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The Egyptian Journal of Laboratory Medicine published by the Egyptian Society of Laboratory Medicine (ESLM) welcomes original papers, review articles, book reviews, abstracts from current literature and technical notes concerning different clinical laboratory procedures. The journal is published three times annually.

Articles to be published should not be published elsewhere, and should be accepted by a referee of the advisory board.

The authors will be responsible for published articles and not the editor.

MANUSCRIPTS:

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2. It is necessary to present the manuscripts type-written, preferably using word processor write on one side of A4 paper only, double spacing, liberal margins and not more than 24 lines per page.
3. Tables and figures should be: Clear, of very good quality and numbered in Arabic numericals. Photo pictures should be either (black and white or colored).
4. Site of the tables and figures in the articles should be marked in the manuscript.
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6. The manuscript should begin with abstract of the work, followed by introduction, material and methods, results, discussion and the references. The last page is an Arabic summary.
7. Author’s names should be written as follows: First name then family name or first name, initials then family name.
8. References at the end of the paper should be arranged alphabetically in the following order: number, name of the author(s) each followed by initials, year in brackets, title of the subject, abbreviation of the journal name, volume number and page.
9. References within the article are referred to using the number of reference between brackets in superscript typing.
10. Authors are requested to condense their papers.
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EVALUATION OF β-LACTAM AND AMINOGLYCOSIDES SYNERGISTIC EFFECT ON MULTIDRUG RESISTANT PSEUDOMonas

Mona AbdelAziz Wassef, Maha Mohammed Gaafar, Dina Mohammed Hassan and Noha Salah ElDein

ABSTRACT

Background: Multidrug resistant Pseudomonas is one of the rapidly spreading bacteria causing infections with serious outcomes due to limited therapeutic options. Rapid spread of multidrug resistant bacteria (MDR) has become an emerging threat and a matter of concern worldwide. Aim: Our work aimed to screen for carbapenem resistance in 250 isolates of MDR Pseudomonas and evaluate the in-vitro synergistic effect of β-lactam and aminoglycoside combination by using the E- test. Materials and methods: The antimicrobial susceptibility testing for MDR Pseudomonas was done by Kirby-Bauer disk diffusion method and according to the interpretative criteria of CLSI, 2013. Carbapenem resistant Pseudomonas isolates were tested for the synergistic combination of ceftazidime/tobramycin combination using E- test strips for ceftazidime and tobramycin. Results: The distribution of the MDR Pseudomonas showed that the majority were encountered from ICU (47.6%) compared to inpatient wards (43.2%). Among different samples showed that the highest prevalence were in sputum (46.8%), followed by urine (26.8%), pus (21.6%) while the samples with the lowest prevalence were blood (4%) and ear discharge (2.8%). The antibioticogram for MDR Pseudomonas showed that β-lactams demonstrated the highest percentage of resistance 100%, followed by quinolones (88.8%) and aminoglycosides (86.4%). The Screening for carbapenem resistance showed 68% resistance. The results of testing ceftazidime/tobramycin combination have shown a synergistic efficacy in 35 (70%) of the isolates. Conclusion: our study showed a great increase in the spread of carbapenem resistant MDR Pseudomonas evidenced by the screening for carbapenem resistance that showed a high percentage. This necessitates more attention to control infections, by rationalizing the use of antibiotics and adherence to infection control measures. The use of antibiotic combinations exhibiting synergistic effects can be a valuable addition to the standard treatment to overcome treatment failures. Key words: MDR Pseudomonas – Carbapenem resistance - synergistic combinations – E-test

INTRODUCTION

Multidrug resistant Pseudomonas disseminates rapidly and causes considerable morbidity and mortality due to limited therapeutic options(7).

Carbapenem has always been the first option of empirical treatment of many severe infections(13). However, lack of antibiotic policy and abuse of antibiotics in hospitals has been reported to be involved in selection of resistant mutants and causing life threatening problems(9). The use of antibiotic combinations exhibiting synergistic effects is a valuable addition to the standard treatment to overcome treatment failure. The combination of β-lactam and aminoglycosides has been reported to exhibit in-vitro synergism against MDR Pseudomonas specially Ceftazidime with Tobramycin(2). Recently, E- test for in-vitro evaluation of synergism in combinations has been successfully used as an alternative method for the other time and material consuming methods like checkerboard and time kill methods(2).

MATERIALS AND METHODS

Isolates collection:

This cross-sectional study was conducted on 250 clinical isolates of multi-drug resistant Pseudomonas that were collected from hospitalized patients at Kasr El-Aini Hospital during the period from April 2013 to July 2014.

The study included Multi-drug resistant Pseudomonas isolates defined as resistant to one anti-microbial agent in three or more anti-pseudomonal anti-microbial classes (carbapenems, fluoroquinolones, penicillins/cephalosporins and aminoglycosides) and which were taken from clinical specimens(6).

The study excluded any Pseudomonas isolate not fulfilling the defined criteria of multi-drug resistance.

Department of Clinical & Chemical Pathology, Faculty of Medicine, Cairo University
Isolation, identification and susceptibility testing:

Pseudomonas isolates were collected from various clinical specimens and identified by conventional methods such as culture characteristics, oxidase and biochemical reactions. Susceptibility testing was done by Kirby-Bauer method and susceptibility was tested to different antimicrobial agents: Ceftazidime (CAZ) (30ug/disk), Aztreonam (ATM) (30 ug/disk), Cefepime (CPM) (30 ug/disk), Cefoxitin (FOX) (30ug/disk), Amikacin (AK) (30ug/disk), Gentamicin (GM) (10 ug/disk), Piperacillin-Tazobactam (TZP), flouroquinolones (Levofloxacin and Ciprofloxacin) each (5ug/disk), Norfloxacin (10ug/disk-only for urine samples), polymixin (PB) (Oxoid Co. England). Also, Meropenem, and Imipenem susceptibility were determined by standard disk diffusion (SDD) using commercially available disks (Oxoid Co. England) and all were categorized as sensitive, intermediate and resistant according to Clinical Laboratory Standard Institute (CLSI) guidelines 2013.

Testing synergistic combination of β-lactam & aminoglycoside by E-test:

Among the total 250 MDR Pseudomonas, carbapenem resistant isolates were selected and tested for the synergistic combination of ceftazidime/tobramycin by E-test. The E test strips for ceftazidime with concentration range 0.016 to 256 μg/ml and tobramycin 0.016 to 256 μg/ml provided by (BioMérieux) were tested first, individually with every Pseudomonas isolate and MIC readings were taken. The in-vitro activity of the combination of both (ceftazidime and tobramycin) was determined by placing E-test strips of the 2 antimicrobials on the agar at 90° angle with the intersection at the respective MICs for the organism. The agar plates were incubated at 37°C for 18 to 24 hours and the MIC for each antimicrobial combination was read.

Using the results of MICs determined with the antimicrobial alone and in combination, fractional inhibitory concentration (FIC) was calculated for each antimicrobial combination according to the following formulas.

\[ \text{FIC of drug B} = \frac{\text{MIC of drug B when tested in combination with drug A}}{\text{MIC of drug B alone}} \]

\[ \sum \text{FIC index} = \text{FIC}_A + \text{FIC}_B \]

The interpretation of the FIC results was done according to the accepted criteria Sueke et al., (2010) as follows:

- ≤0.5 = synergy
- 0.5-1.0 = addition
- 1.0-4 = indifference
- > 4 = antagonism

RESULTS

Clinical isolates and susceptibility testing:

During the study period from April 2013 to July 2014, MDR Pseudomonas was isolated from different samples in the form of sputum 117 (46.8%), urine 62 (26.8%), wound pus 54 (21.6%), blood 10 (4%), ear discharge 7(2.8%). Out of 250 MDR Pseudomonas isolates, 170 (68%) were resistant to both Imipenem and Meropenem. By testing the susceptibility of Pseudomonas to other groups of antibiotics, among the 250 MDR Pseudomonas, the highest percentage of resistance was recorded for β-lactams (100%) and quinolones (88.8%), followed by aminoglycosides (86.4%) and the least was for carbapenems (68%) as shown in figure (1).

Sources of samples from which MDR Pseudomonas were isolated:

The highest percentage were from samples collected from ICU (47.6%) as shown in figure (2).

Results of testing the in-vitro effect of using (ceftazidime/ tobramycin) combination in MDR Pseudomonas:

The 170 MDR Pseudomonas isolates that were carbapenem resistant were selected for studying the in-vitro efficacy of (ceftazidime/tobramycin) combination.

The synergistic effect of ceftazidime/tobramycin combination was demonstrated in 119 (70%) out of 170 MDR carbapenem resistant Pseudomonas isolates. However, the combination showed indifference in 51 (30%) out of 170 isolates, figure (3) and figure (4).
**Fig (1):** Comparing percentage of resistance among different antibiotic groups

**Fig (2):** The sources of isolates of MDR Pseudomonas

**Fig (3):** In-vitro effect of ceftazidime/tobramycin combination

**Fig (4):** An isolate of MDR Pseudomonas showing synergistic effect of (ceftazidime/tobramycin)
DISCUSSION

The emergence of MDR Pseudomonas aeruginosa is a challenging clinical problem worldwide associated with increase in rates of morbidity and mortality\(^{(14)}\).

Classic agents that used to treat MDR Pseudomonas have become outdated. Of the new drugs available, many have already become targets for bacterial mechanisms of resistance\(^{(8)}\).

Over the last few years, carbapenem drugs have become important therapeutic resources for the control of Pseudomonas aeruginosa infections. However, growing resistance to imipenem and other carbapenems has been observed and multidrug resistance has become more common\(^{(11)}\).

In the present study, the majority of MDR Pseudomonas isolates were encountered from ICU (47.6%) compared to inpatient wards (43.2%) and outpatient (9.2%). This was close to another study in Japan done by Shrestha et al., that demonstrated MDR Pseudomonas with the highest prevalence in ICU (57%) in comparison to other medical wards\(^{(15)}\).

As referred to a previous study done by Boyer et al., (2011), the high prevalence of MDR Pseudomonas in ICU is related to the patient colonization by Pseudomonas which is attributed to both chronological component along with selective antibiotic pressure\(^{(4)}\).

In our study, among 250 MDR Pseudomonas isolates, B-lactams showed the highest percentage of resistance 100%, followed by quinolones (88.8%) and aminoglycosides (86.4%). This typically matched with a similar study done on P. aeruginosa clinical isolates collected from eight hospitals from 2007 to 2009 within five regions of Thailand. It demonstrated the highest resistance with ceftazidime (95.79%), followed by ciprofloxacin (92.34%) and gentamicin (87.36%)\(^{(9)}\). However, another study done in 900-bed university-affiliated teaching hospital in Houston, Texas, showed the highest resistance for Carabpenems (100%), followed by Cephalosporins (91%) and Aminoglycosides (21%)\(^{(17)}\).

As regards screening for Carbapenem resistance, we chose to screen for Carbapenem resistance by the use of imipenem and meropenem disk diffusion method being a reference method used in a previous study done by Babay et al in King Khalid University Hospital (KKUH) Riyadh, Saudi Arabia\(^{(1)}\). In that study, a comparison between various methods of screening, demonstrated that there was a difference in susceptibility testing using E-test versus Microscan in comparison to disk diffusion as a reference method. E-test showed error for imipenem and meropenem (34.9%-34.2%) respectively while Microscan showed 10% error\(^{(1)}\).

The results of screening for carbapenem sensitivity according to the interpretative criteria of CLSI, 2013 have shown 68% resistance among 250 MDR Pseudomonas to both imipenem and meropenem. In a study done in Thailand in 2012 conducted on 261 MDR Pseudomonas isolates, met an agreement with our results showing 71% Carbapenem resistance in MDR Pseudomonas\(^{(9)}\). This was also in accordance with another study done in Hospital of the Lithuanian University of Health Sciences demonstrating high percentage of resistance to Imipenem 87.8%\(^{(19)}\).

Another study done in India by Behera et al., 2008 demonstrated a matching result of 69% Carbapenem resistance in Pseudomonas, however, it screened for Carbapenem resistance by Imipenem E-test\(^{(3)}\).

In our study, we examined in vitro efficacy of combined (β-lactam/ Aminoglycosides), Ceftazidime as a representative of β-lactam, and Tobramycin as a representative of aminoglycosides by E-test on 170 Carbapenem resistant MDR Pseudomonas isolates. The results have shown a synergistic efficacy in 70% out of the 170 Carbapenem resistant MDR Pseudomonas isolates. However, the same combination showed indifference in 30% of these isolates.

This was in accordance with a study done in 2010 that examined the in-vitro efficacy of several antibiotic combinations one of which was (Ceftazidime/ Tobramycin) combination, where it expressed the highest synergistic efficacy 68% in comparison to 50% with ( Piperacillint azo-
ß-lactam and Aminoglycosides: Effect on MDR Pseudomonas

A therapeutic strategy against Pseudomonas aeruginosa is the use of antimicrobial combinations to delay the selection of resistant bacterial clones and to obtain a synergistic antibacterial action. The combination of β-lactam antibiotics and aminoglycosides has been recommended for the treatment of infected patients\(^{(11)}\).

Several studies concerned with testing the susceptibility effect of these combined drugs in-vitro by different methods as (E-test, checkerboard and time kill methods) and many of them reported synergistic effect of using combined drugs, a matter that generated hope in treatment of MDR Pseudomonas. However, limited studies have applied for in-vivo trials to test the effect of using combined drugs in improving the clinical outcome which in turn made the use of combination antimicrobial therapy controversial\(^{(18)}\).

A study involving 28 ICUs was conducted to evaluate the therapeutic value of using empiric combination therapy (ß-lactam and Aminoglycosides) compared with ß-lactam monotherapy. The empiric combination therapy revealed decrease in mortality 36% versus 29% respectively\(^{(10)}\).

However other opinions discouraged the use of combination therapy because of the potential negative consequences as (nephrotoxicity, ototoxicity and additional monitoring requirements). Also, this was supported by studies that showed no significant correlation between in-vitro synergy testing and clinical outcome\(^{(18)}\).

Among several studies, there was no significant difference between testing for synergistic combination by checkerboard technique or E-test. A study was done by Balke et al in 2006 to compare the reliability and the reproducibility of E-test versus checkerboard technique in testing the synergistic efficacy of Ceftazidime/Tobramycin and both recovered synergy of 67%. The agreement between E-test and checkerboard was excellent >90%.4 Also, this was supported by another study which used checkerboard technique and recovered synergistic efficacy of Ceftazidime/Tobramycin combination (67%), which is close to our results that were recovered by E-test\(^{(6)}\).

**Conclusion**

Due to the limited therapeutic options in treating MDR Pseudomonas infections, the use of antibiotic combinations exhibiting synergistic effect is a valuable addition to the standard treatment to overcome treatment failure. In our study, among the total 250 MDR Pseudomonas, 170 carbapenem resistant isolates were tested for the synergistic combination of ceftazidime/tobramycin using E- test strips for ceftazidime and tobramycin. The results have shown a synergistic efficacy in 70% of the isolates. Further studies should be done to evaluate the effect of antibiotic combinations in vivo and its relation to patient outcome.

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8. Kanj S. and Kanafani Z., 2011; Current concepts in Antimicrobial therapy against Resistant Gram-Negative organisms: Extended spectrum ß-lactamase-producing Enterobacteriaceae, Carbenpenem –Resistant Enterob-


THE ROLE OF INTERCELLULAR ADHESION MOLECULE -1 GENE POLYMORPHISM IN THE DEVELOPMENT OF CARDIOVASCULAR DISEASE IN EGYPTIAN PATIENTS WITH END-STAGE RENAL DISEASE


ABSTRACT

Context End-stage renal disease (ESRD) is the most serious consequence of chronic kidney disease. The commonest cause of mortality in ESRD is cardiovascular diseases. Objective To explore the relation between K469E (rs5498) polymorphism of the ICAM-1 gene and the susceptibility of cardiovascular disease in Egyptian patients with ESRD. Materials and methods The study included 50 ESRD patients with cardiovascular diseases and 50 ESRD patients without cardiovascular diseases. They were subjected to routine laboratory investigations and genotyping for ICAM-1 gene K469E polymorphism by Real-Time polymerase chain reaction using taqman probes technology. Results KK genotype frequency was significantly higher in ESRD patients with cardiovascular diseases as compared to ESRD patients without cardiovascular diseases; p=0.003. Also K allele frequency was significantly higher in ESRD patients with cardiovascular diseases as compared to ESRD without cardiovascular diseases; carriers of the K allele were 3.08 times more prone to develop cardiovascular disease. Frequency of combined (KK&KE) genotypes was significantly higher in ESRD patients with cardiovascular diseases as compared to ESRD patients without cardiovascular diseases; p= 0.001. Discussion The pathogenesis of cardiovascular diseases affecting ESRD patients has been extensively studied focusing on genetic factors which have a major role. One of these genetic factors is ICAM-1 gene. Studying the various polymorphisms in this gene will be of great benefit to ESRD patients. Conclusion The results of this study suggest that, ICAM-1 K496E gene polymorphism may exhibit a significant influence on cardiovascular diseases susceptibility in ESRD Egyptian patients. Key words End-stage renal disease – Cardiovascular diseases – ICAM-1

INTRODUCTION

End-stage renal disease is a major public health concern worldwide, the total number of ESRD patients requiring renal replacement therapy has been growing dramatically(20). End-stage renal disease is the 9th leading cause of death in the United States, ranking as 18th cause of death globally and it is considered the most serious outcome of chronic kidney disease which results from a lot of causes; the most common causes are Diabetes mellitus, hypertension and chronic glomerulonephritis(16).

Cardiovascular disease is the leading cause of morbidity and mortality in patients with chronic kidney disease(11). Among dialysis patients, 40% of deaths are due to cardiovascular causes and 60% are from other causes(17).

This increased incidence of cardiovascular disease in ESRD cannot be explained by traditional risk factors alone, indicating that genetic factors play an important role in cardiovascular disease pathogenesis in ESRD(15).

There is an increasing evidence that an inflammatory process plays a central role in the development and progression of atherosclerosis, a common entity underlying the pathogenesis of cerebral and cardiac ischemia(4).

Intercellular adhesion molecule-1 (ICAM-1), a cell surface glycoprotein, is a member of immunoglobulin like superfamily of adhesion molecules. It mediates the adhesion of circulating leukocytes to the activated endothelium which is one of the earliest events in the pathogenesis of atherosclerosis. ICAM-1 is expressed on vascular endothelium, smooth muscle cells, macrophages and lymphocytes. Its expression can be upregulated by inflammatory mediators(18).

Variations in the ICAM-1 gene have been reported to be involved in inflammatory diseases and atherosclerosis(6).

The aim of the present work was to study if K469E polymorphism of the ICAM-1 gene is associated with increased cardiovascular disease risk in Egyptian patients with ESRD.
MATERIALS AND METHODS

This study was carried out from January 2013 till October 2014 on 100 ERSD Egyptian patients attending the dialysis unit of Theodore Bilharz Research Institute for regular hemodialysis. They were divided into two groups.

Group one included 50 ERSD patients with cardiovascular diseases in the form of ischemic cerebral stroke, coronary artery disease, left ventricular hypertrophy and congestive heart failure. Their ages ranged from 45 – 70 years (mean age 57.5±6.6) they were 35 males and 15 females. Group two included 50 ERSD patients without cardiovascular diseases, their ages ranged from 45 – 70 years (mean age 57.5±6.6) they were 34 males and 16 females.

The study was approved by the local research ethics committee and an informed written consent was taken from each participant.

All patients were subjected to full history taking, full Clinical examination, routine laboratory investigations (liver, kidney function tests, total cholesterol and triglycerides) and DNA analysis for ICAM-1 gene K469E polymorphism by Real-Time polymerase chain reaction (RT-PCR) using taqman probes technology.

Specimen collection and storage:

- Five ml venous blood were withdrawn from all subjects and divided into 2 parts:
  1- Two ml were collected in sterile vacutainers containing ethylene diamine tetraacetic acid “EDTA” which were used for DNA extraction. Samples were kept frozen at - 20°C till time of DNA extraction and analysis using GeneJET Whole blood Genomic DNA Purification Mini Kit (Thermo Fisher, USA). Quantity of DNA was assessed using Ultrospec 3000 UV/Visible spectrophotometer (Pharmacia Biotech, UK).
  2- Three ml were collected on plain tubes, left for 10 minutes to clot and then centrifuged at 3000 rpm for 5 minutes, the separated serum was used for routine laboratory investigations. They were all analyzed on the Beckman Coulter Synchron CX4 (Beckman Coulter, Ireland).

Statistical analysis

The Results were analyzed using the Software SPSS (Statistical Package for Social Science) Version15. Quantitative data were presented as mean ±SD. For comparison of the two groups means, the Student’s t-test was used. Non parametric quantitative data were expressed as median (range), Mann- whitney tests were used for comparison of medians. Qualitative data was expressed as frequency and percentage. Association between qualitative data was done using Chi- square test. Risk estimate was done by odds ratio (OR) and their 95% Confidence intervals (CIs) were calculated. P value < 0.05 was considered significant.

RESULTS

Results of the current study are presented in tables 1, 2.

No significant difference could be detected upon comparing gender between the 2 studied groups, p=0.829.

ESRD patients with cardiovascular diseases had significantly higher BMI and presence of chronic disease as compared to ESRD patients without cardiovascular diseases (p= 0.000, 0.001), respectively.
Role of ICAM-1 Gene Polymorphism in CVD in ESRD

Percentage of smokers was higher in ESRD patients with cardiovascular diseases group than ESRD patients without cardiovascular diseases group; however this difference didn’t reach statistical significance, \( p = 0.058 \), table 1.

Detection of ICAM K469E SNP in the ICAM-1 gene was analyzed in the two groups using real time - PCR. Three genotypes were recognized; KK, KE and KE, table 3.

It was found that both KK genotype percentage and combined KK&KE genotypes frequencies were significantly higher in ESRD patients with cardiovascular diseases as compared to ESRD patients without cardiovascular diseases \( (p=0.003, 0.001) \) respectively.

Regarding the K allele frequency, it was found that K allele percentage was significantly higher in ESRD patients with cardiovascular diseases as compared to patients without cardiovascular diseases \( (p=0.000) \).

As regards risk estimation for the ICAM-1 K469E genotypes, combined KK&KE genotypes versus EE genotype were of statistical significance \( (OR=5.924, 95\% CI = 1.825-19.229, p =0.001) \) which means that patients with KK&KE genotypes are 5.9 times susceptible to develop CVD. Regarding risk estimation for the ICAM-1 K469E alleles, K versus E allele was of statistical significance \( (OR=3.083, 95\% CI = 1.701-5.588, p =0.000) \) which means that K allele carriers are 3.08 times susceptible to develop CVD.

Table 1 Demographic and clinical data among the 2 studied groups:

<table>
<thead>
<tr>
<th></th>
<th>Group (1) ( n=50 )</th>
<th>Group (2) ( n=50 )</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)**</td>
<td>57.5±6.6</td>
<td>57.5±6.6</td>
<td>1.00</td>
</tr>
<tr>
<td>Gender***</td>
<td>Males ( n=69 )</td>
<td>Males ( n=69 )</td>
<td>0.829</td>
</tr>
<tr>
<td></td>
<td>Females ( n=31 )</td>
<td>Females ( n=31 )</td>
<td></td>
</tr>
<tr>
<td>Weight (Kg)**</td>
<td>83.7±12.39</td>
<td>70.8±13.17</td>
<td>0.000*</td>
</tr>
<tr>
<td>BMI (Kg/m(^2))**</td>
<td>28.7±4.33</td>
<td>28.66±4.47</td>
<td>0.000*</td>
</tr>
<tr>
<td>Systolic blood pressure**</td>
<td>135.6±14.8</td>
<td>132±11.7</td>
<td>0.182</td>
</tr>
<tr>
<td>(mm/Hg)</td>
<td>84±8.57</td>
<td>84.4±10.52</td>
<td>0.835</td>
</tr>
<tr>
<td>Diastolic blood pressure**</td>
<td>84±8.57</td>
<td>84.4±10.52</td>
<td></td>
</tr>
<tr>
<td>(mm/Hg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Presence of chronic disease</strong>*</td>
<td>Known to have diabetes mellitus ( n=27 ) (57%)</td>
<td>Known to have diabetes mellitus ( n=9 ) (18%)</td>
<td>0.001*</td>
</tr>
<tr>
<td></td>
<td>Known to have hypertension ( n=14 ) (28%)</td>
<td>Known to have hypertension ( n=23 ) (46%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Known to have both diabetes and hypertension ( n=21 ) (42%)</td>
<td>Known to have both diabetes and hypertension ( n=4 ) (8%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Systemic lupus erythematosis ( n=1 ) (2%)</td>
<td>Systemic lupus erythematosis ( n=0 ) (0%)</td>
<td></td>
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<tr>
<td></td>
<td>Behcet’s disease ( n=1 ) (2%)</td>
<td>Behcet’s disease ( n=0 ) (0%)</td>
<td></td>
</tr>
<tr>
<td>Smoking***</td>
<td>No chronic disease smoker ( n=5 ) (10%)</td>
<td>No chronic disease smoker ( n=14 ) (28%)</td>
<td>0.058</td>
</tr>
<tr>
<td></td>
<td>Non smoker ( n=28 ) (56%)</td>
<td>Non smoker ( n=38 ) (76%)</td>
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</tr>
<tr>
<td></td>
<td>Ex smoker ( n=5 ) (10%)</td>
<td>Ex smoker ( n=5 ) (10%)</td>
<td></td>
</tr>
</tbody>
</table>

* \( p \) value was considered significant at \( < 0.05 \).

** Data are represented as mean ± SD

*** Data are represented by median (25th-75th percentile).
Table 2 Comparison of biochemical data of the 2 studied groups

<table>
<thead>
<tr>
<th></th>
<th>Group (1)</th>
<th>Group (2)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=50)</td>
<td>(n=50)</td>
<td></td>
</tr>
<tr>
<td>Urea (mg/dl)**</td>
<td>94±21.03</td>
<td>89.62±20.43</td>
<td>0.204</td>
</tr>
<tr>
<td>Creatinine (mg/dl)**</td>
<td>7.78±1.26</td>
<td>8.11±1.23</td>
<td>0.183</td>
</tr>
<tr>
<td>Uric acid (mg/dl)**</td>
<td>5.70±1.63</td>
<td>6.0480±1.5</td>
<td>0.288</td>
</tr>
<tr>
<td>Phosphorus (mg/dl)**</td>
<td>5.58±1.17</td>
<td>4.6±1.21</td>
<td>0.000*</td>
</tr>
<tr>
<td>Sodium (mmol/l)**</td>
<td>138.08±3.34</td>
<td>137.94±3.59</td>
<td>0.841</td>
</tr>
<tr>
<td>Potassium (mmol/l)**</td>
<td>4.3100±0.60</td>
<td>4.4600±0.526</td>
<td>0.191</td>
</tr>
<tr>
<td>Albumin (g/dl)**</td>
<td>3.72±0.472</td>
<td>3.73±0.393</td>
<td>0.890</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)**</td>
<td>238.36±64.54</td>
<td>188.88±49.13</td>
<td>0.000*</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)**</td>
<td>145.7600±39.07</td>
<td>115.92±27.03</td>
<td>0.000*</td>
</tr>
<tr>
<td>Calcium (mg/dl)**</td>
<td>8.28±0.76</td>
<td>8.75±0.72</td>
<td>0.002*</td>
</tr>
<tr>
<td>Glucose (mg/dl)**</td>
<td>136(95.5-178.5)</td>
<td>100(85-127)</td>
<td>0.007*</td>
</tr>
<tr>
<td>ALT (U/L)**</td>
<td>29(18.2-34.2)</td>
<td>25(19-34)</td>
<td>0.748</td>
</tr>
<tr>
<td>Total protein (gm/dl)***</td>
<td>7.75(6.9-8.12)</td>
<td>7.35(6.9-7.8)</td>
<td>0.337</td>
</tr>
<tr>
<td>AST (U/L)**</td>
<td>25(19-34)</td>
<td>20(16-33)</td>
<td>0.150</td>
</tr>
</tbody>
</table>

*P value was considered significant at < 0.05.
**Data are represented as mean ± SD
***Data are represented by median (25th-75th percentile).

Table 3 Frequency Distribution of ICAM-1 K469E genotypes and alleles among the 2 studied groups

<table>
<thead>
<tr>
<th>ICAM-1 K469E genotypes</th>
<th>Group 1 (n=50)</th>
<th>Group 2 CVD (n=50)</th>
<th>Odds ratio (OR)</th>
<th>95% Confidence Interval (CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>KK</td>
<td>28(56%)</td>
<td>15(30%)</td>
<td></td>
<td></td>
<td>0.003</td>
</tr>
<tr>
<td>KE</td>
<td>18(36%)</td>
<td>18(36%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EE</td>
<td>4 (8%)</td>
<td>17(34%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KK&amp;KE</td>
<td>46(92%)</td>
<td>33(66%)</td>
<td>5.924</td>
<td>(1.825-19.229)</td>
<td>0.001</td>
</tr>
<tr>
<td>EE</td>
<td>4(8%)</td>
<td>17(34%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K allele</td>
<td>74(74%)</td>
<td>48(48%)</td>
<td>3.083</td>
<td>(1.701-5.588)</td>
<td>0.000</td>
</tr>
<tr>
<td>E allele</td>
<td>26(26%)</td>
<td>52(52%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results were presented as number (n) of patients and percentage. P value of the difference was considered significant at < 0.05.
ICAM-1 belongs to the super-family of immunoglobulins, it is composed of 507 amino acids and expressed on the surface of endothelial cells, macrophage and activated leukocytes. It promotes the adhesion of vascular endothelial cells and white blood cells through combining with function associated antigen of its ligand lymphocyte and plays an essential role in the inflammatory mechanism of atherogenesis\(^{(10)}\). Its coding gene is located in p13.3- p13.2 of the human chromosome 19 and consists of 7 exons and 6 introns. The sixth exon has a common A to G, also called K469E polymorphism, influencing 469th codon and finally causing a change from lysine to glutamic acid. And such gene variant may affect the Plasma concentration and activity of ICAM-1\(^{(3)}\).

Results of the present study revealed no significant difference between the 2 study groups as regards sex, smoking, blood pressure and age but the first group had significantly higher BMI. This comes in accordance with Gazi et al, Buraczynska et al and Jiang et al\(^{(5,3,8)}\).

Moreover the current study showed that ESRD patients with cardiovascular diseases had significantly higher serum cholesterol, glucose and phosphorus levels and lower calcium levels as compared to ESRD patients without cardiovascular diseases, similar findings were detected by Liu et al, Buraczynska et al and Palmer et al\(^{(10,3,15)}\).

On studying ICAM-1 K469E gene polymorphism it was found that the KK genotype frequency was significantly higher in ESRD patients with cardiovascular diseases as compared to ESRD patients without cardiovascular diseases; \(p=0.003\). Also patients with K allele are 3.08 times susceptible to develop CVD; \(p=0.000\). Moreover, frequency of combined (KK&KE) genotypes was significantly higher in ESRD patients with cardiovascular diseases as compared to ESRD patients without cardiovascular diseases; (OR=5.924, 95% CI = 1.825-19.229) which means that patients with KK&KE genotypes are 5.9 times susceptible to develop CVD; \(p=0.001\).

These results are in accordance with the study done by Buraczynska et al\(^{(3)}\), who found an association of K469E polymorphism in the ICAM-1 gene with cardiovascular disease occurrence in patients with end-stage renal disease. The KK genotype of the K469E polymorphism was significantly associated with cardiovascular disease comorbidity in ESRD patients (OR 9.90, 95% CI 6.17–15.88; \(p=0.006\)).

ICAM-1 gene and particularly ICAM-1 K469E gene polymorphism has been reported to be associated with development of ESRD, and may serve as risk factor for ESRD\(^{(4)}\).

Similarly, Jiang et al\(^{(8)}\) found that K469E polymorphism of the ICAM-1 gene may be involved in the pathogenesis of coronary atherosclerosis. The results of this study suggested that the KK and KE genotypes of the K469E polymorphism in ICAM-1 gene may determine susceptibility to coronary heart disease (OR 2.21 95% CI 1.20–4.07; \(P=0.011\)).

Zhang et al\(^{(21)}\) and Mohamed et al\(^{(14)}\) found that the K allele may serve as a genetic risk factor of coronary heart disease in two studies done on a Chinese and an Egyptian population.

The K469E polymorphism in ICAM-1 gene leads to amino-acid exchange from glutamic acid (negative polar, acidic amino-acid) to lysine (positive polar, basic amino-acid), this polymorphism has been shown to influence the binding of ICAM-1 on endothelial cells, lymphocyte function-associated antigen-1 (LFA-1) and Mac-1 on leucocytes, mediating leukocytosis and its migration in an inflammatory environment and thus enhancing inflammation and atherosclerosis\(^{(12)}\). The K allele of the ICAM-1 K469E gene polymorphism could alter the structure and function of the ICAM-1 5th immunoglobulin like domain, which was implicated in the ICAM-1 molecular dimerization, adhesion function and can change the combination of ICAM-1 and ligands such as the LFA-1 and the complement receptor 3. The combined alterations contributed to leukocytes adhering to vascular endothelial cells more strongly and passing through the vascular wall more easily, and thus enhancing atherosclerosis\(^{(19)}\).
A recent meta analysis by Ji et al(7) which included 12 studies, including 2,157 cases and 1,952 controls showed that ICAM-1 K469E SNP is associated with coronary heart disease risk and K allele is a more significant risk factor for developing coronary heart disease among Asian and Caucasian population.

On the other hand, Li et al(9) reported that EK and EE genotype frequencies were significantly higher in stroke patients than healthy controls (OR 1.68, 95% CI = 1.2-2.35, p=0.002, OR 2.51, 95% CI = 1.3-4.85, p=0.005 respectively). Also they found that allele (E) patients had a significantly increased risk of ischemic stroke (OR=1.79, 95% CI= 1.30-2.46; p= 0.000).

Also, McGlinchey et al(13) and Aminian and Arandi(2) found no association between the K469E polymorphism in the ICAM-1 gene and coronary artery disease in a well-defined Irish and Iranian population.

In conclusion, the results in the present study suggest that ICAM-1 K469E KK, KE genotypes and K allele may influence the susceptibility to acquire cardiovascular diseases in ESRD patients in our population. Replication of the reported association in larger patient cohorts is needed to validate these results.

**Declaration of interest**

The author reports no conflict of interests.

**Acknowledgements**

Special thanks to my colleagues who helped through this work.

**REFERENCES**

Role of ICAM-1 Gene Polymorphism in CVD in ESRD


دور تعدد الأشكال الجيني في الجين المسنول عن جزيء الإنجاب بين الخلايا رقم في حدوث أمراض القلب والأوعية الدموية في المرضى المصريين المصابين بالفشل الكلوي المزمن: دعاء ممدوح على - رانيا كمال درويش - عفاف أحمد عبد الهادي - امل اسماعيل صبري - فاطمة أحمد فتحي الموجي

الفشل الكلوي المزمن هو واحد من الأمراض الأكثر إشكالية في بلدينا. وقد وجد أن المرضى الذين يعانون من الفشل الكلوي المزمن لديهم ارتفاع بشكل كبير في نسبة الوفاة المبكرة نتيجة لأمراض القلب والأوعية الدموية. وتزايد نسبة حدوث أمراض القلب مع انتفاخ وظائف الكلي، وذلك فإن هناك ضرورة فرضية للتعامل المبكر لمنع تطور وتقدم الأعراض الجانبية الإضافية.

الأدلة الحديثة تشير إلى أنه كلاً من الأمراض الأرضية وتوتر الدورة الدموية والالتهابات المتطرفة قد تؤدي إلى ارتفاع خطر حدوث أمراض القلب والأوعية الدموية. نتيجة ارتفاع خطر حدوث أمراض القلب والأوعية الدموية في مرضى الفشل الكلوي المزمن، معدلات الاندماج المختلفة الحالية لهذه الأمراض بين مرضى الفشل الكلوي المزمن في جميع أنحاء العالم تشير إلى أن العوامل الوراثية تلعب دورًا هامًا في حدوث أمراض القلب والأوعية الدموية في مرضى الفشل الكلوي المزمن. هناك تقارير مختلفة تظهر وجود علاقة بين ICAM-1 K496E وحمض الديوكسيجليسيرول في مرضى الفشل الكلوي المزمن. وقد أجريت هذه الدراسة لتحليل توزيع ICAM-1 K496E في مرضى الفشل الكلوي المزمن ومرضى الفشل الكلوي في مجموعة أخرى من المرضى المصريين المصابين بالفشل الكلوي المزمن. وتشمل الدراسة الحالية 50 مريضًا أصليًا مصريًا مزمنًا. وتطورت النتائج الحالية يشير إلى أن فشل كلوي مزمن يعاني من هذه الأمراض بنموذج ICAM-1 K496E

Conclusions:ICAM-1 K496E وحمض الديوكسيجليسيرول في مرضى الفشل الكلوي المزمن. وقد أجريت هذه الدراسة لتحليل توزيع ICAM-1 K496E وحمض الديوكسيجليسيرول في مرضى الفشل الكلوي المزمن. وتشمل الدراسة الحالية 50 مريضًا أصليًا مصريًا مزمنًا. وتطورت النتائج الحالية يشير إلى أن فشل كلوي مزمن يعاني من هذه الأمراض بنموذج ICAM-1 K496E

SERUM AND SYNOVIAL FLUID PLEIOTROPHIN LEVEL IN RHEUMATOID ARTHRITIS
Samia H Fadda*, Iman H Bassyouni*, Rania H Khalifa** and Nora Y El-said*

ABSTRACT
Background: Recent studies have suggested that Pleiotrophin (PTN) might be a good monitoring biomarker and candidate for target therapy in osteoarthritis (OA). However little is known about the role of PTN in Rheumatoid arthritis (RA). Its proposed role in RA is acquired from scarce data that reported its expression in synovial tissues. This study is to measure PTN in the sera and synovial fluids in RA and assess its relation to activity, functional class and radiological staging. Subjects and Methods: Ninety samples were collected as follows: 55 serum samples from 35 RA patients and 20 controls and 35 synovial fluid samples from the RA patients. Demographic, clinical and serological data were prospectively assessed. Functional and radiographic grades were also assessed. Serum and synovial fluid PTN levels were measured using enzyme-linked immunosorbent assay (ELISA). Results: The mean synovial fluid level of PTN in patients was significantly higher than mean serum level (p<0.005). There was significant correlation between serum PTN level and both morning stiffness duration (p=0.008) and MHAQ score (p=0.039). Conclusion: We firstly report a serological pattern of PTN in the sera and synovial fluids of RA patients. However its implementation as a disease marker or a potential target therapy awaits larger studies and further investigations. Key words: Pleiotrophin; Rheumatoid arthritis; Serum; Synovial fluid; ELISA.

INTRODUCTION
Rheumatoid arthritis (RA) represents the most common form of chronic inflammatory joint disease leading to cartilage and bone destruction (20). Leukocytes emigrate into the synovium through the vascular endothelium resulting in synovial inflammation with subsequent joint destruction. The inflammatory process causes diffuse thickening and hyperplasia of the rheumatoid arthritis synovium (21).

Concurrent to degradation of the articular cartilage, there is invasion of the cartilage by blood vessels derived from the subchondral bone (23). RA synovial tissue is rich in newly formed vessels where angiogenesis promotes leukocyte extravasation into the synovium (19).

Chronic inflammation in RA is also characterized by a constant expression of inflammatory cytokines. This understanding is important for the diagnosis, prevention and treatment of RA. We still do not know the full picture of the cytokines that play role in the pathogenesis of RA. However, various cytokines (e.g., TNF-α, IL-1, IL-6, IL-8 and IL-15, TFG-β, bFGF and PDGF) are already established to be involved in the inflammatory reaction. Several studies suggest that Pleiotrophin (PTN) and midkine (MK) should be included in this list, although their precise role in RA is not completely revealed (12, 16).

PTN is a secreted growth factor of 136 amino acids, which form with MK, a distinct family of heparin-binding growth factors. It is secreted by BM-derived endothelial cells and expressed in mesodermal and neuroectodermal cells during development, but rarely in adult tissues. It is called “pleiotrophin” because of its function as a differentiation factor and growth factor for a variety of cell types (12).

PTN is a potent angiogenic factor acting on endothelial cell survival, proliferation, migration, as well as capillary-like structure formation (16). PTN has been also shown to up regulate the mRNA of the potent proangiogenic cytokine; vascular endothelial growth factor (VEGF) in the synovium of RA, and recent reports anticipate that combination of anti-VEGF with anti-PTN therapy will dramatically reduce the angiogenesis process (10).

In addition, PTN has been found to induce the expression of inflammatory cytokines including TNF-α, IL-1b and IL-6 in quiescent human peripheral blood mononuclear cells (PBMC) which points up its importance in the regulation of inflammatory processes (11). PTN is expressed in the infant cartilage. In the adult cartilage, its expression is limited to pathological conditions, including RA and osteoarthritis (OA) (9) where developmentally regulated factors often reappear during disease (16).

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Midkine, which shares 45% sequence identity with PTN, has been found elevated in the synovial fluid and sera of RA patients and it is proposed to be used as a screening aid for RA\(^{(11)}\). However, until now, no data are available about the soluble concentrations of PTN in the synovial fluid and serum of RA patients.

In sight of the above mentioned, our study aimed to investigate whether PTN could be found in the sera and synovial fluid in patients with RA and its relation to disease activity, functional class and radiological staging.

**SUBJECTS AND METHODS**

**Study Population**

This study was carried on 35 adult RA patients (21 females, 14 males); mean age of 42.23 ± 9.32 years, fulfilling the ACR/EULAR 2010 classification criteria of RA\(^{(2)}\). The study also included 20 age, sex and ethnically matched healthy subjects served as normal control group.

All patients were recruited from the Outpatient Clinic of the Rheumatology and Rehabilitation Department, Faculty of Medicine, Cairo University. Exclusion criteria of the present study included arthritis of other etiologies’, hemochromatosis, preceding fractures, preceding infection in joint, systemic inflammatory or autoimmune diseases, malignancies, intra-articular administration of steroids for at least three months before joint aspiration.

Patients were subjected to full history taking, complete physical examination as well as assessment of disease activity and functional ability.

Disease activity was assessed by using the Disease Activity Score 28 (DAS28)\(^{(15)}\). RA functional ability was assessed by using the modified version of the health assessment questionnaire (MHAQ) which was validated previously\(^{(14)}\).

Plain radiographs for hands, wrists and feet were taken for each RA patient at the time of blood sampling. Radiographic joint damage was assessed according to Larsen score\(^{(9)}\); with the number and size of bone erosions and the extent of joint space narrowing related to the cartilage damage being evaluated. Treatment modalities were determined by combining information provided by the patients and the medical records. A maximum daily dose of 7.5 mg of prednisolone was accepted. The presence of the extra-articular manifestations and previous joint surgery were recorded.

The study was approved by the local ethics committee and informed consents were obtained from all patients according to the Declaration of Helsinki.

**Laboratory Investigations**

Blood samples were withdrawn from the RA patients and controls. Whereas, synovial fluid was collected from the RA patients.

Routine biochemistry tests were collected from patients’ records. Complete blood count (CBC) was done using a Coulter counter (T660) and erythrocyte sedimentation rate (ESR) was detected by the Westergren method. Rheumatoid factor (RF) was determined by the latex fixation method. Anti-Citrullinated cyclic peptide (Anti-CCP) was measured using the microparticle enzyme immunoassay (MEIA) method with the Abbott AxSym (Chicago, IL, USA).

**Serum and Synovial Fluid Pleiotrophin**

Serum and synovial fluid pleiotrophin was assayed by quantitative enzyme immunoassay technique (ELISA) according to the recommendations of the manufacturers. A monoclonal antibody specific for pleiotrophin had been precoated onto a microplate. Standards and samples were pipetted into the wells and any pleiotrophin present was bound by the immobilized antibody. After washing away unbound substances, an enzyme-linked monoclonal antibody reagent specific for pleiotrophin was added to the well, then unbound antibody enzyme reagent was washed away. The substrate solution was then added to the wells and color developed in proportion to the amount of pleiotrophin bound in the initial step and then the intensity of color is measured. The PTN concentrations were analyzed in accordance with the manufacturer’s instructions and with an ELISA reader at 450 nm. Both standards and samples were evaluated in duplicates and the inter-assay variations were shown to be within the range given by the manufacturers.

**Statistical analysis**

Results are expressed as mean ± standard deviation (SD) or number and percentage(%). Comparison between categorical data was per-
Serum and Synovial Fluid Pleiotrophin Level in Rheumatoid Arthritis

Comparison between values of different variables in the two studied groups was performed using either unpaired t test or Mann Whitney test whenever it was appropriate while Wilcoxon matched paired test was used for matched serum and synovial fluid level of PTN in the two studied groups. Correlation between PTN either in serum or in synovial fluid and different variables in RA was done using Spearman’s rho correlation test. Statistical Package for Social Sciences (SPSS) computer program (version 19 windows) was used for data analysis. A probability value (p value) less than 0.05 was considered statistically significant.

RESULTS

RA patients’ characteristics are displayed in table 1. Ten RA patients (28.5%) had extra-articular manifestations. Subcutaneous nodules were found in 4 patients (11.4%), and 6 patients had secondary Sjogren syndrome (17.1%). RF was positive in 28 RA patients (82%) with a mean of 38.62±41.62 IU/ml. Anti-CCP antibody was positive in 13 RA patients (37.1%) with a mean of 54.6±44.8 U/ml. Regarding the medical treatment, all our RA patients were on methotrexate (100%), 25 patients were on steroids (71.4%), 9 patients on Leflunomide (25.7%) and 10 on antimalarial therapy (28.5%).

Comparison between serum PTN levels in RA patients and healthy controls

There was no statistical significant difference (p>0.05) on comparing the mean PTN level in sera of RA and healthy controls (22.51±18.49 and 22.44±2.82 ng/ml respectively(Fig. 2).

Comparison between serum and synovial fluid PTN levels in RA patients

PTN levels were significantly higher (p<0.001) in synovial fluid (34.30±31.75 ng/ml) than serum levels in RA patients (22.51±18.49 ng/ml). To confirm these results, we used the Wilcoxon matched paired test for matched serum and synovial fluid PTN levels. The test revealed that PTN level in synovial fluid was significantly higher than that in serum(p<0.001)(Fig. 2). Serum PTN levels correlated positively with its synovial fluid levels in RA patients (r=0.0378, p=0.025).

Association of serum and synovial fluid PTN level with RA disease characteristics

There was a significant correlation between serum PTN level with morning stiffness duration (r=-0.443, p=0.008) and MHAQ score (r=-0.351, p=0.039). However no correlation was found between PTN level in either serum or synovial fluid of RA patients with DAS 28 score, and the modified Larsen score (p>0.05) (Table 2).

Furthermore, we did not find significant correlation between both serum and synovial PTN levels with the presence of RF, SC nodules or other clinical, and laboratory, parameters (p>0.05; table 3). PTN levels were similar in both males and females and were not dependent on the age of the patients or on the duration of arthritis.

There was no statistically significant difference on comparing mean PTN level in serum and synovial fluid level of RA patients who received and those who didn’t receive steroid, leflunomide and antimalarial medications as shown in Table 3.

Table 1Disease characteristics of RA patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>RA (n=35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>42.23±9.32</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>5.21±4.28</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>42.54±15.57</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>10.90±1.98</td>
</tr>
<tr>
<td>Leucocytes (10³/µL)</td>
<td>8.12±2.67</td>
</tr>
<tr>
<td>Platelet count (10⁴/µL)</td>
<td>308.29±82.59</td>
</tr>
<tr>
<td>ALT(U/L)</td>
<td>23.86±10.41</td>
</tr>
<tr>
<td>AST(U/L)</td>
<td>21.40±9.71</td>
</tr>
<tr>
<td>Urea (mg/dl)</td>
<td>22±6.54</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.74±0.24</td>
</tr>
<tr>
<td>Morning stiffness duration (hrs)</td>
<td>0.7±0.63</td>
</tr>
<tr>
<td>DAS-28</td>
<td>4.59±0.94</td>
</tr>
<tr>
<td>mHAQ</td>
<td>1.34±0.52</td>
</tr>
<tr>
<td>Modified Larsen score</td>
<td>38.54±31.75</td>
</tr>
</tbody>
</table>

Data are expressed as Mean± SD, DAS: Disease activity score, mHAQ: Modified health assessment questioner, ESR: Erythrocyte sedimentation rate.
Table 2: Correlation between serum and synovial fluid PTN with disease parameters in RA patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>RA patients (no=35)</th>
<th>Serum PTN (ng/ml)</th>
<th>Synovial fluid PTN (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
<td>r</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.378</td>
<td>0.252</td>
<td>0.131</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>0.166</td>
<td>0.341</td>
<td>0.034</td>
</tr>
<tr>
<td>Morning stiffness duration (hours)</td>
<td>-0.443</td>
<td>0.008**</td>
<td>-0.116</td>
</tr>
<tr>
<td>Extra articular manifestations</td>
<td>-0.227</td>
<td>0.190</td>
<td>-0.257</td>
</tr>
<tr>
<td>DAS 28</td>
<td>0.032</td>
<td>0.856</td>
<td>-0.105</td>
</tr>
<tr>
<td>MHAQ score</td>
<td>-0.351</td>
<td>0.039*</td>
<td>0.042</td>
</tr>
<tr>
<td>Modified Larsen score</td>
<td>0.134</td>
<td>0.444</td>
<td>-0.024</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>-0.077</td>
<td>0.659</td>
<td>-0.162</td>
</tr>
<tr>
<td>Hemoglobin concentration (g/dl)</td>
<td>-0.056</td>
<td>0.751</td>
<td>-0.052</td>
</tr>
<tr>
<td>WBCs (10^3/μL)</td>
<td>0.023</td>
<td>0.897</td>
<td>-0.153</td>
</tr>
<tr>
<td>Platelet count (10^3/μL)</td>
<td>0.116</td>
<td>0.505</td>
<td>-0.191</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>0.081</td>
<td>0.645</td>
<td>-0.053</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>0.071</td>
<td>0.686</td>
<td>0.027</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.043</td>
<td>0.806</td>
<td>0.074</td>
</tr>
<tr>
<td>Rheumatoid factor titer (IU/ml)</td>
<td>0.136</td>
<td>0.482</td>
<td>0.045</td>
</tr>
<tr>
<td>Anti-CCP antibody titre (IU/ml)</td>
<td>-0.179</td>
<td>0.311</td>
<td>-0.155</td>
</tr>
</tbody>
</table>

DAS: Disease activity score, mHAQ: Modified health assessment questioner, *p < 0.05 is statistically significant

Table 3: Comparison between mean values of serum and synovial PTN level in RA patients classified according to medications intake subgroups

<table>
<thead>
<tr>
<th>RA patients (no=35)</th>
<th>Serum PTN (ng/ml)</th>
<th>Synovial PTN (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients on</td>
<td>Patients not on</td>
</tr>
<tr>
<td>Steroids</td>
<td>20.97 ± 15.40</td>
<td>26.34 ± 25.22</td>
</tr>
<tr>
<td>Leflunomide</td>
<td>26.92 ± 25.09</td>
<td>20.98 ± 15.94</td>
</tr>
<tr>
<td>Antimalarial</td>
<td>18.46 ± 2.20</td>
<td>24.12 ± 21.75</td>
</tr>
</tbody>
</table>

Data are expressed as Mean± SD
p < 0.05 is statistically significant
Several reports have focused on PTN effect in cancer angiogenesis\(^{(17)}\). Accordingly, a bulk of researches has studied PTN as an attractive target for tumor therapy and data suggest that PTN may hold promise for breast and prostate cancers \(^{(10,22)}\). In addition to cancer, this embryonic growth and differentiation factor was found to be expressed in adults with inflammatory diseases, with a proposed role in the angiogenesis and growth of synovial cells\(^{(16)}\).

In view of the introductory, we aimed to highlight the role of PTN in RA and to delineate whether it could be a potential target for the development of new therapeutic approaches or implemented as a possible disease biomarker.

To the best of our knowledge, the concept of the current study was investigated only once...
by Pufe et al., who determined the PTN expression in the synovial membranes of patients with RA\(^{(16)}\). However, the basic premise of the ease and rapidity of the serological tests had urged us to assess the PTN concentrations in the sera and synovial fluids of a group of RA patients and to study its correlation with clinical features of the disease, activity and laboratory parameters.

On studying the difference between the serum and synovial fluid levels of PTN in the RA patients group, we found a statistically significant higher PTN levels in the synovial fluid compared to that of the serum (\(p<0.001\)). This is consistent with the results reported by Pufe et al. who found that PTN mRNA expression and PTN protein were considerably up-regulated in the synovial tissues of patients with RA\(^{(16)}\).

However, we did not find any statistical difference between the serum levels of PTN in RA patients group and controls (\(p>0.05\)). This insignificant difference between serum PTN in RA and healthy controls was not expected. Nevertheless, it is known that PTN can act in a paracrine manner as it is released and consumed locally, close to the site where the immune reaction occurs\(^{(16)}\).

Similarly, a number of inflammatory cytokines related to the pathogenesis of RA were also not detected in the serum. Of which that have been reported are the TNF-\(\alpha\), IL-1\(\beta\) and IL-6\(^{(18,5,7)}\). Multiple explanations for the inability to detect a cytokine, when actually it is expected to be found, have been proposed. The presence of specific or nonspecific inhibitors or excessive consumption of a cytokine or diurnal variations are among causes\(^{(4)}\). Another clarification was found regarding low levels of ILs-2 and -7 which were reported in RA serum. This was attributed to the presence of high serum soluble levels of their receptors (sIL-2R and sIL-7R)\(^{(4,6)}\).

From another aspect, blood may not be the appropriate material of choice. The half-life of many cytokines is less than 10 minutes; hence, the time lapse between the collection and the processing of samples may be a significant factor limiting the use of levels of cytokines as biomarkers\(^{(4)}\).

In the present research, we studied the association of PTN levels in serum and synovial fluid with some clinical parameters and disease activity score in RA. We found a significant correlation between serum PTN level with morning stiffness duration and MHAQ score. On the other hand, no correlation was found with age, disease duration, DAS 28 score, or the presence of extra articular manifestations.

In conclusion, the results of the present study emphasize the role of PTN in RA. We were the first to report a serological study of this cytokine in RA.

However, it is important to bear in mind that there are several limitations in our study. Firstly, the number of RA patients was relatively small. Secondly, the cross sectional pattern of our study makes it difficult to assess the effect of treatment and medications taken by the patients and PTN levels in serum and synovial fluid.

In this respect, it is noteworthy that a recent research was carried out by Kaspiris et al. to detect the serum PTN levels in a pilot study of 16 OA patients. They reported that, although the limited number of patients might not provide definitive conclusions, the findings suggest that serum PTN could prove to be a good biomarker for monitoring disease progress and a promising applicant for more studies to develop targeted therapeutic regimens for OA\(^{(8)}\).

Accordingly, further studies on PTN and RA with larger numbers of patients and prolonged follow up are needed to reach reliable conclusions and unveil its exact role in the disease process.

**REFERENCES**


Serum and Synovial Fluid Pleiotrophin Level in Rheumatoid Arthritis


اقترحت الدراسات الحديثة أن البليوتروفين قد يكون من العلامات البيولوجية الجيدة للمتابعة والمرشح للعلاج المستهدف في التهاب المفاصل. ومع ذلك لا يعرف الأقل عن دور البليوتروفين في التهاب المفاصل الروماتويدي والدور المتغير لبليوتروفين في مرض التهاب المفاصل الروماتويدي يأتي من البيانات القليلة التي أبلغت عن وجوده في أنبوب المفصل وتشير هذه الدراسات إلى قياس البليوتروفين في مصل الدم والسائل الزليلي لمرضى التهاب المفاصل الروماتويدي وتقري علاقته بنشاط المرض والدرجة الوظيفية والتصنيف بالتصوير الإشعاعي. وفي هذه الدراسة تم جمع 90 عينية تمثل 35 عينية من مصل مرضى التهاب المفاصل الروماتويدي تم عمل تقييم مستقل للبيانات الديموغرافية وال今生ية والعملية كما تم أيضاً تقييم درجات التهاب المفصل والتصنيف بالتصوير الإشعاعي. وقد تم قياس مستوى البليوتروفين في مصل الدم وفي سائل المفصل باستخدام الفحص المناعي بالانزيم وقد وجدنا أن متوسط مستوى البليوتروفين في السائل الزليلي لمرضى التهاب المفاصل الروماتويدي أعلى بكثير من مستوى المصل (القيمة الاحتمالية = 0.005). كما كان هناك ارتباط كبير بين مستوى البليوتروفين في مصل المرضى وكلا من مدة التقييم الساكن (القيمة الاحتمالية = 0.008) وقيمة mHAQ ولذلك نحن من الأولى الذين يقدمون تقريباً عن النمط المصلي لمستوى البليوتروفين في مصل الدم والسائل الزليلي لمرضى التهاب المفاصل الروماتويدي ولكن استخدامه كعلامة للمرض أو للعلاج يحتاج إلى المزيد من الدراسات والتحقيق.
CD4+ MONOCYTES EXHIBIT DIFFERENT SURFACE EXPRESSION OF TOLL-LIKE RECEPTORS 2 AND 4 IN SYSTEMIC LUPUS ERYTHEMATOSUS

Samar Mohamed Fawzy* and Rania Hassan Khalifa**

ABSTRACT

Background: Systemic Lupus Erythematosus (SLE) is a prototype autoimmune disease of multifactorial origin mainly allocated to defects in the adaptive immune system. However, evidences supported the crucial role of the innate immune system in its pathogenesis. Toll-like receptors (TLRs) have been proposed as important pathways in disease development. This relatively new idea holds promise for new therapeutic strategies. The aim of this work is to measure surface expression of TLR2 and TLR4 on CD4+ monocytes in SLE patients and compare it with healthy controls, also to find out their relation with disease activity and organ damage.

Subjects and Methods: The current study was carried out on forty Egyptian female SLE patients and 20 matched control subjects. Expression of TLR2 and 4 on CD4+ monocytes was studied using flow cytometry. Results: Significant increase of TLR2 surface expression and a significant decrease of TLR4 surface expression on CD4+ monocytes in SLE patients compared to the control group (p=0.006, 0.004 respectively) was observed. No statistically significant associations were detected with both activity and damage indices.

Conclusion: This study suggests that TLR2 and TLR4 play a role in the pathogenesis of SLE but have no impact on disease activity or organ damage.

Key words: Systemic Lupus Erythematosus; Innate immunity; Toll-like receptors; Flow cytometry; SLEDAI

INTRODUCTION

Systemic lupus erythematosus (SLE) is a multifactorial chronic autoimmune disease of connective tissue with a variety of clinical manifestations that differ from patient to patient and affects multiple organs leading to serious complications. To be noted, it is currently accepted that its onset and development are associated with several genetic, environmental, and hormonal factors (13). Previously, chronic arthritis, which is a hallmark in SLE and other autoimmune diseases, was mainly attributed to deregulations in the adaptive immune system. However, evidences supported the important role of the innate immune system in the development of arthritis (15).

Toll-like receptors (TLRs) are a large family of innate immune receptors. They act as a key link between infection, injury and inflammation. TLRs recognize a variety of pathogen and danger-associated molecular patterns (PAMPs and DAMPs) (12). TLRs expression has been revealed on various immunocompetent cells, such as macrophages and dendritic cells (DC), as well as on non-immune cells and this expression is either constitutive or in an inducible manner (9).

At least eleven TLRs have been identified in humans. All TLRs are synthesized in the endoplasmic reticulum (ER) and are secreted in response to stimulation. Most TLRs reside on the cell surface, however, there are also intracellular TLRs that are expressed almost exclusively in the endosomal compartments of cells and are specialized in recognition of nucleic acids (11, 17). TLRs, signals directly regulate the intracellular mechanisms that allow the antigen presenting cells to process an antigen and display it in the context of MHC. Also, TLRs, engagement stimulates DC maturation, resulting in induction of expression of co-stimulatory molecules and chemokine receptors and production of cytokines. These effects allow subsequent antigenic peptide presentation and activation of T lymphocytes. Thus, TLRs play a crucial role in both the activation of innate immune responses and the subsequent development and shaping of adaptive immune responses (2).

The role of TLRs mediated inflammation does not only imply host defense, but also is related to the pathogenesis of several autoimmune diseases (4). A number of TLRs has been studied in the development of many autoimmune diseases (9). Of these are the TLR2 and TLR4. These

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are cell-surface receptors. TLR4 was the first TLR to be characterized. TLRs 2 and 4 mainly recognize cell wall components of various Gram positive and Gram-negative pathogens, as the lipoteichoic acid and LPS respectively, stress proteins and cell decomposition products(10).

Recent studies have elucidated the role of TLRs in the pathogenesis of autoimmune diseases(18), which is attributed to the production of pro-inflammatory cytokines such as IL-1β, TNF-α or IL-6 by the peripheral blood mononuclear cells (PBMCs) and DCs in response to receptor- ligand engagement(6,20).

Hence, the aim of this work is to measure the expression of TLR-2 and TLR-4 on the surface of CD14+ monocytes in patients with SLE and compare it with normal controls, and also to find out the relationship between their expression and the disease activity and organ damage.

SUBJECTS AND METHODS

Study Population

Forty female SLE patients, diagnosed according to Systemic Lupus International Collaborating Clinics classification criteria for SLE, were included in the study(21). Disease activity was assessed with the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI)(3). Disease damage was recorded according to the Systemic Lupus International Collaborating Clinics/ American College of Rheumatology (SLICC/ ACR) Damage Index(5). Concerning treatment, 27 patients (67.5%) received Azathioprine, all the patients received corticosteroids in a dose of 5-60 mg/day (mean: 25.6±14 mg/day). Twenty age and sex matched healthy subjects were enrolled as controls.

Local institutional research board approval and informed consent were undertaken from all the subjects prior to participation in the study. Patients were excluded if they had any autoimmune disease other than SLE, or if they had fever or any infectious disorders that could affect the white blood cells count.

Assessment of TLR expression in peripheral blood

Blood samples were withdrawn on EDTA anticoagulant. Samples were divided into 3 tubes. In each tube one hundred µL of whole blood sample was mixed with ten µL of PE-conjugated anti-CD14 monoclonal antibody (R&D SYSTEMS, catalog number: FAB3832P, Lot number: LGG04, USA). Ten µL of monoclonal antibodies against TLR2 (R&D SYSTEMS, catalog number: FAB2616F, Lot number: ABCY01, USA) or TLR4 (R&D SYSTEMS, catalog number: FAB6248F, Lot number: ABUN02, USA) conjugated with fluorescein isothiocyanate (FITC) were added. Samples were incubated at 2-8°C for 30 min.

Analysis was performed using Epics XL coulter (Beckman Coulter). Intact monocytes were identified by their size and granularity as assessed by their logarithmic amplification of the FSC and SSC signals and thus they were gated upon for furthermore selection of CD14+ monocytes.

Percentages of expression besides mean fluorescence intensities (MFI) were acquired. Isotype-matched antibody controls were used to detect non-specific staining.

Statistical Analysis

All statistical analysis was performed with SPSS Version 20. Results were presented as mean, standard deviation, range and median. Comparison of quantitative variables was performed using an independent Student’s t-test. Correlation was assessed by Spearman coefficient of correlation. p<0.05 was considered statistically significant.

RESULTS

The current study was carried out on forty Egyptian SLE female patients, as well as 20 age matched healthy females. The age of the patients ranged between 16-56 years with a mean ± SD of 31.6±9.2 years. Clinical and laboratory data of the patients are summarized in tables 1 and 2. All patients (100%) were ANA positive, 31 (77.5%) were anti-dsDNA positive, 22 (55%) were anti-Ro positive, 24 patients (60%) were lupus anticoagulant positive and 13 (32.5%) were anticardiolipin positive. The level of C3 ranged between 16-160 G/L (mean: 88±37), C4 ranged between 2.8-90 G/L (mean: 21.6±17.8) and the SLEDAI ranged between 2-30 (median 14).
CD14+Monocytes and TLR 2&4 in SLE

A statistically significant increase of TLR2+CD14+ monocytes (p=0.006) and mean fluorescence intensity (MFI) of TLR2+CD14+ monocytes (p= 0.03) were found in SLE patients than in control group. A statistically significant decrease of expression as well as the MFI of TLR4+CD14+ monocytes (p=0.004, < 0.001 respectively) was also noticed in patients compared to the control group (Table 3).

Within the study group, a tendency towards a decrease of MFI of TLR4+CD14+ monocytes in nephritis subgroup of patients than in non-nephritis was noticed although it did not reach statistical significance (p = 0.054). No other statistically significant correlations between the two groups as regards expression of TLR4+CD14+ monocytes, TLR2+CD14+ monocytes and MFI of TLR2+CD14+ monocytes could be detected. Regarding serositis and non-serositis patients, results revealed a significant decrease of TLR4+CD14+monocytes in serosis SLE patients than in non-serositis ones (p=0.017). As in case of nephritis, no other significant differences could be noticed between the two groups.

Correlation of TLR2 and TLR4 results with SLEDAI and SLICC/ACR criteria are summarized in Table 4. No statistically significant correlations were detected between the expressions and MFI of TLR4+ and TLR2+ and both indices.

<table>
<thead>
<tr>
<th>Table 1. Clinical data of SLE patients</th>
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<tbody>
<tr>
<td>Clinical data</td>
</tr>
<tr>
<td>-------------------------------------</td>
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<tr>
<td>n (%)</td>
</tr>
<tr>
<td>Pleurisy</td>
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<td>Pericarditis</td>
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<tr>
<td>Nephritis</td>
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<tr>
<td>CNS affection</td>
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<tr>
<td>Vasculitis</td>
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<td>Arthritis</td>
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<table>
<thead>
<tr>
<th>Table 2. Laboratory data of SLE patients</th>
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<tbody>
<tr>
<td>Variable</td>
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<tr>
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<td></td>
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<tr>
<td>Hemoglobin(g/dL)</td>
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<td>Platelets (x10^3/cmm)</td>
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<td>ALT(U/L)</td>
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<td>Creatinine mg/dl</td>
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<table>
<thead>
<tr>
<th>Table 3. Results of TLR2 and TLR4 in SLE patients and controls</th>
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<tbody>
<tr>
<td>Parameter</td>
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<tr>
<td>--------------------</td>
</tr>
<tr>
<td>TLR2/CD14 (%)</td>
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<tr>
<td>TLR4/CD14 (%)</td>
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<td>TLR2/CD14 MFI</td>
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<td>TLR4/CD4 MFI</td>
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<tr>
<th>Table 4. Correlation of TLR expression with SLEDAI and SLICC/ACR</th>
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<tr>
<td>R</td>
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<tr>
<td>TLR2+CD14+%</td>
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<td>SLEDA</td>
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<td>SLICC/ACR</td>
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</table>
**DISCUSSION**

It has been noted that there is a lack of identification of a new FDA-approved lupus treatment for 40 years. This is probably issued to the failure of detection of a “common denominator,” for all lupus patients, owing to the complexity of the disease. The importance of TLRs in the pathogenesis of SLE has been reported in many previous studies, and therapeutic interventions targeting these molecules or their signaling pathways warrant high expectation.

Our study revealed significant differences between the patients and control subjects regarding the expression and MFI of TLR2+CD14+, TLR4+CD14+ monocytes with significant increase in the levels of the former and significant decrease in those of the latter receptor. The surface expression of TLR2 and 4 in SLE was not clearly elucidated in previous studies. Our results are in accordance with that of Kirchner et al. who demonstrated a significantly reduced level of cell surface TLR4 expression on CD14+ monocytes compared to that of healthy control subjects. However their results were different in respect to TLR2 expression where no significant difference was observed between the SLE patients and the control group. In contrast, Migita et al. reported that although TLR4 expression on CD14+monocytes was not significantly different between healthy subjects and patients with SLE, TLR2 expression on monocytes was reduced in patients with SLE compared to healthy subjects.

Regardless of the discrepancies observed between the reported results, the significant increase in TLR2 expression detected in our study goes with the proposed role of TLRs in the pathogenesis of autoimmune diseases. Also it supports the findings reported by Komatsuda et al. which showed that relative TLR2, TLR7, TLR9 mRNA expression levels were significantly higher in SLE patients than in control subjects. However the decrease in TLR4 expression recorded in our results was in accordance with previous in vivo and in vitro observations which demonstrate that upon ligand engagement, internalization of TLR4 occurs with subsequent down regulation of surface expression.

Hence our study suggests that the association between infection and SLE is often caused by TLR-mediated induction of proinflammatory cytokine and chemokine expression, upregulation of co-stimulatory molecule expression by APCs and production of autoantibodies by hyperactive B cells. Indeed, Lartigue et al. observed that TLR2-and TLR4-deficient B6lpr/lpr mice expressed lower titers of autoantibodies. Also inhibition of TLRs and their signaling pathways have been shown to be effective in lupus-prone mouse models and successfully inhibit production of IFN by human pDC in vitro.

From another aspect, the TLR2 and TLR4 genotypes in SLE patients were investigated in a previous study done by Kirchner et al. The aim of the study was to evaluate whether functional polymorphisms of these TLR genes affect the surface protein expression as reported in other autoimmune diseases as rheumatoid arthritis. However results did not confirm any role of these SNPs on TLR protein expression levels on monocytes inpatients with SLE.

In our study, no statistically significant associations were detected with SLEDAI and SLICC/ACR indices. However, it is noteworthy that a novel observation revealed by Houssen et al., is the negative correlation between serum soluble TLR2 (sTLR2) levels and SELDAI score in SLE patients. This negative correlation was attributed to the role of sTLR2 in down regulation of TLR2 signaling through various mechanisms.

**Conclusions**

In this study, we concluded that despite different expression levels of TLR2 and 4 on the surface of CD14+monocytes, assertions point to the involvement of the investigated TLRs in the pathogenesis of SLE. Hence further experiments targeting TLRs and their downstream effectors may hold promise to ascertain new reliable treatment modalities.

**REFERENCES**


سلم خلايا الوحدات الحاملة للنسي دي 14 تحمل تعبيرات مختلفة للTLR-2 وTLR-4 في مرض الذئبة الحمراء

العنوان

المرض الذئبي الحمراء هو مرض من أمراض المناعة الذاتية التي يسببها العديد من العوامل من أهمها الخلل في نظام المناعة التكيفي، ولكن البراهين دعمت الدور الحاسم للنظام المناعي الفطري في التسبب بها وقد اقترحت مستقبلات في كمسارات مهمة في تطور المرض وتعتبر هذه الفكرة الجديدة نسبية وأعدت لوضع استراتيجيات علاجية جديدة.

والهدف من هذا العمل هو قياس التعبير السطحي لكل من TLR2 , TLR4 على خلايا الوحدات التي لديها CD14. TLR2 , TLR4 على خلايا الوحدات التي لديها TLR4 وانخفاض مباشر في التعبير السطحي لTLR2.

وتلقيت التعبير السطحي لكل من TLR2 , TLR4 من خلال استخدام التدفق الخلوي. وقد وجدت هذه الدراسة أن هناك زيادة كبيرة في التعبير السطحي لTLR4 على خلايا الوحدات في مرض الذئبة الحمراء مقارنة مع مجموعلا الأصحاء. للقيمة الاحتمالية 0.006، 0.004 على التوالي) ولكن لا يوجد ارتباط ذو دقة احصائية بين التعبير السطحي لTLR4 , TLR2 مع كل من مؤشرات النشاط والضرر وقد استنتجنا من خلال هذه الدراسة أن مستقبلات TLR4 وTLR2 تلعب دوراً في التسبب في مرض الذئبة الحمراء ولكن ليس لها تأثير على نشاط المرض أو ضرر أعضاء الجسم.

المؤلف

سامح محمد فوزي - رانيا حسن خليفه
ASSESSMENT OF THE RISK OF AUTOIMMUNE DISEASES IN HCV INFECTED PATIENTS PRIOR TO INTERFERON ALPHA TREATMENT

Soha A. Nasr, Eman Mahmoud, Amany H Abd Elrahman and Eman Awadallah

ABSTRACT

Background: Autoantibodies are often produced in HCV patients treated with alpha-interferon, but it still remains controversial if HCV patients can trigger the development of autoantibodies by itself before interferon treatment. We studied the presence of organ specific and non organ specific autoantibodies in HCV patients prior to interferon treatment. Subjects and Methods: the study included 36 HCV infected patients and 10 controls. Organ specific autoantibodies as Thyroid peroxidase antibody and antithyroglobulin antibody by Automated Analyzer, and Non organ specific autoantibodies as Antinuclear antibody (ANA), Antismooth muscle antibody (ASMA), Antimitochondrial antibody (AMA), Liver kidney microsomal antibody (LKM) and Antiparietal antibody (APA) by Indirect Immnofluorescence Results: Non organ specific autoantibodies including ASMA only have been significantly observed in HCV patients (72.2%) than in controls (0%) (p=0.001). The prevalence of Anti-thyroid antibodies (organ specific antibodies) as ATG (22.2%) and anti-TPO antibodies (13.9) in HCV patients vs controls was not significant but the observed percentage in our study was higher than the percentage of antithyroid antibodies in the general population screened in other studies. Conclusion: in agreement with most studies, our materials clearly demonstrated a solid relation between HCV and the development of autoantibodies which might develop autoimmune diseases in the future, even if without statistical significance in case of thyroid autoantibodies, for the small number of patients HCV triggers an α-IFN response, which is one of the factors that might lead to the development of autoantibodies. Recommendation: A close follow up is needed in patients HCV infected patients to control autoantibodies if present as thyroid autoantibodies and to rule out concomitant autoimmune diseases if present especially after antiviral treatment which might exacerbates autoimmune diseases in those patients. Keywords: HCV, Autoantibodies, before interferon alpha treatment.

INTRODUCTION

HCV infected patients usually have numerous extrahepatic manifestations, this is due to the development of lymphoproliferative disorders and metabolic abnormalities by HCV infection[20].

Examples of autoimmune disorders triggered by HCV infection, cryoglobulinemia, and autoimmune thyroid disorders[5]. It is well known that HCV infection stimulates non-organ-specific (NOSA) autoantibodies production, as antinuclear antibodies and smooth muscle antibodies, and organ-specific autoantibodies such as thyroid autoantibodies. However, there is still no clear correlation between the clinical significance of these autoantibodies associated with the extrahepatic manifestations caused by HCV infection[10].

Auto-immune thyroid disease (AITD) is due to multiple interaction between genetic, endogenous and exogenous factors[16]. Old studies showed that AITD occurred during alpha-interferon (α-IFN) treatment of chronic viral C hepatitis (HCV)[5]. However, late studies proved the presence of anti-thyroid antibodies in patients with HCV prior to (α-IFN) therapy, implying that AITD could be induced by HCV infection. Therefore, HCV might be one of the environmental elements involved in the evolvement of AITD in patients with genetic predisposition[6].

Different mechanisms were suggested to clarify the induction of autoimmune diseases by HCV for example the generation of (α-IFN) response[12]. There is a discordancy between studies results done on AITD patients. A number of studies showed the high prevalence of HCV antibodies in AITD patients which confirm the idea that HCV infection by itself can trigger the development of AITD[4,15]. But no epidemiological association was found between thyroid autoantibodies and HCV.
The presence of NOSA as antinuclear antibodies (ANAs) and smooth muscle antibodies (SMAs) is related to the severity of necroinflammation and fibrosis in HCV patients’ liver. It is obvious that autoantibodies titers are not dependent on HCV genotypes or HCV (RNA) quantitation. The development of these autoantibodies had no effect on antiviral treatment. However, we have to exclude concomitant autoimmune hepatitis (AIH) from patients with HCV infection seropositive for NOSA, because antiviral treatment occasionally exacerbates AIH in those patients.

The aim of this work is to study the prevalence of organ specific (thyroid autoantibodies) and non organ specific antibodies in patients with HCV prior to interferon therapy.

MATERIALS AND METHODS

Selection of patients and control samples: this study comprised a total of 36 HCV infected patients referred to the National Research Centre Outpatient Clinic, and 10 HCV negative, age and sex matched subjects as control group.

HCV infection was confirmed by HCV positive antibody and HCV RNA positive by PCR.

The HCV patients were 22 (61%) males and 14 (39%) females. Their age ranged between 27 and 58 years with mean age of 43.6 years. The normal control group were 5 (50%) males and 5 (50%) females, their age ranged between 29 and 51 with mean age of 44.3 years. The diagnosis of HCV was based on HCV Ab test.

All participants of this study (HCV patients and control group) were subjected to:

a-Routine laboratory investigations:
• Complete blood picture.
• Liver functions (ALT and AST)
• HCV antibody by Eliza method (Enzyme linked immunoassay method)
• HCV RNA by using real time PCR (polymerase chain reaction) for cases and controls.

b-Special investigations:
• Thyroid peroxidase antibody and antithyroglobulin antibody by Automated Analyzer.
• Antinuclear antibody (ANA), Antismooth muscle antibody (ASMA), Antimitochondrial antibody (AMA), Liver kidney microsomal antibody (LKM) and Antiparietal antibody (APA) by Immunofluorescence.

Sample Collection

Six mL of venous blood were withdrawn aseptically from all subjects by sterile venipuncture, 6ml was collected in serum separating tubes. Serum samples were centrifuged and serum is either stored frozen or kept refrigerated and used within 7 days.

Procedures

I-HCV RNA by using real time PCR (polymerase chain reaction) for cases and controls.

II-Circulating anti-thyroglobulin (ATG) antibodies and antithyroid peroxidase (TPO) antibodies by Architect ci8200 immunoassay analyzer, Abbott diagnostics (The Architect is a two-step immunoassay using CMIA technology with flexible assay protocols, referred to as Chemiflex®).

III- Antinuclear antibody (ANA), AntisMOOTH muscle antibody (ASMA), Antimitochondrial antibody (AMA), Antiparietal antibody (APA) were assessed by immunoﬂuorescence Diasorin kit. ANA,ASMA,AMA,APA on Rat liver kidney stomach tissue by Diasorin.: Serum dilution 1:20 in phosphate buffer saline (50uL of serum in 1950 uL of phosphate buffer saline) was overlaid on each well for 30min at room temperature. slides were washed twice for 5 min each with phosphate buffer saline, overlaid with fluorescently labeled conjugate(antihuman IgG), and incubated for an additional 30min. After a slide was washed twice, a coverslip was placed over the slide with mounting media, and the slides were read with a fluorescence microscope at x40 power.

RESULTS

This study comprised a total of 36 HCV positive patients referred to the National Research Centre Outpatient Clinic, and 10 HCV negative, age and sex matched subjects as control group.

The HCV patients were 22 (61%) males and 14 (39%) females. Their age ranged between 27 and 58 years with mean age of 43.6±12.9 years. The normal control group were 5 (50%) males and 5 (50%) females, their age ranged between 29 and 51 with mean age of 44.3±10.3 years.

In the group of 36 patients HCV Ab and HCV
RNA by PCR was tested positive, and 10 control group tested negative for HCV Ab.

4 (11.1%) of patients were positive for ANA while 1 (10%) of controls were positive for ANA (p=0.69), 26 (72.2%) positive for ASMA while 0 (0%) of controls were positive (p=0.001), 4 patients (11.1%) positive for AMA while none of the control were positive (p=0.36), 6 (16.7%) positive for LKM and none of the control were positive (p=0.21). APA 2 (5.6%) positive while none of controls were positive (p=0.61). (Table 1)

ATG was tested for patients and controls, 7(22.2%) from patients were positive and 0(0%) among controls (p=0.15). Anti TPO antibody was positive in 5 (13.9%) of patients and 1 of the controls was positive (p=0.6). (Table 2)

In the group of 36 patients 10 (27.7%) had antithyroid antibodies and 26 (72.3) were negative for antithyroid antibodies. Mean age was not significant between patients with thyroid antibodies and patients with absent thyroid antibodies.

Positive thyroid antibodies were clearly linked to female sex (p=0.02).

10(100%) of the thyroid antibodies positive patients had ASMA antibodies (p=0.02), and 4(40%) had LKM antibodies positive (p=0.03). (Table 3)

Table (1): Indirect Immunofluorescence results for cases and controls

<table>
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<tr>
<th>36 cases</th>
<th>ANA</th>
<th>APA</th>
<th>ASMA</th>
<th>AMA</th>
<th>LKM</th>
<th>ATG</th>
<th>TPO</th>
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<th>AMA</th>
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Table 2: Autoantibodies by indirect immunofluorescence for HCV positive patients and controls

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<th>Patient (%)</th>
<th>Control (%)</th>
<th>Significance</th>
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<tbody>
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<td>ANA -Positive</td>
<td>4 (11.1)</td>
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</tr>
<tr>
<td></td>
<td>32 (88.9)</td>
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<tr>
<td>ANA -Negative</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASMA -Positive</td>
<td>26 (72.2)</td>
<td>0 (0)</td>
<td>0.001*</td>
</tr>
<tr>
<td></td>
<td>10 (27.7)</td>
<td>10 (100)</td>
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</tr>
<tr>
<td>ASMA -Negative</td>
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<td></td>
</tr>
<tr>
<td>AMA -Positive</td>
<td>4 (11.1)</td>
<td>0 (0)</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>32 (88.9)</td>
<td>10 (100)</td>
<td></td>
</tr>
<tr>
<td>AMA -Negative</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LKM -Positive</td>
<td>6 (16.7)</td>
<td>0 (0)</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>30 (83.3)</td>
<td>10 (100)</td>
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</tr>
<tr>
<td>LKM -Negative</td>
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<tr>
<td>APA -Positive</td>
<td>2 (5.6)</td>
<td>0 (0)</td>
<td>0.61</td>
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<td></td>
<td>34 (94.4)</td>
<td>10 (100)</td>
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<td>APA -Negative</td>
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-ANA= antinuclear antibody, ASMA=anti-smooth muscle antibody, AMA= anti-mitochondrial antibody, LKM=liver kidney microsomes antibody, APA= antiparietal antibody. *Statistically significant

Table 3: Antithyroid antibodies by Architect for HCV positive patients and controls.

<table>
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<th>Patient (%)</th>
<th>Control (%)</th>
<th>Significance</th>
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</thead>
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<tr>
<td></td>
<td>(n=36)</td>
<td>(n=10)</td>
<td></td>
</tr>
<tr>
<td>1-ATG -Positive</td>
<td>7 (22.2)</td>
<td>0 (0)</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>29 (77.8)</td>
<td>10 (100)</td>
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</tr>
<tr>
<td>1-ATG -Negative</td>
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</tr>
<tr>
<td>2-Anti-TPO -Positive</td>
<td>5 (13.9)</td>
<td>1 (10)</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>31 (86.1)</td>
<td>9 (90)</td>
<td></td>
</tr>
<tr>
<td>2-Anti-TPO -Negative</td>
<td></td>
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</table>

-ATG=anti-thyroglobulin antibody, anti-TPO=anti-thyroid peroxidase antibody.

*Statistically significant

Table 4: Comparison of age, sex, non organ specific autoantibodies status in HCV patients with and without thyroid autoantibodies.

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<th>TAB Present</th>
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<td>10 (27.7)</td>
<td>26 (72.3)</td>
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<td>Age</td>
<td>43.6</td>
<td>40.6±11.6</td>
<td>44.8±13.4</td>
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<td>Female sex</td>
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<td>26</td>
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<td>2 (7.7)</td>
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<td>LKM</td>
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<td>4 (40)</td>
<td>2 (7.7)</td>
<td>0.03*</td>
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<td>APA</td>
<td>2</td>
<td>1 (7.6)</td>
<td>1 (10)</td>
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TAB= Thyroid antibody, ANA= antinuclear antibody, ASMA=anti-smooth muscle antibody, AMA= anti-mitochondrial antibody, LKM=liver kidney microsomes antibody, APA= antiparietal antibody.

*Statistically significant
DISCUSSION

Previous studies have shown the prevalence of autoantibodies in patients infected with HCV virus and under alpha interferon therapy and the occurrence of autoimmune diseases was also reported\(^5\). Also prior to (α-IFN) therapy, implying that autoimmune diseases could be induced by HCV infection Therefore, HCV might be one of the environmental elements involved in the development of autoimmune diseases in patients with genetic predisposition\(^6\) as HCV infection triggers lymphoproliferative disorders and metabolic abnormalities\(^7\).

In our study we reviewed the prevalence of autoantibodies in HCV prior to alpha interferon therapy, because antiviral treatment occasionally exacerbates autoimmune diseases in those patients.

HCV infection stimulates the production of organ specific and non organ specific autoantibodies. Hereby we investigated the presence of ANA, ASMA, APA, LKM antibodies and AMA as non organ specific antibodies. Anti-thyroid antibodies (ATG and anti-TPO antibodies) as organ specific antibodies in patients and controls. Non organ specific autoantibodies including ASMA only have been significantly observed in HCV patients (72.2%) than in controls (0%) (p=0.001) in agreement with Marconcini ML et al, 2013\(^13\). While in other studies ANA and ASMA have been observed in patients with HCV\(^19\), other Studies show that NOSA as antinuclear antibodies (ANAs) and smooth muscle antibodies (SMAs) are related to the severity of necroinflammation and fibrosis in HCV patients liver\(^3\).

The prevalence of Antithyroid antibodies (organ specific antibodies) as ATG (22.2%) and anti-TPO antibodies (13.9) in HCV patients vs controls was not significant. In agreement with other studies\(^8\) where antithyroid antibodies was found only in (6.7%) of patients with HCV before interferon therapy.

Although an increased prevalence of thyroid autoantibodies has been reported in patients with HCV infection before alpha-interferon therapy\(^11,14,15\). Other studies have not corroborated these findings\(^13\). However it was not possible to prove a difference in the prevalence of HCV antibodies, testing only 36 HCV patients and 10 controls in agreement with Wong et al, 1996\(^16\). Comparably, Lo-viselli et al. surveyed the general population of two villages in Italy, found the presence of HCV antibodies and anti-TPO in a small number of candidates (0.65%). Showing that the observed percentage in our study was higher than the percentage of antithyroid antibodies in the general population screened in other studies.

The remarkable link between HCV infection and autoimmune thyroid disease proposes that HCV, could be one of the environmental elements breaking the immunological tolerance.

Comparing HCV patients with Thyroid autoantibodies (ATG and/or TPO vs those with negative thyroid antibodies (ATG or TPO) the following was observed: the difference in the mean age between the two groups was not significant. Positive thyroid antibodies were clearly linked to female sex (p=0.02). 10(100%) of the thyroid antibodies positive patients had ASMA antibodies (p=0.02), and 4(40%) had LKM antibodies positive (p=0.03%). Compared to Marazuela M, et al.\(^8\) study where the mean age among thyroid diseased patients where higher than patient with no thyroid dysfunction, thyroid antibodies were also linked to female sex (P <0.01), and only ANA was significantly found in patients with thyroid dysfunction (p=0.01).

In conclusion, in agreement with most authors, our results clearly highlighted a remarkable association between HCV and the development of autoantibodies which might develop autoimmune diseases in the future, even if without statistical significance in case of thyroid autoantibodies, for the small number of patients. HCV most likely producing an α-IFN response, might be one of the environmental factors contributing in the development of autoantibodies.

Recommendation:

A close follow up is needed in HCV infected patients to control autoantibodies if present as thyroid autoantibodies and to rule out concomitant autoimmune diseases if present especially
after antiviral treatment which might exacerbates autoimmune diseases in those patients.

REFERENCES
8- Marazuela M, Garcia-buey L, Gonzalez-Fernandez, et al. (1996) ;“Thyroid autoimmune disorders in patients with chronic hepatitis C before and during interferon-alpha therapy”. Clinical Endocrinology 44: 635

after antiviral treatment which might exacerbates autoimmune diseases in those patients.

REFERENCES
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ASSESSMENT OF CD71 EXPRESSION AND ITS PROGNOSTIC SIGNIFICANCE IN ACUTE LYMPHOBLASTIC LEUKEMIA

Nihal Salah Ibrahim* and Abdulrahman AlShehri **

ABSTRACT

Introduction: Transferrin receptor 1 (CD71) is a transmembrane glycoprotein responsible for cellular iron uptake because it is related to cell proliferation & may offer useful information about tumor characteristics and treatment outcome. The aim of this study was to evaluate CD71 expression on de novo acute lymphoblastic leukemia cells and to follow its possible effect in response to treatment & prognosis. Methods: A total of 102 patients with ALL. We divide our patients into two groups According to the age pediatric group & adult group. CD71 expression was analyzed on the bone marrow blast cells by flow cytometry. Results: CD71 expression on the leukemia blasts was variable. All blast cells showed expression of CD71 with dMFI median of 30.20 & 28.20 in pediatric and adult group respectively. No significant association exist between CD71 expression on the blast cells and initial laboratory & clinical data of both groups, also its expression did not differ between our patients (pediatric and adult group) in response to treatment (p value= 0.979 & 0.734 respectively). In both groups significant Higher level of CD71 expression was detected in T.ALL followed by B.ALL and the lowest expression exists in biphenotypic ALL (p<0.001) .CD71 expression was found to be highly significant in correlation with relapsed ALL patients (P<0.001) in pediatric and adult group. T. lineage ALL show higher incidence of relapse. Conclusion: Our data suggest that Transferrin receptor 1 (CD71) expression by blast cells in ALL could serve as a biological marker of poor prognosis. The role of TfR1 requires further investigation in this context. Key words: ALL, transferrin receptor 1, CD71. Short Title: CD71 expression in patients with Acute lymphoblastic leukemia

INTRODUCTION

The transferrin receptor (CD71) is an integral membrane protein that mediates the uptake of transferrin-iron complexes(1). Two transferrin receptors have been cloned (TfR1 and TfR2); however, TfR1 is considered the major protein responsible for iron uptake owing to its higher affinity and expression pattern(28).

CD71 is a homodimeric glycoprotein containing 760 amino acids, and it binds to diferric transferrin at the cell surface(16). This binding is followed by internalization, a process that is regulated in part by interaction with the HFE protein, which is mutated in patients with hereditary hemochromatosis(26).

The transferrin receptor is most highly expressed on cells with high iron demand, such as maturing erythroid cells and placental syncytiotrophoblasts, and on cells with a high proliferation rate, such as cells of the basal epidermis and intestinal epithelium(23,5). Also activated peripheral blood mononuclear cells express high levels of TfR(23).

CD71 overexpression has been reported in many neoplasms, including solid tumors such as lung(14,36), bladder(30), colon(24), pancreas(27) and breast cancer(31,9), gliomas(25) as well as hematological malignancies(33).

There is still very little information on the possible importance of CD71 in acute lymphoblastic leukemia (ALL), and the data that is available is conflicting (8,13).

Aim of the work:

The aim of this study was to evaluate CD71 expression on de novo acute lymphoblastic leukemia cells and to follow its possible effect in response to treatment & prognosis.

MATERIAL AND METHODS

The study included 102 patients (54 of our patients their age <16 years old enrolled as a pediatric group & 48 patients their age > 16 years old enrolled as adult group ) diagnosed with de novo ALL and 20 age and sex matched healthy participants as a control group. Patients were recruited from Medical Oncology Clinics, Asser Central Hospital, Abha, KSA. All participants provided informed consent before enrolment in the study. The research protocol was approved by the Research Ethics Committee. And the

Departments of Clinical and Chemical Pathology*, Faculty of Medicine, Cairo University, Cairo, Egypt, and Oncology**, Asser Central Hospital, Abha, Kingdom of Saudi Arabia
work was conducted in accordance with the Helsinki Declaration.

The only inclusion criteria were diagnosis of de novo acute lymphoblastic leukemia and material availability. The patients were subjected to Complete history taking, Clinical examination with careful notation and assessment of clinical signs relevant to leukemia, White blood cell (WBC), hemoglobin (Hgb) and platelet (PLT) count was routinely evaluated using cell-counter (Cell-DYNE 1700 or Sysmex XE 2100 analyzer).

The bone marrows were routinely assessed and the leukemic blasts were classified according to their morphology (following French-American-British classification: L1–L3) and immunophenotype. For identification of B lineage leukemia cytoplasmic CD22, cytoplasmic CD79a, CD10 & CD19 were used. For identification of T Lineage leukemia cytoplasmic CD3, CD7, CD5, CD2 &CD1a were used. CD34, HLA Dr & TDT were used as an early marker detection. For identification of biphenotypic leukemia, myeloperoxidase or CD11c&CD14 for myeloid lineage, cytoplasmic CD3 for T lineage and CD19& cytoplasmic CD22 for B lineage.

The mean value of blast cells in our bone marrow samples were 48.98±13.54 and 55.21±12.86 in pediatric & adult group respectively. In pediatric group of our patients, 23 patients were diagnosed with B-lineage ALL, 29 patients had T-lineage ALL, and 2 patients biphenotypic ALL. 50.0% show L1 morphology, 48.1% show L2 and 1.9%with L3 morphology. In adult group, 20 patients were diagnosed with B-lineage ALL, 17 patients had T-lineage ALL, and 11 patients biphenotypic ALL. 45.8% show L1 morphology, 25% show L2 and 29.2%with L3 morphology. Induction therapy response was assessed by microscopic analysis of the bone marrow specimens (<5% blasts cells in the BM, complete remission).

CD71 surface expression was measured on bone marrow blasts cells. Staining to identify blast cells (either CD19/CD10 or CD19/CD34 for B-lineage ALLs or cyto CD3/CD34 for T-lineage ALLs) was chosen based on the routine bone marrow immunophenotyping performed for diagnostic purposes. Bone marrow mononuclear cells were stained with the following antibody conjugates: anti-CD71 FITC/anti-CD19 (or anti-CD10) PE or anti-CD71 FITC/anticyt CD3 PE/anti-CD34 PE. 5 μl of each monoclonal antibody was added to the appropriate tubes and incubated for 30 min. Finally, the cells were washed and analyzed by flow cytometry. Each sample was run with an appropriate isotype control. Samples were analyzed by flow cytometry using a Coulter Epi6on XL equipped with a 488-nm argon laser.

Mean fluorescence intensity (MFI) was used as a measure of antigen surface expression. For individual samples, an anti-CD71 FITC dMFI was calculated: dMFI = MFI of the population of interest – MFI of the appropriate isotype control.

**Statistical analysis**

Data were coded and entered using the statistical package SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 22. Data was summarized using mean, standard deviation, median minimum and maximum in quantitative data and 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 test for comparing categorical data, Chi square (c2) test was performed. Exact test was used instead when the expected frequency is less than 5. Correlations between quantitative variables were done using Spearman correlation coefficient. P-value less than 0.05 were considered as statistically significant.

**RESULTS**

Comparison between pediatric and adult group regarding; clinical data, initial laboratory data, CD71 expression and achievement of CR was studied. No statistically significant correlation could be established with any of the studied parameters except for lymph node & bone marrow blasts (Table 1&2).

There is no statistical significant association found between CD71 expression and the re-
CD71 expression in Patients with ALL

CD71 expression was statistically higher on T-lineage leukemia in pediatric and adult groups.

The median of CD71 expression was higher in T-lineage ALL followed by B-lineage ALL and the lowest for biphenotypic ALL are shown in table (4).

Within a follow up period, a significant difference in CD71 expression existed between 30/54 (55.6%) of relapsed pediatric patients as well as 24/48 (50%) of relapsed adult patients to those show complete morphological remission in both groups 24/54 (44.4%) &24/48 (50%) respectively (p value = <0.001) table (5). All relapsed patients in both groups show significant association with T lineage ALL. 18 out of 30 (60%) relapsed pediatric patient show significant association with L2 morphology.

Table (1) : comparison between pediatric and adult group regarding clinical data.

<table>
<thead>
<tr>
<th>Sex</th>
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<td>%</td>
<td>Count</td>
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<tr>
<td>Remission</td>
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Table (2): Comparison between pediatric and adult group regarding initial laboratory data & CD71 expression.

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<td>13.54</td>
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<td>CD71</td>
<td>33.79</td>
<td>16.68</td>
<td>30.20</td>
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</table>

P-value <0.05 is considered significant, Abbreviations: Hb: Hemoglobin, TLC: Total leucocytes count.

CD71 expression was statistically higher on T-lineage leukemia in pediatric and adult groups.

The median of CD71 expression was higher in T-lineage ALL followed by B-lineage ALL and the lowest for biphenotypic ALL are shown in table (4).

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Table (1) : comparison between pediatric and adult group regarding clinical data.

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<tr>
<td>Relapse rate</td>
<td>Relapse</td>
<td>24</td>
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<tr>
<td>Remission</td>
<td>24</td>
<td>50.0%</td>
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</table>

P-value <0.05 is considered significant, Abbreviations: Hb: Hemoglobin, TLC: Total leucocytes count.
DISCUSSION

The CD71 antibody is specific for the human transferrin receptor, with a molecular weight of 180 kilodaltons (kDa)\(^{(20)}\). The CD71 antigen expression is low on normal resting lymphocytes\(^{(20)}\) and is expressed on all cells upon activation\(^{(10)}\). The transferrin receptor is essential for iron transport into proliferating cells\(^{(11)}\) such as mitogen-activated\(^{(15)}\) and alloantigen-activated\(^{(22)}\) lymphoblast. The transferrin receptor is also present on early erythroid cells but is lost as reticulocytes differentiate into mature erythrocytes\(^{(17)}\). Dong HY in 2011 stated that CD71 is selectively expressed at high levels in erythroid precursors, including those at early maturation stages\(^{(7)}\). Previous flow cytometric studies have demonstrated that CD71 expression is decreased in nucleated erythroid cells in patients with MDS\(^{(18)}\).

Myeloid line bone marrow precursors are variably CD71 positive: dividing cells on the level of blast to myelocytes are all CD71 positive, while non-dividing late precursors (from metamyelocytes) are predominantly CD71 negative \(^{(4,32)}\).

In normal tissues TR was found in a limited number of sites, notably basal epidermis, the endocrine pancreas, hepatocytes, Kupffer cells, testis and pituitary. In contrast to this limited pattern of expression in normal tissue, the receptor was widely distributed in carcinomas, sarcomas and in samples from cases of Hodgkin’s disease\(^{(12)}\).

CD71 is reported to be widely present on neoplastic cells\(^{(23,12)}\). Yeh et al. (1984) found in-
CD71 Expression in Patients with ALL

increased transferrin binding in peripheral blood mononuclear cells from patients with leukemia, lymphoma and myeloma (35).

The aim of this study was to evaluate CD71 expression on de novo acute lymphoblastic leukemia cells and to follow its possible effect in response to treatment & prognosis.

In our patients, all blast cells showed expression of CD71 we choose the dMFI to detect even the low level of expression this finding is concomitant with Anna Płoszyńska et al. 2012(2).

According to the age of our patients, we have pediatric group 54 patients less than 16 years old & 48 patients more than 16 years old as adult group.

In the present study, CD71 expression level in the ALL pediatric & adult patients at diagnosis were studied in relation to their clinical and laboratory findings. There was no significant association between CD71expression at diagnosis and either of gender, organomegaly, response to induction therapy or any of laboratory data (TLC, Hb concentration, platelets count & bone marrow blasts). Anna Płoszyńska et al. (2), found the same result for gender & organomegaly While Das Gupta et al. in 1996, found correlation of CD71 with initial hyperleukocytosis as Anna Płoszyńska et al. 2012 he said that Patients with initial hyperleukocytosis showed markedly higher CD71 expression on the blast cells compared to the non-hyperleukocytosis patients although it was due to the prevalence of hyperleukocytosis in T-lineage leukemias. When only B-lineage ALLs were analyzed, the difference, although present in patients with hyperleukocytosis, it was not statistically significant

In our study T-lineage ALLs showed higher dMFI of CD71 expression than B-lineage ALL (dMFI median was 41.20 & 40.10) in pediatric & adult group of patients respectively which is in concordance with the previously published data. Koehler et al. 1993(13) investigated CD71 expression in childhood ALL by flow cytometric and found that 92% of T-lineage ALLs and 32% of B-lineage ALLs were CD71 positive(13). Das Gupta et al.1996 performed an immunohistochemical study of CD71 in ALLs and showed 62% of T-lineage ALLs were CD71 positive(6).

Glasova et al. in 1998 addressed CD71 expression in ex vivo ALL specimens from adults and reported CD71 expression on a few common ALLs with less than 10% of positive cells in the PB. Ten cases of T-ALL analyzed in his study showed a higher percentage of CD71 positive cells: from 13% to 75%(8). Wei et al. in 2015 studied the expression of CD71, as a proliferation indicator, on cell proliferation in hematologic malignancy and its correlation with Ki-67, so as to assess the feasibility of CD71 instead of Ki-67 for assaying cell proliferation by flow cytometry(33). He found the expression rate of CD71 on acute T lymphoblastic leukemia cells was (68.40±20.83)% & the expression rate of CD71 on acute B lymphoblastic leukemia was (39.67 ± 18.27)% (34).

Moura et al. in 2004 stated that Transferrin receptor is overexpressed in adult T-cell leukemia, a neoplasm related to human T-lymphotropic virus type 1 (HTLV-1) with a poor prognosis (19,21). And It is also overexpressed in acute myeloid leukemia (3)

We found no statistical significant association between CD71 expression on blast cells and the response to treatment in both group of patients like Anna Płoszyńska et al. in 2012.

We also attempted an analysis of CD71 prognostic value in our patient groups and found more relapse rate in patients with higher CD71 expression, mostly with T lineage ALL. Kohler et al. showed no relation between CD71 positivity and the EFS in a large group of patients(43). Anna Płoszyńska et al. in 2012 found no significant association between CD71 expression in T. lineage ALL in relation to relapses or deaths. Płoszyńska’s divided B-lineage ALL into two groups based on the median, one group below the MFI expression median of CD 71 & the other group above the median and found the DFS and OS probabilities were lower in the MFI above the median group and The mortality rate in the B-lineage ALL with CD71 MFI above the median was higher than the group below the median.

We concluded that a higher CD71 expression at diagnosis is a predictor for relapse in ALL patients. CD71 expression is a valuable and important tool, increasing effort should be done in
order to establish CD71 expression in ALL, as a powerful adverse prognostic factor despite of achievement of morphological remission. Acknowledgment:

The authors thank Asser central Hospital, Ministry of health, Abha, Saudi Arabia for helping in performing this study and also the patients for their willing participation in this research. Conflict of interest: The authors stated that there is no conflict of interest.

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CD71 Expression in Patients with ALL


تقييم تعبير السي دي 71 وعلاقته بالتنبؤ السريري في حالات سرطان الدم الليمفاوي الحاد

نهال صالح إبراهيم - عبد الرحمن الشهري

مستقبلات ترانسفرين هي بروتين سكري موجود في جدار الخلايا وهو مسئول عن امتصاص الحديد الخلوي، وبما أنه مسئول عن تكاثر الخلايا فهو يمكن أن يدل على خصائص الورم ونتائج العلاج. وكان الهدف من هذه الدراسة هو تقييم تعبير مستقبلات ترانسفرين. تم دراسة تعبير مستقبلات ترانسفرين على خلايا سرطان الدم الليمفاوي الحاد في 102 مريض مقسمين على مجموعتين، مجموعات الأطفال وعدهم 54 تحت سن 16 سنة ومجموعه البالغين وعدهم 48 مريض، وتم استخراج عينة الخلايا بطرق التدوير الخلوي. ووضعت هذه الدراسة أن تعبير مستقبلات ترانسفرين على جميع الخلايا المصابة بسرطان الدم تميزها وتؤثر في الاستجابة للعلاج وتطور المرض. وتم دراسة تعبير مستقبلات ترانسفرين على خلايا نخاع الدم السرطاني في 48 مريض، ووضعت هذه الدراسة أن تعبير مستقبلات ترانسفرين على خلايا نخاع الدم السرطاني يمكن الاستنباط من ظهوره في هؤلاء المرضى على التطور السيء، وانتكاس المرض، ودور مستقبلات ترانسفرين تحتاج لمزيد من الدراسات على نطاق أوسع لتحقق هذا السياق.
CORRELATION BETWEEN SOME BIOLOGIC AND OTHER PROGNOSTIC MARKERS OF CHILDHOOD NEUROBLASTIC TUMORS
Ehteasam M. El-Gezawy*, Osama B Sedik*, Marwa Abdelgawad**, Ahmed M Morsy***
and Samy Al Gizawy**

ABSTRACT

Background: The mutated Anaplastic lymphoma kinase (ALK) gene has been identified as a potential and major predisposition oncogene in human neuroblastomas (NBLs). However, the frequency of mutation is only 5-8%. Purpose: The present study was performed to determine the level of ALK mRNA gene expression in primary neuroblastoma and to assess its relation to other prognostic factors of neuroblastoma. Methods: Quantitative real-time RT-PCR was applied to examine the expression level of ALK mRNA in seventy nine primary neuroblastoma patients, and its prognostic value in those patients. Immunohistochemical staining was used to check the expression level of ALK proteins. Results: In analysis of 79 patients with sporadic primary neuroblastoma, we found that high expression level of ALK mRNA was significantly associated with Shimada’s pathological classification (p<0.001), patient’s age (p<0.001), MYCN amplification status (p<0.001), tumor stage (p<0.001) and low TrkA expression level (p=0.0390), all these factors are known to be associated with poor prognosis in neuroblastoma. Of interest, immunohistochemical study revealed positive ALKin ALK-amplified tissues. Furthermore, mutation results showed that ALK mutation represented about 4.6% of cases and ALK amplification represented about 1.5% of cases. So that mutations not only occur among unfavorable cases with low ALK but also in favorable cases with high ALK expression. Conclusion: Our findings suggested that, high expression of ALK gene is associated with poor prognosis of NBL so it can be used as a prognostic factor in NB in clinical practice. Keywords: ALK, neuroblastoma, N-MYC, TrKA

INTRODUCTION

Neuroblastoma (NBL) is one of the most common solid tumors in childhood; it accounts for 7-10% of childhood cancer and around 15% of childhood cancer mortality; it originates from the sympathoadrenal lineage of the neural crest. Clinical courses are highly variable, ranging from spontaneous regression to therapy resistant progression(30). Clinical and biological features, such as age at diagnosis, disease stage, DNA ploidy and structural chromosomal alterations are associated with patient outcome(9,22,25). It was demonstrated that heritable mutations of anaplastic lymphoma kinase (ALK) gene are the main cause of familial neuroblastoma(21). It was described NBL patients with high levels of ALK expression without ALK gene mutations(23). They showed that regardless of mutation status, high ALK levels were strongly correlated with poor prognosis. Moreover, it was demonstrated that RNA interference (RNAi)-based knockdown of ALK, regardless of its genetic status, showed reduced proliferation and increased apoptosis in NBL cells and inhibited NBL tumor growth as well as prolonged survival in vivo(19).

ALK is a 220 kDa transmembrane receptor tyrosine kinase that belongs to the insulin receptor superfamily. It was first described as part of an oncogenic fusion tyrosine kinase, nucleophosmin (NPM)-ALK. It is dominantly expressed in the neural system and the gene encoding it is located on the short arm of chromosome 2 (2p23.2) proximal to the N-MYC amplicon on 2p24.1(7,15).

For years, it was considered that N-MYC (V-Myc Avian Myelocytomatosis Viral Oncogene Neuroblastoma Derived Homolog) is a Protein Coding gene. It was the only oncogene known to be involved recurrently in ~22% of tumors, and the N-MYC protein is highly overexpressed by copy number gains of the gene in neuroblastic tumors with advanced stages and aggressive clinical behavior(27). Analysis of N-MYC remains an essential component of disease evaluation for newly diagnosed NBL patients and serves as a paradigm for the utility of molecular biologic information in cancer treatment stratification(29). N-MYC is proved to be vital for proliferation, migration, and stem cell homeostasis, whereas decreased levels are associated with terminal...
neuronal differentiation. However, downregulation of N-MYC may lead to decreased proliferation and differentiation, emphasizing the importance of N-MYC signaling in NB biology\(^{(27)}\).

Tropomyosin receptor kinase A (TrkA) is a neurotrophic tyrosine kinase receptor that is activated by binding to the nerve growth factor (NGF). Activation of TrkA promotes the growth and differentiation of neural cells. Previous studies have shown that TrkA expression is associated with good prognosis of NB; in fact, low or absent TrkA expression is correlated with poor outcome\(^{(10)}\).

The purpose of our study was to determine ALK expression level in human NBL tissue samples to evaluate its role in disease progression and prognosis.

**MATERIALS AND METHODS**

Seventy nine primary neuroblastoma patients were included in this study, the tumor specimen were obtained during surgery or biopsy after informed consent from the parents of the children with neuroblastoma who were referred to Department of Clinical Oncology, Faculty of Medicine and Department of Pediatric Oncology, South Egypt Cancer Institute, Assiut University, Egypt, during the period from January 2008 to November 2015. The samples were stored at -80°C.

Staging work up including computed tomographic scan of the neck to the pelvis, bone scan and bone marrow biopsy before and after treatment were mandatory. Patients were diagnosed clinically as well as pathologically and tested for DNA ploidy, MYCN amplification and TrkA expression. Tumors were staged according to the International Neuroblastoma Staging System criteria (INSS).\(^{(19)}\) Patients were stage 1, (11 patients) were stage 2, (19) patients were stage 3, (25) patients were stage 4, and (5) patients were stage 4S.

The patients were treated following the protocols proposed by the Pediatric Oncology Group for Treatment of Advanced Neuroblastoma\(^{(20)}\). Regular follow up data should be available for every patient. Our present study was approved by the ethics committee of the Faculty of Medicine, Assiut University. The clinical follow-up ranged from 3 to 93 months, with a median of 42.0 months.

Patient samples: The tumor specimens were sent to Department of Clinical Pathology, Faculty of Medicine, Assiut University, Egypt, after informed consent from the parents of the children. The selection of samples was solely based on the availability of a sufficient amount of tumor tissue, from which DNA and mRNA could be prepared for analyses described below.

RNA extraction: Total mRNA was prepared from Fresh-frozen tissues of primary neuroblastoma by using (Qiagen extraction kit, Valenica, CA, USA).

The RNA quality was verified by gel electrophoresis. Two µg of total RNA was used as started material for complementary DNA (cDNA) synthesis by employed with SuperScript III Reverse Transcriptase (200U/µl, Invitrogen, Carlsbad, Calif.). After reverse transcription reaction was finished, a total volume of 40 µl cDNA was obtained. One µl resulting cDNA was used for further PCR reactions.

Reverse transcription was carried out using random primers and SuperScript II (Invitrogen) following the manufacture’s instructions. Following the reverse transcription, the resultant cDNA was subjected to PCR-based amplification using the following primer sets and annealing temperatures (Ta):

Human GAPDH, 5’ACCTGA CCTGC-CGTCTAGAA-3’ (sense) and 5’ TCCAC-CACCCTGGCTGTA-3’ (antisense), Ta, 58 C; human ALK, 5’AGGACCCGGATGTAAT-CAAC-3’ (sense) and 5’- CTTGTGCAACTC-GAAGGAG-3’ (antisense), Ta, 58 C;

Quantitative RT- PC:. Total RNA prepared from primary neuroblastomas was reverse transcribed into cDNA (SuperScript II kit) and subjected to the real-time PCR. The expression level of GAPDH was measured in all samples to normalize ALK expression according to the manufacturer’s instructions\(^{(14)}\) (Applied Biosystems, 7500 fast real-time PCR System, CA, USA). Oligonucleotide primers and TaqMan probes, which were labeled at the 5’end with the
reporter dye 6-carboxyfluorescein (FAM) and at the 3'end with the quencher dye 6-carboxytetramethylrhodamine (TAMRA), were provided by Applied Biosystems for ALK.

FISH Technique: The tissue samples were tested for MYCN amplification and duplication and TrkA expression using FISH in paraffin-embedded tissue sections.

The MYCN amplification probe was used to detect MYCN gene amplification and copy number changes (duplication). This probe contains two differently labeled probes: a 140 kb N-MYC probe labeled with red covers the N-MYC region at 2p24 and the LAF gene probe, 201 kb and labeled with Spectrum Green, located at 2q11, acts as the control.

Interphase fluorescence in situ hybridization (FISH) was performed on 4 um-thick paraffin sections of the specimens. Briefly, the tissue sections were deparaffinized and heated in 8% sodium thiocyanate for 3 min. The sections were then digested in 0.1% of pepsin solution at 37°C for 20 min and the probe was applied onto the appropriate tissue areas. The slides were incubated at 73°C for 6 min and at 37°C for 16 h, followed by washing in gradient SSC solutions and counter-staining with anti-fade solution containing DAPI. The slides were examined using an Axioscope 2 mot plus fluorescence Microscope (Zeiss, NY, USA) and diagnosed using image system (Leica dg software, Leica, Manhiem, Germany).

In each case, around 100–200 nuclei from at least five to eight areas were examined. Nuclei with apparent overlapping or truncation were excluded from analysis. The cut-off value was established on 16 paraffin slides of the control bone marrow tissue, and was calculated as the mean (8%) plus 3 SD of nuclei counted. All the cut-off values of the probes were less than 11%.

Immunohistochemistry: human neuroblastoma tissue samples were stained with immunoperoxidase method using anti-ALK antibody. Neuroblastoma specimens were fixed in 10% buffered formalin and embedded in paraffin, and 3 µm sections were applied to the immune staining using the DAKO, ALK1 monoclonal. Before incubation with anti-ALK antibody, the sections were treated with 0.05% Pronase in 0.05 mol/L Tris-HCl (pH 7.6) for 5 minutes. The sections were incubated with anti-ALK antibody, which was diluted to 1:200 at 4°C overnight. The biotin-streptavidin method (Nichirei, Tokyo, Japan) was done, and the sections were visualized with diaminobenzidine solution. The nuclei were counterstained with hematoxylin.

Statistical analysis: Student’s t tests were used to explore possible associations between ALK expression and other factors, the difference between high and low levels of ALK expression was based on the mean value obtained from quantitative real-time PCR analysis. Kaplan-Meier survival curves were compared using the log-rank test. Multivariate analysis using a Cox proportional hazards regression model was used to assess the independent predictive importance of ALK expression for survival Statistical significance was declared if P<0.05. The statistical analysis was done using SPSS Statistical Software Release 20.0.

RESULTS

Patients characteristics (Table 1): We collected data from 79 patients with NBL who met study criteria, 46 of them were favorable and 33 unfavorable according to Shimadas’ classification, 34 below one year of age and 45 above one year, 19 patients were stage 1, 11 were stage 2, 19 were stage 3, 25 were stage 4, and 5 were stage 4S. The clinical follow-up ranged from 3 to 93 months, with a median of 42.0 months.

ALK expression and other biomarkers Assay: The tissue samples of all (79) NBL patients were examined. Expression levels of ALK were examined by the quantitative real-time RT-PCR. High ALK expression was reported in 46 patients (58.22%). Other biomarkers assay included 57 patients with a single copy of MYCN and 22 with amplified copies of MYCN, 33 with high TrkA 1 expression and 46 with low TrkA 1 expression.

Correlation between ALK Expression and other biologic and clinical prognostic factors in all patients (Table 1 and Figures 1&2):

We found significantly high expression of ALK mRNA in patients of age >1 year in com-
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In comparison to patients <1 year (p<0.001) with mean value (108.7±25.1) and (35.4±12.5) respectively, unfavorable NBLs histology (p<0.001) with mean value (108.8±20.0) for unfavorable cases and (2.3±1.0) for favorable cases. Regarding other biologic biomarkers, we recorded that significantly high expression of ALK mRNA in patients with amplified copies of MYCN (p<0.001) with mean value (84.8±17.7) as compared with patients with a single copy of MYCN (19.8±10.3), and low TrkA 1 expression level (P =0.0390) with mean value (91.4±16.9) for patients with low TrkA 1 expression and (54.3±15.2) for patients with high TrkA 1 expression.

On the other hand, we reported significantly high expression of ALK mRNA in patients with the tumor stages 3 and 4 (p<0.001) with mean value (101.2±25.5) for patients with stage 3 and 4 and (39.6±12.0) for patients with stage 1, 2 and 4s. Also, we found that low expression of ALK mRNA is significantly related to stage 4s tumor when compared to whole other stages (p<0.001) with mean value (224.4±102.4) for stage 4s and (50.2±11.0) for stage 1, 2, 3 and 4, which may suggest a relation between low expression of ALK mRNA and stage 4s. This may indicate a relationship between low and high expression of ALK mRNA and metastasis of neuroblastoma.

There was no association between high expression of ALK mRNA and DNA index (p=0.4) or tumor origin (p=0.1). These results suggest that high expression of ALK is well associated with conventional markers indicating the unfavorable outcome of neuroblastoma.

**High expression of ALK and other biomarkers associated with poorer survival in NBL:**

As shown in fig. (2), high expression of ALK was significantly associated with unfavorable survival (p=0.04). The univariate analysis demonstrated that Shimadas’ histology (p=0.0003), age (p=0.0227), tumor stage (p=0.0030), MYCN amplification (p<0.0001), TrkA 1 expression (p=0.0120) and DNA index (p=0.0134) were of prognostic importance, supporting the results of log-rank test. On the other hand, the multivariate analysis showed that ALK expression was not significantly associated with survival suggesting that ALK expression was not an independent prognostic factor from the other factors, (Table 2).

**Immunohistochemical findings in NBLs tissues:**

Immunohistochemical staining was applied to examine the ALK protein expression in neuroblastoma human tissue samples Immunohistochemical study revealed positive ALK only in ALK-amplified tissues as shown in fig.(3).

### Table 1: Relation between the expression of ALK and prognostic factors of NBL.

<table>
<thead>
<tr>
<th>Variable</th>
<th>No.</th>
<th>ALK expression (Mean ± SEM)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 1 year</td>
<td>34</td>
<td>108.7 ± 25.1</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>≥ 1 year</td>
<td>45</td>
<td>35.4 ± 12.5</td>
<td></td>
</tr>
<tr>
<td><strong>Shimadas histology</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Favorable</td>
<td>45</td>
<td>108.8 ± 20.0</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Unfavorable</td>
<td>34</td>
<td>2.3 ± 1.0</td>
<td></td>
</tr>
<tr>
<td><strong>ICSS stage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1, 2, 4s (non metastatic)</td>
<td>35</td>
<td>101.2 ± 25.5</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>3, 4 (metastatic)</td>
<td>44</td>
<td>39.6 ± 12.0</td>
<td></td>
</tr>
<tr>
<td><strong>Tumor origin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adrenal</td>
<td>43</td>
<td>76.8 ± 20.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Non adrenal</td>
<td>36</td>
<td>54.4 ± 16.5</td>
<td></td>
</tr>
<tr>
<td><strong>MYCN</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single copy</td>
<td>57</td>
<td>84.6 ± 17.7</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Amplified</td>
<td>22</td>
<td>19.8 ± 10.3</td>
<td></td>
</tr>
<tr>
<td><strong>TrkA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low expression</td>
<td>55</td>
<td>54.3 ± 15.2</td>
<td>0.0390</td>
</tr>
<tr>
<td>High expression</td>
<td>24</td>
<td>91.4 ± 16.9</td>
<td></td>
</tr>
<tr>
<td><strong>DNA Ploidy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diploid/tetraploid</td>
<td>41</td>
<td>60.7 ± 16.8</td>
<td>0.4</td>
</tr>
<tr>
<td>Hyperdiploid</td>
<td>28</td>
<td>82.5 ± 28.3</td>
<td></td>
</tr>
</tbody>
</table>
Table (2) Cox regression model using ALK expression:

<table>
<thead>
<tr>
<th>Univariate analysis</th>
<th>Factor</th>
<th>p-value</th>
<th>HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>ALK mRNA expression (High vs Low)</td>
<td>0.9354</td>
<td>0.99 (0.88 - 1.10)</td>
</tr>
<tr>
<td>B</td>
<td>Age (7 vs 1 vs 1 y)</td>
<td>0.0227 *</td>
<td>0.28 (0.03 - 0.53)</td>
</tr>
<tr>
<td>C</td>
<td>Shimada (F vs UF)</td>
<td>0.0033 *</td>
<td>0.13 (0.04 - 0.41)</td>
</tr>
<tr>
<td>D</td>
<td>MYCN amplification (Single copy vs Amplified)</td>
<td>&lt; 0.0001 *</td>
<td>0.10 (0.03 - 0.26)</td>
</tr>
<tr>
<td>E</td>
<td>Stage (1, 2, 4 vs 3, 4)</td>
<td>0.0030 *</td>
<td>0.11 (0.02 - 0.47)</td>
</tr>
<tr>
<td>F</td>
<td>TrkA expression (High vs Low)</td>
<td>0.0120 *</td>
<td>4.79 (1.41 - 16.32)</td>
</tr>
<tr>
<td>G</td>
<td>DNA Ploidy ( Diploidy vs Hyper diploidy)</td>
<td>0.0134 *</td>
<td>6.50 (1.47 - 28.76)</td>
</tr>
<tr>
<td>H</td>
<td>Origin (Adrenal gland vs Others)</td>
<td>0.9019</td>
<td>2.46 (0.95 - 6.37)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Multivariate analysis</th>
<th>Factor</th>
<th>p-value</th>
<th>HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>ALK mRNA expression (High vs Low)</td>
<td>0.2521</td>
<td>0.99 (0.88 - 1.10)</td>
</tr>
<tr>
<td>B</td>
<td>Age (7 vs 1 vs 1 y)</td>
<td>0.0973</td>
<td>0.27 (0.11 - 1.15)</td>
</tr>
<tr>
<td>C</td>
<td>Shimada (F vs UF)</td>
<td>0.0113</td>
<td>0.09 (0.09 - 1.00)</td>
</tr>
<tr>
<td>D</td>
<td>MYCN amplification (Amplified vs Single copy)</td>
<td>0.03126</td>
<td>0.99 (0.88 - 1.00)</td>
</tr>
<tr>
<td>E</td>
<td>Stage (3, 4 vs 1, 2, 4a)</td>
<td>0.0013</td>
<td>0.26 (0.10 - 0.68)</td>
</tr>
<tr>
<td>F</td>
<td>TrkA expression (High vs Low)</td>
<td>0.0014</td>
<td>0.26 (0.10 - 0.68)</td>
</tr>
<tr>
<td>G</td>
<td>DNA Ploidy (Diploidy vs Hyper diploidy)</td>
<td>0.1050</td>
<td>0.26 (0.10 - 0.68)</td>
</tr>
<tr>
<td>H</td>
<td>Origin (Adrenal gland vs Others)</td>
<td>0.0584</td>
<td>2.52 (0.97 - 6.51)</td>
</tr>
</tbody>
</table>

Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval; *p-value < 0.05
All variables with two categories. Hazard ratio shows the relative risk of death of first category relative to the second.

Figure (1): Expression levels of ALK mRNA examined by the quantitative RT-PCR and relations to other prognostic factors.
Figure (2): Kaplan-Meier survival curves of patients with NBLs based on higher or lower expression of ALK according to the mean value of ALK mRNA expression in 79 primary neuroblastoma patients, relative expression level of ALK mRNA was determined by calculating the ratio between GAPDH and ALK. To evaluate whether a significant relationship could be observed between the expression of ALK in primary neuroblastoma and the patients’ survival.

Figure (3): Immunohistochemistry for ALK in neuroblastoma tissues

DISCUSSION

The evidence that ALK has an important role in both familial and sporadic NBL pathogenesis has been provided by several studies (4,6,13,16,21). ALK is a dependence receptor that homodimerizes in the presence of ligand leading to activation of its tyrosine kinase domain by transphosphorylation and mediating a decrease of apoptosis through various signaling pathways (1,24).

Our results indicate that high expression level of ALK is significantly associated with poorer survival and patient outcome. Moreover, we found that the high expression level of ALK is significantly correlated with other prognostic factors of NBL including patient age, Shimada’s pathological classification, tumor stage, MYCN amplification status and TrkA expression levels. Contrary to our findings, it was described by Lamanet, et al. (2000) in 19 neuroblastoma cases using Western Blotting that there is no correlation between the level of ALK expression and the known neuroblastoma prognostic factors, but this may be due to small number of cases, different methodology and different population (18).

Consistent with our results, earlier studies clearly showed that aggressive and metastatic NBLs exhibited a significantly higher expression level of ALK mRNA compared with localized and favorable NBLs, supporting the oncogenic role of ALK in this disease (3,8,11,23).

The molecular mechanisms how ALK induces aggressive NBL have not yet been fully elucidated. Schonheer et al. (2011), showed that both wild-type and gain-of-function ALK mutants were able to stimulate transcription at the MYCN promoter through the activation of a downstream molecule, ERK, and initiate mRNA transcription of MYCN in both neuronal and NBL cells. Furthermore, Berry et al., 2012 demonstrated that the F1174L mutation of ALK enhanced MYCN protein stabilization and found that endogenous Mycn mRNA was upregulated in the tumors of MYCN/ALKF1174L transgenic mice (3).
Correlation between Some Biologic and Other Prognostic Markers of Childhood

MYCN amplification occurs in approximately 25% of primary NBLs and is one of the most reliable prognostic factors identified to date\(^{(12,14)}\). It is significantly associated with advanced disease stages, rapid tumor progression and poor prognosis. However, the molecular mechanism show MYCN induces aggressive NBL have not yet been fully elucidated. In our present, findings clearly provided the evidence that MYCN-mediated ALK induction promotes cell proliferation, migration and invasion and this is in consistent with Hasan et al. (2013).

In our study, Immunohistochemical study revealed positive ALK expression in ALK-amplified tissues. Previously it was reported that ALK is a dependence receptor, this type of receptors, defined by its functional rather than structural similarity, creates cellular states of dependence on their respective ligands by inducing or favoring apoptosis when unoccupied by ligand, but inhibiting apoptosis in the presence of ligand\(^{(1)}\).

Collectively, our findings suggest that high expression of ALK is associated with poor prognosis of NB, also the expression level of ALK gene might also have some function in cell growth as well as differentiation in neuroblastoma. Also our study revealed that the ALK gene in association with N-MYC amplification in NB are highly expressed. The ALK gene may be a good target for ALK inhibitors in the treatment of NB.

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العلاقة بين بعض العوامل البيولوجية والدلالات المنذرة الأخرى في حالات أورام الجزع العصبي في مرحلة الطفولة

ابتسام الجيزاوي - أسامة بكر صديق - مروة إبراهيم عبد الجواد - أحمد محمد مرسي - سامي محمود الجيزاوي

قد تم التعرف على (ALK) العصبي البشرية (NBLs) والجين المتحور باعتباره الجين الورمي المحتمل والمهدئ الكبير في ورم خلايا الجزع (ALK) العصبي البشرية (NBLs) في حالات الورم العصبي الأولي وتقييم علاقته بالعوامل الأخرى المنذرة (ALK) العصبي البشرية (NBLs) في تسعة وسبعون مريض ذو أورام خلايا الجزع العصبي البشرية (ALK) العصبي البشرية (NBLs) للورم. تم تطبيق تفاعل البلمرة المتسلسل الكمي لفحص مستوى التعبير الجيني (ALK) العصبي البشرية (NBLs) في هؤلاء المرضى. وقد استخدم صبغات الكيمياء المناعية لتحدي التعرف على مستوى بروتينات ALK في هؤلاء المرضى. وتحليل عينات متفرقة للورم من هؤلاء المرضى وجدها ان ارتفاع نسبة ALK فيها دالة إحصائية عالية مع التصنيف الباثولوجي للمريض بواسطة شيمادا (ALK). وجدنا أن ارتفاع نسبة ALK وانخفاض المستوى التعبيري لـ MYCN وكذلك عمر المريض ووجود السيدة للمرض، كما أن نسبة ALK لـ TrKA هي أيضاً يكون لها دور في نمو وتحليل خلايا ورم الجزع العصبي. تشير النتائج تشيد النتائج البادرة إلى أن التعبير الجيني ذو القيمه العالية من الجين ALK يرتبط بسوء تطور أورام خلايا الجزع العصبي البشرية لذلك يمكن استخدامه كعامل منذر ومؤثر في الممارسة العملية الأكلينيكية.
A -174 G/C POLYMORPHISM OF THE INTERLEUKIN-6 GENE PROMOTER AND LOW SERUM LEVEL OF IL6 AS A POSSIBLE RISK FOR RECURRENT SPONTANEOUS ABORTION IN EGYPT

Dina M. Rasheed Bahgat*, Nermin M. Riad* and Fouad A. Abu-Hamila*

ABSTRACT

As certain cytokines may play a role in unexplained recurrent spontaneous abortion (RSA) and also some cytokine gene polymorphisms may affect the level of cytokine production, we aimed in this study to investigate the relationship between (RSA) and IL-6 (−174G/C) gene polymorphism among Egyptians. Women (n = 142) with at least three consecutive spontaneous abortions and 142 healthy matched women with at least one successful pregnancy were included. Genotype was determined using restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction products. Enzyme Linked Immunosorbent Assay (ELISA) was performed to determine the level of IL6 in sera of the two groups. The frequencies of the polymorphic gene IL-6−174CC and the CG genotypes were significantly higher in the RSA group versus the control group (P=0.0008 and P=0.04, respectively) and, at the same time, the frequency of the wild gene IL-6−174GG was significantly lower in the RSA group versus the control group (P=0.003), suggesting that this polymorphism IL-6−174G/C might be a possible genetic factor for RSA in Egyptian population. The levels of IL6 in the RSA women was significantly lower than those of the control group (P<0.05). Allele frequencies among women with RSA and controls were 23.5% and 14.4%, respectively for allele C (mutant type); 95% confidence Interval(CI) =1.29-2.47; P=0.0005). Conclusion: To our knowledge this is the first report on the genetic variant IL-6 −174G/C of the promoter of Il-6 gene among Egyptian women with RSA. Based on our results, IL-6 seems to be a candidate gene for that condition. Keywords: Recurrent spontaneous abortion, Interleukin 6, gene polymorphism

INTRODUCTION

Recurrent spontaneous abortion (RSA) is a serious complication of pregnancy with the frequency of 1-5% among fertile couples trying to conceive(11). It is defined as three or more consecutive pregnancy losses before 20 weeks of gestation(19). RSA is a genetically heterogeneous condition resulting from both maternal and embryonic regulating factors(1). The etiology of RSA is multifactorial including genetic, anatomical, endocrine, placental anomaly, hormonal problems, infection, smoking and alcohol consumption, exposure to environmental factors, psychological trauma and stressful life event. Detection of an abnormality in any of these areas may result in specific therapeutic measures, with varying degrees of success(22). However, the etiology in approximately 50% of the idiopathic recurrent miscarriage cases remains unknown, which might be explained by immunological factors(16,30).

Since, fetus expresses the antigen inherited from both parents, survival of semi-allograft fetus till term, is one of most challenging pregnancy related concepts(10,27). The maternal immune system plays its critical role in successful pregnancy by controlling fertilization, implantation, progression and maintenance of pregnancy products(8,25,32). Therefore, investigation of immune cells, immune mediators/cytokines in the peripheral blood of the mother and their coding genes would open a new window on understanding, at least maternal causes of RSA.

Depending on inflammatory reactions, cytokines are subdivided into pro-inflammatory and anti-inflammatory, which are produced by T helper-1 (Th-1) and Th-2 cells, respectively(21). Th1 and Th2 cells are the major subsets of fully differentiated T helper cells, with distinct functional properties. Th1 cells induce several cytotoxic and inflammatory reactions via interferon-γ (IFN-γ), tumor necrosis factor (TNF)-α, interleukin (IL)-1β while Th2 cells are associated with the production of IL-6 and IL-10 cytokines that are related to B cell response and antibody formation(13). Proinflammatory cytokines inhibit trophoblast growth and differentiation, while anti-inflammatory cytokines promote embryonic development and placentation(20). In this regard, Th-2 type immunity is believed to contribute to

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normal and successful pregnancy and Th-1 type immunity have been shown to be associated with pregnancy failure and may be with RSA\textsuperscript{(24)}. Successful pregnancy is dependent on maintaining a fine balance between Th1 and Th2 immunity\textsuperscript{(15)}. Failure to enable the shifting from Th1 to Th2, as evidenced by a persisting or dominant Th1 immune response, is associated with RSA\textsuperscript{(14)}. There are several studies exploring the associations between cytokine gene polymorphisms and RSA\textsuperscript{(9,39,36,18,38)}.

Interleukin (IL)-6 is a multifunctional pro- and anti-inflammatory 21-28 kDa glycoprotein, produced by different types of cells, mainly T-lymphocytes, macrophages and monocytes\textsuperscript{(29)}. The gene encoded for IL-6 is mapped to the short arm of chromosome 7 (7p21) and composed of 6 exons with only 5 are coding. Anti-inflammatory properties of this Th-2 type cytokine are well recognized\textsuperscript{(34)}. Several polymorphisms in the IL-6 gene have been reported, some of which have been suggested to regulate its expression. Polymorphisms in the IL6 gene are seen in many immunological diseases but a relationship between the polymorphism -174G/C in particular and several abortions has already been proven\textsuperscript{(6,7)}. Single nucleotide polymorphism (SNP) at position -174G/C in the promoter region and at position -634 C/G of the IL-6 gene are known to cause an altered promoter activity thus resulting in a decreased production and secretion of IL-6 by peripheral blood mononuclear cells in vitro\textsuperscript{(5)}. Thus reducing the anti-inflammatory effect needed for the success of pregnancy evolution.

Our study aims at analyzing the polymorphism-174G/C in the promoter gene of IL-6 as well as the blood level of IL-6 in sera of women with RSA and correlating the influence of this polymorphism on the pregnancy evolution in the Egyptian population; this is the first study to be conducted in our country on -174G/C and the second on IL6 polymorphisms after one done on the -634C/G polymorphism.

**MATERIALS AND METHODS**

A total of 142 women aged 20-42 years with RSA and 142 matched normal postmenopausal controls who visited the Obstetrics and Gynecology Department at Cairo University Hospital comprised our study population. Both patients and controls had a single partner during their reproductive age. The diagnosis of idiopathic recurrent spontaneous abortion (RSA) was based on a documented history of at least three spontaneous, consecutive miscarriages before 20 weeks gestation with the same partner. Each woman underwent a diagnostic work-up to rule out a verifiable cause for the recurrent miscarriages. RSA cases were diagnosed clinically by a gynecologist in the Obstetrics and Gynecology Department at Medical School, Cairo University, and diagnosed serologically at the Department of Clinical and Chemical Pathology, Medical School, Cairo University. Diagnostic procedures included hysteroscopy, paternal and maternal karyotyping, cervical cultures for chlamydia and mycoplasma, a comprehensive hormonal status, and evaluation of antiphospholipid syndrome with IgM and IgG anti-cardiolipin antibody assessment and lupus anticoagulant testing. As infection was linked with RSA, all subjects included were confirmed to be negative for the TORCH agents Toxoplasma gondii, Rubella, Cytomegalovirus (CMV), Herpes simplex viruses (HSV-1 and HSV-2), Varicella virus (VZV) and Human immunodeficiency virus (HIV-1 and HIV-2) by indirect enzyme-linked immunosorbent assay (ELISA). Transvaginal ultrasound was performed to confirm spontaneous abortion. None of the women included in the study group were pregnant at the time of blood sampling.

The control group consisted of women with at least two live births and no history of miscarriage. All control women were post-menopausal, to rule out possible future miscarriages after inclusion in the study. To avoid confounding by ethnicity, only Egyptian women from lower Egypt were included in the study and control groups. Written informed consent was obtained from all participating women and the study was approved by the institutional ethical board for human gene and genome studies at Cairo University, School of Medicine.

**Cytokine gene polymorphism**

Genomic DNA was extracted from lymphocytes of peripheral blood samples by the use of standard techniques. Sequence amplification was performed with polymerase chain reaction (PCR). To analyze the −174G/C genotype, PCR amplifications were carried out as described by Bohiltea and Radoi, 2014\textsuperscript{(28)}. Restriction enzyme digestion by N1aiIII endonuclease was employed. The digested products were separated on 2-2.5% agarose gel electrophoresis and identi-
A -174 G/C Polymorphism of the IL-6 Gene Promoter and RSA

fied by ethidium bromide staining. The resulting products of 198-base pair (bp), a 140 and 58-bp fragments and 198+140+58-bp fragments represented the “GG”, “CC” and “CG” genotypes, respectively. The G allele is the wild and the C allele is the mutant one(2).

**QUALITY CONTROL:** 30% of samples were randomly selected to be genotyped a second time to ensure reproducibility. Genotyping was performed blinded to clinical status.

**ELISA**

Another 5 ml venous blood samples were taken from all participants in plain tubes and centrifuged then the serum was stored at -80°C until measurement of IL-6 levels. Serum concentrations of IL-6 were measured using the commercially available enzyme-linked immunosorbent assay (Quantikine®; Human IL-6 Immunoassay; R&D Systems, Minneapolis, MN, USA). All serum IL-6 analyses were performed at the same time, in the same batch, and in duplicate according to manufacturer’s instructions.

**RESULTS**

The demographics of patients and controls were indicated in Table 1. The frequencies of the genotypes/alleles -174 G/C in IL6 gene in study and control people are presented in Table 2. Statistically significant differences were identified in the frequencies of -174 G/C polymorphism when the RSA patients were compared to the controls (P<0.05), with the wild type lower in the RSA group (P=0.003) and the mutant type more frequent (P=0.008) among them.

**RESULTS OF GENETIC STUDY**

Table 1: Demographic and clinical characteristics of RSA patients in comparison with the control group

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Cases</th>
<th>Controls</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20-29</td>
<td>55</td>
<td>38.7</td>
<td></td>
</tr>
<tr>
<td>30-39</td>
<td>86</td>
<td>60.6</td>
<td></td>
</tr>
<tr>
<td>&gt; 40</td>
<td></td>
<td>10.7</td>
<td></td>
</tr>
<tr>
<td>Number of previous pregnancy losses</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;4</td>
<td>43</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Genotype and allele frequencies of polymorphism in IL-6 gene promoter region in RSA patients compared with the control group

<table>
<thead>
<tr>
<th>IL6 Genotype</th>
<th>RSA cases</th>
<th>Controls</th>
<th>Patients versus controls</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>the -174G/C</td>
<td></td>
<td></td>
<td>OR (95% CI)</td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>85</td>
<td>105</td>
<td>1.85 (1.24-2.78)</td>
<td>0.0034</td>
</tr>
<tr>
<td>CG</td>
<td>47</td>
<td>33</td>
<td>2.41 (1.02-5.67)</td>
<td>0.0448</td>
</tr>
<tr>
<td>CC</td>
<td>10</td>
<td>4</td>
<td>1.93 (1.31-2.83)</td>
<td>0.0008</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Allele</th>
<th>RSA cases</th>
<th>Controls</th>
<th>Cases versus controls</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C(mutant)</td>
<td>67</td>
<td>41</td>
<td>1.29-2.47 (95% CI)</td>
<td>0.0005</td>
</tr>
</tbody>
</table>

OR: odds ratio, CI: confidence interval

**Statistical analysis**

Statistical analysis was performed by using SPSS version 20.0 software. Data were presented as median and range or mean standard deviation (S.D.). Data were analyzed using the \( \chi^2 \)-test and Fisher’s exact test where appropriate. All tests were performed two-tailed with a confidence interval (CI) of 95%. Differences at the level of P<0.05 were considered statistically significant.

**RESULTS**

The demographics of patients and controls were indicated in Table 1. The frequencies of the genotypes/alleles -174 G/C in IL6 gene in study and control people are presented in Table 2. Statistically significant differences were identified in the frequencies of -174 G/C polymorphism when the RSA patients were compared to the controls (P<0.05), with the wild type lower in the RSA group (P=0.003) and the mutant type more frequent (P=0.008) among them.
Table 3: Serum level of IL-6 in women with and without RSA

<table>
<thead>
<tr>
<th></th>
<th>RSA cases</th>
<th>Controls</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 (pg/mL)</td>
<td>69.75±23.15</td>
<td>180.43±20.63</td>
<td>&lt;0.05*</td>
</tr>
</tbody>
</table>

Values are mean±SD  P values <0.05* are significant

DISCUSSION

Recurrent spontaneous abortion represents an important medical problem. Numerous researches have been performed for discovering the possible causes of abortion disease; however, at a significant percentage of patients, the exact cause of spontaneous abortions could not be identified. Immunological factors play an important role in affecting the evolution of pregnancy. Several studies have been conducted to establish relationships between the codifying genes for cytokines and the abortion disease(2,28,33). The production of cytokines can be influenced by genetic polymorphisms, especially in the promoter regions and result in high, intermediate or low levels of cytokines(23). Some studies had reported that there was a correlation between such polymorphisms and the production levels of various cytokines important for pregnancy, including TNF-α and -β, IL-6 and IL-10(33).

Our hypothesis to test the IL-6 gene as a candidate gene for RSA was based on existing evidence that immunological processes are involved in the pathogenesis of this condition(22,23,33). Interleukin-6 is a pleiotropic cytokine. Two common SNPs at positions (-174 and -634) of the promoter are known to influence IL-6 expression(12). Ethnicity seems to play a critical role in association studies for RSA and has been considered a major cause of conflicting results(38). One study in Egypt was done on the -634C/G polymorphism and revealed no association between this polymorphism and RSA(26). In the present study, we attempted to establish an association between the other polymorphism in the promoter region of the IL-6 gene (-174G/C), known to alter IL-6 protein expression, and the occurrence of RSA in Egyptian subjects. We included women from lower Egypt and excluded those of upper Egypt to limit ethnicity variations. Our results showed that there was an association between -174 G/C SNP and RSA in Egyptian women. We demonstrated that RSA risk in carriers of the C allele and the CC genotype was higher than that in women with the wild-type (OR = 1.93), which is entirely consistent with the previously published data by other researchers(28, 33, 5, 37) who found a significant correlation between the -174 G/C SNP and recurrent spontaneous abortion and who reported that IL-6 serum level in RSA was lower than normal(33) and claimed that -174 polymorphism might be an important cause of RSA.

In 2002 Saijo et al. reported that it is known that the IL-6 −174G/C polymorphism is frequently found in Caucasians and the IL-6 −634C/G in the Japanese(35).

There have been no reports of other frequent polymorphisms of the IL-6 gene. It is noteworthy that up till now all the studies have been performed based on Caucasian, Chinese or Japanese population, few on Iranian and Romanian populations and there are very few reports on African populations.

In 2014, Bohitea and Radoi studied a group of Romanian RSA cases and found no association between the presence of allele C at position -174 from the region of the IL-6 gene promoter and recurrent pregnancy. The frequency of allele C was 0.06 in the studied lot compared to 0.40-0.45 at the Caucasian population(39).

A Chinese study detected no -174CC or -174GC allele in women with recurrent pregnancy loss or in control women(35). Another report found that -174 G/C polymorphism was rare on Koreans(17). In contrast to many studies on Caucasian population, a study on Middle European
Caucasian Population claimed the IL-6-174G/C polymorphism was not associated with RSA nor to alterations in IL-6 serum levels(37).

Japanese women carrying the -634G allele (mutant) of IL-6 gene had a decreased risk of RSA(36). In a Chinese population, Han population from Jiangsu area (south of China), Ma et al. reported that the frequencies of the polymorphic IL-6 -634GG genotype and -634G allele were significantly lower in the RSA group versus the control group, suggesting the IL-6 -634C ⁄G polymorphism might be a possible genetic protective factor for RSA (4). Iranian women showed an increased risk of recurrent pregnancy loss in patients with -634- GG + GC genotype (OR=5.1, 95%CI: 1.04-25.3, p=0.04) in comparison to CC genotype(31).

Since ethnicity has been considered a major cause of conflicting results, it is necessary to evaluate global frequencies for the variant alleles that may cause cytokine dysfunction and establish a databank of information for future RSA studies.

Considering the facts that decreased expression of IL-6 mRNA was demonstrated in the mid-secretory phase of the menstrual cycle and is associated with habitual abortion(33) and increased IL-6 expression was associated with successful pregnancy, whereas low levels were linked with recurrent fetal loss(39), we measured the level of IL-6 in the sera of RSA women and compared it to that of controls. Our findings were in concordance with those who reported that IL-6 serum level in RSA was lower than normal(33) and claimed that -174 polymorphism might be an important cause of RSA. Our research showed significantly lower levels of IL-6 in the sera of RSA women when compared to normal controls (mean values were 69.75 ± 23.15 and 180.43 ± 20.63 , respectively; P<0.05).

Hence, although further research is necessary to clarify the functional impact of other polymorphisms within IL-6 promoter region, our results together with the previous studies suggest that the presence of the C allele (mutant type) at position -174 and more so the CC genotype may confer a risk for the pathogenesis of RSA. Putting in consideration that the Egyptian population was mixed since ages, due to political situations, might give us an explanation why our results are more in concordance with those of the European populations than to the Far-Eastern populations, agreeing with the fact that the results are influenced strongly by ethnicity.

Women with more than three pregnancy losses have a more severe condition of recurrent pregnancy loss and poorer reproductive outcome in a subsequent pregnancy than do women with three pregnancy losses. This is documented by the fact that women with more than 3 pregnancy losses are mostly those with the CC and the CG genotypes and are those who have the lowest levels of IL-6 in their sera in our study.

Conclusion

Based on the results obtained in this study, as well as the results of previous studies we may conclude that there is not only one genetic factor but possibly several that are involved in the abortion disease etiology as favoring factors(39,2,40,28,35). If the relationship between genetic factors and the immune system disorders is cleared, genetic polymorphisms as the one that is studied on IL-6 may represent a marker for selecting the therapeutic options and for counseling patients with recurrent spontaneous abortions. Our study has proven a possible association of polymorphism -174G/C and the increased frequency of recurrent abortions and confirmed the positive association between low IL-6 levels in sera and the abortion disease in the studied group.

In summary, this is the first report of a genetic variant of the IL-6 -174G/C promoter gene among Egyptian women with RSA. Based on our data, IL-6 seems to be an candidate gene for that condition.

REFERENCES
prehensive review, Am J Reprod Immunol60, 91.
31. Rasti, Z., Nasiri, M., Kohan, L. (2016): The IL-6 -634C/G polymorphism: a candidate genetic marker for the prediction of idiopathic recurrent pregnancy loss,
A -174 G/C Polymorphism of the IL-6 Gene Promoter and RSA


في الدم علي خطر حدوث فقدان الحمل المتكرر مجهول السبب
وقد ذكر أن نسبه انترلوكين 6 فيه محل جدول
دور التعدد الشكلي لجين انترلوكين 6 في أمراض
هؤلاء السيدات وحل مشكلة العقم لديهن. تم إجراء هذه
الدراسية في قسم الباثولوجيا الاكلينيكية و الكيميائية و أمراض
النساء و التوليد.

وصفت السيتوكينات بأنها تلعب دوراً رئيسياً في
توضيح سبب انترلوكين 6 في أمراض التوليد و حماية
الحمل. وضعت هذه الدراسة من منظور علاجية
التكاثر الشكلي لجين انترلوكين 6 في فقدان
الحمل المكتكر مجهول السبب.

الدراسة: تهدف هذه الدراسة إلى البحث عن
مدى علاقة التعدد الشكلي لجين انترلوكين 6
وبفقدان الحمل المتكرر مجهول السبب. تم اختيار
群体 البحث في قسم أمراض النساء و
التوليد تبعاً لمعايير معينة بحيث تكون المريضة
تمت ابتساماع مع الدراسة. تم اختيار
المريضة بفقدان الحمل المتكرر مجهول السبب و
 واحدة ضابطة شملت 142
المريضة. تم اخبار
١٤٢
أصحاء لا يعانون من أمراض و لا يتناولون أدوية
تعارض مع الدراسة.

النتائج: تم تسجيل في مجموعتي البحث تقديرات
الليلج في منطقة المروج من انترلوكين 6. و
جاءت أرقام التكرار هي ٢٣.٦% و ١٤.٤% على
التوالي. و بهما تستطيع القول أنهات هناك ارتباطاً وثيقاً بين حامل
١٤٢
الليلج في منطقة المروج من انترلوكين 6. و
قد حددت هذه الدراسة تكرار هذه الاليل
المتكرر ٩٥% (Cl 95% 1.29-2.47 = P=0.0005) فأصبحت
هذا الخلل الجيني هو السبب في فقدان الأجنة و إنهاء الحمل. الاستنتاج:
لاستنتاجا في أن هذا الخلل
الجيني هو السبب في فقدان الأجنة و إنهاء الحمل. الاستنتاج:
نسبة التكرار الأول على تعدد الشكل الجيني للانترلوكين 6
في موقعة 142. في منطقه المروج في مرضى العقم بسبب
فقدان الحمل المكتكر مجهول السبب في جمهورية مصر العربية.
و قد
أظهر توافق العيب الجيني مع السبب في عصم هؤلاء السيدات الموجودات تحت البحث.
THE RELATIONSHIPS OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA-2 GENE (PPARG2) POLYMORPHISMS PRO12ALA AND C161T WITH DIABETIC NEPHROPATHY AMONG EGYPTIAN TYPE 2 DIABETIC PATIENTS
Moyassar A. Zaki*, Ragaa A. Ramadan*, Lubna M. Desouky** and Marwa A. Madkour***

ABSTRACT
Background and aim of the work: The pathogenesis of diabetic nephropathy (DN) is multifactorial with the contribution of multiple genetic factors. The gene coding for peroxisome proliferator-activated receptor gamma 2 (PPARG2) protein has become a susceptibility locus for type 2 diabetes mellitus yet its role in DN is controversial. We carried out this case control study to investigate the association of the polymorphisms Pro12Ala and C161T in the PPARG2 gene with DN. Methods: 227 Egyptian patients with type 2 diabetes mellitus of whom 120 were suffering from DN along with 100 healthy volunteers were included. Genotyping for PPARG2 polymorphisms was done by polymerase chain reaction restriction fragment length polymorphism. Results: The PPARG2 Ala allele was associated with decreased risk of DN (OR=0.595, 95% CI 0.38–0.94, p=0.027), while no significant association was found between C161T polymorphism and the risk of developing DN. Potential confounders did not alter such a significant association. Urinary albumin to creatinine ratio median value and glycated hemoglobin percent mean value were higher in DN cases with Pro/Pro genotype when compared to DN cases with combined Ala/Ala and Pro/Ala genotypes. Conclusion: A potential reno-protective role for PPARG2 Ala allele is suggested in the pathogenesis of DN. Further studies are needed to implement such findings in the pharmacogenetic aspects of DN to initiate genotyped tailored individualized therapy. Key words: Type 2 diabetes mellitus; diabetic nephropathy; PPARG2 polymorphism

INTRODUCTION
Diabetic nephropathy (DN) is a leading cause of end-stage renal disease worldwide. About 30% of patients with type 1 and 25 to 40% of patients with type 2 diabetes develop diabetic nephropathy irrespective of glycemic control, although the reason why not all patients with diabetes develop this complication is unknown. Its severity usually correlates with glycemic control and duration of the diabetes. Multiple mechanisms contribute to the development and outcomes of DN, such as an interaction between hyperglycemia-induced metabolic and hemodynamic changes and genetic predisposition, which sets the stage for kidney injury.

The role of genetics in predisposition and progression of DN is proved through familial clustering of the disease and occurrence of complications despite of good glycemic control, in addition to the ethnic heterogeneity in prevalence rates. Susceptibility loci are suggested based on their location and their effect on pathways involved in pathogenesis. Genome-wide association studies have identified several DM2 risk loci and the quest is still going for recognition of risk loci for diabetic microvascular complications. Moreover, gene-gene interaction as well as gene-environmental interactions can modulate the risk susceptibility.

The peroxisome proliferator-activated receptor gamma (PPARG) gene, located on chromosome 3p25.3, encodes a protein that belongs to the nuclear hormone receptor superfamily. The two major protein isoforms encoded are PPARG1 with ubiquitous expression in the body and PPARG2 which is predominantly expressed in adipose tissue. PPARG2 is a transcription factor formed by an alternative mRNA splicing pathway. It regulates the transcription and expression of numerous target genes implicated in adipocyte differentiation, lipid metabolism and insulin-mediated glucose uptake in peripheral tissues. The two most common single nucleotide polymorphisms (SNPs) in PPARG2 gene are the non synonymous substitution polymorphism rs1801282 where guanine replaces cytosine at position...
34 (C34G) of exon 2 resulting in the translational substitution of alanine for proline at position 12 (Pro12Ala), and the silent mutation rs3856806 (C161T) located at exon 6 which codes for histidine amino acid.\(^{(12,20,36)}\) The \(PPARG\) gene is recently considered a confirmed type 2 DM susceptibility locus\(^{(14)}\), however reports of its association with DN are usually inconsistent\(^{(1,4,10,16,19,24,29,32)}\).

This case control study was carried out among Egyptian type 2 diabetic patients to determine the possible association of \(PPARG2\) Pro12Ala, and C161T polymorphisms with DN and their relation to markers of glycemic control and renal function.

**MATERIALS AND METHODS**

**Subjects**

Two hundred and twenty seven diabetics were recruited from the Diabetes Clinic in the Teaching Hospital of Medical Research Institute, Alexandria University. The diagnosis of DM2 and DN were established based on the guidelines of the American Diabetes Association\(^{(2)}\), with albumin being measured in a spot urine sample collected as the first urine in the morning to identify nephropathy. Accordingly, diabetics were classified into 107 non diabetic nephropathy (NN) patients who were normoalbuminuric subjects with a urinary albumin to creatinine ratio (UAC) of <30 mg/gm creatinine and hence considered the control group when studying nephropathy status, while the 120 diabetic nephropathy (DN) cases were those with persistent albuminuria with a UAC of ≥ 30 mg/gm, based on consensus of at least two consecutive overnight samples collected over a three to six months period. One hundred non diabetic healthy volunteers were also recruited from the same population. The study was explained to all participants and written informed consents were obtained. The study was approved by the local ethics committee of the Institute in accordance with The Code of Ethics of the World Medical Association for experiments involving humans (Helsinki Declaration as revised in 2013). All diabetics were receiving oral hypoglycemic agents for control of the diabetic state at the time of the study.

General exclusion criteria included DM2 of less than 10 years duration, type 1 diabetes, secondary diabetes, smoking, hypertension, pregnancy, heart failure, and previously diagnosed non-diabetic kidney disease. During urine sample collection; acute fever, diabetic ketoacidosis, significant bacteriuria or hematuria, as well as patients who performed excessive exercise within 24 hours were excluded and repeated after resolution of the condition.

**Clinical examination, anthropometric measurements and radiological studies**

All subjects were subjected to a thorough clinical examination, with a detailed history taking regarding diagnosis of type 2 DM. Anthropometric measurements (weight and height) and calculation of body mass index were done to rule out other non-diabetic organic kidney disease.

**Biochemical analysis**

Following a 12 hours fasting period, concomitant spot urine and venous blood samples were collected. Serum separator vacutainer tubes as well as tripotassium salt of ethylene diamine tetra-acetic acid (K\(_3\)EDTA) coated tubes were used for blood collection. glycated hemoglobin was determined from the EDTA blood using an immunoturbidimetric assay, while serum sample was used for the determination of concentrations of glucose, creatinine, uric acid, triglycerides, total cholesterol and its high density lipoprotein fraction. Urinary albumin and creatinine were determined from the spot urine sample. All analyses were conducted on the Olympus AU400 clinical chemistry analyzer (Beckman Coulter Inc, Brea, CA, USA). Calculations of estimated glomerular filtration rate (eGFR) using the Cockcroft and Gault formula\(^{(7)}\), low density lipoprotein cholesterol using the Friedwald formula\(^{(11)}\) and the UAC ratio\(^{(22)}\) were done.

**Genomic analysis**

Genomic DNA was extracted from the EDTA blood samples using a commercially available kit (Qiagen, Hildenberg, Germany). The concentration and purity of extracted DNA were determined by the NanoDrop\(^{\text{TM}}\) 1000 spectrophotometer (Thermo Fisher Scientific). Polymerase
The Relationships of PPARG Polymorphisms With DN

Chain reactions (PCR) for the PPARG2 gene polymorphisms were carried out separately in a Veriti thermal cycler (Applied Biosystems) according to the methods described by Dongxia et al. (2008)\(^9\). Briefly, in 0.2 mL eppendorf tubes, 50ng of genomic DNA were added to 12.5 μL DreamTaq Green PCR master mix (2x) (Thermo Fermentas, California, USA), 5 pmol of each forward and reverse primers specific for the gene sequence (Thermo Fermentas, California, USA) and completed to a final reaction volume of 25 μL using nuclease free sterile water. Details of primer sequences, thermal cycler conditions, and PCR product sizes are supplied in table-1. The resultant PCR products were visualized by electrophoresis on 2% agarose gel stained with ethidium bromide for visualization under UV light.

Restriction analyses of PCR products were carried out separately in a thermomixer set at 37°C utilizing the fast digest restriction enzymes HpaII and Eco72I (Thermo Fisher Scientific Inc., USA) for Pro12Ala and C161T polymorphisms of PPARG2 gene respectively, according to the manufacturer’s instructions (Table 1). The restriction products were visualized on a 3% agarose gel stained with ethidium bromide, with bands being visualized using an ultraviolet transilluminator. In 5% of the samples genotyping was performed in duplicate and was fully concordant.

Statistical analysis

Statistical Program for Social Sciences (SPSS) version 20 was used for analysis of data\(^{25}\). Appropriate descriptive measures were done for all quantitative variables based on the results of their distribution using the Kolmogorov-Smirnov test of normality, where a p-value less than 0.05 was considered statistically significant. Allele frequencies of polymorphisms were determined using direct gene counting. Chi-square test for goodness of fit was used to compare the observed frequencies of different genotypes among all subjects to expected frequencies according to Hardy-Weinberg equilibrium equation\(^{15}\). The strength of association between variant alleles and their susceptibility to disease were assessed using univariate and multivariate logistic regression analyses. Confounders were identified based on change-in-estimate criterion with a 10% cut-off value. Being a case control study, Odds ratio was used to measure the impact of the PPARG2 SNP genotypes on the risk of developing DN.

RESULTS

The study included 100 healthy adults (56% males) with a median age value of 47 (34-66) years and 227 unrelated type 2 diabetics with a median age value of 51 (32-66) years, stratified according to the presence of DN into 120 (52.9%) with and 107 without DN. Details of selected demographical, anthropometric, and clinical parameters are listed in table 2.

Whole blood percent glycated hemoglobin mean value and median value of UAC ratio were higher in DN group when compared to the NN control group. The lipid profile showed no statistically significant difference among the studied groups, except for higher triglycerides median value in DN compared to NN (Table 2).

All genotype groups obeyed the Hardy-Weinberg equilibrium (p> 0.05) (Table 3). When the genotype distribution of the PPARG2 gene was compared according to the presence or absence of DN, the homozygous Pro genotype of the Pro12Ala SNP was present in 108 DN and 82 NN cases, leaving the combined heterozygous (Pro/Ala) and homozygous mutant Ala genotypes detected in 12 DN and 25 NN cases. As for the C161T, the C/C was detected in 70 DN and 65 NN cases, leaving the combined C/T and T/T genotypes detected in 49 DN and 42 NN cases (Table-4).

The distribution of clinical and metabolic parameters according to the studied genotypes was analyzed. Owing to the small number of homozygous patients (Ala/Ala and TT), they were combined with the heterozygous patients (Pro/Ala and CT) respectively. There were no statistically significant differences among the genotypes in the studied parameters apart from the observation that diabetics with Pro/Pro had significantly higher mean and median values for glycated hemoglobin and UAC ratio respectively when compared to DN cases with combined...
Table (1): Primer sequences, thermal cycler conditions, PCR products, and genotypes of the studied SNPs:

<table>
<thead>
<tr>
<th>SNP (rs number)</th>
<th>Primer sequence</th>
<th>Thermal cycler conditions</th>
<th>(PCR product)</th>
<th>Restriction enzymes and genotypes (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARG2 Pro12Ala (rs1801282)</td>
<td>Forward 5'-CAAGCCCAGTCTTTCTGTG-3'</td>
<td>Initial denaturation 30 cycles: Denaturation 94°C for 3 min</td>
<td>237bp</td>
<td>(HpaII) Pro/Pro (217+20) Pro/Ala (237+217+20) Ala/Ala (237)</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-AGTGAAGGAATCGCTTTCCG-3'</td>
<td>Annealing 56°C for 45 sec</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Extension 72°C for 1 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Final elongation 72°C for 7 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPARG2 C161T (rs3856806)</td>
<td>Forward 5'-CAAGACAACCTGCTACAAGC-3'</td>
<td>Initial denaturation 30 cycles: Denaturation 94°C for 4 min</td>
<td>200bp</td>
<td>(Eco72I) CC (120+80) CT (200+120+80) TT (200)</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-TTCTTGTAGATCTCCTGCAG-3'</td>
<td>Annealing 58°C for 45 sec</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Extension 72°C for 1 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Final elongation 72°C for 7 min</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table (2): Baseline characteristics of subjects according to the presence of diabetic nephropathy

<table>
<thead>
<tr>
<th>Items</th>
<th>Whole diabetics</th>
<th>Nephropathy</th>
<th>Nephropathy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number (%)</td>
<td>NN</td>
<td>DN</td>
</tr>
<tr>
<td>Number (%)</td>
<td>227 (100)</td>
<td>107 (47.1)</td>
<td>120 (52.9)</td>
</tr>
<tr>
<td>Gender (% men)</td>
<td>110 (48.2)</td>
<td>49 (45.8)</td>
<td>61 (50.8)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>51 (32-66)</td>
<td>52 (38 – 65)</td>
<td>51 (32 – 66)</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>12 (10-22)</td>
<td>12.0 (10.0-20.0)</td>
<td>12.0 (10.0-22.0)</td>
</tr>
<tr>
<td>Body mass index (Kg/m²)</td>
<td>31.2 (21.5-39.5)</td>
<td>32.0 (21.5-39.5)</td>
<td>31.0 (23.4-39.1)</td>
</tr>
<tr>
<td>eGFR (ml/min)</td>
<td>85.7 (14.6-155)</td>
<td>91.5 (62.50-155.0)</td>
<td>72.0 (14.6-132.73)***</td>
</tr>
<tr>
<td>UAC (mg/gm)</td>
<td>46 (4.6-1749)</td>
<td>14.5 (4.6 – 29.0)</td>
<td>172.7 (43.3-1749.2)***</td>
</tr>
<tr>
<td>Glycated hemoglobin (%)</td>
<td>8.65 ± 1.83</td>
<td>8.19 ± 1.53</td>
<td>9.07 ± .97***</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>169 (78-346)</td>
<td>177 (110-318)</td>
<td>155 (78-346)**</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>1.0 (0.6-5.7)</td>
<td>0.90 (0.70 – 1.30)</td>
<td>1.10 (0.60-5.70)***</td>
</tr>
<tr>
<td>Uric acid (mg/dl)</td>
<td>4.7 (3.2-10.2)</td>
<td>4.50 (3.20 – 6.70)</td>
<td>5.55 (3.70 – 10.20)***</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>219 (130-286)</td>
<td>219 (168-269)</td>
<td>222 (130-286)</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>168 (68-272)</td>
<td>158 (78-266)</td>
<td>171 (68-272)*</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>43 (25-78)</td>
<td>45 (26-78)</td>
<td>43 (25-67)</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>143.7 ± 29.2</td>
<td>143.3 ± 27.1</td>
<td>144.1 ± 31.1</td>
</tr>
</tbody>
</table>

\( p_1 \): p-value for comparing between NN and DN  
*: p < 0.05, **: p < 0.01, ****: p < 0.001  

**Abbreviations:**  
eGFR: estimated glomerular filtration rate; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol; UAC: Urinary albumin to creatinine ratio.

### Table (3): Agreement of the single nucleotide polymorphisms of PPARG2 gene with the Hardy Weinberg Equilibrium

<table>
<thead>
<tr>
<th>Gene SNP</th>
<th>Genotypes</th>
<th>(observed / expected)</th>
<th>Hardy Weinberg Equilibrium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diabetics</td>
<td>Healthy</td>
<td>Diabetics</td>
</tr>
<tr>
<td>PPARG2</td>
<td>Pro/Pro</td>
<td>190 / 187.9</td>
<td>47 / 45.6</td>
</tr>
<tr>
<td>Pro12Ala</td>
<td>Pro/Ala</td>
<td>33 / 37.3</td>
<td>23 / 25.7</td>
</tr>
<tr>
<td>(rs1801282)</td>
<td>Ala/Ala</td>
<td>4 / 1.9</td>
<td>5 / 3.6</td>
</tr>
<tr>
<td>PPARG2</td>
<td>CC</td>
<td>135 / 131.8</td>
<td>57 / 54.8</td>
</tr>
<tr>
<td>C161T</td>
<td>CT</td>
<td>76 / 82.3</td>
<td>34 / 38.5</td>
</tr>
<tr>
<td>(rs3856806)</td>
<td>TT</td>
<td>16 / 12.8</td>
<td>9 / 6.8</td>
</tr>
</tbody>
</table>

Abbreviation:  
DN: Diabetic Nephropathy  
**p**: p-value for comparing between NN and DN
Table (4): Genotype distribution by clinical subgroups:

<table>
<thead>
<tr>
<th>PPARG2</th>
<th>Whole diabetics</th>
<th>Nephropathy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NN</td>
<td>DN</td>
</tr>
<tr>
<td>Pro12Ala</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala/Ala</td>
<td>4(1.76)</td>
<td>3(2.8)</td>
</tr>
<tr>
<td>Pro/Ala</td>
<td>33(14.54)</td>
<td>22(20.6)</td>
</tr>
<tr>
<td>Pro/Pro</td>
<td>190(83.7)</td>
<td>82(76.6)</td>
</tr>
<tr>
<td>C161T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T/T</td>
<td>16(7.05)</td>
<td>7(6.5)</td>
</tr>
<tr>
<td>C/T</td>
<td>76(33.48)</td>
<td>35(32.7)</td>
</tr>
<tr>
<td>C/C</td>
<td>135(59.47)</td>
<td>65(60.7)</td>
</tr>
</tbody>
</table>

Table (5): Demographic data and biochemical parameters according to the studied genotypes of PPARG2 gene

<table>
<thead>
<tr>
<th>PPARG2</th>
<th>Pro12Ala</th>
<th>C161T</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pro/Pro</td>
<td>Pro/Ala + Ala/Ala</td>
</tr>
<tr>
<td>Number (%)</td>
<td>190</td>
<td>37</td>
</tr>
<tr>
<td>Gender (% men)</td>
<td>(83.7%)</td>
<td>(16.3%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>(32.0-66.0)</td>
<td>(40.0-65.0)</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>12.0</td>
<td>14.0</td>
</tr>
<tr>
<td>in (Kg/m²)</td>
<td>(21.5-39.1)</td>
<td>(21.5-39.1)</td>
</tr>
<tr>
<td>Estimated GFR (ml/min)</td>
<td>(14.6-155.0)</td>
<td>(35.4-155.0)</td>
</tr>
<tr>
<td>UAC ratio (mg/gm)</td>
<td>52.50</td>
<td>18.60***</td>
</tr>
<tr>
<td>Glycated hemoglobin (%)</td>
<td>8.76 ± 1.80</td>
<td>8.09 ± 1.91*</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>168.0</td>
<td>176.0</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>(78.0-346.0)</td>
<td>(98.0-318.0)</td>
</tr>
<tr>
<td>Uric acid (mg/dl)</td>
<td>4.75</td>
<td>4.60</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>(130.0-286.0)</td>
<td>(169.0-269.0)</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>166.0</td>
<td>171.0</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>(68.0-272.0)</td>
<td>(78.0-266.0)</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>45.0</td>
<td>39.0</td>
</tr>
</tbody>
</table>

*: significant at p < 0.05, **: significant at p ≤ 0.01, ****: significant at p ≤ 0.001

Abbreviations:
GFR: Glomerular filtration rate; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol; UAC: Urinary albumin to creatinine ratio.
The Relationships of PPARG Polymorphisms With DN

The prevalence of the PPARG2 Ala allele shows great diversity among populations ranging from 2–18% in healthy people, coinciding with our frequency of 16.5% in non diabetics healthy volunteers, with higher prevalence in Caucasians when compared to Asians. Generally speaking different populations shared the relatively lower frequency of Ala allele in diabetics with nephropathy versus those without nephropathy.

In the present work, the Ala allele had frequencies of 9.03% and 5.4% in all diabetic cases as well as DN cases respectively, while the frequency of Ala allele carriers (Ala/Ala or Ala/Pro) was 23.4% in NN cases. Such an Ala allele frequency was close to that reported in Brazilian diabetics (9%), while the frequencies of their Ala carriers were 20.3% in normoalbuminuric subjects and 10.6% in DN with chronic renal insufficiency.

The meta-analysis by Wang et al. (2013) confirmed the protective role of Ala allele in DN in both Caucasians and Asians (OR=0.778, 95%CI=0.618-0.981, p=0.034). Such a protective role was confirmed in Germans, Brazilians, and Chinese, while denied in Turkish and Southern Indians, and in some cases the association was overshadowed by other predictors such as duration of diabetes and systolic blood pressure. In our study the reno-protective role of Ala allele was still significant after adjustment of potential confounders (OR=0.595, 95%CI=0.38-0.94, p=0.027).

Ahmed et al. (2012) studied this SNP in Egyptians and reported a significant association with DN despite the small sample size (14 NN versus 37 DN).

DISCUSSION

In this case-control study, we demonstrated a significant association between PPARG2 Pro12Ala and the risk of developing DN. There is great heterogeneity in genetic studies concerned with diabetic microvascular complications including ethnicity of the studied population, demographics including different age and gender distribution, as well as non uniformity in selection criteria of cases. This makes comparability of results quite difficult. Besides, the pathogenesis of diabetic microvascular complications is complex and includes multiple genetic risk factors.

The protective role of Ala allele is proposed through several pathways; first through insulin sensitization and suggested inhibition of lipid oxidation that may enhance glucose disposal. Second; lowering oxidative stress, a key player

### Table (6): Allele frequency and Odd’s ratio of the studied genotypes with the risk of developing DN:

<table>
<thead>
<tr>
<th>PPARG2 Gene polymorphisms</th>
<th>Nephropathy</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NN (no/%)</td>
<td>DN (no/%)</td>
<td>p-value</td>
</tr>
<tr>
<td>Pro12Ala Ala allele</td>
<td>28 (13.1)</td>
<td>13 (5.4)</td>
<td></td>
</tr>
<tr>
<td>Pro allele</td>
<td>186 (86.9)</td>
<td>227 (94.6)</td>
<td></td>
</tr>
<tr>
<td>Crude OR</td>
<td>0.380 (0.192 – 0.755)</td>
<td>0.004*</td>
<td></td>
</tr>
<tr>
<td>Adjusted OR</td>
<td>0.595 (0.38-0.94)</td>
<td>0.027*</td>
<td></td>
</tr>
<tr>
<td>C161T T allele</td>
<td>49 (22.9)</td>
<td>59 (24.6)</td>
<td></td>
</tr>
<tr>
<td>C allele</td>
<td>165 (77.1)</td>
<td>181 (75.4)</td>
<td></td>
</tr>
<tr>
<td>Crude OR</td>
<td>1.098 (0.711-1.693)</td>
<td>0.674</td>
<td></td>
</tr>
<tr>
<td>Adjusted OR</td>
<td>0.7 (0.32-1.51)</td>
<td>0.471</td>
<td></td>
</tr>
</tbody>
</table>

A p-value less than 0.05 was statistically significant

Pro/Ala + Ala/Ala (Table 5).

We compared the studied SNPs in relation to DN by comparing NN group to DN group. The Ala allele conferred protection against DN even after considering multiple covariates of age, gender, duration of diabetes, and glycated hemoglobin with an OR of 0.595, 95%CI of 0.38–0.94, and a p-value of 0.027 (Table 6).
in pathogenesis of microvascular diabetic complications, which was proved in PPARG-2 heterozygous mice\textsuperscript{(30,21)}. This enhanced oxidative stress tolerance triggers antioxidant genes and increases a transcription factor, FoxO3a, which regulates reactive oxygen species (ROS) clearance\textsuperscript{(21)}. Consequently, in absence of this protective genotype, the building up of ROS leads to an increase in glomerular albumin permeability and the degree of proteinuria correlates with the development of glomerulosclerosis and tubulointerstitial fibrosis\textsuperscript{(33)}. Besides, oxidative stress itself can cause insulin resistance\textsuperscript{(19)}. Lastly, the ubiquitous expression of insulin resistance\textsuperscript{(19)}. Lastly, the ubiquitous expression of PPARG2 in adipocytes which is a major site for release of adipokines and free fatty acids that modulate inflammation and angiogenesis, thus affecting both structure and function of renal vasculature\textsuperscript{(19)}.

Another polymorphic site examined in the present study was the PPARG2 C161T. The frequency of T allele in our study (26\% in healthy volunteers) was close to that reported in Tunisians\textsuperscript{(5)} but lower values were reported by others; 0–7\% in Caucasians\textsuperscript{(27)} and 12\% in Chinese\textsuperscript{(35)}. The C161T SNP in our study proved not to be associated with DN. This SNP has been studied in relation to coronary artery diseases in diabetic and non diabetic cases, where a protective effect of T allele was suggested through modulation of lipid metabolism, matrix metalloproteinase-9 and tumor necrosis factor-\alpha expression\textsuperscript{(31)}. Still this protective effect was evident among Chinese and absent among Caucasians\textsuperscript{(34)}, and it was even associated with marginally increased risk in meta-analysis\textsuperscript{(6)}. Moreover, the T-allele was suggested as a risk allele in relation to metabolic syndrome in Tunisians\textsuperscript{(36)}. A study by Hishida et al. (2013) reported the lack of a significant association of T allele with chronic kidney diseases including DN\textsuperscript{(17)}.

We investigated whether the studied SNPs were reflected on the related biochemical parameters of the subjects. The Ala carriers had lower glycated hemoglobin and UAC ratio than non Ala carriers. Herrmann et al. (2002) reported lower urinary albumin excretion between Ala carriers, which highlights the reno-protective role of Ala allele\textsuperscript{(16)}. Despite the expected metabolic role of PPARG2, we did not find any association between the genotypes and either BMI or lipid profile which was supported by other studies\textsuperscript{(4,3,26)}.

Nevertheless, certain considerations need to be addressed in this study. The subjects were selected from an outpatient clinic rather than chosen randomly from the surrounding community. In addition, it was hard to differentiate simple association of the polymorphism from the possibility of linkage disequilibrium. Further replication studies targeting polygenic and haplotype-based effects with larger population are thus needed because a single gene has a modest effect size on disease susceptibility which is incremented by interaction with other genes and/or environmental factors.

In conclusion, our study was able to demonstrate a reno-protective role exerted by Ala allele of PPARG2 Pro12Ala SNP, which could benefit in the treatment of DN. In fact, the PPAR-\alpha agonists (fibrates) and PPAR-\gamma agonists (thiazolidinediones; TZDs) are currently used for the respective treatment of hyperlipidemia and insulin resistance\textsuperscript{(17)}. TZDs have been shown to ameliorate microalbuminuria in early DN\textsuperscript{(37)}. Moreover, the effect of PPAR agonists was not limited to DN as they revealed promising results in toxic and immune mediated nephropathies. As a result, the reno-protective role of PPARG2 can be perceived beyond its metabolic effects\textsuperscript{(6,18,23)}. Whether TZDs might be more effective in patients carrying the risky Pro allele of the same SNP may be a point of future research in clinical studies with the concept of personalized medicine.

**Conflicts of interest:** None to declare by the authors of the manuscript.

**REFERENCES**


The Relationships of PPARG Polymorphisms With DN

tes and obesity in a highly consanguineous population. Indian J Endocrinol Metab; 19: 77.


STUDY OF THE ASSOCIATION OF ALOX5AP, ALOX5 AND LTC4S GENES PROMOTER POLYMORPHISMS WITH BRONCHIAL ASTHMA AND THEIR RELATION TO ITS SEVERITY IN A PILOT STUDY OF ASTHMATIC EGYPTIAN PATIENTS

Hanan A. Madani*, Dalia Hamed*, Maha Hosni*, Wafaa Ashur** and Amal Abd-Elwahab*

ABSTRACT

Introduction: Bronchial asthma (BA) is a chronic disorder of airways. 5-Lipoxygenase (5-LO) plays an essential role in biosynthesis of Leukotrienes that induce proinflammatory actions. The aim of this study was to determine association of 5-lipoxygenase pathway genes promoter polymorphisms in asthmatic patients and understanding their role in pathophysiology of BA. Subjects and methods: 50 asthmatic patients and 30 controls were included in this study. Three genes were studied; ALOXAP-169/-146 using real time PCR, ALOX5-176/-147 gene promoter Sp1/Egr1 tandem repeats using fragment length analysis and LTC4S-444A/C using polymerase chain reaction/ Restriction Fragment Length Polymorphism (PCR/RFLP). Results: There was statistical significant increase in the mutant genotype of ALOX5AP-169/-146 gene among the patients group compared to the control group (p=0.004, OR=4.329, 95% CI=1.98-8.78) with 4.5 times risk of bronchial asthma among those carrying the mutant ‘357’ allele of ALOX5AP-169/-146 gene (P<0.001). ALOX5-176/-147 promoter Sp1/Egr1 tandem repeats type 4 is significantly higher in patients’ group than controls (p=0.047, OR= 12.4, 95% CI= 0.00-0.88. LTC4S-444A/C gene promoter showed no statistical significant differences between the study groups (p=0.679). Conclusion: The homozygous 357/375 genotype of ALOX5AP-169-146 gene promoter and ALOx5-Sp1/Egr1 4 repeats are associated with susceptibility to bronchial asthma in Egyptian asthmatics supporting role of LTs in asthma pathogenesis. Key words: Bronchial Asthma pathogenesis, 5-Lipoxygenase genes, LTC4S gene

INTRODUCTION

Bronchial asthma is a heterogeneous disease usually characterized by chronic airways inflammation. It is defined by the history of respiratory symptoms such as wheezes, shortness of breath, chest tightness and cough that vary over time and intensity, together with variable expiratory airflow limitation. Many genetic and environmental factors have been associated with the development of asthma(4).

The complexity in asthma diagnosis and treatment comes in part from the range of potential molecular pathways involved in asthma pathogenesis(19). Among these different mechanisms, Leukotrienes (LTs) which are lipid mediators generated from the metabolism of arachidonic acid via a series of enzymatic reactions that constitute the 5-lipoxygenase pathway(6).

5-lipoxygenase is coded by the arachidonate 5-lipoxygenase (ALOX5) gene on chromosome 10q.11.21(5). The promoter region of ALOX5 gene shows variation in the number of tandem Sp1 binding motifs (5‘GGGCCGG3’)(7). Transcription factors Sp1 and Egr1 bind to this sequence and up regulate ALOX5 transcription. Genetic variants in the promoter region may change the binding of these transcription factors; therefore, change the rate of 5-lipoxygenase transcription and activation of Leukotrienes under inflammatory conditions(14). Mutations in the promoter region of ALOX5 gene lead to variable response to leukotriene inhibitors and leukotriene receptor antagonists(10).

5-lipoxygenase activating protein (FLAP), which is coded by arachidonate 5-lipoxygenase activating protein (ALOX5AP) gene on chromosome 13q12(20), is involved in the conversion of arachidonate to the unstable intermediate LTA4 and metabolism of LTA4 by the enzyme LTA4 hydrolase results in the formation of the dihydroxy acid leukotriene B4 (LTB4). Alternatively, conjugation of LTA4 with glutathione by LTC4 synthase forms LTC4, the first member of the family of cysteinyl-leukotrienes (cys-LTs) LTC4, LTD4 and LTE4(17). It has been established that the cys-LTs play a significant role in bronchoconstriction and airway inflammation in asthma(13).
The gene encoding LTC4 synthase resides on human chromosome 5q35. The -444A>C LTC4S (rs730012) polymorphism has been found to create an additional response element for histone H4 transcription factor-2 and to increase the transcription rate or the gene in vitro and in vivo in a Polish population[15].

In the present study we aimed to study the association of 5-lipoxygenase pathway (ALOX5AP, ALOX5, and LTC4S) genes polymorphisms with bronchial asthma and detect their frequencies as regards the severity of the asthma.

**PATIENTS AND METHODS**

**Study population:** This is a case-control study that was conducted in the molecular unit of Clinical and Chemical Pathology Department Kasr Al Aini Hospital, Cairo University. The study was approved by the Local Ethical Committee after informed consents were obtained from all participants. Fifty asthmatic patients were recruited from Chest Department and Chest Outpatient Clinic, Kasr Al Aini Hospital, Cairo University. They were classified according to Global Initiative for Asthma, 2015[4] into severe and non-severe asthmatic patients (24 severe asthmatic patients; 19 females and 5 males with mean age 51.5±9.6 years, and 26 non severe asthmatic patients; 24 females and 2 males with mean age 49.5±12.8). Inclusion criteria: Presence of symptoms of airway obstruction (cough, dyspnea, wheezes), the airway obstruction was at least partially reversible (demonstrated by spirometry where at any time the forced expiratory volume in the first second (FEV1) is increased by>12% following B-agonist inhalation). Patients with inflammatory, chronic, autoimmune or malignant conditions were excluded from the study. Thirty healthy subjects (16 females and 14 males, mean age 47±8.9 years) without previous history of asthma or allergic diseases were enrolled as healthy controls.

*All patients were subjected to the following:* Full history taking and full clinical examination. Pulmonary function tests using spirometry (basal and 20 minutes after 2 buffs salbutamol 200 µg inhalation); Flow-Volume Loop was performed to all patients using Erich-Jaeger GmbH model No:DP-556BA (Germany).

**Genotyping of 5 LO genes:** Genomic DNA was extracted from peripheral blood leucocytes of EDTA anticoagulant blood samples using innu PREP blood Mini Kit (analytic jena Biometra), the extracted DNA was stored at -20ºC till the time of the assay. The primers used for the assy of the 3 genes are listed in table 1[18]. The following techniques were used for genetic analysis of the three studied genes:

- **Polymerase chain reaction-Restriction fragment length polymorphism (PCR-RFLP):** For LTC4S- 444 SNP detection to amplify a 563-bp fragment, followed by 1.5% agarose gel electrophoresis for the amplified products which then digested by MPS1 restriction enzyme and the digested products are detected in 3.5% agarose gel containing Ethidium Bromide by performing electrophoresis on gel electrophoresis apparatus and visualized by UV trans-illumination (Promega- USA).

- **Fragment Length Analysis (FLA):** ALOX5-176/-147 gene polymorphism analysis was performed using PCR followed by Fragment length analysis using Forward primers labeled with 6-FAM fluorophore, following amplification, the product size was determined using ABI PRISM 310 capillary electrophoresis and Gene Mapper IDX software was run. ALOX5 -176-146 SNP

<table>
<thead>
<tr>
<th>Genes (symbol)</th>
<th>Forward primers (5’-3’)-</th>
<th>Reverse primers (5’-3’)-</th>
<th>Chromosome Number</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALOX5AP-169-146</td>
<td>GGGAAAGTTTCATGAACAC</td>
<td>ACCATTCGACCCACCTGAT</td>
<td>Ch.13</td>
<td>NG_011963</td>
</tr>
<tr>
<td>ALOX5-176-147</td>
<td>AGGACCCAGACACCTCGC</td>
<td>GAGCAGGAGGCCGAGCCTCGGC</td>
<td>Ch.10</td>
<td>NG_011437</td>
</tr>
<tr>
<td>LTC4S-444A/C</td>
<td>TCCATTCTGAAGCCAAAGGC</td>
<td>GTCACAGCAGCCGAGTCAGAC</td>
<td>Ch.5</td>
<td>NG_028161</td>
</tr>
</tbody>
</table>
yielded fragments 210 bp (6 bp deletion), 216 bp (wild type) and 222 bp (6 bp insertion), corresponding to 4, 5, and 6 SP1/Egr1 binding sites respectively.

**-Real time-PCR (RT-PCR):** ALOX5AP-169/-146 allelic discrimination was performed by Real Time PCR using Light Cycler Fast Start DNA Master Plus SYBER Green I (Roche Diagnostics GmbH, Mannheim, Germany) followed by melting curve analysis for product identification.

**Statistical analysis:** The data were coded and entered using the statistical package SPSS version 15 (SPSS Inc., Chicago, IL, USA). Quantitative data were expressed as mean ± SD and compared using t-test when normally distributed, and as median and range using Mann Whitney U test when not normally distributed. Allele frequency was calculated using the allele counting method (each individual is represented by 2 alleles & allele frequency = number of mutated alleles/ total number of alleles). Genotype frequencies fulfilled Hardy Weinberg equilibrium. The Chi-square test was used to compare allele and genotype frequency among different studied groups. P value <0.05 is considered significant Odds ratio (OR) and 95% confidence interval (CI).

**RESULTS**

The phenotypic data of the studied groups are shown in table 2. The forced expiratory volume and forced vital capacity is significantly lower in severe asthma group than non-severe asthma.

The frequencies of different genotypes and alleles of the studied genes are represented in tables 3, 4&5. The genotypes and alleles frequencies of ALOX5AP–169–146 gene were higher in the 2 patient groups compared to the control group (p=0.004 and 0.00 respectively). The presence of the mutant ‘357’ allele of AL-
OX5AP–169–146 was associated 4.5 times with increased risk of bronchial asthma (OR=4.5, 95%CI= 1.7-12.018, P<0.001). ALOX5–147–176 promoter Sp1/Egr1 tandem repeats 4 (6 bp deletion) were significantly higher in the two patient groups compared to the control group (p=0.047), while the wild allele (5 repeats) were significantly higher in the control group than the two patients groups (p=0.004, OR = 12.4), but the 6 bp insertion repeats show no difference between patients and controls (p= 0.25). LTC4S–444 A/C genotypes and alleles showed no statistical significant differences between the three groups (P=0.679, 0.252 respectively).

**DISCUSSION**

The promoter regions of the genes involved in the LT pathway LTC4S, ALOX5, ALOX5AP have been studied for regulatory polymorphisms that may affect gene transcription. Several of these polymorphisms were reported to be associated with bronchial asthma(18).

Results of this study showed that ALOX5AP–169–146 gene had a significantly higher frequency of the (357/357) homozygous mutant genotype and allele in bronchial asthma patients compared to the controls (P=0.004 & 0.000, OR= 13.19 & 4.2) respectively. Individuals carrying the (357) mutant allele have the increased risk of developing bronchial asthma than non-carriers. This agrees with, Kim et al who reported the frequency of ALOX5AP promoter polymorphism in three subject groups [Aspirin Induced Asthma, non-asthmatic control, and Aspirin Tolerant Asthma] in Korean population, where polymorphisms in the promoters of ALOX5AP were associated with asthma phenotypes(8).

Moreover, Koshino et al. documented that, there was significantly increased incidence of the mutant homozygous genotype of ALOX5AP–169–146 in the asthmatics than in the nor-
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However, contrary to the results of this study were Torres-Galván et al who found no association between ALOX5AP –169/–146 promoter polymorphisms and susceptibility to asthma in Spanish population\(^9\). This difference is multifactorial that may be attributed to the multi ethnic variations which are well recognized with ALOX 5AP promoter genotypes or to the population stratification.

ALOX5AP protein is postulated to play an essential role in the transfer of arachidonic acid to ALOX5 with subsequent LTs synthesis. ALOX5AP promoter polymorphism results in over expression of mRNA of ALOX5AP, with increased cys-LT production which contributes to asthma development by promoting inflammation, increasing vascular permeability and persistent airway inflammation\(^12\). This may explain the high frequency of ALOX5AP promoter polymorphism among asthmatic group than control group in our study. This could be used as a predictor of developing asthma in areas with high asthma prevalence.

Genetic analysis of ALOX5–147–176 promoter polymorphism in this study showed that the 4 repeats are significantly higher in patients’ groups compared to control group (P=0.047), While the 5 repeats (wild type) are significantly higher in control group than patients’ groups (p= 0.004, OR= 12.4), which may confer protection from occurrence of asthma. Torres-Galván et al reported that no association was found between the ALOX5–147–176 polymorphisms and asthma, or any form of asthma\(^18\). While, Sanak et al reported that Aspirin intolerant asthma (AIA) development is associated with the LTC4S –444A/C polymorphism. The reason for this could be the multi ethnic variations which are well recognized with LTC4S genotypes as well as small sample size of the bronchial asthma patients in the present study\(^15\).

On comparing the two patients groups (severe versus non-severe asthma) as regards the promoter polymorphisms of the three studied genes, there was no statistical significant difference between the two patients groups (p> 0.05), these may suggest that these polymorphisms may be related to the pathogenesis of asthma rather than the severity of asthma.

In conclusion, this study shows a strong association between ALOX5AP promoter polymorphisms and ALOX5- Sp1/Egr1 4 repeats and susceptibility to asthma. These results provide additional support to the notation that ALOX5AP and ALOX5 promoter polymorphisms and subsequently LTS are likely to be involved in the pathogenesis of bronchial asthma and indicate a potential clinical benefit for therapies based on
LT inhibition over and above those provided by by Cysteinyl LT receptor antagonism. Understanding the molecular mechanisms underlying the disease may lead to new therapies for asthma based on LT inhibition.

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Genes Promoter Polymorphisms and Relation To Severity of BA

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The study aimed to investigate the relationship between the polymorphisms of genes involved in the oxidation of fatty acids and their role in the severity of BA. The study was conducted on a sample of 50 patients suffering from BA in Egyptian patients.

1. The DNA of LTC4 genes was extracted from blood samples of 30 healthy volunteers as a control group.
2. The ALOX5 gene was amplified using a nested PCR reaction.
3. A real-time PCR was performed for the ALOX5 gene.

The study found a significant relationship between the polymorphisms of the ALOX5AP gene and the severity of BA in Egyptian patients. No significant relationship was found between the polymorphisms of the LTC4S gene and the severity of BA.
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