation .USA) were published previously [26].

Amplification was carried out in 20 µl tube of PCR PreMix Reaction Mixture (Bioneer Corporation, USA) containing 5 µl of template DNA, 1 unit DNA polymerase, 2 µl reaction buffer, 2 µl stabilizer and loading-dye, 2 µl dNTPs, and 2µl of each primer(2 µl forward and 2 µl reverse). Distilled water was added to the final volume of 20 µl.

Amplification was performed in a thermal cycler (Cleaver scientific Ltd/UK) programmed for 40 cycles of denaturation at 94°C for 30 seconds, annealing at 66°C for 45 seconds, and extension at 72°C for 45 seconds, preceded by an initial denaturation of 10 min at 95°C. Final extension was for 7 min at 72°C. Finally, the gel electrophoresis method was done according to Sambrook and Russell [27], and 5 µl of each samples was loaded onto 2% agarose gel.

Table 1: Primers pairs used to amplify of single nucleotide polymorphisms in the promoter region of the IL-6 gene (−174 G/C) [26].

Genes	Primers sequences	Product (bp)
Forward primer (G)	5'- GCACTTTTCCCCCTAGTTGTGTCTTACG -3'	121 bp
Forward primer (C, Normal)	5'-GACGACCTAAGCTTTACTTTTCCCCCTA GT TGTGTCTTGAC-3'	136 bp
Reverse primer	5'-ATAAATCTTTGTTGGAGGGTGAGG-3'	

4. Statistical Analysis: Statistical analyses of all results were carried out by the help of SPSS version 17 software statistical package using chi square (P value was considered significant at level less than 0.05).

Results

The study population was included 86 patients with acute appendicitis (The clinical and physical diagnosis was under the supervision of surgeons from the hospital).

Distribution of Cases According to Gender:

The distribution of patients according to gender showed higher rates in male than female but without a significant difference (p> 0.05) between them. The acute appendicitis cases were 47 (54.65%) males and 39 (45.35%) females (Figure 1).

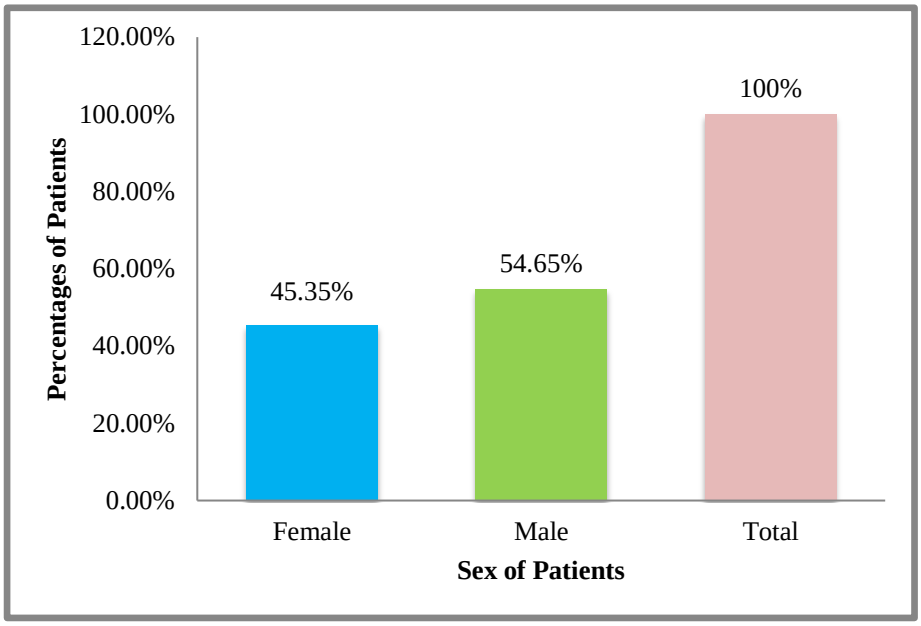

Figure 1: The distribution of patients with acute appendicitis according to gender.

Distribution of Cases According to Age:

Assessment of age presentation of patients revealed that 14 (16.27%) patients were seen in age group 9-15 years, 17 (19.76%) in age group 16-22 years, 11 (12.79%) in age group 23-29 years, 12 (13.95%) in age group 30-36 years, 10 (11.62%) in age group 37-43 years , 12 (13.95%) in age group 44-50 years, and 10 (11.62%) patients in the age group 51-57 years (Figure 2). Their ages ranged from 9 to 57 years, with a mean age of 27.9 years. The estimated incidence of acute appendicitis increased in the first and second age group but without a significant difference in comparison with the other groups.

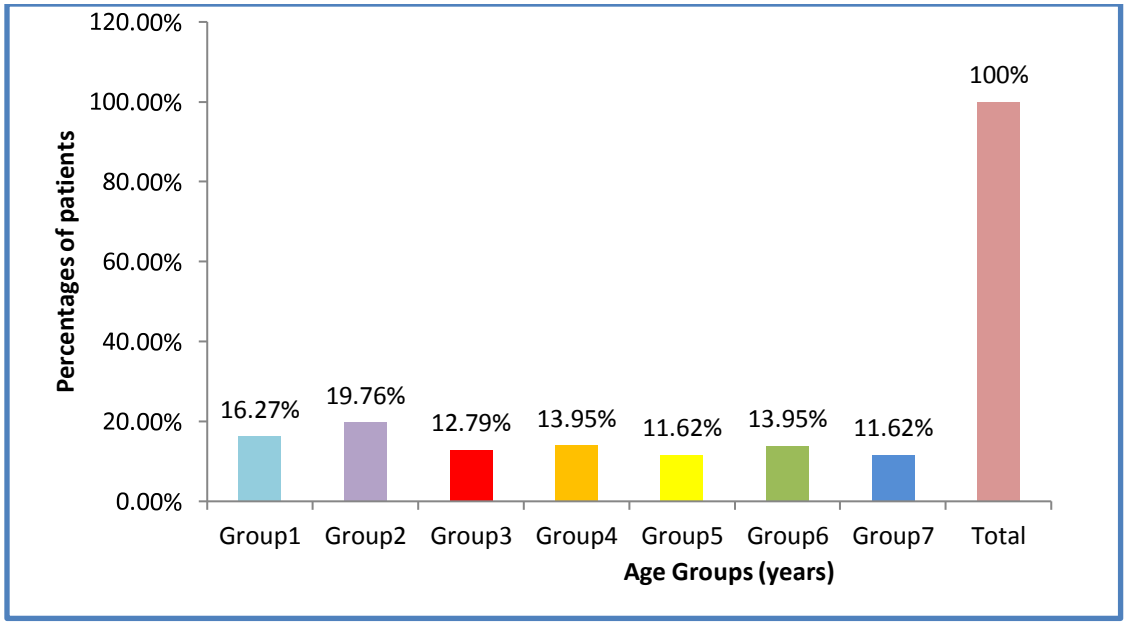

Figure 2: Age Distribution of the patients with acute appendicitis in years groups (Group1: 9-15, Group2: 16-22, Group3: 23-29, Group4: 30-36, Group5: 37-43, Group6: 44-50, Group7: 51-57).

WBC and CRP:

The results of WBC count (Table 2) were showed that 74 (86.05%) patients had WBC count above the normal range in the blood, with a significant difference with control ($p<0.05$).

The serum CRP was positive in 49 (56.97%) patients, while all the controls (100%) were negative. There was a significant difference ($p<0.05$) between positive and negative results (Table 2).

Table 2: The results of WBC count and serum CRP levels in patients and controls.

Tests / Subjects	WBC ($10^3/mm^3$)		CRP (mg/L) (mean± SD)		Total (n=)
	(mean± SD)	n (%)	(mean± SD)	n(%)	
Healthy controls	6.1±2.5	40 (100%)	0.9±3.4	40 (100%)	40
Patients	11.9± 1.3	74 (86.05%)*	28± 4.2	49 (56.97%)*	**86**

***p< 0.05 significant**

Molecular study (polymorphisms in the promoter region of the IL-6 gene):

a) **Healthy controls:** Among the controls (40 healthy persons) 38 (95%) had contain an amplified product in the normal (C) (found as homozygote C / C (95%)), 2 in the normal (C)

and the mutant (G) primer (found as heterozygote G/ C (5%)), but lacks it in the both mutant (G) primer (found as homozygote G/G) (Table 3 & Figure 3).

b) **Acute appendicitis patients:** The genotype distribution among patients with acute appendicitis (86) was: 77(89.53%) had an amplified product in the normal (C) primers which indicating to the normal homozygous genotype, 6 (6.98%) contain an amplified product in both normal (C) and mutant (G) primers; assigning those individual to heterozygous genotype. While 3 (3.49 %) contain an amplified product in the mutant (G) primers indicating to the homozygous genotype (Table 3 & Figure 3).which mean that acute appendicitis Iraqi patients group has either GC or GG (in addition to normal genotype) in the region of promoter in IL-6 gene but without a significant difference with that of individual in control group ($p > 0.05$).

Table 3: The results of the PCR with genotypic distribution and polymorphic variants in the promoter region of IL-6 (at -174 G/C).

Subjects	PCR results			Total
	Homozygote (Normal)	Homozygote (mutant)	heterozygote (N&M)	
Healthy controls	38 95%	0	2 5%	40 32.75%
Acute appendicitis patients	77 89.53%	3 3.49 %	6 6.98%	86 68.25%
Total	113 89.68%	3 2.38%	8 6.35%	126 100%

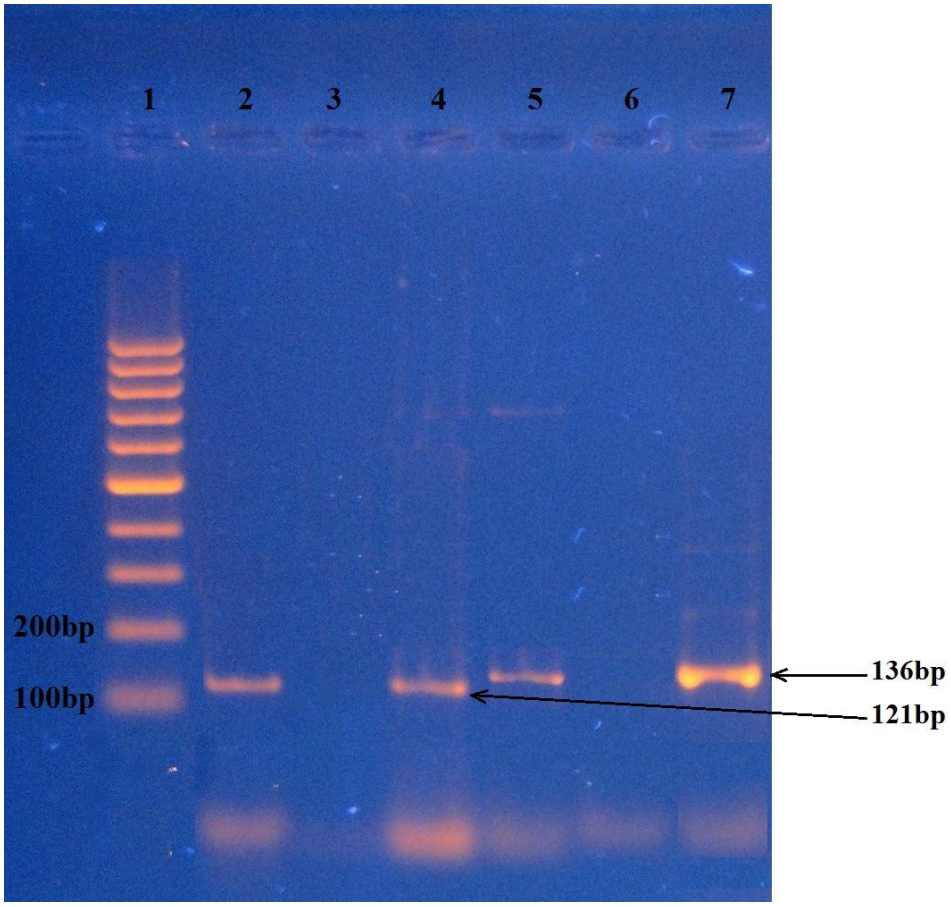

Figure 3: PCR amplified products of IL-6 promoter polymorphism (-174 G/C). Lane1:100 bp DNA ladder. Lane 2&3: Homozygous G (121 bp). Lane4&5: Heterozygous G/C (121 and 136 bp). Lane6&7: Homozygous C (136 bp).

Discussion

Inflammation plays a major role in acute appendicitis, so inflammatory markers had predictive and diagnostic value in confirm the preoperative acute appendicitis diagnosis.

Interleukin-6 (IL6) is an early indicator of inflammatory response which plays an important role in release of CRP, maturation and differentiation of immune cell especially T cells.

The study population was included 86 patients confirmed with acute appendicitis. The distribution of patients according to gender showed higher incidence rates in male than female but without a significant difference. This result agreed with another an Iraqi study [20] and with many other studies [28, 29, 30], which found that males were more susceptible than females to appendicitis.

Assessment of age presentation revealed that the estimated incidence of acute appendicitis increased in the first (9-15 years) and second age group (16-22 years). Other studies had documented a higher incidence of acute appendicitis in preadolescents and young adults [31, 32].

In these age groups, a proliferation of submucosal lymph tissue was observed in the appendix. An increase in the amount of lymphoid tissue in the appendiceal wall is thought to be the key determinant of local immunological and inflammatory responses to infectious or environmental agents, resulting in acute appendicitis [33, 34].

This study also showed that the patients had significantly higher mean WBC counts and CRP when compared to controls.

These findings are in accord with four Iraqi studies [20, 35, 36, 37], which showed that measurement of WBC counts and CRP can improve the accuracy of acute appendicitis diagnosis.

Finally, acute appendicitis Iraqi patients group has either GC or GG in the region of promoter in IL-6 gene, but allelic distributions of SNPs in the regulatory (promoter) region of IL-6 showed no significant difference between acute appendicitis patients and controls. That means polymorphism plays no role in acute appendicitis prediction so this SNP cannot use to confirm the clinical and physical diagnosis of acute appendicitis

These results agreed with other studies [38, 39], that mentioned one explanation "This SNP may be a population specific SNP", that mean it may be associated with appendicitis in other populations but not in population of study, furthermore, there could be other population specific SNP(s) in IL-6, which could be associated with appendicitis. While Almagor *et al*. [40] reported that there were associated between this SNP and diagnosis of appendicitis.

In conclusion, the SNPs (-174G/C) in the regulatory (promoter) region of IL-6 gene cannot be considered as marker to diagnosis of acute appendicitis, while other inflammatory markers (WBC count and serum CRP levels) were useful in the prediction of acute appendicitis in Iraqi population.

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دراسة تعدد أشكال النوكليوتيدات المفردة في منطقة الباديء لجين IL-6 وغيرها من علامات الالتهاب كمتنبئ لالتهاب الزائدة الدودية الحاد في مرضى عراقيين

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الخلاصة:

أجريت هذه الدراسة لمعرفة ما إذا كان تعدد أشكال النوكليوتيدات المفردة (-174 G / C) في المنطقة التنظيمية (الباديء) لجين IL-6 يمكن اعتباره علامة مفيدة وتسهم في التنبؤ لالتهاب الزائدة الدودية الحاد جنبا إلى جنب مع علامة التهابات أخرى مثل عدد كرات الدم البيضاء ومستويات بروتين CRP في الدم في 86 من المرضى العراقيين الداخلين في قسم الطوارئ في مستشفى الصدر التعليمي في محافظة النجف الذين أظهر التشخيص السريري والجسدي اصابتهم بالتهاب الزائدة الدودية الحاد، و 40 من الأشخاص الأصحاء دون أي اضطرابات التهابية أو مظاهر سريرية من أي مرض كمجموعة سيطرة. أظهر توزيع المرضى وفقا لنوع الجنس والسن ارتفاع معدلات الإصابة في الذكور وفي الفئة العمرية الثانية (16-22 سنة) ولكن دون فرق معنوي ($p> 0.05$) . كما أظهرت هذه الدراسة أن المرضى لديهم ارتفاع معنوي ($p> 0.05$) في معدل عدد كريات الدم البيضاء و بروتين CRP بالمقارنة مع السيطرة. وأخيرا أظهرت التوزيعات الأليلية لتعدد أشكال النوكليوتيدات المفردة (-174G / C) في منطقة الباديء لجين IL-6 عدم وجود فرق معنوي ($p> 0.05$) بين مرضى التهاب الزائدة الدودية الحاد و السيطرة. وهذا يعني أن تعدد الأشكال الجيني لا يلعب أي دور في التنبؤ لالتهاب الزائدة الدودية الحاد لذلك فان تعدد أشكال النوكليوتيدات المفردة لا يمكن استخدامها لتأكيد التشخيص السريري والجسدي لالتهاب الزائدة الدودية الحاد، بينما كانت العلامات الأخرى للالتهاب (معدل اعداد كريات الدم البيضاء و مستويات CRP المصلي) مفيدة في التنبؤ لالتهاب الزائدة الدودية الحاد في المجتمع العراقي.