NOTES TO THE CONTRIBUTORS

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Articles to be published should not be published elsewhere, and should be accepted by a referee of the advisory board.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

1. An original manuscript and a photocopy plus one soft copy on a CD in Microsoft words format should be sent to the editor. (Clinical Pathology Dept., Kasr El-Eini, Faculty of Medicine, Cairo University), Tel: 02-23654480
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.
5. The first page should only include (a) Title of paper (b) Authors (c) Institution in which the work was carried out (d) Complete address for mailing purposes (e) Mobile Phone and e-mail.
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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A379V (ALA379VAL) GENETIC VARIANT OF (PLA2G7) IN ACUTE ISCHEMIC STROKE
Ghada Saddek Sabbour, Heba M. Adel Abou Zaghla and Ramy Mahmoud

ABSTRACT
Phospholipases are a group of enzymes that hydrolyze phospholipids into fatty acids and other lipophilic molecules. They are ubiquitously expressed and have diverse biological functions including roles in inflammation, cell growth, signaling and maintenance of membrane phospholipids. PLA2G7 is a protein-coding gene. The protein encoded by this gene is the secreted enzyme, platelet-activating factor acetylhydrolase, that catalyzes the degradation of platelet-activating factor to biologically inactive products. Platelet-activating factor is a biologically active phospholipid that provokes inflammation by its ability to activate polymorphonuclear neutrophils and increase vascular permeability. Genetic study of the human Lp-PLA2 shows that it is encoded by the PLA2G7 gene, which was located on chromosome 6p21-p12 by fluorescence in situ hybridization and was originally assigned as the human leukocyte antigen region and contains 12 exons. One non-synonymous polymorphism A379V (Ala379Val) in exon 11, has previously been found to be associated with platelet-activating factor acetylhydrolase deficiency (P AF AD), coronary artery disease and ischemic stroke in Chinese and USA population. Our aim was to investigate the association of A379V (Ala 379 Val) genetic variant of PLA2G7 gene in patients with acute ischemic stroke in the first 48 hours and diagnosed according to clinical neurological and radiological examinations. The other group included in our study was a control group of eleven (11) apparently healthy, age and sex matching subjects. Assay of A379V genetic variant of PLA2G7 was by PCR-RFLP analysis. In this study, we found that the genotypic and allelic frequencies of A379V were found in higher percentages in patients with acute ischemic stroke than controls, yet they didn’t show statistically significant association with the presence of acute ischemic stroke regarding the distributions of genotypes (χ2 = 2.488, P value = 0.115) of A379V between acute ischemic stroke patients and controls. The AV genotype (35 % in patients and 9 % in controls) of A379V was represented at an increased frequency in the group of patients but the frequency of A379V V allele wasn’t significantly higher in patients with acute ischemic stroke than in the control group (OR= 2.69, 95% CI= 0.29-24.74, χ2 = 0.461, P value = 0.497). A379V variant also showed no statistically significant association with a certain age, sex, as well as hypertension, diabetes mellitus and hyperlipidemia in the Egyptian population. Thus our results provide the first insight into the contribution of A379V in PLA2G7 gene to acute ischemic stroke susceptibility in the Egyptian population.

INTRODUCTION
Stroke is defined as “neurological deficit of cerebrovascular cause that persists beyond 24 hours or is interrupted by death within 24 hours”. This can be due to ischemia caused by thrombosis, embolism or hemorrhage. Approximately 79 percent of strokes are ischemic in nature (9). The 24-hour limit differentiates stroke from transient ischemic attack (TIA), which is a related syndrome of stroke symptoms that resolve completely within 24 hours(9).

Cerebrovascular stroke is the most frequent cause of permanent disability in adults worldwide(4) and the second most common cause of death (after ischemic heart diseases) and one of the leading causes of death in Egypt, according to the WHO(21). Annually, 15 million people worldwide suffer a stroke. Of these, 5 million are left permanently disabled. The World Health Organization (WHO) estimates that a stroke occurs every 5 seconds worldwide(20). Globally, the average 30-day case fatality following first ischemic stroke is about 22.9% with the exception of Japan (17%) and Italy (33%) (8). Ischemic stroke is the most common type of stroke in Egypt and worldwide, is accounting for 43% to 79% of all stroke types(8).

Inflammation is an essential process in the pathogenesis of ischemic stroke. Therefore, genes involved in inflammatory responses are under investigation to look for variants predisposing to ischemic stroke(10).

Genetic study of proteins, such as lipoprotein-associated phospholipase A2 (Lp-PLA2), also known as platelet-activating factor acetylhydrolase (PAF-AH), shows that it is a calcium ion (Ca2+) independent phospholipase which belongs to phospholipase A2 superfamily and is composed of 441 amino acids(3).
Genetic study of the human Lp-PLA2 shows that it is encoded by the PLA2G7 gene, which was located on chromosome 6p21-p12 by fluorescence in situ hybridization and was originally assigned as the human leukocyte antigen region and contains 12 exons\(^{(17)}\). One non-synonymous polymorphism A379V (Ala379Val) in exon 11, has previously been found to be associated with platelet-activating factor acetylhydrolase deficiency (PAFAD), coronary artery disease and ischemic stroke in Chinese and USA population\(^{(11,18)}\). Our aim was to investigate the association of A379V (Ala 379 Val) genetic variant of PLA2G7 gene in patients with acute ischemic stroke in Egyptian population.

**MATERIALS AND METHODS**

**Study subjects:**

The study population was composed of twenty 20 patients presented with acute ischemic stroke in the first 48 hours and diagnosed according to clinical, neurological and radiological examinations. The patients were consecutively hospitalized in the Department of Neurology and stroke units of Ain Shams University hospitals (Al-Demerdash and Ain Shams Specialized hospitals) between October 2014 and January 2015. Exclusion criteria included: Patients with transient ischemic attacks, patients with hemorrhagic cerebral stroke or cerebral hemorrhage and other diseases like autoimmune diseases, tumors, chronic infectious diseases. A control group of eleven\(^{(11)}\) apparently healthy, age and sex matching subjects were included in the study and were recruited from the out-patient clinics of Ain Shams University hospitals. Oral informed consent was obtained either from the patients or their relatives as well as from the controls before enrollment in the study and the study was approved by the Ethical Committee of Faculty of Medicine, Ain Shams University.
Full laboratory investigations including complete blood picture, coagulation profile, blood sugar level and lipid profile were done to patients and control. Diabetes mellitus was defined according to the American Diabetes Association or if the participant was taking insulin or oral hypoglycemic agents. Hypercholesterolemia was conservatively defined as ≥ 200 milligrams per deciliter for adult according to the recommendation of National Cholesterol Education Programme (NCEP) \(^{(15)}\). Plasma cholesterol, triglycerides and blood glucose were measured by routine enzymatic methods. HDL-cholesterol was determined after precipitation of the apolipoprotein B-containing lipoproteins. LDL-C was calculated using the Friedewald formula (LDL = TC - HDL - TG/5.0 (mg/dL)).

**Genotyping analysis:**

Assay of A379V genetic variant of PLA2G7 was done by PCR amplification and restriction analysis. Genomic DNA was extracted from EDTA- anticoagulated peripheral whole blood by a DNA purification kit (Thermo Scientific, USA). During extraction, 20ul proteinase K (nucleases-inactivator) was added to 200 μL blood and mixed by vortexing, 400 μL of lysis solution was then added and mixed thoroughly by vortexing to obtain a uniform suspension. Samples were incubated at 55°C for 10 minutes until the cells were completely lysed. Then, 200 μL of ethanol (96-100%) was added and mixed by pipetting. The mixture was transferred to a spin column and centrifuged for 1 minute at 6000 x g (8000 rpm). The collection tube containing the flow-through solution was discarded and the column was placed into a new 2 mL collection tube. 500 μL of Wash Buffer WB I (with ethanol) was added and the mixture was centrifuged for 1 minute at 6000 x g (8000 rpm). The collection tube containing the flow-through solution was discarded and the column was placed into a new 2 mL collection tube. Then, 500μL of Wash Buffer II (with ethanol) was added to the column and the mixture was centrifuged for 3 minutes at maximum speed (> 20,000 x g, >14,000 rpm), then the collection tube containing the flow-through solution was discarded and the column was transferred to a sterile 1.5 mL microcentrifuge tube. 200 μL of the Elution buffer was added to the center of the column membrane to elute the genomic DNA and incubated for 2 minutes at room temperature and centrifuged for 1 minute at 8000 x g (10,000 rpm). Finally, the purification column was discarded and the purified DNA was stored at -20°C till amplification.

**PCR Amplification:**

PCRs (total volume, 25 μL) contained 10 μg of DNA template, 12.5 μL of master mix (chemically modified Maxima hot start Taq DNA polymerase, optimized hot start PCR buffer, Mg2+, and dNTPs), 0.5 μL nuclease-free water (Thermo Scientific, USA) and 1 μL of each primer (forward and backward) (Applied Biosystems, USA). Hot start Taq DNA polymerase is inactive at room temperature avoiding extension of non-specifically annealed primers or primer dimers and providing higher specificity of DNA amplification. PCR included 35 cycles of amplification (4 min at 94°C followed by 30 s at 95°C, 40 s at primer-specific annealing temperature (51°C) and 50 s at 72°C) with a final extension of 10 min at 72°C. PCR amplification was performed with the primers 5’-TGACTTTTAAATGTCTTGTT-3’ (forward) and 5’-CTGGTTTAGGT-CATGAAAAA-3’ (reverse).

**Restriction Fragment-Length Polymorphism Analysis (RFLP):**

PCR products were digested with the restriction enzyme Fnu4h1 (FastDigest, Thermo Scientific, USA). The following reaction components were combined together at room temperature in the following order (total volume, 30 μL): 17 μL nuclease-free water, 2 μL green buffer, 10 μL DNA and 1 μL of the restriction enzyme. The components were mixed gently, spun down and incubated at 37°C for 30 minutes. Finally an aliquot of the reaction mixture was loaded on 2% high-resolution agarose gel and the DNA fragments were separated by electrophoresis (100 volts for 30 minutes). The digestion produced 85- and 126-bp fragments for the 379C allele and a non-digested 211-bp fragment for the 379T allele (Figure 3).
**Statistical Analysis:**

Statistical analysis was done using software version SPSS (Statistical Package for the Social Sciences). Continuous parametric variables as age, were presented as mean +/- 2SD and compared using Student’s t-test, while categorical variables of the PLA2G7 genotypes were presented as percentages and compared by χ² test (Chi square test). Differences of the distributions of alleles and genotypes between cases and controls were analyzed using χ² test. Allel-genotype frequencies were calculated by the gene-counting method and were checked for Hardy–Weinberg analysis through χ² test.

Hardy–Weinberg equilibrium (HWE) is the state of the genotypic frequency of two alleles of one autosomal gene locus after one discrete generation of random mating in an indefinitely large population: if the alleles are A and a with frequencies p and q (q=1-p), then the equilibrium gene frequencies are simply p and q and the equilibrium genotypic frequencies for AA, Aa and aa are p², 2pq and q² (12).

The association of the PLA2G7 gene polymorphism with acute ischemic stroke was estimated by computing the odds ratios (OR) and 95% confidence intervals (CI) from logistic regression analyses after adjusting for confounding risk factors. An odds ratio (OR) is a measure of association between an exposure and an outcome. The OR represents the odds that an outcome will occur with a given particular exposure, compared to the odds of the outcome occurring in the absence of that exposure.

\[
\text{OR}=\frac{(n) \text{ exposed cases} \times (n) \text{ unexposed controls}}{(n) \text{ exposed controls} \times (n) \text{ unexposed cases}} \quad (2)
\]

OR=1 Exposure does not affect odds of outcome

OR>1 Exposure associated with higher odds of outcome OR<1 Exposure associated with lower odds of outcome

The 95% confidence interval (CI) is used to estimate the precision of the OR. A large CI indicates a low level of precision of the OR, whereas a small CI indicates a higher precision of the OR. 95% CI is calculated as follows:

\[
95\% \text{ CI} = P \pm 1.96 \times SE (\text{Standard Error of Population}) \quad (5)
\]

P values were calculated by two-sided test; the probability (P) value for the calculated χ² value was then deduced, with the degrees of freedom being equal to the sum of the two sample sizes minus 2. P values > 0.05 indicate a non-significant difference, P < 0.05 indicates a significant difference for all statistical analyses(7).

**RESULTS**

A total of 20 acute ischemic stroke patients and 11 healthy controls were enrolled in this study. The characteristics of the patients and controls are shown in table (1). There were no significant differences in age and sex by χ² (P= 0.285 and 0.939 consecutively) and there was no significant difference regarding the prevalence of conventional risk factors for acute ischemic stroke, such as hypertension (P=0.332), diabetes mellitus (P=0.135) and hyperlipidemia (P=0.809) between the acute ischemic stroke patients and the controls (Table1).

The genotype and allele frequencies of PLA2G7 polymorphism in patients and control subjects were shown in tables (2 and 3). Allele frequencies were calculated by gene counting method and were checked for Hardy-Weinberg equilibrium.

![Fig. (3): The digestion produced 85- and 126-bp fragments for the 379C allele (Valine) and a non digested 211-bp fragment for the 379T allele (Alanine).](image-url)
As for A379V polymorphism, the AV genotype (35% in patients and 9% in controls) of A379V was represented at an increased frequency in the group of patients with acute ischemic stroke than in controls, yet they didn’t show statistically significant association with the presence of acute ischemic stroke regarding the distributions of genotypes ($\chi^2 = 2.488$, P value = 0.115) (Table 2) of A379V in acute ischemic stroke patients and controls.

The data was further confirmed by stepwise forward logistic regression analysis to evaluate the association between PLA2G7 polymorphism and the risk of acute ischemic stroke. Logistic regression analysis revealed that after adjusting for the confounding factors (hypertension, diabetes mellitus and hyperlipidemia), no variables entered significantly into the model (OR= 5.39, 95% CI=0.56-51.17).

As for the allelic distribution of PLA2G7 alleles, the frequency of A379V (V) allele wasn’t significantly higher in patients with acute ischemic stroke than in the control group (OR= 2.69, 95% CI= 0.29-24.74, $\chi^2 = 0.461$, P value = 0.497) (Table 3).

The distribution of risk factors of AIS among PLA2G7 phenotypes is shown in table (4), we found no significant differences among the PLA2G7 phenotypes in age or sex, as well as hypertension, diabetes mellitus and hyperlipidemia in both patients and controls.

### Table (1): Descriptive and comparative statistics between the control and patient groups regarding the risk factors of AIS.

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Control group (%) (Total= 11)</th>
<th>Patient group (%) (Total= 20)</th>
<th>$X^2$/t*</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>4 (36.4%)</td>
<td>7 (35%)</td>
<td>0.006</td>
<td>0.939</td>
</tr>
<tr>
<td>Male</td>
<td>7 (63.6%)</td>
<td>13 (65%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2- Age*</td>
<td></td>
<td></td>
<td>1.090*</td>
<td>0.285</td>
</tr>
<tr>
<td>Mean +/- 2SD</td>
<td>61.91 +/- 10.31</td>
<td>57.15 +/- 12.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>45-77</td>
<td>30-75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3- Hypertension</td>
<td></td>
<td></td>
<td>0.94</td>
<td>0.332</td>
</tr>
<tr>
<td>Negative</td>
<td>3 (27.3%)</td>
<td>9 (45%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>8 (72.7%)</td>
<td>11 (55%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4- Diabetes mellitus</td>
<td></td>
<td></td>
<td>2.232</td>
<td>0.139</td>
</tr>
<tr>
<td>Negative</td>
<td>6 (54.5%)</td>
<td>16 (80%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>5 (45.5%)</td>
<td>4 (20%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5- Hyperlipidemia</td>
<td></td>
<td></td>
<td>0.969</td>
<td>0.809</td>
</tr>
<tr>
<td>Negative</td>
<td>9 (81.8%)</td>
<td>15 (75%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>↑LDL-C</td>
<td>1 (9.1%)</td>
<td>3 (15%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>↑LDL-C, ↑VLDL-C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>↑VLDL-C</td>
<td>0 (0%)</td>
<td>1 (5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 (9.1%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Independent t-test  P > 0.05: NS, P < 0.05: S
Table (2): Descriptive and comparative statistics between the control and patient groups regarding PLA2G7 phenotype and genotype frequencies (Checked by HWE after gene-counting method).

<table>
<thead>
<tr>
<th>PLA2G7 Phenotype</th>
<th>Genotype</th>
<th>Control group (%) (Total= 11)</th>
<th>Patient group (%) (Total= 20)</th>
<th>OR (95% CI)</th>
<th>Χ²</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Valine</td>
<td>AV (CT)</td>
<td>1 (9.1%)</td>
<td>7 (35%)</td>
<td>5.39 (0.56-51.17)</td>
<td>2.488</td>
<td>0.115</td>
</tr>
<tr>
<td>Alanine</td>
<td>AA (TT)</td>
<td>10 (90.9%)</td>
<td>13 (65%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P > 0.05: NS, P < 0.05: S   A (Alanine), V(Valine), C (Cytocine) and T (Thiamine)

Table (3): Descriptive and comparative statistics between the control and patient groups regarding PLA2G7 allele frequencies (Checked by HWE after gene-counting method).

<table>
<thead>
<tr>
<th>Allele</th>
<th>Control group (%) (Total= 22)</th>
<th>Patient group (%) (Total= 40)</th>
<th>OR (95% CI)</th>
<th>Χ²</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>V (C)</td>
<td>1 (4.5%)</td>
<td>7 (17.5%)</td>
<td>2.69 (0.29-24.75)</td>
<td>0.461</td>
<td>0.497</td>
</tr>
<tr>
<td>A (T)</td>
<td>10 (95.5%)</td>
<td>33 (82.5%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P > 0.05: NS, P < 0.05: S   A (Alanine), V(Valine), C (Cytocine) and T (Thiamine)

Table (4): Descriptive and comparative statistics between PLA2G7 phenotypes regarding the risk factors of AIS

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Valine (AV) (%) (Total= 7)</th>
<th>Alanine (VV) (%) (Total= 13)</th>
<th>Χ²/t*</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- Sex</td>
<td>Female</td>
<td>3 (42.9%)</td>
<td>4 (30.8%)</td>
<td>0.292</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>4 (57.1%)</td>
<td>9 (69.2%)</td>
<td></td>
</tr>
<tr>
<td>2- Age*</td>
<td>Mean+/−2SD</td>
<td>57.86+/−11.58</td>
<td>56.77+/−13.08</td>
<td>0.184*</td>
</tr>
<tr>
<td>3-Hypertension</td>
<td>Negative</td>
<td>4 (57.1%)</td>
<td>5 (38.5%)</td>
<td>0.642</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>3 (42.9%)</td>
<td>8 (61.5%)</td>
<td></td>
</tr>
<tr>
<td>4- Diabetes mellitus</td>
<td>Negative</td>
<td>6 (85.7%)</td>
<td>10 (76.9%)</td>
<td>0.220</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>1 (14.3%)</td>
<td>3 (23.1%)</td>
<td></td>
</tr>
<tr>
<td>5-Hyperlipidemia</td>
<td>Negative</td>
<td>6 (85.7%)</td>
<td>9 (69.2%)</td>
<td>4.176</td>
</tr>
<tr>
<td></td>
<td>↑LDL-C</td>
<td>0 (0%)</td>
<td>3 (23.1%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>↑LDL-C, ↑VLDL-C</td>
<td>0 (0%)</td>
<td>1 (7.7%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>↑VLDL-C</td>
<td>1 (14.3%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
</tbody>
</table>

*Independent t-test   P > 0.05: NS, P < 0.05: S
DISCUSSION

Cerebrovascular stroke is the second most common cause of death (after ischemic heart disease) and the leading cause of disability worldwide and one of the leading causes of death in Egypt, according to the WHO media center (21). Ischemic stroke is the most common type of stroke in Egypt, as in other countries, accounting for 43% to 79% of all stroke types (8).

Acute Ischemic stroke is diagnosed by full history, clinical examination including general and neurological examinations, emergent brain imaging for confirming the diagnosis including CT and MRI, and the conventional baseline laboratory testing that is often limited to blood glucose, coagulation studies, complete blood count (CBC) and lipid profile. Additional laboratory tests may include cardiac biomarkers, homocysteine level, antinuclear antibody (ANA), & rheumatoid factor. Emerging studies of the genetic basis of enzymes like matrix metalloproteinases and Angiotensin converting enzyme may be essential for early diagnosis and determining the prognosis of acute ischemic stroke (8).

There are some traditional factors that increase the risk of ischemic stroke such as hypertension and hyperlipidemia, but genetic risk factors, suggested by evidence from inheritance-based studies, might also contribute to a predisposition to ischemic stroke (6).

Inflammation is an essential process in the pathogenesis of ischemic stroke. Therefore, genes involved in inflammatory responses are under investigation to look for variants predisposing to ischemic stroke (10).

Genetic study of proteins, such as lipoprotein-associated phospholipase A2 (Lp-PLA2), also known as platelet-activating factor acetylhydrolase (PAF-AH), shows that it is a calcium ion (Ca2+) independent phospholipase which belongs to phospholipase A2 superfamily and is composed of 441 amino acids (3).

The platelet-activating factor (PAF) is a phospholipid with a variety of biological functions. Lp-PLA2 was primarily recognized as an anti-inflammatory enzyme because of its capability of degrading platelet-activating factor. Intriguingly, this anti-inflammatory activity is gradually opposed by its pro-inflammatory action. It is involved in the signaling and activation of proinflammatory cells such as platelets, neutrophils, and macrophages (14) and also was shown to inhibit apoptosis of B cells (19). Furthermore, it has been reported to alter vascular permeability and to induce hypotension, platelet-dependent broncho-constriction and smooth-muscle contraction (13).

Lp-PLA2 also participates in the oxidative modification of low-density lipoprotein (LDL) by cleaving oxidized phosphatidylcholines, generating lysophosphatidylcholine (lyso-PC) and oxidized non-esterified fatty acids (oxNEFAs), which are two important pro-inflammatory mediators (1).

Genetic study of the human Lp-PLA2 shows that it is encoded by the PLA2G7 gene, which is located on chromosome 6p21-p12 originally assigned as the human leukocyte antigen region (17).

One non-synonymous polymorphism A379V (Ala379Val) in exon 11, has previously been found to be associated with coronary artery disease & ischemic stroke in Chinese and USA population (11,18). However, little is known about whether the PLA2G7 gene polymorphism is associated with the risk of ischemic stroke in Egyptian population or not. So, our study aimed at investigating the association of A379V (Ala379 Val) genetic variant of PLA2G7 gene in patients with acute ischemic stroke.

Our study was conducted on a group of twenty 20 patients presented with acute ischemic stroke in the first 48 hours and diagnosed according to clinical neurological and radiological examinations. Samples were collected from patients admitted to the Neurology Department and stroke units of Ain Shams University hospitals (Al-Demerdash and Ain Shams Specialized hospitals). Oral informed consents were obtained either from the patients or their relatives before
enrollment in the study according to the Ethical Committee of Faculty of Medicine, Ain Shams University.

In this study, we found that the genotypic and allelic frequencies of A379V was found in higher percentages in patients with acute ischemic stroke than controls, yet they didn’t show statistically significant association with the presence of acute ischemic stroke regarding the distributions of genotypes ($\chi^2 = 2.488$, P value = 0.115) of A379V between acute ischemic stroke patients and controls. The AV genotype (35 % in patients and 9 % in controls) of A379V was represented at an increased frequency in the group of patients but the frequency of A379V V allele wasn’t significantly higher in patients with acute ischemic stroke than in the control group (OR= 2.69, 95% CI= 0.29-24.74, $\chi^2 = 0.461$, P value = 0.497). A379V variant was also showed no statistically significant association with a certain age, sex, as well as hypertension, diabetes mellitus and hyperlipidemia in the Egyptian population.

Thus, the ability of the Lp-PLA2 to cause acute ischemic stroke may be mediated through a mechanism that is at least partially independent of its polymorphic activity, although we cannot exclude the potential role of Lp-PLA2 gene in modulating the steps of atherosclerosis that contribute in the pathogenesis of acute ischemic stroke.

Our study demonstrated also that there was no significant difference regarding the prevalence of conventional risk factors for acute ischemic stroke, such as hypertension (P=0.332), diabetes mellitus (P= 0.135) and hyperlipidemia (P=0.809) between the acute ischemic stroke patients and the controls.

The genotypic and allelic frequencies of A379V were found in higher percentages in patients with acute ischemic stroke than controls, yet they didn’t show statistically significant association with the presence of acute ischemic stroke in our study. Also we cannot exclude the potential role of PLA2G7 gene in modulating the steps of atherosclerosis that contribute in the pathogenesis of acute ischemic stroke. Our study also suggests that there’s no significant association of A379V polymorphism genotype with certain age, sex, as well as hypertension, diabetes mellitus and hyperlipidemia.

REFERENCES


تيمكين V (A379Val) للفوسفوليباز المرتبط بالبروتين الدهني (PLA2G7) في مرضى السكتة الدماغية الحادة.

تعد السكتة الدماغية السبب الأول للوفاة بعد أزمة تخصص تربوية للطب والأسیس الرئيسي للإعاقة في جميع أنحاء العالم وإحدى الأسباب الرئيسية للوفاة في مصر وذلك لتفاوت تطور المرض في مصر مما يساهم في النمو ويعزى إلى أنواع السكتات الدماغية المختلفة. وتم تبني نصائح السكتات الدماغية呼唤ة وعمر الرياضة المرتبط بالحمض العضبي في مرضى السكتة الدماغية المتواجدون في تأثير على أن يسبب السكتة الدماغية الحادة في التشخيص المبكر ولأسباب عدة رسومتها: ما هي الأسباب ؟ وهما من المواد الوسيطة المهمة الموالية للالتهابات ويعود إلى ازدياد معدل تكرار الأليل المرتبط بالبروتين الدهني حمض أميني. وللعامل المنشط للصفائح الدموية (، وهما من المواد الوسيطة المهمة الموالية للالتهابات. ويرمز للفوسفوليباز المتغيرات المسببة للسكتة الدماغية. والفوسفوليباز المرتبط بالبروتين الدهني يشارك أيضا في التعديل المؤكسد للبروتين الدهني منخفض الكثافة A2 المتغير الجيني للفوسفوليباز للدراسة. وبالتالي فإن قدرة ٩٧١٤٤ في اكسون ٦٩ من الضوابط هو السبب وراء عدم وجود المتغير AV في السكتة الدماغية الحادة و ٢٠١٤ من الضوابط، وهو النوع الأكثر شيوعاً من أنواع السكتات الدماغية في مصر. وعلامة此类 السكتات الدماغية الحادة، وتعتبر السكتة الدماغية الحادة، وهي النوع الأكثر شيوعاً من أنواع السكتات الدماغية في مصر. وتعتبر السكتة الدماغية الحادة، وهي النوع الأكثر شيوعاً من أنواع السكتات الدماغية في مصر. وتعتبر السكتة الدماغية الحادة، وهي النوع الأكثر شيوعاً من أنواع السكتات الدماغية في مصر.
EPCA-2: COULD IT BE A PROMISING MARKER FOR EARLY DIAGNOSIS OF PROSTATE CANCER?

Mona F. Youssef, Abeer I. Abd El Mageed, Mohamed O. El Maraghy and Menat Allah A. Shaaban

ABSTRACT

Background: Prostate cancer (PC) is the second most common cancer in men and the third most prevalent cancer worldwide. Its diagnosis relies initially on digital rectal examination (DRE), assay of serum prostate specific antigen (PSA) level and confirmed by biopsy and histopathological examination. However, PSA has low diagnostic specificity leading to a large number of unnecessary biopsies in patients who are suspected to have PC. Early prostate cancer antigen-2 (EPCA-2) is expressed throughout the tissue prostate of patients with PC but not in healthy prostate tissue. Objective: Aim of the present study was to evaluate the clinical utility of EPCA-2 in PC in comparison to the traditional marker; PSA. Subjects and Methods: This study was conducted on 90 patients suffering from PC; 40 patients suffering from benign prostatic hyperplasia (BPH) and 30 healthy age matched control group. Patients with PC were re-classified according to TNM staging into 40 organ confined PC patients and 50 organ non confined PC patients. Blood samples were collected, then sera were separated and stored at -20°C till assay of total PSA (tPSA), free PSA (fPSA) and EPCA-2. Assay of serum EPCA-2 was carried out using an enzyme linked immunosorbent assay technique (ELISA). While, tPSA and fPSA were determined using chemiluminescent immunoassay. Results: The results of the present study revealed that serum EPCA-2 level showed statistically high significant increase in whole PC group or its subgroups (organ confined and organ non confined) when compared to each of controls and BPH groups. The diagnostic sensitivity and specificity of EPCA-2 for discrimination between PC patients and control group were 93.3% and 95.3% at a cut off level of 27 ng/mL. Moreover, the diagnostic sensitivity and specificity of EPCA-2 for discrimination between organ confined PC patients and BPH group were 87% and 85% at a cut off level of 30 ng/mL. In conclusion: Assay of serum EPCA-2 could be considered a promising marker in early diagnosis of PC with better specificity than tPSA and f/tPSA ratio , thus, EPCA-2 can spare unnecessary prostate biopsies.

INTRODUCTION

Prostate cancer (PC) is one of the most common cancers in men worldwide and one of the leading cause of cancer-related death among men(36). Thus, early detection of PC is a very important issue for managing patients with PC and is associated with an improved outcome(13).

Currently, the screening for PC essentially involves DRE, determination of serum concentrations of PSA, and transrectal ultrasonography (TRUS). Definitive diagnosis of PC requires histopathological examination of prostate biopsies or operative specimens(9).

A major noted limitation for PSA is lacking specificity as high levels are found in benign conditions as BPH and prostatitis. This may lead to unnecessary biopsies for definitive diagnosis(2).

Early prostate cancer antigen-2 (EPCA-2), a nuclear matrix protein of 40 KD, is identified as being expressed throughout the prostate of patients with PC, but not in benign diseases involving the prostate(13).

Aim of the Work:

The aim of the present study was to assess the clinical utility of EPCA-2 in early diagnosis of PC in comparison with the currently traditional marker; PSA.

SUBJECTS AND METHODS

I. Subjects:

This study was conducted on 90 patients suffering from PC; 40 patients suffering from BPH and 30 healthy age matched control group. Patients with PC were re-classified according to TNM staging into 40 organ confined PC patients and 50 organ non confined PC patients.

Subjects included in this study were classified into three groups:

A. Group I (PC patients’ Group, n=90):

This group included 90 patients suffering from PC. Their ages ranged from 54 to 80 years with a median age of 65 years. The diagnosis of PC was based on histopathological examination.
of prostatic biopsy. PC patients were classified according to TNM staging into two subgroups:

**a) Subgroup Ia (organ confined PC patients; n=40):**

This subgroup included 40 organ confined PC patients. Their ages ranged from 54 to 80 years with a median age of 65 years. According to TNM staging, 12 patients were T1 and 28 patients were T2.

**b) Subgroup Ib (organ non confined PC patients; n=50):**

This subgroup included 50 organ non confined PC patients. Their ages ranged from 58 to 80 years with a median age of 65 years. The metastatic lesions were detected by bone scan and/or pelvic CT. According to TNM staging, 26 patients were T3 and 24 patients were T4.

**B. Group II (Patients with BPH; n=40):**

This group included 40 patients with BPH. Their ages ranged from 60 to 79 years with a median age of 66.5 years.

**C. Group III (Control group; n=30):**

This group included 30 apparently healthy individuals. Their ages ranged from 57-76 years with a median age of 67 years.

All individuals included in this study were subjected to laboratory investigations including assay of tPSA and fPSA by chemiluminescent technique, and assay of serum EPCA-2 by ELISA method.

**II. Methods:**

**A) Analytical Methods:**

Five mL of venous blood samples were drawn into vacutainer tubes from each subject. Samples were left to clot for 10 minutes, then centrifuged at 2000 r.p.m for 20 minutes, serum was separated and stored in aliquots at -20 °C until the assay of tPSA, fPSA and EPCA-2.

Serum tPSA and fPSA were assayed on the Immulite (Diagnostic Products Corporation, DPC)” by chemiluminescent immunometric assay using reagent products of DPC (Diagnostic Product Corporation: 5700 West 96 Street. Los Angeles).

The EPCA-2 assay was done using an ELISA kit supplied by Glory Science (2400Veterans Blvd Suite 16 – 101, Del Rio, TX 78840.USA).

In this technique, the solid phase anti-human EPCA-2 antibody was pre-coated to the inner wall of ELISA wells plate. Standards & samples were pipetted into the wells and EPCA-2 present was bound by the immobilized antibody. After washing away any unbound substances, the second antibody, labeled with biotin to which Streptavidin-horse radish peroxidase (HRP), was combined forming immune complex. The reaction was stopped by adding stop solution to each well. Addition of chromogen results in color development that is measured spectrophotometrically at a wavelength of 450 nm. The concentration of EPCA-2 was proportional to the color intensity of the test sample. A standard curve was constructed from which the concentrations of EPCA-2 in the samples were determined.

**B) Statistical Methods:**

Statistical analysis was performed by standard complete program of SPSS, version 15.0, Echo soft corporation, USA, 2007. Data was expressed descriptively as mean ± standard deviation (SD) for quantitative parametric data and median and interquartile range for quantitative skewed data. Comparison between each two groups was done using Mann-Whitney U test for skewed data. p< 0.05 was considered significant and p< 0.01 was considered highly significant and p> 0.05 was considered non-significant. Furthermore, the diagnostic performance of the studied parameters was evaluated using receiver operating characteristic curve analysis, in which sensitivity % was plotted on the Y axis and 100-specificity on the x-axis. The best cut off value (the point nearest to the left upper corner of the curve) was determined.

**RESULTS**

Results of the present study are shown in tables (1-4) and figures (1 - 2).
Descriptive statistics of the various studied parameters among all studied groups is shown in table (1).

Statistical comparison between the whole patients versus controls regarding the various studied parameters is shown in table (2). This revealed a statistically highly significant increase in serum EPCA-2 in patients with PC & BPH when compared to control group using Mann-Whitney U test \( Z = -7.961, -2.9, P < 0.001 \) respectively. A statistically highly significant increase in serum tPSA was found in patient with PC & BPH when compared to the control group \( Z = -7.82, -4.514, P > 0.001 \) respectively. A statistically highly significant decrease in f/tPSA ratio was recorded when patients with PC & BPH compared to control group \( Z = -4.11, -4.492, P < 0.001 \) respectively.

A statistically highly significant increase in serum EPCA-2 and tPSA in PC group when compared to control group using Mann-Whitney U test \( Z = -9.623, -10.086, P < 0.001 \) respectively and a significant decrease in f/tPSA ratio \( Z = -2.105, P < 0.05 \). A statistically highly significant increase in serum tPSA was recorded in patients with PC when compared to BPH \( Z = -7.237, -8.284, P < 0.001 \) respectively, while a non significant difference in f/tPSA ratio was observed in organ non confined PC when compared with control, BPH and organ non confined PC \( Z = -0.333, P > 0.05 \).

Statistical comparison between all studied groups versus organ confined PC and organ non confined regarding the various studied parameters is shown in Table (2). This revealed a statistically highly significant increase in serum EPCA-2 in patient with organ confined PC when compared to control group and BPH using Mann-Whitney test \( Z = 7.237, 6.678, 5.798, P < 0.001 \) respectively. A statistically highly significant increase in serum tPSA was found in patient with organ confined PC when compared to the control group, BPH and organ non confined PC \( Z = 7.347, 6.413, 6.181, 5.5832, P > 0.001 \) respectively. There was a non significant difference in f/tPSA ratio recorded in organ confined PC when compared with control, BPH and organ non confined PC \( Z = 0.012, -1.386, -1.738, P > 0.05 \).

A statistically highly significant increase in serum EPCA-2 in patient with organ non confined PC when compared to control group and BPH using Mann-Whitney test \( Z = -5.56, -5.23, -4.19, P < 0.001 \) respectively. A statistically highly significant increase in serum tPSA was recorded in patient with organ non confined PC when compared to the control, BPH and organ non confined PC \( Z = -6.36, -5.278, -5.76, P > 0.001 \) respectively. There was a non-significant difference in f/tPSA ratio observed in organ non confined PC when compared with control, BPH and organ non confined PC \( Z = -1.93, -1.453, -0.17, P > 0.05 \). While there was a non-significant difference of EPCA-2 between organ confined PC and organ non confined PC \( Z = -0.96, P > 0.05 \).

Receiver operating characteristic curve (ROC) analysis was applied to assess the diagnostic performance of serum EPCA-2& tPSA in discriminating patients with PC from the control group as shown in Table (3) and (Figure 1). The best diagnostic cut off for serum EPCA-2 was 27 ng/mL. The diagnostic sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and diagnostic efficacy were 93.3%, 95.3%, 95.5%, 91.7% and 93.8% respectively. The best diagnostic cut off for serum tPSA was 15 ng/mL. The diagnostic sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and diagnostic efficacy were 93.3%, 84.7%, 89.4%, 90.9% and 90%, respectively.

Receiver operating characteristic curve (ROC) analysis was applied to assess the diagnostic performance of serum EPCA-2, tPSA and f/tPSA ratio in discriminating patients with organ confined PC from BPH as shown in table (4) and (Figure 2). The best diagnostic cut off for serum EPCA-2 was 30 ng/mL. The diagnostic sensitivity, specificity, PPV, NPV and total efficacy were 87%, 85%, 85%, 85% and 85% re-
Table (1): Descriptive statistics of the various studied parameters in the all studied groups:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Whole PC Group (n =45)</th>
<th>Organ confined PC (n = 20)</th>
<th>Organ non confined PC (n = 25)</th>
<th>BPH (n=20)</th>
<th>Control Group (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (IQR) *</td>
<td>Median (IQR) *</td>
<td>Median (IQR) *</td>
<td>Median (IQR) *</td>
<td>Median (IQR)*</td>
</tr>
<tr>
<td>Age (years)</td>
<td>65 (62-71.25)</td>
<td>65 (59.25-73.5)</td>
<td>65 (62-70)</td>
<td>66.5 (60-70)</td>
<td>67 (62-70)</td>
</tr>
<tr>
<td>tPSA (ng/ml)</td>
<td>129.35 (68.47-380.37)</td>
<td>67.65 (27.92-119)</td>
<td>281.9 (131.7-518)</td>
<td>4.5 (2.6-10.97)</td>
<td>1.5 (1.3-2)</td>
</tr>
<tr>
<td>fPSA (ng/ml)</td>
<td>21.45 (7.57-72.2)</td>
<td>8.5 (5.55-55)</td>
<td>37.8 (15-88.5)</td>
<td>0.6 (0.425-1.1)</td>
<td>0.5 (0.4-0.7)</td>
</tr>
<tr>
<td>f/tPSA ratio</td>
<td>0.171 (0.096-0.33)</td>
<td>0.201 (0.09-0.58)</td>
<td>0.161 (0.09-0.19)</td>
<td>0.1589 (0.1-0.23)</td>
<td>0.35 (0.21-0.46)</td>
</tr>
<tr>
<td>EPCA-2 (ng/ml)</td>
<td>50 (42.25-100)</td>
<td>50 (40-90)</td>
<td>60 (44.5-105)</td>
<td>20.5 (7.5-28.5)</td>
<td>7 (4.5-18)</td>
</tr>
</tbody>
</table>

* IQR: Inter quartile range for non-parametric data

Table (2): Statistical comparison between all groups regarding the various studied parameters using Mann-Whitney U test for non parametric data:

<table>
<thead>
<tr>
<th>Compared groups</th>
<th>tPSA (ng/ml)</th>
<th>f/tPSA Ratio</th>
<th>EPCA-2 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Z</td>
<td>P</td>
<td>Z</td>
</tr>
<tr>
<td>PC / BPH</td>
<td>-7.82</td>
<td>&lt;0.001</td>
<td>-4.11</td>
</tr>
<tr>
<td>PC / Control</td>
<td>-4.514</td>
<td>&lt;0.001</td>
<td>-4.492</td>
</tr>
<tr>
<td>Organ Non Confined/ BPH</td>
<td>-5.76</td>
<td>&lt;0.001</td>
<td>-0.017</td>
</tr>
<tr>
<td>Organ Non confined PC/ Control</td>
<td>-5.278</td>
<td>&lt;0.001</td>
<td>-1.453</td>
</tr>
<tr>
<td>Organ Confined/ BPH</td>
<td>-6.181</td>
<td>&lt;0.001</td>
<td>-1.386</td>
</tr>
<tr>
<td>Organ confined PC/ Control</td>
<td>-6.413</td>
<td>&lt;0.001</td>
<td>-1.734</td>
</tr>
<tr>
<td>Organ Confined PC/Organ Non Confined PC</td>
<td>-55.832</td>
<td>&lt;0.001</td>
<td>-1.738</td>
</tr>
</tbody>
</table>

p<0.05: Significant difference,  p<0.001: Highly significant difference,  p>0.05: Non-significant difference
Table (3): Diagnostic performance of serum EPCA-2 & tPSA (ng/mL) in discrimination between PC and control group:

<table>
<thead>
<tr>
<th></th>
<th>Cutoff</th>
<th>Diagnostic Sensitivity (%)</th>
<th>Diagnostic Specificity (%)</th>
<th>Positive predictive value (%)</th>
<th>Negative predictive value (%)</th>
<th>Diagnostic Efficacy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum EPCA-2</td>
<td>27</td>
<td>93.3</td>
<td>95.3</td>
<td>95.5</td>
<td>91.7</td>
<td>93.8</td>
</tr>
<tr>
<td>(ng/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum tPSA</td>
<td>15</td>
<td>93.3</td>
<td>84.7</td>
<td>89.4</td>
<td>90.9</td>
<td>90</td>
</tr>
<tr>
<td>(ng/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table (4): Diagnostic performance of serum EPCA-2, tPSA (ng/mL) & f/tPSA ratio in discrimination between organ confined PC patients and BPH subgroup:

<table>
<thead>
<tr>
<th></th>
<th>Cutoff</th>
<th>Diagnostic Sensitivity (%)</th>
<th>Diagnostic Specificity (%)</th>
<th>Positive predictive value (%)</th>
<th>Negative predictive value (%)</th>
<th>Diagnostic Efficacy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum EPCA-2</td>
<td>30</td>
<td>87</td>
<td>85</td>
<td>85</td>
<td>85</td>
<td>85</td>
</tr>
<tr>
<td>(ng/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum tPSA</td>
<td>11.3</td>
<td>90</td>
<td>80</td>
<td>81.8</td>
<td>88.9</td>
<td>80</td>
</tr>
<tr>
<td>(ng/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>f/tPSA ratio</td>
<td>0.209</td>
<td>50</td>
<td>75</td>
<td>66.7</td>
<td>60</td>
<td>62.5</td>
</tr>
</tbody>
</table>

Figure (1): ROC curve analysis showing the diagnostic performance of EPCA2, tPSA & f/tPSA ratio for discriminating patients with PC from the control group.

AUC
- tPSA: 0.812
- f/tPSA Ratio: 0.386
- EPCA-2: 0.982

Figure (2): ROC curve analysis showing the diagnostic performance of EPCA2, tPSA & t/fPSA ratio for discriminating Organ Confined PC from the BPH subgroup.

AUC
- tPSA: 0.896
- f/tPSA Ratio: 0.466
- EPCA-2: 0.792

respectively. The best diagnostic cut off for serum tPSA was 11.3 ng/mL. The diagnostic sensitivity, specificity, PPV, NPV and total efficacy were 90%, 80%, 81.8%, 88.9% and 80% respectively.

**DISCUSSION**

Several laboratory markers are used for early detection of PC. PSA is widely applied in clinical diagnosis; however, major disadvantage is its false positive rates in case of benign conditions of the prostate as inflammation and BPH that may lead to unnecessary biopsies (17).

The alterations in the protein components of the nuclear structure in various tissues have been implicated in carcinogenesis. EPCA-2 – a 40 KD
nuclear matrix protein - is one of these biomarkers on focus in researches nowadays\textsuperscript{(12)}.

The results of the present study revealed that tPSA were significantly higher in the whole PC patient than control and BPH groups. These significant high results were also apparent in organ confined and organ non confined cancer patients when compared with the control group. Our results agreed with those of Ahmed and Aref\textsuperscript{,(1)} Moreover, our study found a significant increase in tPSA in organ non confined PC patients versus organ confined PC. This is in agreement with Daniels et al.\textsuperscript{(3)}, who stated that all previous findings can be attributed to great tissue mass presented in malignant neoplasm. Moreover, PSA can diffuse in the systemic circulation in a markedly increased amount only after significant changes have occurred in the architectural pattern of prostate gland.

However, the present study recorded a non significant difference in f/tPSA ratio between organ confined PC and organ non confined PC. Our results go with the findings of Miyake et al.\textsuperscript{(8)}.

Our study showed no significant difference in f/t PSA ratio between PC patients when compared to control group. These results were in agreement with the study conducted by Yamamoto et al.\textsuperscript{(14)}.

In the present study, it was found that serum EPCA-2 level showed statistically high significant increase in whole PC group or its subgroups (organ confined and organ non confined) when compared to each of controls and BPH groups. This is in agreement with the results found by Thompson et al.\textsuperscript{(10)} The alterations in the protein components of the nuclear structure have been associated with carcinogenesis in a variety of tissues, including prostate. The nuclear matrix protein, EPCA-2, causes the DNA within cells to become deformed, increasing the tendency for cells to aggregate into tumors and reproduce rapidly. Therefore, the greater the protein is expressed, the greater the capacity for a cell to rapidly reproduce and become cancer-like\textsuperscript{(4)}.

When EPCA-2 level was compared between organ confined PC and organ non confined PC subgroups, no significant difference was recorded between the 2 subgroups. This is in accordance with Uetsuki et al.\textsuperscript{(13)} who stated that the alteration in EPCA-2 level appears to be preserved through malignant progression. However, other studies as that obtained by Leman et al.\textsuperscript{(7)} recorded that EPCA-2 was able to differentiate between organ confined PC from organ non confined PC.

The study of the diagnostic performance of studied parameters in discriminating whole PC patients from control group using ROC curve revealed that EPCA-2 at a cut off level of 27 ng/mL had the best discriminating power (sensitivity, specificity, PPV, NPV and diagnostic efficacy of EPCA-2 were 93.5\%, 95.3\%, 95.5\%, 91.7\% and 93.8\%, respectively). The sensitivity, specificity, PPV, NPV and diagnostic efficacy of tPSA at a cut off level of 15 ng/mL were 93.3\%, 84.7\%, 89.4\%, 90.9\% and 90 \%, respectively.

Similar results were obtained by Leman et al.\textsuperscript{(6)} who tested the diagnostic performance of serum EPCA-2 to discriminate between PC group and control group at cut off 30 ng/mL and they found that EPCA-2 at this cut off had a sensitivity 92\% and specificity 94\%.

Paul et al.\textsuperscript{(9)} had similar results and have attributed the reason that 100\% sensitivity was not reached in his results due to the fact that even at tissue level, not all PC expresses EPCA-2.

The evaluation of the discriminating power in organ confined PC patients versus BPH group using ROC curve revealed that EPCA-2 at a cut off level of 30 ng/mL had the best discriminating power (sensitivity, specificity, PPV, NPV and diagnostic efficacy of EPCA-2 were \textit{87\%, 85\%, 85\%, 85\% and 85\%}, respectively). As for tPSA, sensitivity, specificity, PPV, NPV and diagnostic efficacy were 90\%, 80\%, 81.8\%, 88.9\% and 80\%, respectively at a cut off level of 11.3 ng/mL. As regarding f/tPSA ratio, sensitivity, specificity, PPV, NPV and diagnostic efficacy were 50\%, 75\%, 66.7\%, 60\% and 62.5\%, respectively at a cut off 0.209.

\textbf{In conclusion:} EPCA-2 has a great potential value as a serum based biomarker for early diagnosis of PC and can spare unnecessary prostate
biopsies with a comparable sensitivity and better specificity than tPSA and f/t PSA ratio.

REFERENCES
في التشخيص المبكر لسرطان البروستاتا

مني فتحي يوسف – عبير إبراهيم عبد المجيد – محمد عمر المراغي – منة الله علي شعبان

يعد سرطان البروستاتا من أكثر الأورام انتشارا على مستوى العالم وأحد الأسباب الرئيسية المسببة للوفاة. لذلك، هناك حاجة ملحة للتشخيص المبكر لمرضى سرطان البروستاتا. يعتمد التشخيص المبكر على الفحص الأكلينيكي للمستقيم وتحليل بروتين البروستاتا الخاص ثم يكون التشخيص النهائي بأخذ وفحص نسيج البروستاتا من أكثر دلالات الأورام استخداماً في تشخيص سرطان البروستاتا على الرغم من وجود العديد من العوائق مثل ارتفاع درجة في العديد من الأمراض الأخرى كورم البروستاتا الحميد والتهابات البروستاتا، لذلك فإن من الضروري البحث عن دلالات أورام أخرى تتغلب على عيوب بروتين البروستاتا الخاص.

لذا كان الهدف من هذه الدراسة هو تقييم الاستخدام الاكلينيكي لبروتين سرطان البروستاتا المبكر-2 في التشخيص المبكر لسرطان البروستاتا ومقارنته ببروتين البروستاتا الخاص. تم قياس بروتين سرطان البروستاتا المبكر-2 باستخدام التحليل المناعي الإنزيمي وبروتين البروستاتا الخاص الكلي والحر بالوميض الكيميائي في تسعةين مريضاً منحناً من سرطان البروستاتا و أربعين مريضاً منحناً من بروستاتا الحميد. وتمت مقارنة النتائج بناءً على دراسة أفضل قيم فيROC لبجاير لبروتين البروستاتا الخاص الكلي من الأصحاء في نفس المرحلة العمرية كعينة جامعية. وباستخدام منحنى ROC للفرق بين سرطان البروستاتا والمجموعة الضابطة، كان أفضل مستوى لبروتين البروستاتا الخاص الكلي هو 20 نانو جرام لكل ملليلتر، والحساسية التشخيصية 100%، والشفافية التشخيصية 95.2%، والحساسية التشخيصية 89.3%، والحساسية التشخيصية 78.2%.

أما بالنسبة للبروتين المبكر-2، فهو 22 نانو جرام لكل ملليلتر والحساسية التشخيصية 100%، والشفافية التشخيصية 94.5%، والحساسية التشخيصية 81.7%، والحساسية التشخيصية 41.2%. وقد أثبت ذلك دراسة الأداء التشخيصي لبروتين البروستاتا الخاص الكلي للفترة بين مرضاً سرطان البروستاتا في المرحلة المبكرة والمسابرون ببجقرأ البروستاتا الحميد. أن أفضل مستوى لبروتين البروستاتا الخاص الكلي هو 11.6 نانو جرام لكل ملليلتر، والحساسية التشخيصية 95.5%، والشفافية التشخيصية 93.3%، والحساسية التشخيصية 81.5%، والشفافية التشخيصية 78.9%.

وأما بالنسبة للبروتين المبكر-2، فهو 20 نانو جرام لكل ملليلتر والحساسية التشخيصية 100%، والشفافية التشخيصية 95.2%، والحساسية التشخيصية 89.3%، والشفافية التشخيصية 81.7%.
CLINICAL UTILITY OF TELOMERASE mRNA ASSAY IN BLOOD IN THE EARLY DIAGNOSIS OF HEPATOCELLULAR CARCINOMA

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and is considered the third cause of cancer related deaths. The prognosis of patients with HCC is poor when diagnosed at advanced stages. With early diagnosis and treatment, the 5-year survival rate may reach up to 70-80%. Spiral CT scan is considered the routinely used method for diagnosis. As regard serological screening, alpha fetoprotein (AFP) is still the currently routinely used marker, though its diagnostic performance is unsatisfactory. Telomerase is a ribonucleoprotein enzyme. The telomerase catalytic subunit (hTERT) exerts important cellular functions, including telomere homeostasis, genetic stability, cell survival and perhaps differentiation. hTERT mRNA in blood was reported in different types of cancer but not in benign diseases. Thus, hTERT mRNA can be focused on as a diagnostic marker for HCC patients. Objective: Aim of the present study was to evaluate the clinical utility of assessment of hTERT mRNA in peripheral blood in diagnosis of HCC and to correlate its level with AFP.

Subjects and Methods: This study was conducted on 50 patients with liver diseases; 30 adult patients with HCC and 20 patients with chronic liver disease (CLD). Patients with HCC were re-classified according to Barcelona staging into 10 Barcelona A subgroup patients, 10 Barcelona B subgroup patients and 10 Barcelona D subgroup patients. Patients with CLD were re-classified according to Child classification into 7 Child A subgroup patients, 7 Child B subgroup patients and 6 Child D subgroup patients. In addition, 20 age-and sex- matched controls were included in the study. Blood samples were collected for determination of ALT, AST, albumin, total and conjugated bilirubin, PT and INR. Assay of hTERT mRNA in whole blood was carried out using real-time polymerase chain reaction (RT-PCR) technique. Results: The results of the present study revealed that hTERT mRNA was significantly higher in HCC patients as compared to both CLD patients and controls. Also, a significant elevation in HCC patients compared to control group was revealed, and significant elevation in CLD patients was found as compared to controls. The diagnostic sensitivity and specificity of hTERT mRNA was 100 % for both by using cut-off 2 (2-ΔΔCT) for discrimination between HCC patients and control groups. Moreover, the diagnostic sensitivity and specificity of hTERT mRNA was 100 % for both by using cut-off 18 (2-ΔΔCT) for discrimination between HCC patients and CLD patients. In conclusion: Assay of hTERT mRNA in whole blood can be considered a promising marker in diagnosis of HCC with an excellent diagnostic efficiency compared to patients with CLD and healthy groups as well. Moreover, it can differentiate between various Barcelona stages, thus, it can diagnose HCC at an early stage and hence improve the prognosis and survival rate of the HCC patients.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a major challenge in medicine. The incidence of HCC is increasing and it is becoming more and more significant both clinically and epidemiologically. Now, HCC represents the fifth most common cancer in the world and the third most frequent cause of mortality among oncological patients.

The development of HCC is a multi step process associate with alteration in several genes and, depending also on external factors, such as infection by hepatitis B or C; chronic alcohol intake and exposure to certain chemicals.

Although the modalities such as spiral CT scan and conventional tumor markers such as AFP are widely used and important for HCC detection in clinical practice, they still do not provide an entirely satisfactory solution to detect HCC at the early stage.

Telomeres are specialized DNA-protein structures that cap the ends of linear chromosomes. The telomeric structure is crucial for protecting linear chromosomes from fusion by cellular DNA repair processes and thus telomeres are essential for maintaining the integrity and stability of genomes.

Telomerase are situated at the ends of linear chromosomes and protect them from degradation as well as end-to-end fusions. In humans, telomerase are typically 10 kb long and consist of TTAGGG repeats.

Telomerase is a ribonucleoprotein enzyme, consists of human telomerase RNA component, human telomerase protein 1 and human telomer-
The telomerase catalytic subunit (hTERT) exerts important cellular functions, including telomere homeostasis, genetic stability, cell survival and perhaps differentiation. Thus, telomerase plays an important role in the development of cellular immortality and oncogenesis.

**Aim of the Work:**

The aim of the present study was to evaluate the clinical utility of assessment of hTERT mRNA in peripheral blood in diagnosis of HCC and to correlate its level with the conventionally used serological marker; AFP.

**SUBJECTS AND METHODS**

**I. Subjects:**

Subjects included in this study were classified into two groups:

**A. Group I (Patients’ Group, n=50):**

This group included fifty patients with liver diseases who were further classified into 2 subgroups:

1) **Subgroup Ia (HCC group; n=30):**

This subgroup included 30 adult patients with HCC. Their ages ranged from 50 to 67 years, with a median age of 59 years. The diagnosis was based on spiral CT scan. Diagnosis was based on the presence of the typical hallmark of HCC (hyper-vascular in the arterial phase with washout in the portal venous or delayed phases).

This group was further classified into 3 subgroups according to Barcelona staging:

- a- Barcelona A subgroup included 10 patients. Their ages ranged from 57 to 62 years with a median age of 60 years.
- b- Barcelona B subgroup included 10 patients. Their ages ranged from 51 to 67 years with a median age of 58.5 years.
- c- Barcelona D subgroup including 10 patients. Their ages ranged from 50 to 65 years with a median age of 55.5 years.

2) **Subgroup Ib (CLD group; n=20):**

This subgroup included twenty patients with chronic viral hepatitis. Their ages ranged from 29 to 68 years with a median age of 51.5 years. The diagnosis was based on:

- a- Clinical picture: such as ascities, variceal bleeding from portal hypertension, hepatic encephalopathy and jaundice of the eyes or skin.
- b- Ultrasonography: shows nodular contour of the liver with coarse echo texture ± hypoechoic nodules.
- c- Laboratory diagnosis including liver function tests and markers of viral hepatitis.

Patients with CLD were further classified according Child classification into 3 subgroups:

- i. Child A subgroup included 7 patients. Their ages ranged from 45 to 60 years with a median age of 49 years.
- ii. Child B subgroup included 7 patients. Their ages ranged from 35 to 63 years with a median age of 54 years.
- iii. Child C subgroup included 6 patients. Their ages ranged from 29 to 68 years with a median age of 49 years.

**B. Group II (Control Group; n=20):**

This group included twenty age- and sex-matched healthy subjects. They were 11 males and 9 females. Their ages ranged from 50 to 67 years with a median age of 59 years.

All individuals included in this study were subjected to laboratory investigations including liver function tests (AST, ALT, total and conjugated bilirubin, PT and INR), markers of CLD as viral markers, serum AFP and assay of hTERT mRNA in peripheral blood using RT-PCR technique.

**II. Methods:**

**A- Analytical Methods:**

Four mL of blood were collected in EDTA vaccutainers for TERT mRNA assay. Samples were transported on ice in order to avoid any possible RNA degradation and were processed within 30 minutes from collection using RNeasy Mini Kit provided by Qiagen (Qiagen incorporation, 28159 Avenue, Stanford Valencia. CA 91355, USA).

RNA was extracted following the manufacturer instructions and the standard protocol.
The eluted RNA was collected immediately and stored at -70°C for 24-48 hours for further processing.

Real-Time two-step RT-PCR synthesis was performed on the extracted RNA using QuantiTect Reverse Transcription Kit provided by Qiagen. Telomerase (hTERT)-encoding mRNA is reverse transcribed and the generated complementary DNA (cDNA) is amplified using quantifast probe where the amplicon is detected by fluorescence using a specific pair of hybridization probes. The Hybridization Probes consist of two different oligo-nucleotides that hybridize to an internal sequence of the amplicon during the annealing phase of the amplification cycle. One probe is labeled at the 5'-end and modified at the 3'-end by phosphorylation. The other probe is labeled at the 3'-end with fluorescein. Only after hybridization to the template cDNA, the two probes come in close proximity, resulting in fluorescence resonance energy transfer (FRET) between the two fluorophores. The emitted fluorescence is then measured by the LightCycler Instrument.

The PCR cycler was programmed to start with an initial incubation for 5 minutes at 95°C, then 40 cycles of denaturing step at 95°C (30 seconds), annealing step at 60°C (10 seconds), and finally an extension step where fluorescence data collection was performed at 60°C (30 seconds).

Results were reported in relative quantification which is based on the expression levels of a target gene (TERT mRNA) versus a reference gene (housekeeping gene) which was GAPDH in our study. Calculations are based on the comparison of a distinct cycle in real-time PCR determined by threshold values (CT) at a constant level of fluorescence.

The ΔCT value for each sample was then determined by calculating the difference between the CT value of the target gene and the CT value of the housekeeping gene. This was determined for each unknown sample as well as for the calibrator samples.

Then, ΔΔCT was calculated, where ΔΔCT = ΔCT (sample) – ΔCT (calibrator)

Finally, the normalized level of target gene expression was calculated by using the formula: 

\[ 2^{-\Delta \Delta CT} \]

B) Statistical Methods:

Statistical analysis was done using IBM SPSS software package, version (V. 20.0, IBM Corp., USA, 2011). Data was expressed descriptively median and interquartile range for quantitative skewed data. Comparison between each two groups was done using Mann-Whitney U test for skewed data, p<0.05 was considered significant and p<0.01 was considered highly significant and p>0.05 was considered non-significant. Furthermore, the diagnostic performance of the studied parameters was evaluated using receiver operating characteristic curve analysis, in which sensitivity % was plotted on the Y axis and 100-specificity on the x-axis. The best cutoff value (the point nearest to the left upper corner of the curve) was determined.

RESULTS

Results of the present study are shown in tables (1-5) and figures (1 - 2).

The descriptive statistics of all studied groups (HCC, CLD and control group) regarding different studied parameters are shown in table (1).

The comparative statistics between each two groups regarding hTERT mRNA and AFP using Mann-Whitney U test is shown in table (2). Regarding the comparison between CLD group versus control group, hTERT mRNA shows a significantly higher difference between both groups (z= -5.395, p<0.01). However, a non significant difference is observed between both groups regarding AFP (z= -1.762, p>0.05). Regarding the comparison between HCC group versus CLD group, AFP and hTERT mRNA are significantly higher in HCC group compared to CLD group (z= -4.417, -5.941, p<0.01, respectively). Regarding the comparison between HCC group versus control group, hTERT mRNA and AFP are significantly higher in HCC group compared to control group (z= -5.955, -4.215, p<0.01, respectively).

The comparative statistics between each two Barcelona stages regarding different stud-
ied parameters using Mann-Whitney U test are shown in table (3). Regarding the comparison between Barcelona A and Barcelona B, hTERT mRNA are significantly higher in Barcelona A compared to Barcelona B subgroup \((z=-0.269, p<0.01)\). However, AFP showed no statistically significant difference between the 2 subgroups \((z=-0.1741, p>0.05)\). The comparison between Barcelona A and Barcelona D revealed that hTERT mRNA and AFP are significantly higher in Barcelona D compared to Barcelona A subgroup \((z=-0.378\) and \(-2.347, p<0.01\) and \(<0.05\), respectively). Regarding the comparison between Barcelona B and Barcelona D, hTERT mRNA are significantly higher in Barcelona D compared to Barcelona B subgroup \((z=-0.378, p<0.01)\). However, AFP showed no statistically significant difference between the 2 subgroups \((z=-0.605, p>0.05)\).

Receiver operating characteristic curve (ROC) analysis was constructed to assess the diagnostic performance of serum AFP and hTERT mRNA for discrimination between HCC and control groups as shown in table (4) and figure (1). The best cut off level of AFP was 10 IU/mL. This revealed diagnostic sensitivity 70%, specificity 80%, positive predictive value (PPV) 84%, negative predictive value (NPV) 64% and total efficacy 74%. On the other hand, The hTERT mRNA best cut off value for discrimination between HCC group and control group was \(2^{-\Delta \Delta CT}\) This had diagnostic sensitivity 100%, specificity 100%, PPV 100%, NPV 100% and total efficacy 100%.

Receiver operating characteristics (ROC) curve analysis of serum AFP and hTERT mRNA for discrimination between HCC group and CLD group as shown in table (5) and figure (2). This revealed that the best cut off value of AFP was 14 IU/mL. This had a diagnostic sensitivity 70%, specificity 60%, PPV 72.4%, NPV 57.1% and total efficacy 66%. The hTERT mRNA best cut off value was 18 \(2^{-\Delta \Delta CT}\). This revealed diagnostic sensitivity 100%, specificity 100%, PPV 100%, NPV 100% and total efficacy 100%.

Table (1): Descriptive statistics of all studied groups (HCC, CLD and control groups) regarding different studied parameters:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HCC group (n=30)</th>
<th>CLD group (n=20)</th>
<th>Control Group (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median (IQR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>87 (29 –110)</td>
<td>51 (35 – 87)</td>
<td>21 (15 – 29)</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>48 (29 – 102)</td>
<td>37 (30 – 62)</td>
<td>27 (19 – 39)</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>2.6(2 – 3)</td>
<td>2.5 (2.2 – 2.8)</td>
<td>4 (3.8 – 4.4)</td>
</tr>
<tr>
<td>Total Bilirubin (mg/dL)</td>
<td>1.8 (1.2 – 7.4)</td>
<td>1.6 (0.8 – 4.4)</td>
<td>0.9 (0.6 – 0.9)</td>
</tr>
<tr>
<td>Conjugated Bilirubin (mg/dL)</td>
<td>0.9 (0.4 – 4.6)</td>
<td>0.6 (0.2 – 2.3)</td>
<td>0.1 (0.0 – 0.2)</td>
</tr>
<tr>
<td>PT(sec.)</td>
<td>14 (14 – 16.6)</td>
<td>16(13.2 –17.7)</td>
<td>13(12 – 13.5)</td>
</tr>
<tr>
<td>INR ratio</td>
<td>1.2 (1.1 – 1.4)</td>
<td>1.5 (1.2 – 1.77)</td>
<td>1(0.85 – 1.05)</td>
</tr>
<tr>
<td>Alpha fetoprotein(IU/mL)</td>
<td>23.2 (3.2- 1000)</td>
<td>9.5 (1.7 – 57)</td>
<td>1 (0.1 – 6)</td>
</tr>
<tr>
<td>hTERT mRNA (2^{-\Delta \Delta CT})</td>
<td>43 (29- 89)</td>
<td>4.6 (2.7 – 13.5)</td>
<td>1.15(1-2)</td>
</tr>
</tbody>
</table>

HCC: Hepatocellular carcinoma. CLD: Chronic liver disease. IQR: Inter-quartile range.
Table (2): Comparative statistics between each two groups regarding hTERT mRNA and AFP using Mann-Whitney U test:

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HCC group (n=30) Versus Control group (n=20)</th>
<th>CLD group (n=20) Versus Control group (n=20)</th>
<th>HCC group (n=30) Versus CLD group (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>z P</td>
<td>z P</td>
<td>z P</td>
</tr>
<tr>
<td>AFP (IU/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hTERT mRNA ((2^{\Delta\Delta C_t}))</td>
<td>-4.215 &lt;0.01</td>
<td>-1.762 &gt;0.05</td>
<td>-4.417 &lt;0.01</td>
</tr>
</tbody>
</table>

P >0.05: Non significant difference  
P <0.05: significant difference  
P <0.01: Highly significant difference

Table (3): Comparative statistics between each two Barcelona subgroups regarding TERT mRNA and AFP using Mann-Whitney U test:

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Barcelona A (n=10) Versus Barcelona B (n=10)</th>
<th>Barcelona A (n=10) Versus Barcelona D (n=10)</th>
<th>Barcelona B (n=10) Versus Barcelona D (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>z P</td>
<td>z P</td>
<td>z P</td>
</tr>
<tr>
<td>AFP (IU/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hTERT mRNA ((2^{\Delta\Delta C_t}))</td>
<td>-1.174 &gt;0.05</td>
<td>-2.347 &lt;0.05</td>
<td>-0.605 &gt;0.05</td>
</tr>
</tbody>
</table>

P >0.05: Non significant difference  
P <0.01: Highly significant difference

Table (4): The diagnostic performance of AFP and hTERT mRNA in discriminating HCC group from control group:

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Cut off</th>
<th>AUC</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha-fetoprotein (IU/mL)</td>
<td>10</td>
<td>0.752</td>
<td>70</td>
<td>80</td>
<td>84</td>
<td>64</td>
<td>74%</td>
</tr>
<tr>
<td>hTERT mRNA ((2^{\Delta\Delta C_t}))</td>
<td>2</td>
<td>1.000</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100%</td>
</tr>
</tbody>
</table>

Table (5): The diagnostic performance of AFP and hTERT mRNA in discriminating HCC group from CLD group:

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Cut off</th>
<th>AUC</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha-fetoprotein (IU/mL)</td>
<td>14</td>
<td>0.746</td>
<td>70</td>
<td>60</td>
<td>72.4</td>
<td>57.1</td>
<td>66%</td>
</tr>
<tr>
<td>hTERT mRNA ((2^{\Delta\Delta C_t}))</td>
<td>18</td>
<td>1.000</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100%</td>
</tr>
</tbody>
</table>
DISCUSSION

Hepatocellular Carcinoma (HCC) is the fifth most common form of malignant diseases worldwide (accounting for > 5% of all human cancers) and the third most common cause of cancer-related death[2]. The most common risk factors for HCC are chronic hepatitis and cirrhosis in association with hepatitis B or C viral infection[4].

The clinical course of HCC is usually asymptomatic. Therefore, most patients are presented with tumors that are often large and too advanced to be subjected to effective and radical therapy[1].

Although serum AFP level is a widely used tumour marker for the detection and monitoring of HCC, however, its sensitivity is still limited (41-65%), in small well-differentiated HCC and even in patients with advanced HCC. In addition, AFP may show false positive results reaching as high as 40%[6].

Thus, the development of new non invasive biomarkers for the early diagnosis and improved prognosis of HCC is urgently required[11].

Telomerase is a large ribonucleoprotein (RNP) complex that consists of an RNA component subunit, which serves as a template for the synthesis of telomere sequence and the reverse transcriptase (TERT), which is the catalytic subunit[16].

Telomerase plays a major role in the maintenance of telomere DNA at the ends of linear chromosomes, thus, maintaining chromosomal stability and accordingly facilitating immortal proliferation of cells, thus it is revealed to be involved in the multistep process of carcinogenesis in various types of cancers including HCC[9].

The results of the present study revealed that AFP levels were significantly higher in HCC patients compared to CLD patients. This goes with results obtained by Othman et al.[12].

The statistical comparison between different Barcelona stages in HCC patients showed that AFP levels were significantly higher in Barcelona D compared to Barcelona A, however, no statistical significance was found between Barcelona A versus B and Barcelona B versus D. Our results are in agreement with Francesco et al.[7] and Peng et al.[14] who stated that AFP levels vary in a non regular pattern throughout the dif-
Different stages of HCC. They have also observed that among patients diagnosed with HCC, AFP levels may be normal in up to 40% of patients, particularly in the early stages of the tumor (low sensitivity).

In our study, no significant difference was found between child subgroups of CLD patients regarding AFP. This agreed with Di Carlo et al. who claimed that AFP levels vary during the different phases of the disease, but it does not follow a regular pattern.

On the other hand, hTERT mRNA in peripheral blood showed a highly significant increase in HCC patients compared to patients with CLD and healthy controls. Our results are in accordance with Mizuno et al. and El-Idrissi et al. They claimed that telomerase level changes occur early, at the cirrhotic stage, and persist to the tumor stage, which suggests that telomerase activity contribute to both tumor development and tumor progression. On the contrary, Miura et al. observed that there was a slight increase in expression of hTERT mRNA level in cirrhotic patients as compared to controls, but that does not reach a significant level. They attributed this to the fact that normal hepatocytes may express a negligible amount of hTERT-mRNA and that inflamed hepatocytes may still express more weakly than HCC cells.

Moreover, the statistical comparison between different Barcelona stages in HCC patients regarding hTERT mRNA levels revealed significant difference between each two groups of the three HCC subgroups. Comparable results were obtained by Miura et al. and EL-Mazny et al. who revealed correlation between levels of hTERT mRNA with tumor stage, including tumor size and number.

The study of the diagnostic performance of serum AFP for discrimination between HCC and CLD patients revealed that serum AFP at a cut off value 14 IU/mL showed a diagnostic sensitivity 70%, specificity 60%, PPV 72.4%, NPV 57.1% and diagnostic efficacy 66%.

On the other hand, the diagnostic performance of hTERT mRNA in whole blood for discrimination between HCC from CLD patients revealed that hTERT mRNA at a cut off value 18 (2-ΔΔCT) showed a diagnostic sensitivity 100%, specificity 100%, PPV 100%, NPV 100% and diagnostic efficacy 100%.

In conclusion, TERT mRNA is a promising sensitive and specific marker for HCC. It has a great potential value in distinguishing between various Barcelona stages that can detect HCC at an early stage and thus improve the prognosis and survival rate of patients suffering from HCC.

REFERENCES


11) Mizuno H.; Honda M.; Shirasaki T. et al. (2012): Heterogeneous nuclear ribonucleoprotein A2/B1 in association with hTERT is a potential biomarker for hepa-
الآليهة الأكليميكية لقياس التيلوميراز (TERT) بالدم في التشخيص المبكر لمرض سرطان الكبد

بخل سرطان الكبد المركز الخامس في قائمة الأورام الخبيثة الأكثر شيوعاً في جميع أنحاء العالم، وأحد الأسباب الرئيسية
المسببة للوفاة. ويلتزم تشخيص أورام الكبد بالأساس على طرق التشخيص مثل الأشعة المقطعية و لا يزال الألفا فيتو بروتين في
العصب مستخدم في التشخيص عن سرطان الخلايا الكبدية. على الرغم من افتقار هذه الطرق للحساسية الكافية للكشف عن السرطان في المراحل
المبكرة، واللهوم صغرى الحجم. كما يقلد الألفا فيتو بروتين خصوصيته للورم بسبب زيادة في الإبلدات الكبدية. في مرضى تليف
الكبد، لذلك هناك حاجة ملحّة إلى الكشف عن دلالات أخرى جديّة تكون أكثر حساسية في التشخيص المبكر لسرطان الكبد وأكثر دقة
في التمييز بينه وبين الأورام الحميدة. إذا كان الألفا فيتو بروتين في الدم في التشخيص المبكر لسرطان الكبد ومقارنته بالألفا فيتو بروتين.

وقد تم قياس التيلوميراز باستخدام التقنيات البلمرة الفيتوغيني في ثلاثين مريضاً مصاباً بسرطان الكبد، وعشرين مريضاً من
عائنين من سرطان الكبد، وعشرين مريضاً من عائنين من تليف الكبد. وقد تم مقارنة النتائج مع نتائج عشرين من الأصحاء في نفس
المرحلة العمرية كعينة ضابطة. باستخدام منحنى ROC لدراسة أفضل قيمة للألفا فيتو بروتين للفرقة بين سرطان الكبد والمجموعة
المصابة. كان أفضل مستوى للألفا فيتو بروتين 10 وحدة دولية لكل مليلتر و الحساسية التشخيصية 80%، والخصوصية 100%، والخصوصية
العاجلية الإيجابية 84%، والخصوصية السلبية 44%، والقياسية التشخيصية 74%، بينما أفضل مستوى للتيوميراز
هو 2 –ΔΔCT% و الحساسية التشخيصية 100%، والخصوصية 100%، والقياسية التشخيصية 74%، والقياسية التشخيصية 100%، والقياسية التشخيصية 74%، والقياسية التشخيصية 100%، والقياسية التشخيصية 74%، والقياسية التشخيصية 100%، والقياسية التشخيصية 74%، والقياسية التشخيصية 100%، والقياسية التشخيصية 74%، والقياسية التشخيصية 100%
OBJECTIVE: To evaluate the clinical utility of serum neopterin and highly sensitive CRP in diagnosis and assessment of the severity of pre-eclampsia.

METHODS: This study was conducted on 30 patients with mild pre-eclampsia (Group Ia), 30 patients with severe pre-eclampsia (Group Ib) and 20 healthy pregnant females (Group II). Fasting serum samples, blood samples on EDTA and morning urine were collected for all subjects. Alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen, creatinine, fasting blood glucose and complete blood picture were assayed. Serum neopterin and hs-CRP were estimated by ELISA technique and urine protein was evaluated by dipstick test. Results: The levels of the measured neopterin and hs-CRP in group Ia and group Ib were significantly higher than those of group II. Moreover, the elevated levels of hs-CRP could differentiate between group Ia and group Ib. A significant positive correlation was found between neopterin and both Hb and SBP in group Ia, while in group Ib a significant positive correlation was found between serum neopterin and hs-CRP. Diagnostic performance was studied for both serum neopterin and hs-CRP. Serum neopterin could discriminate group Ia from group II (at a cut-off 2.95 nmol/L, sensitivity was 96.7% and specificity was 55%) and group Ib from group II (at a cut-off level 3.25 nmol/L, sensitivity was 96.7% and specificity was 70%). On the other hand, hs-CRP showed better performance, it can discriminate group Ia from group II (at a cut-off level 1.2 mg/L, sensitivity was 76.7% and specificity was 70%), group Ib from group II (at a cut-off level 2.5 mg/L, sensitivity was 96%, specificity was 100%); and fortunately, group Ia from group Ib (at a cut-off level 5 mg/L, sensitivity was 73.3%, specificity was 100%). Conclusion: Maternal serum hs-CRP and neopterin can be used for the early diagnosis of pre-eclampsia. Moreover, serum hs-CRP can strongly help obstetricians for evaluation of the severity of pre-eclampsia to avoid serious complications for mother and fetus.

INTRODUCTION

Pre-eclampsia (PE), is a serious syndrome that develops after 20 weeks of gestation. It is featured as newly diagnosed hypertension in conjunction with proteinuria(3). Worldwide, the incidence of pre-eclampsia ranges from 5% to 10%(12).

It can cause multi-system dysfunction including renal failure, hepatic failure, coagulopathy and central nervous system disorders to the mother and it may lead to fetal growth restriction, prematurity and perinatal death(5). It is considered to be one of the principal causes of preterm birth, resulting in annual death of approximately 63000 maternal and 500000 infant(18).

Although pre-eclampsia remains a disease of theories, an improved understanding of the pathogenesis of pre-eclampsia has raised the possibility that deficient remodelling of the spiral arteries during the interaction between maternal and fetal sides at the time of trophoblast invasion has been postulated as a cause of placental insufficiency. This would lead to the release of inflammatory factors in the systemic maternal circulation which are involved in the endothelial dysfunction; the hallmark pathological finding in pre-eclampsia(21).

Neopterin is a protein derived from activated monocytes and macrophages, present in body fluids. It is secreted after IFN-gamma stimulation leading to cellular immunity enhancement(19). Studies have shown that neopterin can be found in different body fluids following immune system activation. It has reported high levels in various conditions including, various autoimmune diseases, and also in some cancer types, organ transplants and nephritic syndrome(13).

C-reactive protein (CRP) is an acute-phase protein found in the blood. Its level rises in response to inflammation, tissue damage, infection and neoplasia. Hs-CRP is highly sensitive immunoassay for CRP with detection limit down to 0.05 mg/L; so, its measurement provides a clinical tool that may have an important role in the prediction, identification and assessment of diseases such as cardiovascular or metabolic diseases(22).
Aim of the work:

The aim of the present study was to assess serum neopterin and highly sensitive CRP in a group of pregnant females with pre-eclampsia and to evaluate their clinical utility in diagnosis and assessment of severity of the disease.

SUBJECTS AND METHODS

Subjects

This study was conducted on 60 pregnant females suffering from pre-eclampsia at the third trimester, recruited from Ain Shams University Hospitals (Group I). They were diagnosed according to the current American College of Obstetricians and Gynecologists Guidelines\(^1\). Group I was further classified according to the severity of pre-eclampsia into two subgroups; subgroup Ia which consisted of 30 patients with mild pre-eclampsia; SBP≤160 mmHg, DBP≤110 mmHg on at least two occasions, at least 6 hours apart and proteinuria (dipstick showing +1 or +2 in a random urine sample), aged 25.2± 3.07 years, subgroup Ib which consisted of 30 patients with severe pre-eclampsia presenting with (at least one of the following): SBP>160 mmHg, DBP>110 mmHg on at least two occasions, at least 6 hours apart, proteinuria (dipstick showing +3 or +4 in a random urine sample), pulmonary oedema, seizures, oliguria (<400mL/day), thrombocytopenia (platelet count <100,000/μL), abnormal liver enzymes associated with persistent epigastric or right upper-quadrant pain, persistent and severe central nervous system symptoms e.g., altered mental status, headaches, blurred vision, blindness or the presence of fetal growth restriction, aged 25.9±3.87 years. In addition to 20 healthy normotensive age-matched pregnant females at the third trimester, aged 24.9±2.73 years. After patients consent, all individuals included in the study were subjected to complete history taking, clinical examination, and determination of gestational age.

Patients with diabetes mellitus, chronic hypertension, liver disease, renal disease, cardiovascular disease, pre-existing chronic inflammatory diseases, acute inflammatory diseases, and smokers were excluded from this study.

Samples

Blood samples were collected from all participants: first; a 7 mL venous blood sample was withdrawn under complete aseptic conditions into a plain vacutainer. Five milliliters were put in plain test tubes, and the remaining two milliliters were put in a test tube with ethylene diamine tetraacetic acid (EDTA) (1.2 mg/ml) as an anticoagulant, to be used for performing CBC.

After clotting, samples were centrifuged at 1500xg for 15 minutes. Part of the separated serum was used to perform fasting blood glucose (FBG), kidney and liver function tests.

The rest of the serum was divided into two aliquots which were stored at -20°C. One aliquot was used for the subsequent assay of hs-CRP and the second aliquot for the assay of serum neopterin. Hemolysed samples were discarded. Repeated freezing and thawing was avoided.

Second; a 10 mL of morning urine samples were collected for urinary protein estimation by dipstick test.

Methods

The fasting blood glucose, kidney and liver function tests was done on the Synchron CX-9 autoanalyser (Beckman Instruments Inc.; Scientific Instruments Division, Fillerton, CA 92634, 3100, USA) using the hexokinase reaction applying a timed endpoint method for glucose analysis. For serum blood urea nitrogen (BUN) urease reaction was applied using an enzymatic rate method. Serum creatinine was assayed by a modified rate Jaffè method. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity were measured by coupling the transaminase reactions to specific dehydrogenase reactions using an enzymatic rate method. Complete blood count (CBC) was done using Gen-S Coulter counters (Coulter Corporation, Florida, and USA). Protein detection in urine was performed using urine dipstick based on the protein error of pH indicators.
Highly sensitive C-reactive protein (hs-CRP) concentrations were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit supplied by Accu-Bind, Inc (Monobind Inc, Lake Forest, USA, Ca 92630) by sandwich ELISA. Whereas neopterin concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) kit supplied by IBL, Inc (Immuno-biological laboratories, Germany, Flughafenstrasse 52A, D-22335) applying competitive ELISA technique.

**Statistical Methods**

Statistical analysis was performed using statistical software programme SPSS version 9.02. The parametric data are expressed as mean and standard deviation, whereas the non-parametric data are expressed as median and interquartile range. Comparative statistics were done using ANOVA, Kruskal Wallis test, Student’s t test and Wilcoxon rank sum test. Correlation analysis was performed using Spearman’s rank correlation coefficient (rs). p<0.05 was considered significant and p<0.01 was considered highly significant. Receiver operating characteristic curve (ROC) analysis was applied to assess the overall diagnostic performance of neopterin and hs-CRP.

**RESULTS**

The results of the present study are shown in the tables (1) and (2) and the figures (1) and (2). As regards the SBP, DBP, AST, ALT, BUN, creatinine, neopterin and hs-CRP a highly statistically significant difference was found (p<0.01) on comparing the 3 groups (Tables 1 and 2).

A statistically significant positive correlation was found between neopterin and Hb (rs=0.46, p<0.01) and SBP (rs=0.58, p<0.01) in group Ia. In group Ib, there is a statistically significant correlation between neopterin and hs-CRP (rs=0.38, p<0.05).

Correlation study between hs-CRP and the other studied parameters in group Ia, group Ib and group II, revealed a statistically significant positive correlation between hs-CRP and DBP (rs=0.64, p<0.01) and FBG (rs=0.62, p<0.01) as regards group Ia. In addition to a statistically significant correlation (p<0.05) between hs-CRP and FBG (rs=0.48, p<0.05) in group Ib.

Assessment of the diagnostic performance of neopterin and hs-CRP in pre-eclampsia patients versus the healthy control group using ROC curve analysis is shown in figures (1) and (2), respectively.

In discriminating group Ia and group II, the best diagnostic cut off level for neopterin was 2.95 nmol/L, with a diagnostic sensitivity of 96.7%, specificity 55%, negative predictive value 91.7%, positive predictive value 76.3%, efficacy 80% and area under the curve (AUC) of 0.721. In discriminating group Ia and group Ib, the best diagnostic cut off level for neopterin was 6.79 nmol/L, with a diagnostic sensitivity of 63.3%, specificity 60%, negative predictive value 62.1%, positive predictive value 61.3%, efficacy 61.7% and AUC of 0.566. In discriminating group Ib and group II, the best diagnostic cut off level for neopterin was 3.25 nmol/L, with a diagnostic sensitivity of 96.7%, specificity 70%, negative predictive value 93.3%, positive predictive value 82.9%, efficacy 86% and AUC of 0.871.

As regards the hs-CRP, upon discriminating group Ia and group II, it was found that the best cut off value was 1.20 mg/L with a diagnostic sensitivity of 76.7%, specificity 70%, negative predictive value 66.7%, positive predictive value 79.3%, efficacy 74% and AUC of 0.677. In discriminating group Ia and group Ib, it was found that the best cut off value was 5.00 mg/L with a diagnostic sensitivity of 73.3%, specificity 100%, negative predictive value 78.9%, positive predictive value 100%, efficacy 86.7% and AUC of 0.892. In discriminating group Ib and group II, it was found that the best cut off value was 2.50 mg/L with a diagnostic sensitivity of 96%, specificity 100%, negative predictive value 95.2%, positive predictive value 100%, efficacy 98% and AUC of 0.999.
Table (1): Descriptive and comparative statistics between the three studied groups as regards the demographic data, neopterin and hs-CRP using ANOVA for parametric data and Kruskall Wallis test for non-parametric data:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group Ia (n=30)</th>
<th>Group Ib (n=30)</th>
<th>Group II (n=20)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \bar{X} \pm SD )</td>
<td>Median and (IQR)</td>
<td>( \bar{X} \pm SD )</td>
<td>Median and (IQR)</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>154.83±4.04</td>
<td>171±6.6176</td>
<td>115.25±9.24</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>98.5±2.33</td>
<td>111.17±5.2</td>
<td>73.05±8.41</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>11.54±0.99</td>
<td>11.1±0.8175</td>
<td>11.835±0.85</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>FBG (mg/dl)</td>
<td>79.767±9.57</td>
<td>81±6.953</td>
<td>77.665±7.24</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>19 (15.5-23)</td>
<td>32 (24.5-38)</td>
<td>18 (15.25-21.5)</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>18 (16-22)</td>
<td>30 (24.75-40)</td>
<td>18.5 (12.5-20.75)</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>6.95 (6.3-9)</td>
<td>9 (7.15-10.35)</td>
<td>6.8 (6.12-8.17)</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>CR (mg/dl)</td>
<td>0.7 (0.5-0.8)</td>
<td>0.8 (0.67-0.92)</td>
<td>0.5 (0.42-0.71)</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Neopterin (nmol/L)</td>
<td>8.60 (3.54-11.82)</td>
<td>11.79 (4.43-17.73)</td>
<td>2.95 (2.25-3.84)</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Hs_CRP (mg/L)</td>
<td>2.44 (1.18-3.12)</td>
<td>28.12 (5.00-50.00)</td>
<td>1.00 (0.75-1.25)</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

p < 0.01 Highly Significant
p > 0.05 Non Significant

Table (2): Statistical comparison between each two of the studied groups as regards the studied parameters using Student’s t test for parametric data and Wilcoxon rank sum test for non-parametric data:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group Ia vs. Group II</th>
<th>t / z •</th>
<th>p</th>
<th>Group Ib vs. Group II</th>
<th>t / z •</th>
<th>P</th>
<th>Group Ia vs. Group Ib</th>
<th>t / z •</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP (mmHg)</td>
<td></td>
<td>1.9092</td>
<td>&lt; 0.01</td>
<td>1.9092</td>
<td>&lt; 0.01</td>
<td>-1.7076</td>
<td>&lt; 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td></td>
<td>1.5731</td>
<td>&lt; 0.01</td>
<td>1.5731</td>
<td>&lt; 0.01</td>
<td>-1.407</td>
<td>&lt; 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td></td>
<td>0.2588</td>
<td>&gt; 0.05</td>
<td>0.2588</td>
<td>&lt; 0.01</td>
<td>0.2315</td>
<td>&gt; 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FBG (mg/dl)</td>
<td></td>
<td>2.34</td>
<td>&gt; 0.05</td>
<td>2.34</td>
<td>&gt; 0.05</td>
<td>2.09</td>
<td>&gt; 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AST (U/L)</td>
<td></td>
<td>-0.57</td>
<td>&gt; 0.05</td>
<td>-5.05</td>
<td>&lt; 0.01</td>
<td>-5.38</td>
<td>&lt; 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td></td>
<td>-0.33</td>
<td>&gt; 0.05</td>
<td>-4.95</td>
<td>&lt; 0.01</td>
<td>-5.63</td>
<td>&lt; 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td></td>
<td>-0.74</td>
<td>&gt; 0.05</td>
<td>-2.95</td>
<td>&lt; 0.01</td>
<td>-2.85</td>
<td>&lt; 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR (mg/dl)</td>
<td></td>
<td>-1.378</td>
<td>&gt; 0.05</td>
<td>-2.96</td>
<td>&lt; 0.01</td>
<td>-1.93</td>
<td>&gt; 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neopterin (nmol/L)</td>
<td>-4.428</td>
<td>&lt; 0.01</td>
<td>-5.134</td>
<td>&lt; 0.01</td>
<td>-1.615</td>
<td>&gt; 0.05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hs_CRP (mg/L)</td>
<td></td>
<td>-3.957</td>
<td>&lt; 0.01</td>
<td>-5.94</td>
<td>&lt; 0.01</td>
<td>-5.848</td>
<td>&lt; 0.01</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

p < 0.01 Highly Significant
p > 0.05 Non Significant
DISCUSSION

Data of the present study revealed a statistically highly significant increase (p < 0.01) in serum levels of neopterin in both mild pre-eclamptic women (group Ia) (median [IQR]: 8.60 [3.54-11.82] nmol/L), and severe pre-eclamptic women (group Ib) (median [IQR]: 11.79 [4.43-17.73] nmol/L) when compared to the normal pregnant controls (group II) (median [IQR]: 2.95 [2.25-3.84] nmol/L). Also, there is a statistically highly significant increase (p < 0.01) in serum neopterin in group Ib when compared with group II alone, but no statistically significant difference was found when group Ia was compared with group Ib.

This comes in accordance with Kaleli et al.\(^6\) and Frauke et al.\(^4\) who reported that plasma neopterin concentrations were elevated in women with pre-eclampsia compared to normal pregnant women. This could be explained by the fact that normal pregnancy is characterized by the shift from a Th1/Th2 balance to Th2 predominance, so, exaggerated Th1 response appears to be harmful and may lead to pre-eclampsia, which is characterized by excessive immune activation\(^4\).

Also, Ozler et al.\(^14\) had stated that patients with pre-eclampsia had higher serum neopterin levels than healthy pregnant women, but on the contrary to our study, serum levels of neopterin showed a statistically significant difference between mild and severe pre-eclampsia vs control group. Furthermore, they reported that patients with HELLP syndrome have higher serum levels of neopterin than patients with mild and severe pre-eclampsia.
On the contrary to our findings, Kronborg et al.\(^8\) showed that there were no significant differences (p > 0.05) in neopterin concentrations between normal pregnant women and women with pre-eclampsia, however, most of the pre-eclamptic patients in this previous study were diagnosed with mild pre-eclampsia, also, smoking was not an exclusion criterion in that study and may increase serum neopterin level\(^\text{[15]}\).

Results of the present study concerning levels of hs-CRP revealed a highly statistically significant increase (p < 0.01) in serum of both mild pre-eclamptic women (group Ia) (median [IQR]: 2.44 [1.18-3.12] mg/L), and severe pre-eclamptic women (group Ib) (median [IQR]: 28.12 [5.00-50.00] mg/L) when compared to their matched controls (group II) (median [IQR]: 1.00[0.75-1.25] mg/L). Additionally, it revealed a highly statistically significant increase (p < 0.01) in serum levels of hs-CRP in group Ia when compared to group II alone, group Ib when compared with group II alone, and in group Ib when compared with group Ia alone.

These results are in agreement with the previous reports of Luis et al.\(^9\), Yusuf et al.\(^\text{[23]}\) and Selahattin et al.\(^\text{[16]}\) and Anil et al.\(^\text{[2]}\), Nanda et al.\(^\text{[11]}\) who had recorded an elevation of plasma hs-CRP among pre-eclamptic women when compared to their matched controls and positive significant correlation between hs-CRP levels and the severity of pre-eclampsia. These results support the hypothesis that systemic inflammation is involved in the pathogenesis of pre-eclampsia. Although maternal levels of CRP are elevated in overt pre-eclampsia, there is still a debate about its usefulness as a predictive marker for pre-eclampsia during the first and second trimester of pregnancy\(^\text{[10]}\).

On the opposite side, a cross sectional study done by Makrina et al.\(^\text{[10]}\) presents a different picture where, the serum CRP concentration in women who subsequently developed pre-eclampsia was not significantly different from that in women with uncomplicated pregnancies. This lack of difference in the levels of CRP suggests either that the maternal inflammatory response in the second trimester of pregnancy is not exaggerated before the onset of the clinical signs of pre-eclampsia, or that CRP is not a part of the inflammatory response that might exist. But the fact that CRP levels, as measured by routine assays, have been reported to be increased in pregnant women with established pre-eclampsia suggests that the latter is unlikely, and these findings indicate that the onset of clinical signs of pre-eclampsia may not be preceded by a maternal inflammatory response\(^\text{[10]}\).

Our study also revealed a statistically significant positive correlation (rs=0.38, p < 0.05) between hs-CRP levels and serum neopterin levels in pre-eclampsia women (group Ib). These results are in agreement with the previous reports of Frauke et al.\(^\text{[4]}\) who stated that neopterin was significantly correlated with hs-CRP in patients with pre-eclampsia in the third trimester. This shows that there is a positive association between the cellular inflammatory marker neopterin and the acute phase inflammatory marker CRP and strengthens the hypothesis that pregnancy is marked by a significant activation of the innate immune system (soluble and cell-mediated inflammatory factors) and that this activation is further exaggerated in pre-eclampsia specially near the end of pregnancy\(^\text{[4]}\).

Our correlation study showed also in pre-eclampsia women (group Ia) a statistically highly significant correlation between serum neopterin and SBP (rs=0.58, p<0.01) and Hb (rs=0.46, p <0.01) and a non significant correlation with all other Parameters. In pre-eclampsia women (group Ib), and the healthy pregnant control (group II), there is a non significant correlation was found between serum neopterin and all other parameters. This comes in accordance with Frauke et al.,\(^\text{[4]}\) and Ozler et al.,\(^\text{[14]}\) who also did not find significant correlation between serum neopterin in pre-eclampsia women and other studied parameters as body mass index (BMI), serum creatinine levels, and gestational age.

On the opposite side, Von Versen and Powers\(^\text{[20]}\) and Spencer et al.,\(^\text{[17]}\) stated that serum neopterin levels are influenced by some factors such as age, body mass index (BMI), serum creatinine levels, and gestational age.

Also, in pre-eclampsia women (group Ia), a statistically highly significant correlation was
found between serum hs-CRP and DBP (rs=0.64, p < 0.01) and FBG (rs=0.62, p < 0.01) and a non significant correlation with all other parameters. In the healthy pregnant control (group II), a statistically significant correlation only found between hs-CRP and FBG (rs=0.48, p < 0.05). In pre-eclampsia women (group Ib), a statistically non significant correlations are found between serum hs-CRP and all other parameters. On the contrary to this results, Anil et al.,(2) recorded a statistically positive significant correlation between hs-CRP levels and blood pressure which shows that the elevation of hs-CRP level is proportional to severity of pre-eclampsia and supported his results by the hypothesis that systemic inflammation is involved in the pathogenesis of pre-eclampsia and serum hs-CRP level may be the marker to predict severity of disease.

Assessment of the diagnostic performance of neopterin showed an acceptable discriminatory power as regards group Ia vs group II, and group Ib vs group II. Unfortunately, it lacked this power for discrimination between mild and severe pre-eclampsia (group Ia vs Ib) as evidenced by an AUC of 0.566. In discriminating group Ia and group II, the best diagnostic cut off level for neopterin was 2.95 nmol/L, with a diagnostic sensitivity of 96.7 %, specificity 55%, and AUC of 0.721. In discriminating group Ib and group II, the best diagnostic cut off level for neopterin was 3.25 nmol/L, with a diagnostic sensitivity of 96.7 %, specificity 70%, and AUC of 0.871.

Hs-CRP showed a much better diagnostic performance, with the best performance being achieved upon discriminating group Ib vs group II with an AUC of 0.999, while group Ia vs II showed an AUC of 0.677. The ROC curve analysis as regards mild and severe pre-eclampsia yielded a good discriminating power with an AUC of 0.892. In discriminating group Ia and group II, it was found that the best cut off value was 5.00 mg/L with a diagnostic sensitivity of 73.3%, specificity 100%. In discriminating group Ib and group II, it was found that the best cut off value was 2.50mg/L with a diagnostic sensitivity of 96%, specificity 100%.

This comes in accordance with Kashanian et al.(7) who stated that using serum hs-CRP with a cut-off value of 4 mg/L during the first trimester of pregnancy can predict pre-eclampsia with sensitivity of 78.1%, specificity of 72.1%, positive predictive value of 25%, and negative predictive value of 96.5% and diagnostic accuracy of 72.8%.

In conclusion, serum neopterin can early predict the inflammatory process in pre-eclampsia even before the onset of clinical signs, as its release begins 3 days before T cells proliferation reaches its maximum. Hs-CRP can early predict pre-eclampsia, as it detects minimal elevation in CRP levels even when its level is within ranges previously considered to be normal, differentiate mild and severe pre-eclamptic patient from normal healthy pregnant female and correlate well with the severity of the disease. Thus the use of both neopterin and hs-CRP could help clinicians hand in hand with conventional parameters for diagnosis of mild and severe pre-eclampsia.

REFERENCES
6- Kaleli, I.; Demir, M.; Yildirim, B.; et al. (2005): Serum levels of neopterin and interleukin-2 receptor in women with severe pre-eclampsia. Journal of Clinical Laboratory Analysis; 19:36.


الدلاله الأكلينكيه لبروتين سى التفاعلى عالى الحساسيه و النيوبترين فى مصل الدم لمرضى تسمم الحمل

من مصطفي عثمان - هدى أحمد عبد الستار

يتمثل تسمم الحمل حالة خطيره لا تزال تثير بشكل كبير على معدلات الاعتلال والوفيات بالنسبه لأم ولوليد حيث تحتوي هذه الدراسه على معدلات عالية لدلاله الأكلينكيه لبروتين سى التفاعلى عالى الحساسيه و النيوبترين فى مصل الدم لمرضى تسمم الحمل منى مصطفى عثمان - هدى أحمد عبد الستار


LIPOCALIN-2: A BIOMARKER FOR CORONARY ARTERY DISEASE AND ITS ASSOCIATION WITH DISEASE SEVERITY
Arig Aly Seif*, Ahmed Mohamed El Mahmoudy** and Doaa Mostafa Awad*

ABSTRACT
Objective: To assess serum lipocalin-2 (NGAL) levels in patients with coronary artery disease (CAD) and to investigate the association between circulating Lipocalin-2 levels and disease severity. Subjects and Methods: The study included 60 participants serving as patient group 1 and was further subdivided according to the result of coronary angiography into 19 patients with one vessel disease (subgroup 1a), 24 patients with two vessel disease (subgroup 1b) and 17 patients with three vessel disease (subgroup 1c). 20 age and sex matched participants with normal un-occluded vessels served as a control group (group 2). All the 80 participants were examined by coronary angiography and serum samples were assayed for total cholesterol, low density lipoprotein (LDL-C), high density lipoprotein (HDL-C), triglycerides (TG), fasting glucose to exclude diabetes and serum creatinine to exclude renal affection. Serum lipocalin-2 was estimated using ELISA technique and expressed as ng/mL. Results: Data of the present study were expressed as mean ± standard deviation or median [interquartile range] in case of skewness. Serum levels of lipocalin-2 assayed in whole patient group (group 1) showed a highly significant increase compared to its levels in control group (group 2). A highly significant progressive increase in the marker level was recorded through progressively increasing disease severity among subgroups 1a, 1b and 1c. Moreover, lipocalin-2 serum levels can highly significantly differentiate between subgroup 1a, 1b, 1c and between each subgroup and the control group. A highly significant positive correlation was found between serum levels of lipocalin-2 and gensenki score for assessment of CAD severity among the whole patient group 1. Serum levels of total cholesterol, LDL-C, HDL-C and TG showed a statistically significant difference between patient group 1 and the control group 2. Conclusion: Serum lipocalin-2 serves as a promising non-invasive marker for the assessment of atherosclerotic CAD severity.

INTRODUCTION
Coronary artery disease (CAD) and specially its acute form, acute coronary syndrome (ACS), is one of the leading causes of death all over the world. It is responsible for about one-third of all deaths in individuals over the age of 35 (12,9). It is predicted to be the leading contributor to morbidity and mortality worldwide over the next coming decades. It is responsible for about 45% of all deaths in the Middle East(4). Coronary atherosclerosis with subsequent superimposition of an arterial thrombus over an underlying disrupted atherosclerotic plaque represents the major pathogenic process in CAD. Therefore identification of atherosclerosis at the subclinical stages would essentially facilitate earlier selection of more effective treatments which may lead to a better prognosis(10). Laboratory and clinical evidence have demonstrated that atherosclerosis is not simply a disease of lipid deposits. Systemic inflammation also plays an important role in atherosclerosis development and progression and there is considerable evidence supporting the involvement of neutrophils in this inflammatory process. Several biomarkers seemed to be related to CAD development within the last years, such as highly sensitive c reactive protein (hsCRP), matrix metalloproteinase 9 (MMP-9), fibrinogen, pregnancy associated plasma protein A (PAPP-A) and leukocyte count. All these markers contribute to plaque destabilization, acute phase response and atherosclerosis(13) which raised the possibility for their role in assessment of CAD in relation to coronary angiography; the gold standard method for diagnosing the presence and the extent of CAD(8). Among these markers is lipocalin-2, also known as neutrophil gelatinase–associated lipocalin (NGAL), a 25-kDa secretory glycoprotein. It is a member of the lipocalin superfamily of proteins that was originally identified in mouse kidney cells and human neutrophil granules(15). Recent evidence demonstrates a diversity of functions of lipocalin-2. It has been implicated as a protective factor against apoptosis and oxidative stress. Besides, it has been associated...
with the development of insulin resistance, pre-eclampsia, chronic kidney disease and lupus nephritis\textsuperscript{(17)}. It is also involved in the process of endothelial dysfunction and development of atherosclerosis\textsuperscript{(14)}. The demonstration of increased lipocalin-2 expression in atherosclerotic plaques supports a direct role of lipocalin-2 in the development and progression of CAD\textsuperscript{(5)}.

In view of the previous observations and postulations, our study aimed at assessing serum lipocalin-2 levels in patients with CAD and investigating the association between circulating lipocalin-2 levels and disease severity.

**SUBJECTS AND METHODS**

**Subjects**

This study was conducted on eighty (80) participants who were all selected from the Cardiology Department in Ain Shams University Hospitals.

**Subjects were classified into the following groups:**

**Patient group1 (n=60):**

This group included sixty (60) patients undergoing coronary angiography for suspected CAD. They were 50 males and 10 females. They had a mean age of $58.3 \pm 9.3$ years. According to the angiographic gensini scoring system, they were further divided into the following subgroups:

- **a) Subgroup 1a: One Vessel Disease Group (n=19):**
  
  This group included nineteen\textsuperscript{(19)} patients with one vessel disease. They were 18 males and 1 female. Their ages had a mean of $58.6 \pm 8.1$ years.

- **b) Subgroup 1b: Two Vessel Disease Group (n=24):**
  
  This group included twenty four\textsuperscript{(24)} patients with two-vessel disease. They were 20 males and 4 females. Their ages had a mean of $56.7 \pm 8.3$ years.

- **c) Subgroup 1c: Three Vessel Disease Group (n=17):**
  
  This group included seventeen\textsuperscript{(17)} patients with three-vessel disease. They were 12 males and 5 females. Their ages had a mean of $60.2 \pm 11.9$ years.

**Control group2 (n=20):**

This group included twenty (20) age and sex matched patients with normal unoccluded coronary arteries as revealed by coronary angiography. They served as the control group.

Patients with histories of malignant neoplasm, recent myocardial infarction, unstable angina, major trauma or surgery, renal insufficiency, acute or chronic infectious disease, or any kind of immune-mediated disease were excluded from this study.

After an informed consent, all individuals in this study were subjected to the following:

- a. Detailed history taking.
- b. Thorough clinical examination.
- c. Coronary angiography.
- d. Laboratory investigations including:
  - Lipid profile including: Total cholesterol, LDL-C, HDL-C and triglyceride (TG).
  - Serum creatinine.
  - Fasting serum glucose.
  - Serum lipocalin-2 assay using ELISA.

**Sampling:**

Five milliliters of venous blood were collected under complete aseptic precautions after 12 hours fasting from each of controls and patients in plain test tubes without anticoagulant. After coagulation, samples were centrifuged at 2000-3000 rpm for 20 minutes. The separated serum was divided into two aliquots. One aliquot was used for the immediate assay of total cholesterol, TG, HDL-C, LDL-C, creatinine and fasting serum glucose. The second aliquot was stored at -20°C for the assay of lipocalin-2. Hemolysed samples were discarded. Repeated freezing and thawing was avoided.

**C) Methods:**

1) **Analytical Methods:**

- **a) Routine investigations** including: total cholesterol, LDL-C, HDL-C, TG, serum creatinine and fasting serum glucose were assayed on Synchro CX9 system auto-analyzer (Beckman synchro CX-systems, 2500 Harbor Boulevard,
Lipocalin-2: A Biomarker of CAD Severity

P.O.Box 3100, Fullerton, California 92634-3100, USA.

b) Serum Lipocalin-2 assay: Serum samples taken from all subjects included in this study were assayed for lipocalin-2 by sandwich enzyme-linked immunosorbent assay (ELISA) technique using reagents provided by Quantikine R&D International, Inc. (R&D International, Inc., 614 McKinley Place N.E., Minneapolis, MN 55413 USA). Following the manufacturer instructions, standards and samples were pipetted into wells that were pre-coated with a monoclonal antibody specific for lipocalin-2, thus allowed lipocalin-2 present in samples to be bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for lipocalin-2 was added to the wells. Following a wash step to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color developed which was directly proportional the amount of lipocalin-2 bound in the initial step. The color development was stopped by stopping solution and the intensity of the color was measured at 450 nm on ELISA reader.

c) Calculation of results. To deduce the concentration of lipocalin-2 in the serum samples and control material, a standard curve using logarithmic scale was constructed by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis, the best fit curve was drawn through the points on the graph. Zero standard absorbance was subtracted from all recorded absorbances. The concentration of the samples was read directly from this standard curve by using their average optical density.

2) Statistical Methods. IBM SPSS statistics (V. 22.0, IBM Corp., USA, 2013) was used for data analysis. Data were expressed as mean±SD for quantitative parametric data. In addition, median and interquartile range (Q1-Q3) were used for quantitative non-parametric data. Multiple-group comparisons were done using analysis of variance (ANOVA) for parametric data and Kruskal – Wallis test for non-parametric data. Meanwhile, two group comparisons were done using Wilcoxon’s rank sum test for non-parametric data and Student t test for parametric data. Spearman’s Rank correlation coefficient (rs) was used to assess the correlation between two sets of variables if one or both of them had a skewed distribution. P value < 0.05 was considered significant, whereas P < 0.01 was considered highly significant.

RESULTS

The results of the present study are shown in tables 1-7 and figure 1-2.

Table 1: Descriptive statistics of the different studied parameters in the whole patient group and the control group.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group (n=60)</th>
<th>Control group (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>195±31*</td>
<td>178±34*</td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>0.94±0.16*</td>
<td>0.89±0.16*</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>Median 115</td>
<td>Median 98</td>
</tr>
<tr>
<td></td>
<td>Q1-Q3** 101-144</td>
<td>Q1-Q3** 61-175</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>37</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>28-45</td>
<td>33-52</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>137</td>
<td>152</td>
</tr>
<tr>
<td></td>
<td>128-172</td>
<td>134-175</td>
</tr>
<tr>
<td>Fasting serum glucose (mg/dL)</td>
<td>88</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>78-118</td>
<td>76-92</td>
</tr>
<tr>
<td>Lipocalin-2 (ng/mL)</td>
<td>15</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>10-20</td>
<td>0.3-0.5</td>
</tr>
</tbody>
</table>
* =± SD
Q1-Q3**=Interquartile range
Table 2: Descriptive statistics of the different studied parameters in the different studied subgroups.

<table>
<thead>
<tr>
<th>Laboratory investigations</th>
<th>CAD patient group (n=60)</th>
<th>1 Vessel disease (n=19)</th>
<th>2 Vessel disease (n=24)</th>
<th>3 Vessel disease (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol (mg/dL)</td>
<td>Median/ Mean*</td>
<td>Q1-Q3 /SD**</td>
<td>Median/ Mean*</td>
<td>Q1-Q3 /SD**</td>
</tr>
<tr>
<td>197*</td>
<td>25*</td>
<td>190*</td>
<td>25*</td>
<td>198*</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>119</td>
<td>112-147</td>
<td>112</td>
<td>86-132</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>40</td>
<td>25-46</td>
<td>38</td>
<td>31-44</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>138</td>
<td>128-164</td>
<td>137</td>
<td>132-185</td>
</tr>
<tr>
<td>Lipocalin-2 (ng/mL)</td>
<td>10</td>
<td>6.5-11</td>
<td>15</td>
<td>12.5-18.5</td>
</tr>
</tbody>
</table>

Q1-Q3 = Interquartile range. SD** = standard deviation.

Table 3: Statistical comparison between the whole patient group and the control group regarding the different studied parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>t*/Z</th>
<th>P</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-Chol (mg/dL)</td>
<td>2.1*</td>
<td>&lt;0.05</td>
<td>S</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>2.3</td>
<td>&lt;0.05</td>
<td>S</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>2.2</td>
<td>&lt;0.05</td>
<td>S</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>1.1</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Lipocalin-2 (ng/mL)</td>
<td>6.7</td>
<td>&lt;0.01</td>
<td>HS</td>
</tr>
</tbody>
</table>

t* = Student t Test. Z = Wilcoxon’s Rank Sum Test. NS: Non-significant difference. S: Significant difference. HS: Highly significant difference.

Table 4: Wilcoxon’s Rank Sum test for the statistical comparison of serum level of lipocalin-2 in the different studied groups.

<table>
<thead>
<tr>
<th>Lipocalin-2</th>
<th>Z</th>
<th>P</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 vessel disease / control</td>
<td>5.4</td>
<td>&lt;0.01</td>
<td>HS</td>
</tr>
<tr>
<td>2 vessel disease / control</td>
<td>5.7</td>
<td>&lt;0.01</td>
<td>HS</td>
</tr>
<tr>
<td>3 vessel disease / control</td>
<td>5.2</td>
<td>&lt;0.01</td>
<td>HS</td>
</tr>
<tr>
<td>1 vessel disease / 2 vessel disease</td>
<td>4.3</td>
<td>&lt;0.01</td>
<td>HS</td>
</tr>
<tr>
<td>2 vessel disease / 3 vessel disease</td>
<td>2.8</td>
<td>&lt;0.01</td>
<td>HS</td>
</tr>
<tr>
<td>1 vessel disease / 3 vessel disease</td>
<td>4.9</td>
<td>&lt;0.01</td>
<td>HS</td>
</tr>
</tbody>
</table>

HS: Highly significant difference.
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Table 5: Statistical comparison of all studied parameters in the different patient subgroups using Kruskall Wallis test and ANOVA:

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
<th>H ratio/F ratio</th>
<th>P</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-Chol (mg/dL)</td>
<td>1 vessel disease</td>
<td>1.7*</td>
<td>&gt; 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>2 vessel disease</td>
<td>2.7</td>
<td>&gt; 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>3 vessel disease</td>
<td>3.8</td>
<td>&gt; 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td></td>
<td>0.09</td>
<td>&gt; 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Lipocalin-2 (ng/mL)</td>
<td></td>
<td>32</td>
<td>&lt; 0.01</td>
<td>HS</td>
</tr>
</tbody>
</table>


Table 6: Spearman’s Rank correlation between lipocalin-2 and other studied parameters in the whole patient group:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>rs</th>
<th>P</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-Chol (mg/dL)</td>
<td>0.1</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>0.08</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>-0.03</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>0.001</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS: Non-significant correlation.

Table 7: Spearman’s Rank correlation between lipocalin-2 and gensini score among the whole patient group:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>rs</th>
<th>P</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gensini Score</td>
<td>0.5</td>
<td>&lt;0.01</td>
<td>HS</td>
</tr>
</tbody>
</table>

HS: Highly significant correlation.

Figure (1): Bar chart showing median levels of serum lipocalin-2 in different studied groups and the control group.
DISCUSSION

Results of our study confirmed the presence of a significant statistical difference between serum levels of total cholesterol, HDL-C and LDL-C in the studied CAD patients compared to the control group. These go in agreement with results of Jie Ni et al., who assured that low levels of HDL-C and elevated LDL-C levels contribute strongly to atherosclerotic CAD development and that disruption of lipid metabolism is involved in the mechanism of atherosclerosis. On the other hand, a non significant difference was found between atherosclerotic CAD patients and the control group regarding TG levels. These findings are consistent with Yang et al., who didn’t observe a significant difference in TG serum levels between atherosclerotic CAD patients and control group\(^7,18\).

Results of this study highlighted the possibility of lipocalin-2 being a promising marker for the assessment of CAD. A highly significant statistical rise in lipocalin-2 levels was observed among studied atherosclerotic CAD patients when compared to the control group. Similar results were obtained by Nikolaos et al.,\(^11\) who measured serum lipocalin-2 levels in 140 CAD patients with atherosclerosis undergoing coronary angiography compared to 20 healthy control. Higher levels of serum lipocalin-2 were found in their atherosclerotic CAD patients compared to their healthy controls. Also Jie Ni et al.,\(^7\) assayed serum lipocalin-2 levels in 261 CAD patients undergoing coronary angiography. Their results assured that increased serum lipocalin-2 levels are associated with the presence of atherosclerotic CAD. The underlying mechanism of connection between circulating lipocalin-2 and atherosclerosis was supported by earlier studies done by Roudkenar et al.\(^14\) who claimed that lipocalin-2 over expression has been detected in atherosclerotic plaques and damaged myocardium. He added that atherosclerosis, hypoxia and myocardial infarction induce lipocalin-2 mRNA expression with accompanying increase in lipocalin-2 serum levels.

In 2011, Andrew et al. demonstrated induction of lipocalin-2 expression by an acute vascular inflammatory regulator NF-kB in response to angioplastic injury. Their data suggested that lipocalin-2 is upregulated in chronic inflammation associated with atherosclerosis\(^11\).

A different explanation for the potential role of lipocalin-2 in the pathogenesis of CAD was given by Houard et al. who postulated that lipocalin-2 has been shown to form a complex with MMP-9, protecting the latter from degradation and thereby preserving MMP-9 enzymatic activity which has been implicated in vascular remodeling, atherogenesis and plaque rupture\(^6\).

Nickolas et al. and Jie Ni et al. assured that increased serum lipocalin-2 levels are associated with the presence of atherosclerosis. They added that biomarkers of neutrophil activation as lipocalin-2 could be of prognostic and even diagnostic importance as it reflects the extent of neutrophil activation in various stages of ACS\(^7,12\).

Interestingly, in this study lipocalin-2 serum levels showed a significant statistical rise with increased number of affected coronaries assessed by gensini score. The association between lipocalin-2 and disease severity was further emphasized by the assessment of the correlation
study between lipocalin-2 serum levels and gensini score. Our results revealed a highly significant positive correlation between lipocalin-2 and gensini score. Similar correlations were previously assessed by Choi et al.\(^{(3)}\) and Theodoros et al.\(^{(16)}\) who measured serum lipocalin-2 levels in 140, 284 patients with angiographically confirmed CAD patients and stated that serum lipocalin-2 levels were increased with higher gensini score. These findings add more value to the role of lipocalin-2 in assessment of atherosclerotic CAD patients at different stages of disease severity.

On performing Kruskall Wallis test between the different studied patient subgroups and the different studied analytes, lipocalin-2 had the highest H value indicating that it is an excellent parameter to discriminate between patients with one, two and three vessel obstruction disease. Similar results were obtained by Nikolaos et al.\(^{(11)}\) who observed that lipocalin-2 is more sensitive to differentiate between CAD patients.

In conclusion, our study highlights the promising future role of lipocalin-2 as a noninvasive serum marker for the assessment of atherosclerotic CAD disease severity based on the results of this study and supported by previously performed studies by other authors.

REFERENCES


اليبوكالين: كدليل حيوي لمرض الشريان التاجي والارتباط بينه وبين شدة المرض

أريج علي سيف - أحمد محمد المحمودي - دعاء مصطفى عوض

يتمثل تصلب الشرايين وما يلحق به من تكون الجلطة الشريانية على اللويحة العضدية المتمزقة الآليه المركزة المسؤوله عن حدوث أمراض الشريان التاجي. وقد أثبتت الأدلة العملية والسريريه أن تصلب الشرايين ليس مجرد مرض ترسب الدهون فقط وإنما يلعب الالتهاب العام دوراً مهمًا في تنمية وتطور هذا التصلب وتوجد أداة كثيرة تدعم مشاركة الخلايا العضدية في هذه العملية الالتهابيه.

هدفت هذه الدراسة إلى قياس مستوي الليبوكالين في مرضى الشريان التاجي وتحقيق مراقبه للثقب مستوي الليبوكالين.

وقد استوجب هذا دراسة مستويات الليبوكالين في المصل في (60) من مرضى الشريان التاجي، ووفقا لنتائج تصوير الأوعية التاجية تم تصنيف المرضى طبقاً لعدد الشرايين المصابة إلى 3 مجموعات فرعية.

19 من المرضى الذين يعانون من المرض في وعاء واحد، 24 من المرضى الذين يعانون من المرض في وعاء واحد ووعاء واحد، 17 من المرضى الذين يعانون من مرض ثلاثة أوعية.

وقد اشتملت المجموعة الضابطة على 20 من المرضى الذين يعانون من مرض ثلاثة أوعية سليمة، وتم استخدام الطرق الانزيمية المناعية لقياس مستوي الليبوكالين.

كانت نتائج دراسة على ارتفاع مستوي الليبوكالين في المصل بشكل ملحوظ للغاية في مرضى الشريان التاجي عند مقارنتهم بالمجموعة الضابطة ومستويات الليبوكالين. المرتفعة ترجع إلى حقيقة أن الالتهاب والخلايا العضدية تلعب دوراً محوريا في تصلب الشرايين ومزيف اللويحة العضدية. كما أن وجود في الدراسة الفعلية ارتفاع مستوي الليبوكالين بين الأوعية التاجية لعب دوراً محوريا في تصلب الشريان، وتم تعريف الصلة بين الليبوكالين والخلايا العضدية في مرضى الشريان التاجي، وتم تأكيد هذه النتائج بوجود علاقة إيجابية بين الليبوكالين والخلايا العضدية.

وقد كشفت الدراسة عن ارتفاع نسبة الليبوكالين في مرضى الشريان التاجي مقارنة بالمجموعة الضابطة، مما يدعم نظرية أن مستويات أعلى من الليبوكالين تعكس درجة أعلى من الالتهاب في مرضى الشريان التاجي الشديد.

ومن أجل هذا، نجحت الدراسة في معرفة العلاقة بين الليبوكالين والخلايا العضدية في مرضى الشريان التاجي، وتم تأكيد هذه النتائج بوجود علاقة إيجابية بين الليبوكالين والخلايا العضدية.

وقد أظهرت الدراسة النتائج الإيجابية التي تدعم نظرية نشاط الليبوكالين كدليل حيوي لمرض الشريان التاجي، وتشير بيانات البحث أيضاً أن الليبوكالين يلعب دوراً محورياً في تطور مرض الشريان التاجي، وتشير البيانات أيضاً أن الليبوكالين يلعب دوراً محورياً في تطور مرض الشريان التاجي.

وقبل أن تكون نتائج هذا البحث، يشير النتائج الإيجابية إلى أن الليبوكالين يلعب دوراً محورياً في تطور مرض الشريان التاجي، وتشير النتائج الإيجابية إلى أن الليبوكالين يلعب دوراً محورياً في تطور مرض الشريان التاجي. وتشير نتائج الدراسة إلى أن الليبوكالين يلعب دوراً محورياً في تطور مرض الشريان التاجي.
DISCRIMINATION BETWEEN BACTEREMIC AND COMMENSAL ISOLATES OF COAGULASE NEGATIVE STAPHYLOCOCCI: ROLE OF IcaA AND IcaD GENES
Hala Mahmoud Hafez and Marwa Abd El-Rasoul El-Ashry

ABSTRACT

Coagulase negative staphylococci (CoNS) are the most commonly isolated contaminants from blood cultures. However, they are the most frequent cause of true bacteremia particularly in patients with compromised host defenses who have implanted medical devices. The ability to differentiate between pathogenic and commensal isolates of CoNS has practical importance because of its therapeutic implications in terms of unnecessary use of antibiotics and emergence of resistance. Furthermore, failure to recognize and treat cases of true bacteremia due to CoNS can lead to higher rates of morbidity and mortality. Objective: The aim of the present study was to evaluate the role of icaA and icaD genes as pathogenic determinants for bacteremic isolates of CoNS. Also, to determine the relationship between the presence of icaA and icaD genes, in bacteremic isolates of CoNS, and their ability to produce slime when subcultured on Congo red agar. Methods: The study included 50 isolates of CoNS (35 from bacteremic patients and 15 from healthy controls). All isolates were subcultured on Congo red agar to detect slime production. Moreover, detection of icaA and icaD genes was carried out, on all studied isolates, using Syber green real-time PCR. Results: 88.6% of CoNS isolates recovered from bacteremic patients and 100% of the control strains grew black colonies on Congo red agar denoting positive slime production with no statistically significant difference detected between both groups (P>0.05). There was a highly significant difference between the pathogenic and control isolates regarding the detection of the icaA gene (P<0.01). The icaA gene was detected in 100% and 66.6% of bacteremic isolates and control isolates, respectively. Also, a highly significant difference was observed between the pathogenic and control isolates regarding the detection of the icaD gene (P<0.01). The icaD gene was detected in 100% and 13.3% of the pathogenic and control groups, respectively. Both icaA and icaD genes were co-detected in all of the pathogenic isolates (100%) and in only 6.7% of the control isolates. A highly significant difference was observed between both groups regarding the co-expression of icaA and icaD genes (P<0.01). There was a non-significant association between the slime production on Congo red agar and the detection of both icaA and icaD genes (P>0.05). Conclusion: Concomitant expression of both icaA and icaD genes can significantly discriminate between pathogenic and commensal isolates of CoNS. No association was observed between the presence of both icaA and icaD genes and the ability of the pathogenic isolates to produce slime on Congo red agar.

INTRODUCTION

Coagulase-Negative Staphylococci (CoNS) are widely distributed over the surface of the human body, where they constitute the majority of the commensal bacterial microflora(19). Although they were formerly regarded as contaminants of clinical specimens, they have been increasingly recognized as nosocomial pathogens in patients whose defenses are compromised by an implanted foreign body or by the administration of immunosuppressive drug(25).

Infections caused by CoNS are often persistent and relapsing. CoNS are responsible for the majority of device-related infections. They are responsible for 50-70% of catheter-related blood stream infections, 40-50% of prosthetic cardiac valve infections, and 20-50% of joint replacement infections(19). In addition, CoNS have been isolated with increasing frequency as the causative pathogens of nosocomial sepsis, and account for approximately 30% of all nosocomial blood stream infections(23). The prevalence of methicillin resistance among CoNS and the emergence of vancomycin resistance among these isolates constitute a major threat to the clinician for the management of these infections(22).

The differentiation between pathogenic and commensal isolates of CoNS has practical importance because of its therapeutic implications in terms of unnecessary use of antibiotics and emergence of resistance. Also, failure to treat true bacteremia can lead to higher rates of morbidity and mortality especially in the critically ill or immunocompromised patients(12).

Several indicators have been investigated in order to differentiate true bacteremia from...
Coagulase-Negative Staphylococci strains associated with disease were found to produce a wider range of extracellular toxins and enzymes as compared to commensal strains. More importantly, they produce an extracellular polysaccharide (slime) and have the ability to form biofilm on polymeric surfaces to which they adhere and colonizes. This is believed to facilitate the establishment of infection on the surfaces of implanted foreign bodies\(^{(9,13,21)}\).

Synthesis of the extracellular polysaccharide is mediated by the ica operon. Upon activation of this operon, a polysaccharide intercellular adhesin (PIA) is synthesized. This supports cell-to-cell bacterial contacts by means of a multilayered biofilm. The synthesis of the PIA is encoded by the intercellular adhesion (ica) locus, in particular by the icaA gene\(^{(24)}\). However, the sole expression of icaA induces only low enzymatic activity, but co-expression of icaA with icaD leads to a significant increase in activity and is related to phenotypic expression of the capsular polysaccharide\(^{(2,29)}\).

**Aim of the Work:**

The aim of the present work was to evaluate the hypothesis that bacteremic isolates of CoNS are more likely to be positive for icaA and icaD genes as compared to non-pathogenic (commensal) CoNS isolates. Also, to determine the relationship between the presence of the icaA and icaD genes, in bacteremic isolates of CoNS, and the ability of these isolates to produce slime when subcultured on Congo red agar.

**MATERIALS AND METHODS**

**Methods:**

The present study was carried out at the main Microbiology Laboratory, Clinical and Chemical Pathology Department, Faculty of Medicine, Ain Shams University over the period between May 2014 and November 2014.

The study included 50 isolates of CoNS that were divided to two groups as follows:

1. **Potentially pathogenic group:** included 35 isolates that were recovered from blood cultures of bacteremic patients (single isolate/patient). Selected isolates met the following inclusion criteria:
   
i) Isolates recovered in pure growth from two or more positive blood cultures obtained within the same collection day, or
   
ii) Isolates recovered in pure growth within 48 hours from blood collection, or
   
iii) Isolates recovered from immunocompromised patients with clinical signs and symptoms of bacteremia.

On the other hand, isolates growing after >48 hours from blood collection, or isolates from mixed cultures were excluded from the study.

2. **Control group:** included 15 isolates of CoNS that were recovered from skin swabs obtained from apparently healthy individuals.

Selected isolates were subcultured on blood agar and incubated overnight at 36 ±1 °C under aerobic conditions in order to obtain pure and separate colonies. Growing colonies had their identity confirmed using Gram stain, catalase test, oxidase test, Bacitracin (0.04U) sensitivity test, and DNase test.

**The selected isolates of CoNS were subjected to:**

**A. Subculture on Congo red agar to detect slime production:**

Fresh isolates obtained from subculture on blood agar were subcultured onto the Congo red agar and incubated at 36 ±1°C under aerobic conditions. A positive result was indicated by the appearance of black colonies with a dry crystalline consistency. Non-slime producers usually remained pink, though occasional darkening at the center of the colonies was observed and this gave a bull’s eye appearance. An indeterminate result was indicated by a darkening of the colonies but with the absence of a dry crystalline colonial morphology.

**B. Detection of icaA and icaD genes using Syber-Green Real Time PCR:**
Bacterial DNA was extracted using the Thermo Scientific Gene JET DNA Purification Kit K0721 (Sigma) in accordance to manufacturer’s instructions.

DNA amplification and detection was done using Maxima SYBR Green qPCR Master Mix (2X) (Thermo Scientific, EU). The primers for amplification of icaA and icaD genes were designed in accordance to Arciola (2)(Table 1).

Table (1): Primers for the detection of icaA and icaD genes

<table>
<thead>
<tr>
<th>icaAgene</th>
<th>Reverse</th>
<th>188bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>5-ACACCTTGCTGGCGCAGTCAA-3</td>
<td></td>
</tr>
<tr>
<td>Reverse</td>
<td>5-TCTGGAACCAACATCCAACA-3</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>icaDgene</th>
<th>Reverse</th>
<th>198bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>5-ATGGGCAAGCCCAGACAGAG-3</td>
<td></td>
</tr>
<tr>
<td>Reverse</td>
<td>5-AGTATTTTCAATGTTTAAAGCAA-3</td>
<td></td>
</tr>
</tbody>
</table>

The thermal cycler was programmed according to recommendations by Alper et al. (1). Initial denaturation at 95°C for 10 min (1 cycle), denaturation at 95°C for 15 sec (40 cycles), annealing at 60°C for 30 sec (40 cycles) and extension at 72°C for 30 sec (40 cycles). The amplification program was followed immediately by a melt program consisting of 1 minute at 95°C, 30 sec at 55°C then again for 30 sec at 95°C. Results were interpreted according to Muldrew (17) where the greenish horizontal line in the graph (Figure 1) is the threshold line at which the fluorescence begins to be detected. The point at which the amplification plot crosses the threshold is the cycle threshold (Ct).

Figure (1): Results of Syber Green real-time PCR in amplification plot with cycle’s number on x axis and fluorescence on y axis.
Statistical Analysis:

Data was analyzed using IBM SPSS statistics (V. 23.0, IBM Corp., USA, 2015). Categorical data were described by the number and percentage. Chi-square test was used to study the association between each 2 variables or comparison between 2 independent groups as regards the categorized data. The probability of error at 0.05 or more was considered non-significant, while at 0.01 and 0.001 are highly significant.

RESULTS

Using Congo red agar, 88.6% (31/35) of the CoNS isolates recovered from bacteremic patients grew black colonies on the agar medium denoting positive slime production, whereas 11.4% (4/35) isolates grew red colonies denoting a negative slime production (Figure 2). All of the control isolates of CoNS (15/15, 100%) grew black colonies on the Congo red agar medium denoting positive slime production. There was no statistically significant difference between the pathogenic group and the control group as regards the growth appearance on Congo red agar (P > 0.05) (Table 2).

Concerning the detection of icaA gene by Real Time PCR, all pathogenic isolates (35/35, 100%) were icaA gene positive. Among the control isolates, 10 cases (10/15, 66.6%) were positive and 5 cases (5/15, 33.3%) were icaA gene negative. Comparison between the pathogenic isolates and the control isolates regarding the presence of icaA gene revealed a highly significant difference (P < 0.01) (Table 3).

As regards icaD gene, the gene was detected in all of the 35 pathogenic isolates (100%). Yet among the control isolates, 2 (2/15, 13.3%) were positive for the icaD gene and 13 (13/15, 86.7%) were negative. Comparison between the pathogenic isolates and control isolates regarding the presence of icaD gene revealed a highly significant difference (P < 0.01) (Table 4).

Both icaA and icaD genes were co-detected in the pathogenic isolates (35/35, 100%). Among control isolates, both genes were detected in only one isolate (1/15, 6.7%). Comparison between the pathogenic and control isolates regarding the co-detection of both icaA and icaD genes showed a highly significant difference (P < 0.01) (Table 5).

A non-significant association was observed between the results of culture on Congo red agar and the detection of both icaA and icaD genes (P > 0.05) (Table 6). Out of the 50 studied isolates, 32 (32/50, 64%) were positive by both tests, 14 (14/50, 28%) were positive by Congo red only and 4 (4/50, 8%) were positive by PCR only.

Table (7) shows the diagnostic performance of the different investigations done on the 50 selected CoNS isolates. From the present study, it was found that slime production on CRA had the lowest specificity (0%), whereas the co-expression of both genes had the highest specificity (93.3%) in differentiating between bacteremic and commensal isolates of CoNS. The sensitivity of CRA was 88.6%, whereas detection of icaA genes, icaD genes and co-expression of both genes had a sensitivity of 100%, 100%, and 100%; respectively.

Table (2): Comparison between the control group and the pathogenic group regarding the appearance on Congo red agar.

<table>
<thead>
<tr>
<th>Growth on Congo red agar</th>
<th>Control group</th>
<th>Pathogenic group</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative (red colonies)</td>
<td>Count 0</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>%</td>
<td>0.0%</td>
<td>11.4%</td>
<td>8.0%</td>
</tr>
<tr>
<td>Positive (black colonies)</td>
<td>Count 15</td>
<td>31</td>
<td>46</td>
</tr>
<tr>
<td>%</td>
<td>100.0%</td>
<td>88.6%</td>
<td>92.0%</td>
</tr>
<tr>
<td>Total</td>
<td>Count 15</td>
<td>35</td>
<td>50</td>
</tr>
<tr>
<td>%</td>
<td>100.0%</td>
<td>100.0%</td>
<td>100.0%</td>
</tr>
<tr>
<td>Pearson Chi-Square</td>
<td>1.863</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>0.172 (NS*)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*NS=non-significant.
### Table (3): Comparison between the control group and the pathogenic group regarding the detection of icaA gene by real-time PCR

<table>
<thead>
<tr>
<th>icaA gene</th>
<th>Control group</th>
<th>Pathogenic group</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Count</td>
<td>%</td>
<td>Count</td>
</tr>
<tr>
<td>Negative</td>
<td>5</td>
<td>33.3%</td>
<td>0</td>
</tr>
<tr>
<td>Positive</td>
<td>10</td>
<td>66.7%</td>
<td>35</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>100.0%</td>
<td>35</td>
</tr>
</tbody>
</table>

**P value** .000 (S)*

*S=significant.

### Table (4): Comparison between the control group and the pathogenic group regarding the detection of icaD gene by real-time PCR

<table>
<thead>
<tr>
<th>icaD gene</th>
<th>Control group</th>
<th>Pathogenic group</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Count</td>
<td>%</td>
<td>Count</td>
</tr>
<tr>
<td>Negative</td>
<td>13</td>
<td>86.7%</td>
<td>0</td>
</tr>
<tr>
<td>Positive</td>
<td>2</td>
<td>13.3%</td>
<td>35</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>100.0%</td>
<td>35</td>
</tr>
</tbody>
</table>

**P value** .000 (S)*

*S=significant.

### Table (5): Comparison between the control group and the pathogenic group regarding the co-detection of icaA and icaD genes by real-time PCR

<table>
<thead>
<tr>
<th>icaA and icaD genes</th>
<th>Control group</th>
<th>Pathogenic group</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Count</td>
<td>%</td>
<td>Count</td>
</tr>
<tr>
<td>Negative</td>
<td>14</td>
<td>93.3%</td>
<td>0</td>
</tr>
<tr>
<td>Positive</td>
<td>1</td>
<td>6.7%</td>
<td>35</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>100.0%</td>
<td>35</td>
</tr>
</tbody>
</table>

**P value** .000 (S)*

*S=significant.

### Table (6): Association between the results of culture on Congo red agar and combined icaA and D gene detection in both pathogenic and control groups.

<table>
<thead>
<tr>
<th>PCR icaA/icaD</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>Congo_red</td>
<td>Count</td>
</tr>
<tr>
<td>negative</td>
<td>0</td>
</tr>
<tr>
<td>positive</td>
<td>14</td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
</tr>
</tbody>
</table>

**Pearson Chi-Square** 1.691

**P value** 0.193 (NS)*

*NS=non-significant.
Table (7): Diagnostic performance:

<table>
<thead>
<tr>
<th>Item</th>
<th>TN</th>
<th>FN</th>
<th>FP</th>
<th>TP</th>
<th>SP</th>
<th>SN</th>
<th>P-</th>
<th>P+</th>
<th>Eff</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congo Red</td>
<td>0</td>
<td>4</td>
<td>15</td>
<td>31</td>
<td>0.0</td>
<td>88.6</td>
<td>0.0</td>
<td>67.4</td>
<td>62.0</td>
</tr>
<tr>
<td>ica A</td>
<td>5</td>
<td>0</td>
<td>10</td>
<td>35</td>
<td>33.3</td>
<td>100.0</td>
<td>100.0</td>
<td>77.8</td>
<td>80.0</td>
</tr>
<tr>
<td>ica D</td>
<td>13</td>
<td>0</td>
<td>2</td>
<td>35</td>
<td>86.7</td>
<td>100.0</td>
<td>100.0</td>
<td>94.6</td>
<td>96.0</td>
</tr>
<tr>
<td>ica A &amp; D</td>
<td>14</td>
<td>0</td>
<td>1</td>
<td>35</td>
<td>93.3</td>
<td>100.0</td>
<td>100.0</td>
<td>97.2</td>
<td>98.0</td>
</tr>
</tbody>
</table>

TN: true negative, FN: false negative, FP: false positive, TP: true positive, SP: specificity, SN: sensitivity, P-: predictive negative, P+: predictive positive, Eff: efficacy.

Figure (2): Congo red agar showing an isolate of CoNS(a: positive slime production, b: negative slime production).

**DISCUSSION**

In the present work, using Congo red agar, 88.6% of the CoNS isolates recovered from bacteremic patients (pathogenic isolates) showed positive slime production. This percent goes in accordance with those previously reported in the literature, in which the percent of slime producing strains of pathogenic CoNS ranged from 31-89% (4,8,15,30). However, lower percentage was reported by Zhou et al. (2013) who found that only 16% of their studied pathogenic CoNS were slime producers on Congo red agar(29). Bacteremic isolates that were negative slime-producing constituted 11.4% in the present study. Similarly, Liduma and his coworkers found that in about 50% of their studied clinical isolates the production of extracellular slime was not registered(15). Also, Zhou and his colleagues (29) found that some CoNS isolated from blood had reduced slime production and this could be explained by two hypotheses:1. there are many differences in nutrients, pH, oxygen radicals, osmotic pressure and antibiotics between in vivo and in vitro environmental conditions which may confer a positive selective pressure on slime production; and 2. certain genes such as icaA or icaD may be lost or mutated.

All of the control isolates of CoNS, in the present work, showed positive slime production. There was no statistically significant difference between the pathogenic isolates and the control isolates regarding the growth appearance on Congo red agar. This finding agreed with the study carried out in Pakistan by Hassan et al., who found that Congo red method has no significance to differentiate between pathogenic and non-pathogenic groups when compared with tissue culture plate method(13). Also, Oliveira and Cunha showed no difference in the frequency of biofilm production between isolates obtained...
Bacterimic or Commensals CoNS: Role of IcaA and IcaD

from clinical samples and those obtained from the nares of healthy subjects\(^\text{20}\). On the other hand, Liduma et al. found a statistically significant difference between the clinical isolates and the control isolates regarding the production of extracellular slime\(^\text{15}\). This was further confirmed by the study of Zhou and co-workers who found a statistically significant difference in slime production between the clinical isolates recovered from the patient catheter blood specimens and those isolates obtained from the healthy volunteers\(^\text{29}\).

In the current work, there was a highly significant difference between the pathogenic isolates and the control isolates regarding the presence of icaA gene. This is in accordance with the previous results of Liduma and colleagues who observed that 92% of their studied clinical biofilm-producing strains and 43% of their commensal (control) strains were positive for the icaA gene\(^\text{15}\). Also, Tektook and his colleagues were able to detect icaA gene in 34% of their studied bacteremic isolates of CoNS and in only 2% of their studied control isolates\(^\text{27}\). Yet, Darwish and Asfour detected the icaA gene in only 5.9% of CoNS isolated from bovine mastitis\(^\text{6}\).

Also, the present study was also able to demonstrate a highly significant difference between the bacteremic isolates of CoNS and the control isolates regarding the expression of icaD gene. Similar findings were also reported by Tektook and his colleagues who found that icaD gene was positive in 38% of their studied bacteremic CoNS isolates and in 4% of their control isolates\(^\text{27}\). However, Klingenberg and associates found no statistically significant difference between invasive isolates and contaminants regarding the icaD gene carriage\(^\text{14}\).

All of our studied bacteremic isolates (100%) had concomitant expression of icaA and icaD genes. Yet, among the control isolates, both genes were co-detected in only one strain (6.7%). Comparison between the pathogenic and control isolates regarding the co-expression of both icaA and icaD genes showed a highly significant difference. This is in agreement with previous studies which reported that the co-expression of icaA and icaD is necessary for slime production\(^\text{5,29}\). Also, Arciola et al. reported that all Staph. epidermidis biofilm positive strains isolated from intravenous catheters were positive for both icaA and icaD genes\(^\text{3}\). Moreover, Gad et al. revealed that both icaA and icaD genes were either present or absent in pathogenic strains and no single strain had shown the presence of one gene. The authors concluded that their results confirm the fact that both genes are part of one operon and so the entire operon was either present or absent\(^\text{29}\).

In the current work, there was a non-significant association between the slime production on Congo red agar and the detection of both icaA and icaD genes. Out of the 50 studied isolates, 32 (64%) were positive by both CRA and PCR, 14 (28%) were positive by Congo red only and 4 (8%) were positive by PCR only. Similarly, other studies demonstrated that the presence of the ica genes did not always correlate with biofilm production\(^\text{7,18}\).

The sensitivity of CRA, in the present work, was found to be 88.6%, the specificity was 0%, the predictive ability to detect positive cases was 67.4%, the predictive value to detect negative cases was 0% and the efficacy was 62%. This was not in accordance with the study of Mathur and his co-workers who found that CRA had a sensitivity of 6.8%, 90.2% specificity, 40.9% efficacy, positive predictive value 66.6% and negative predictive value 25.3%\(^\text{16}\). Also, Oliveira and Cunha found that CRA method had 89% sensitivity and 100% specificity as compared to PCR recognizing the concomitant presence of the icaA and icaD genes\(^\text{20}\). In another study done by Hassan and his colleagues, the sensitivity of CRA was 11%, the specificity was 92% and the efficacy was 41%\(^\text{11}\).

The sensitivity of icaA gene detection, in the current work, was 100%, specificity was 33.3%, the predictive value to detect positive cases was 77.8%, the predictive value to detect negative cases was 100% and the efficacy was 80%. Vandecasteele and his colleagues reported that the sensitivity, specificity and positive predictive value of icaA gene detection for differentiating between colonizing isolates and skin isolates were 88%, 62% and 56%, respectively\(^\text{28}\). In the present study, the sensitivity of icaD
gene detection was 100%, specificity was 86.7%, the predictive value to detect positive cases was 94.6%, the predictive value to detect negative cases was 100.0% and the efficacy was 96.0%. Oliveira and Cunha reported that the sensitivity and specificity of icaD gene was 100% and 100%, respectively as compared to CRA and TCP methods.

In the current work, the co-expression of both icaA and icaD genes was found to have 100% sensitivity, 93.3% specificity, 97.2% PPV, 100% NPV and 98% efficacy. García and co-workers, found that the sensitivity and specificity of both icaA and icaD were 63% and 74%, respectively.

**Conclusion:**

From the present study, we concluded that concomitant expression of both icaA and icaD genes can significantly discriminate between pathogenic and commensal isolates of CoNS. Unfortunately, no association was observed between the co-expression of these genes and the ability of the bacteremic isolates to produce slime on Congo red agar. This finding confirms the fact that biofilm elaboration is a complex process in which different proteins play a role, with adhesion molecules, accumulation-associated proteins and factors responsible for cell-to-cell interactions among them.

**Recommendations:**

We do recommend further studies including larger number of isolates and evaluating the role of other CoNS virulence factors (AtlE gene, SarA family and agr quorum sensing system) in organism pathogenicity in true bacteremia.

**REFERENCES**


Bacterimic or Commensals CoNS: Role of IcaA and IcaD


ANTIBIOTIC RESISTANCE THREATS AMONG PSEUDOMONAS AERUGINOSA ISOLATES
Malaka Zakaria Amer and Marwa Abd El-Rasoul El-Ashry

ABSTRACT
Background: The prevalence of infections caused by multidrug resistant pseudomonas aeruginosa has steadily been increased. Pseudomonas aeruginosa producing AmpC β-lactamases (AmpCs) have become a major therapeutic challenge. Objectives: The aim of this study is to evaluate different methods to detect AmpC β-lactamase, to determine the prevalence of both inducible and derepressed expression of of AmpC β-lactamase among P. aeruginosa and to detect the antibiotic susceptibility pattern of Pseudomonas aeruginosa strains isolated to anti-pseudomonal antibiotics. Materials and methods: This study was conducted on 150 pseudomonas isolates collected from different clinical specimens referred to the Central Microbiology Laboratory of Ain Shams University Hospitals. All isolates had been subjected to the following tests: antibiotic susceptibility, Disk antagonism, Disk potentiation, AmpC disk and Modified three dimensional (MTDT). Results: The resistance pattern of the isolated Pseudomonas spp. to different antibiotics at our hospital was highest to Ceftazidime (44.7%), and lowest to Imipenem. AmpC induction screening assay showed that best inducer/substrate combination was Imp/Tzp with best sensitivity. 44% of isolates were shown to be inducible for AmpC and 36.4% of them were partially derepressed. Only 1.3% of isolates were non inducible AmpC producers. MTDT test showed 100% sensitivity and specificity for detection of derepressed AmpC β-lactamases. Specificity of AmpC disk test and Disk potentiation test was 98.4% & 92.7% respectively and sensitivity was 80.0% & 92.3% respectively. Conclusion: There was high prevalence of inducible AmpC β-lactamase production among pseudomonas aeruginosa isolates from Ain Shams University Hospitals. This warranted its detection as routine laboratory work for patients with serious infections to avoid therapeutic failure. MTDT test is the most efficient test for detection of derepressed AmpC β-lactamases.

INTRODUCTION
Pseudomonas aeruginosa(P.aeruginosa) has now clearly emerged as a major nosocomial pathogen in immunocompromised and debilitated patients, as well as in cystic fibrosis patients. It is a common cause of healthcare-associated infections (HAIs) including pneumonia, bloodstream infections, urinary tract infections, and surgical site infections(23). Multidrug resistant P. aeruginosa has emerged in Egypt in recent years and is mainly seen in HAIs(1). The selection of appropriate treatments for Paeruginosa infection is a major challenge due to high level resistance to antibiotics and is therefore associated with significant morbidity and mortality(9).

Pseudomonas aeruginosa is intrinsically resistant to many structurally unrelated antimicrobial agents because of the low permeability of its outer membrane, the constitutive expression of various efflux pumps with wide substrate specificity and the naturally occurring chromosomal AmpCβ-lactamase(13).

AmpC low basal level expression can be induced to significantly higher levels in the presence of certain β-lactamases Cefoxitin and imipenem which are strong inducers and stable for hydrolysis(10). Constitutive hyperproduction of AmpCβ-lactamase is the most frequent mechanism by which the wild type of P. aeruginosa gain resistance to the antibiotic that are naturally susceptible to them (carboxypenicillins, ceftazidime and aztreonam)(2).

Several genes are known to be involved in AmpC induction control. In pseudomonas, there are three AmpD homologues, AmpD, AmpDh2 and AmpDh3 co-ordinately repress AmpC expression and they are responsible for a stepwise AmpCupregulation mechanism. Consequently, several form of expression of AmpCare noted related to degree of chromosomal mutation(11).

In addition to plasmid mediated constitutive AmpC β-lactamase expression that had been reported, several form of chromosomal AmpC β-lactamase expression was detected: basal-level inducible expression (wild type), partially derepressed(either moderate-level hyperinducible expression with increased antipseudomonal β-lactam resistance or high-level hyperinducible
expression with high-level \( \beta \)-lactam resistance) and fully derepressed (non-inducible constitutive) \((10,11,12)\).

AmpC \( \beta \)-lactamases producing organisms are on rise and lead to therapeutic failure with third generation cephalosporins when given empirically or not tested in the laboratory for AmpC \( \beta \)-lactamases production\((9)\).

Phenotypic detection methods for detection of AmpC \( \beta \)-lactamases are simple and easy to implement in comparison to molecular methods which are expensive and not available in all clinical laboratories although highly sensitive and specific\((19)\).

Aim of the Work:

The aim of the present work was to evaluate different methods to detect AmpC \( \beta \)-lactamase, to determine the prevalence of both inducible expression and depressed expression of AmpC \( \beta \)-lactamase among \( P. \) aeruginosa isolated from different clinical samples from hospitalized patients in Ain Shams University Hospitals and to detect its’ antibiotic susceptibility pattern to anti-pseudomonal antibiotics.

MATERIALS AND METHODS

I. MATERIALS:

This study was conducted on a total number of 150 pseudomonas isolates collected from different clinical specimens referred to the Central Microbiology Laboratory of Ain Shams University Hospitals for routine culture and sensitivity from March to September 2013.

II. METHODS:

Collected isolates had been subjected to the following:\( P. \) aeruginosa organism identification (through Gram stain and Conventional biochemical tests), antibiotic susceptibility test, disk antagonism test as screening test for detection of inducible AmpC beta lactamases. Also, Disk potentiation test, modified three dimensional test and AmpC disk test for detection of derepressed AmpC beta lactamases.

A. Antibiotic Susceptibility Test:

Isolates were screened by disc diffusion method and interpreted according to zone of inhibition as listed by CLSI.\((5)\). Isolates were tested against Imipenem(10\( \mu \)g), Meropenem (10\( \mu \)g), Amikacin(30\( \mu \)g), Ciprofloxacin(5\( \mu \)g), Piperacillin/Tazobactum(100/10\( \mu \)g), Ceftazidime (30\( \mu \)g) and Cefepime (30\( \mu \)g).

B. Disk Antagonism Test:

Imipenem (inducer) was placed in the center of Mueller-Hinton agar (MHA) plates while ceftazidime, cefotaxime and piperacillin–tazobactam(substrate) were placed at a distance of 25 mm from center.

After incubation, zones of inhibition were measured on both the induced (adjacent to the inducer disk) and the un-induced side of the substrate disk from disk edge to zone edge. A test was considered positive if the zone of inhibition was reduced by \( \geq 2 \) mm on the induced side of the substrate disk; (Figure. 1)(7).

\( \textbf{Figure (1): } \) Pseudomonas isolate showing positive screening test.

C. AmpC Disk Test:

This test is based on use of Tris-EDTA to permeabilize a bacterial cell and release \( \beta \)-lactamases into the external environment. AmpC disks (i.e., filter paper disks containing Tris-EDTA were prepared by applying 20 \( \mu \)l of a 1:1 mixture of saline and 100\( \times \) Tris-EDTA to sterile filter paper disks (5mm), after disk became dry they were stored in refrigerator. A suspension of the cefoxitin susceptible strain of \( E. \) coli (American Type Culture Collection, ATCC 25922) was prepared by inoculation of organism in 1mL of sterile saline (suspension turbidity was adjusted to 0.5 McFarland turbidity standard), and then the \( E. \) coli suspension was poured on MHA. The plates were then incubated.
for 15 min. at 37°C for drying. The sterile AmpC discs were moistened with 20 µL of sterile saline and then several colonies of each of the tested organisms were applied. A 30 µg of cefoxitin disk was then placed almost touching the inoculated disk on the inoculated MHA. Plate was then inverted and incubated aerobically over night at 37°C.

Incubated plates were examined for either an indentation or a flattening of the zone of inhibition of cefoxitin, indicating AmpC production (positive result), or absence of a distortion, indicating negative result(Figure 2)(4).

**D. Potentiation Test with Boronic Acid:**

Boronic acids (BAs) were reported as reversible inhibitors of AmpCs. 5 µl of a 80g/l stock solution of 3-aminophenyl boronic acid was added to ceftazidime disk to have final concentration of BA on the disk of 400 µg. A test strain was inoculated on MHA plates. Discs containing ceftazidime 30 µg and ceftazidime plus 400 µg of BA were placed apart from each other on the MHA plates. The plates were incubated overnight at 37°C. An increase in the zone size of ≥ 5 mm for ceftazidime plus BA compared with that of the drug alone was considered a positive result, absence of that increase was considered a negative result; (Figure. 3)(20).

**E. Modified Three-Dimensional Test:**

Test was done as described by Coudron et al.(6). Briefly, fresh pseudomonas isolate was inoculated on peptone water in a micro centrifuge tube and centrifuged at 3000 rpm for 15 min. Crude enzyme extract was prepared by repeated freeze thawing in -80°C for seven times. A lawn culture of E.coli ATCC 25922 was prepared on MHA plates and cefoxitin (30 µg) discs are placed on the plate, by using a sterial scalpel blade, a slit beginning 5 mm from the edge of the disc was cut in the agar plate in outward radial direction, 30 ul from the enzyme preparation was dispensed into the slit, beginning near the disc and moving in outward direction by using a micropipette, overfill of the slit was avoided, then they were incubated at 37°C for overnight. Clear distortion of zone of inhibition of cefoxitin was taken as Amp C beta- lactamase producers (Figure. 4).
RESULTS

One hundred and fifty Pseudomonas spp. isolates were isolated from Ain Shams University hospitals' inpatients (49.3% from surgical unit, 24.7% from internal medicine wards and 26% from Intensive care unit). Isolates were recruited from variable clinical sites. The resistance pattern of the isolated Pseudomonas spp. to different antibiotics was Ceftazidime (44.7%), Cefepime (36.7%), Piperacillin +Tazobactam (36%), Amikacin (25.3%), Meropenem (14.7%) and Imipenem (14%). MDR pseudomonas was 14.7%

Out of 150 P. aeruginosa isolates, 66 isolates (44%) were shown to be inducible for AmpC by one or more of the inducer /substrate combinations. The sensitivity of inducer/substrate combinations among Pseudomonas isolates were IMP/TZP is 100%, IMP/CAZ is 90.9% and IMP/CTX is 42%;

AmpC derepressed P. aeruginosa isolates were detected by Disk potentiation test, modified three dimensional test, and AmpC test. Results are summarized in table (1).

In the current study we considered the isolate as AmpC derepressed positive if any two of the three used tests (disk potentiation test, MTDT and AmpC disk test) were positive. 26 out of 150( 17.3%) isolates were AmpC derepressed and Out of 26 cases, 24 (92.3%) were partially derepressed and only 2 isolates (7.7%) were fully derepressed ( non-inducible). 82 isolates were negative for AmpC production(Figure. 5).

Our results showed no significant difference between the antibiotic resistance pattern of inducible and derepressed AmpC producers (Pvalue >0.05) but there was a highly significant difference between antibiotic resistance pattern of total AmpC producers and negative AmpC producers (P value <0.05) except with Amikacin which showed non-significant association between them (P value >0.05).

In this study, specificity of MTDT, AmpC test and Disk potentiation test was (100%, 98.4% & 92.7%) respectively, sensitivity was (100%, 80.0% & 92.3%) respectively, negative predictive value was (100%, 91.3% & 98.3 %) respectively, positive predictive value was (100%, 96.1% & 72.7%) respectively and the efficacy was (100%, 95.3% & 92.7%) respectively.

In addition there was statistically significant correlation between MTDT and both disc potentiation and AmpC disc tests (P <0.05), table (2).
Table (1): Summary for results of all studied methods to detect AmpC B lactamases among tested isolates of pseudomonas aeruginosa (150 isolates)

<table>
<thead>
<tr>
<th>Method</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Screening test</td>
<td>66 44%</td>
<td>84 56%</td>
</tr>
<tr>
<td>Disk potentiation test</td>
<td>33 22%</td>
<td>117 78%</td>
</tr>
<tr>
<td>Modified three dimensional test</td>
<td>26 17.30%</td>
<td>124 82.70%</td>
</tr>
<tr>
<td>AmpC disk test</td>
<td>23 15.30%</td>
<td>127 84.70%</td>
</tr>
</tbody>
</table>

Table 2: Correlation between MTDT and both disc potentiation and AmpC disc tests

<table>
<thead>
<tr>
<th>Method</th>
<th>Modified three dimensional test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>Disc potentiation</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>No 115</td>
</tr>
<tr>
<td>Positive</td>
<td>No 9</td>
</tr>
<tr>
<td>Total</td>
<td>No 124</td>
</tr>
<tr>
<td>Chi-square test</td>
<td>X² 90.601</td>
</tr>
<tr>
<td>AmpC disk</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>No 122</td>
</tr>
<tr>
<td>Positive</td>
<td>No 2</td>
</tr>
<tr>
<td>Total</td>
<td>No 124</td>
</tr>
<tr>
<td>Chi-square test</td>
<td>X² 103.735</td>
</tr>
</tbody>
</table>

DISCUSSION

At the turn of the third millennium, P. aeruginosa clearly represents one of the most challenging pathogenic bacteria with constant evolution of resistance that affect proper patient management and force for the development of appropriate diagnostic tools(13).

In our study, the resistance pattern of the isolated Pseudomonas spp. to different antibiotics was variable. A similar resistance pattern was reported by a study in Egypt, done on a larger number of pseudomonas isolates (2593 isolates), that showed comparable resistant pattern of pseudomonas to ceftazidime (43.9%) and MDR.
rate of pseudomonas (12.3\%)^{27}. Another study by Zafer et al., showed comparable resistance pattern; 60.6\% to ceftazidime, 32.8\% to amikacin, and 25.4\% to piperacillin + tazobactam, but they showed higher resistance to Meropenem (45.9\%) and Imipenem (39.3\%)^{28}. The higher resistance to Carbapenens may be attributed to low number studied, increase infection rate with MDR pseudomonas at time of study and selection of studied patients, as isolates were collected from Kasr El Aini Hospital and National Cancer Institute, Cairo University may be more cancer patients that are known by increased resistance of bacterial isolates^{19}. Several resistance pattern of pseudomonas to different antibiotics have been reported worldwide that are higher or lower than ours^{3,17,25}.

In our study, the screening method done by disk antagonism test detected 44\% inducible AmpC producers, which correlated well with a study in India that showed 42\% inducible AmpC producers^{14}. A comparable study in Egypt, reported 51.6\% inducible AmpC beta-lactamases among gram negative bacilli^{26}. However another study done by Wassef and her colleagues in Egypt revealed lower rate of inducible AmpC among pseudomonas aeruginosa (25.5\%)^{27}. This may be attributed to the large number of P. aeruginosa isolates in their study (2593 isolates) and probably endemicity of AmpC producer and/or high burden of antibiotic usage of third generation cephalosporines in surgical units as 48.7\% of our pseudomonas isolates were isolated from their patients. On the contrary, Dunne and Hardin, in USA demonstrated that out of 134 P. aeruginosa collected, 85.8\% were inducible for AmpC^{7}. This difference in rate of inducible AmpC may be due to the increased usage of third generation cephalosporines and subsequent increased in prevalence of chromosomal AmpC beta lactamases.

The sensitivity of AmpC induction screening assay for each inducer/substrate combination showed Imp/Tzp with best sensitivity and least sensitivity with Imp/CTX. This comes in agreement with a previous study that showed sensitivity of Imp/Tzp (100\%), Imp/caz (100\%) and Imp/Ctx (69\%)^{7}.

In the current study for the detection of derepressed AmpC, 3 techniques were used; disk potentiation test, MTDT and AmpC disk. It was found that 17.3\% isolates were derepressed AmpC as they were positive by two or more of the three used tests. Basak and his coworkers detected 17.2\% derepressed AmpC B-Lactamases among P. aeruginosa through using MTDT^{19}. Also, Tam et al., detected AmpC in 18.4\% of their isolates through molecular technique^{22}. Feglo and Opoku, in Ghana found 84/187 (44.9\%) positive by MTDT^{26}. Different detection rates of derepressed AmpC were reported in other studies through using disk potentiation test and AmpC disk test. Dunne and Hardin showed 15/134 (11\%) positive by using disk potentiation test^{7}, Salimi and Eftekharin Iran also found 26/135 (19.2\%) and Sreeshma and his associates reported only 2\% positive by AmpC disk test^{21}. These differences in results may be due to the differences in the geographical distribution with different burden of use of third generation cephalosporines and resistance patterns in the different localities, and it may be also attributed to the difference in the test technique used.

Modified three-dimensional test showed 100\% sensitivity and specificity. This was in agreement with Upadhyay and his associates, where they considered MTDT as the gold standard for phenotypic detection of derepressed AmpC B-Lactamases^{22}. Moreover, another studies reported sensitivity of 100\% and 93.6\% of MTDT test to detect derepressed AmpC B-Lactamases^{3,14}.

In this study, there was no statistically significant difference between antibiotic resistance pattern of the inducible and derepressed AmpC beta lactamases, the cause could be that most of derepressed group were partially derepressed (24/26), so level of AmpC production was not so high and it affected also the antibiotic resistance pattern of the inducible group (24/66). But there is significant difference between the antibiotic resistance pattern of AmpC producers and non inducible non derepressed group except with amikacin, which confirms that the presence of AmpC beta lactamases changes the resistance pattern of the organism toward the beta lactams antibiotics.
Antibiotic Resistance Among PA Isolates

Option of treatment for AmpC producer is 4th generation cephalosporins as cefepime, carbapenems and other non beta lactam agents. But in our study 46.9% were only susceptible to cefepime which may be due to beta-lactamases: ESBL production or another lactamases as those belong to group 2ce with RTG-4 as representative enzyme, even presence of fully derepressed AmpC, or another mechanism of resistance \(^{11,16}\).

**Conclusion:**

In conclusion, high rate of AmpC expression mainly inducible production was noted among pseudomonas isolates in Ain Shams University Hospitals, this may result in high clinical treatment failures with broad-spectrum cephalosporins. The best inducer/substrate combination used in screening test for detection of inducible AmpC beta lactamase, was impenem/tazobactam that should be considered as routine test for detection of AmpC inducer to monitor the panel of resistance among P. aeruginosa and to avoid treatment failure with third generation cephalosporin, at least, for critically ill patients. Modified three dimensional test is the most sensitive, specific, interpretable and efficient test for detection of derepressed AmpC beta lactamases in clinical isolates of P. aeruginosa. The best option for treatment of inducible AmpC producers in P. aeruginosa is carbapenems and Amikacin. Our findings raise the necessity to control the use of antimicrobial agents and to implement prompt infection control procedures in our hospital.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

**Acknowledgments**

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Amer, M . Z.&El-Ashry,M.A


GENE MUTATION IN PROGRESSIVE FAMILIAL INTRAHEPATIC
CHOLESTASIS AND BILE SALT EXPORT PUMP DYSFUNCTION IN A
COHORT OF EGYPTIAN CHILDREN
Marianne S. Makbool*, Amaal A, Abdelaal* and Hanaa M, El-Karaksy**

ABSTRACT

Background: Progressive familial intrahepatic cholestasis (PFIC) refers to a heterogeneous group of autosomal recessive disorders of childhood that disrupt bile formation and present with cholestasis of hepatocellular origin. The ABCB11 gene encodes the ATP-dependent canalicular bile salt export pump (BSEP) of human liver. PFIC2 is a rare inherited disease due to mutations in ABCB11 encoding BSEP of hepatocyte which is the major transporter responsible for bile acid excretion. Impaired biliary excretion leads to bile salt accumulation in hepatocytes, ongoing hepatocellular damage leading to hepatocellular carcinoma. Aim of work: To detect mutations in exon 8 and exon 9 of ABCB11 gene in patients with suspected PFIC2 among studied Egyptian population in order to confirm diagnosis of PFIC2 and to detect mutations that impair BSEP protein function and bile acid excretion. Subjects and methods: This study was conducted on 33 subjects including 11 suspected PFIC2 patients and 22 healthy control subjects. ABCB11 genotyping was performed by DNA extraction followed by PCR amplification, purification then sequencing of exon 8 and exon 9 of the gene. Results: No mutations or variations in sequence results involving exon 8 or exon 9 of the ABCB11 gene of studied Egyptian PFIC2 patients and healthy subjects. Conclusion: this work detected no mutations or variations involving exon 8 and exon 9 of ABCB11 gene in studied Egyptian patients with suspected PFIC2 as a cause of severe BSEP deficiency, so further investigations of other exons of the gene are necessary to confirm diagnosis of PFIC2. Key words: Progressive Familial Intrahepatic Cholestasis (PFIC2) - Bile Salt Export Pump (BSEP) - ABCB11 gene- exon 8-exon 9.

INTRODUCTION

Progressive familial intrahepatic cholestasis (PFIC) is a group of inherited liver disorders of childhood in which cholestasis of hepatocellular origin mostly presents in the neonatal period and leads to death from liver failure at ages usually ranging from infancy to adolescence. This disorder is autosomal recessive in inheritance. The incidence is from 1/50,000 to 1/100,000 births. This disorder equally affects both genders and has been reported all over the world. PFIC are divided into three types-PFIC 1, PFIC 2 and PFIC 3 according to clinical presentation, laboratory findings, liver histology and genetic defect.

It represents 10% to 15% of causes of cholestasis in children and 10% to 15% of indications of liver transplantation in children. PFIC1 and PFIC2 represent 2/3 of PFIC cases, and PFIC3 represents 1/3 of cases.

The diagnosis of PFIC need the co-operation of clinical, laboratory, radiological, and liver histological parameters; and also need specific investigations to exclude other causes of neonatal cholestasis.

The PFIC-2 is caused by mutations in the ABCB11 gene. The ABCB11 gene encodes the ATP-dependent canalicular bile salt export pump of human liver and is located on human chromosome 2.

Bile acids secretion is the main ongoing force for bile flow in humans. The described adenosine triphosphate (ATP)-dependent bile acid transporter, bile salt export pump (BSEP), is the transporter of bile acids through the hepatocellular canalicular membrane into bile. It was recognized that mutations in the gene that encode this protein (ABCB11) are responsible for a subgroup of infants and children with PFIC-2.

The Bile Salt Export Pump consists of 1321 amino acids that its molecular mass is about 160 kDa. Like other full-length transporters of the ABC superfamily, BSEP is predicted to be a double structure with each half of the molecule consisting of six predicted TMD and a large cytoplasmic NBD in a TMD–NBD–TMD–NBD organization.
Patients with PFIC-2 present with severe jaundice, hepatomegaly, failure to thrive, and pruritis. Laboratory investigations show direct hyperbilirubinemia, high level of serum aminotransferases, and unexpectedly normal serum gamma glutamyl transpeptidase. There is rapidly progressive course to liver cirrhosis, liver failure and hepatocellular carcinoma (HCC), which can only be treated by liver transplantation\(^{11}\).

Severe phenotypical forms are often associated with mutations leading to premature truncation of protein or failure of protein production. Insertion, deletion, nonsense and splicing mutations lead to damaging effects and patients exhibited little or no detectable BSEP at the hepatocyte canaliculus. Also common defects are missense mutations that either affect protein processing and trafficking or disrupt functional domains and protein structure\(^{12}\).

Detection of ABCB11 gene mutations is the only way to confirm diagnosis of PFIC-2 in suspected patients and close follow up of these patients that usually progress to liver cirrhosis and HCC\(^{12}\).

**SUBJECTS AND METHODS**

**Study Participants**

The study was conducted on 11 children suspected as PFIC2 and 22 non diseased subjects (control group). The PFIC-2 subjects were selected from the Pediatric Hepatology Unit at Cairo University Children’s Hospital, after approval by the Ethical Committee of Faculty of Medicine; Cairo University.

PFIC-2 was suspected in children with a clinical history of chronic cholestasis of unknown origin after exclusion of the other main causes of cholestasis (e.g. biliary atresia, α-1 antitrypsine deficiency, cystic fibrosis, sclerosing cholangitis, viral or autoimmune hepatitis, and extra hepatic bile duct obstruction). Diagnosis of PFIC-2 was made in patients with chronic cholestasis in the form of recurrent episodes of jaundice from first months of life that become permanent later in the course of the disease. Severe pruritis is usually observed. The clinical manifestations with normal level of GGT, very high serum bile acid concentration, radiological and histological approaches helped the diagnosis.

**All patients were subjected to:**

Full history taking including: age at presentation, familial pedigree, familial illness, current age. Clinical features including: continuous jaundice or intermittent, pruritis, diarrhea, hepatomegaly, splenomegaly, hearing lose, manifestation of vitamins deficiency like A, D, E, K. Laboratory investigation: GGT, AST, ALT, Bilirubin, serum bile acids. Radiological examination including: abdominal U/S. Histological examination in the form of: liver biopsy. Finally molecular investigation included DNA sequencing of ABCB11 gene (exon 8 and 9) for patients and controls.

**Methodology:**

Three ml of venous blood were collected from each subject in sterile EDTA vacutainers for the genotyping technique. DNA was extracted from samples to be used for performing the PCR for ABCB11 gene study. Sequencing of exon 8 and 9 of ABCB11 gene required the following steps: DNA extraction then amplification using the PCR, detection of amplified products followed by DNA purification then Cycle Sequencing, capillary electrophoresis and finally analysis of data.

**Amplification of exon 8 and 9 of ABCB11 gene by Polymerase Chain Reaction (PCR):**

DNA was extracted from peripheral EDTA blood samples by QIAamp DNA extraction kit (QIAGEN GmbH. Germany) according to manufacturer’s instructions. Amplification of the extracted DNA was done according to the protocol proposed by Thompson et al\(^{13}\), followed by detection of PCR amplification products using 1.5% agarose gel electrophoresis containing ethidium bromide and ultraviolet light transillumination. Using HOT FIREPol (10X) Master Mix (Solis BioDyne, Tartu.Estonia) which con-
tain: ready to use HotStart Taq DNA polymerase mixture, PCR buffer, MgCl<sub>2</sub>, and dNTPs. Forward and reverse primers (supplied by Bioscience GmbH, Jena, Germany) and annealing temperature for exon 8 and 9 of ABCB11 gene are described in table (1).

Reactions were performed in a total volume of 25 µL containing: 2µl PCR HotStart Master Mix (10X), 1 µl forward primer (100 pmol /µl), 1 µl reverse primer, 14 µl nuclease-free water and 7 µl purified DNA solution. Thermal cycler (Professional Thermocycler, Biometra; Applied Biosystems, Foster City, CA, USA) was programmed according to the following conditions: Initial activation at 95°C for 15 minutes, then 34 cycles of denaturation at 94°C for 45 seconds, annealing at 62°C for both exon 8 & 9 for 45 seconds, extension at 72°C for 1 minute. Final elongation was done at 72°C for 1 minute. The quality of DNA was examined using 1.5% Agarose gel electrophoresis, the amplified DNA ran as a single bright band on an agarose gel which indicated successful amplification.

**Cycle Sequencing and Capillary Electrophoresis (on the ABI 3500 Genetic Analyzer):**

The amplified DNA was purified using QIAquick PCR purification kit supplied by Qiagen, Germany. Then Frederick Sanger’s enzymatic dideoxy DNA sequencing technique was applied which was based on the chain-terminating dideoxynucleotide analogues<sup>(9)</sup> using ABI PRISM Big Dye Terminator cycle Sequencing Ready Reaction Kit v 3.1.(contain dideoxynucleotides) and BigDye 5X dilution buffer supplied by Applied Biosystem (LifeTechnologies Corporation, California, USA). For each reaction the following reagents were added to a separate tube: 2.0 µl of BigDye Terminator, 1.0 µl of 5X Dilution buffer, 2.0µL of the forward Primer used for PCR reactions, 2.0µL of template DNA, and 3.0µL Nuclease free water to complete the total volume to 10µL.

The Thermal cycler was adjusted to the following conditions: Initial denaturation at 96°C for 1 minute followed by 25 cycles of: denaturation at 95°C for 10 seconds, annealing temperature 62°C for both exon 8& 9 for 5 seconds, and extension at 72°C for 4 minutes. Then Purification step was performed using BigDye X terminator purification kit supplied by Applied Biosystem to sequester unincorporated dye terminators and dNTPs to prevent their co-injection with dye-labeled extension products. The “cleaned up” sequencing reactions were resuspended in 15µL of Hi Di formamide for denaturing DNA before injection, then it was injected in Applied Biosystems 3500 Genetic Analyzers (Figures1&2).

**Analysis of data**

Sequences were compared to the published sequence (NM_003742.2). According to NM_003742.2 exon 8 is from738 to 909 and for exon 9 is from 910 to 1034 nucleotide base .According to Homo sapiens ATP-binding cassette, sub-family B (MDR/TAP), member 11 (ABCB11), mRNA by NCBI (National Centre of Biotechnology Information). Reference Sequence: NM_003742.2 which is 4775 bp mRNA and its translated protein (bile salt export pump) NP_003733.2 which is 1321 amino acid analy-

<table>
<thead>
<tr>
<th>EXON 9</th>
<th>EXON 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>5’-TGAGAATCTAATATTGTATTAA AACCCATGCC -3’</td>
</tr>
<tr>
<td>Reversed primer</td>
<td>5’-CAAGGTGGGTCTGCCGCTT-3’</td>
</tr>
<tr>
<td>Amplification protocol</td>
<td>-Initial activation at 95°C for 15 minute -34 cycles of: 94°C for 45 seconds. 62°C for 45 seconds. 72°C for 1 minute. -Final elongation at 72°C for 1 minute</td>
</tr>
<tr>
<td>Number of nucleotides</td>
<td>340 base</td>
</tr>
</tbody>
</table>
sis was done by BLAST [Basic Local Alignment Search Tool] (www.ncbi.nlm.nih.gov) and the CLC-BIO sequence viewer 6 program (www.clcbio.com).

Results of PCR product of Exon 9 of suspected PFIC2 patient number (10).

Figure 1: The sequence results of PCR product of Exon 9 of suspected PFIC2 patient number (10).

Statistical methods: Quantitative data were summarized as mean and standard deviation when normally distributed and as median and (25th-75th) when abnormally distributed, qualitative data were summarized as number and percentages and compared by Chi square (X²) test. P-value was considered significant if <0.05. Data were analyzed using the software SPSS (Statistical package for social science) version 17. (SPSS, Inc, Chicago, IL, USA).

Figure (2): The sequence results of PCR product of Exon 8 of suspected PFIC2 patient no (10).

Results

This cross-sectional study was performed in the Molecular Biology Unit in Chemical Pathology Department, Faculty of Medicine Cairo University. Eleven suspected PFIC-2 patients (their current age 1-6 years), and twenty two control subjects were enrolled in this study. They were suspected to be diagnosed as PFIC-2 before the age of 3 years; (9/11) at less than 6 month, 1/11 at the age of 1 year, and 1/11 at the age of 3 years. Ten cases (90.9%) were males and 1/11 (9.1%) was a female. Eight of cases were positively consanguineous (72.7%), one of these families there was one sibling died at 6 month & he was jaundiced. The (control group) consisted of 22 subjects, 11 males (50%) and 11 (50%) females. Demographic and clinical data of subjects are presented in table 2.

All patients had high bilirubin levels (total and direct), normal GGT, and elevated transaminases (AST, ALT) with characteristic increased level of serum bile acid concentration as shown in table 2.

Radiologically, two patients (18.2%) had average size liver and spleen, seven of them (63.6%) had hepatomegaly, and two had hepatosplenomegaly (18.2%) detected by abdominal ultrasound. Pathologically, one of the patients (number 10 among subjects) showed developed chronic hepatitis with impending fibrosis. Two
Gene Mutation in PFIC and BSEP in Egyptian Children

DISCUSSION

Type 2 PFIC is an autosomal recessive condition that disrupts bile transport. BSEP deficiency is among disorders with low serum concentrations of GGT activity despite conjugated hyperbilirubinemia, as in familial intrahepatic cholestasis 1 deficiency caused by mutations in ATP8B1. DNA sequence analysis of exon 8 and exon 9 of the ABCB11 gene revealed no mutations or variations in sequence results involving phenotypically normal control group and suspected PFIC-2 patients as shown in table 4.

Table (2): Demographic and clinical data of subjects

<table>
<thead>
<tr>
<th>Groups</th>
<th>Patients</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (among cases)</td>
<td>Males</td>
<td>10/11 (90.9%)</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>1/11 (10.1%)</td>
</tr>
<tr>
<td>Familial illness</td>
<td></td>
<td>1/11 (9.09%)</td>
</tr>
<tr>
<td>Familial pedigree</td>
<td>+ve consanguinity</td>
<td>8/11 (72.7%)</td>
</tr>
<tr>
<td></td>
<td>No consanguinity</td>
<td>3/11 (27.3%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>3.4 (1-6)</td>
<td></td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>100.3 (47-350)</td>
<td></td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>129.7 (55-512)</td>
<td></td>
</tr>
<tr>
<td>Total bilirubin (mg/dl)</td>
<td>T: 8.0 (3.5-14.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D: 5.25 (2.7-10)</td>
<td></td>
</tr>
<tr>
<td>Serum bile acids (µmol/L)</td>
<td>223.7 (19-522)</td>
<td></td>
</tr>
</tbody>
</table>

Table (3): Ultrasound and liver biopsy results among patients

<table>
<thead>
<tr>
<th>DATA</th>
<th>FREQUENCY</th>
<th>PERCENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>U/S Findings</td>
<td>Hepatomegaly</td>
<td>7/11 63.6%</td>
</tr>
<tr>
<td></td>
<td>Hepatosplenomegaly</td>
<td>2/11 18.18%</td>
</tr>
<tr>
<td></td>
<td>Average size liver and spleen</td>
<td>2/11 18.18%</td>
</tr>
<tr>
<td>Liver biopsy result</td>
<td>Neonatal hepatitis</td>
<td>6/11 54.54%</td>
</tr>
<tr>
<td></td>
<td>PIBD</td>
<td>2/11 18.18%</td>
</tr>
<tr>
<td></td>
<td>Not available</td>
<td>3/11 27.27%</td>
</tr>
</tbody>
</table>

Table (4): Sequence results of ABCB11 gene (exon 8&9) of suspected PFIC-2 patients and control subjects

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>PATIENT</th>
<th>CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency</td>
<td>11/33 (33.3%)</td>
<td>22/33 (66.7%)</td>
</tr>
<tr>
<td>Nucleotide/amino acid changes for Exon 8&amp;9</td>
<td>0/11 (0.0%)</td>
<td>0/22 (0.0%)</td>
</tr>
</tbody>
</table>

It was explained that young patients with severe BSEP deficiency syndrome are at risk to subsequently develop HCC and cholangiocarcinoma, with the reported cases of HCC being much greater than cholangiocarcinoma. These patients frequently present with splice site changes, deletion, insertions, and nonsense mutations that result in the absence of functional...
protein. This absence results in elevated levels of intracellular bile salts that have been shown to influence many aspects of cell function, including mitochondrial function, cell cycle, DNA repair, cell polarization and differentiation \(^\text{[14]}\).

Many polymorphisms in ABCB11 have been described in a population with different ethnic distribution all over the world with respect to allele number, frequency of common and population-specific sites. These polymorphisms are located in exons and introns, as well as in 5'-flanking regions, but no effect on the mRNA or protein has been determined. However, Population genetic analysis suggested some selective pressure against changes in the protein, supporting the important endogenous role of these transporters \(^\text{[8]}\).

Many different ABCB11 disease causing variants have been detected worldwide, most of them are mutations grouped as missense, nonsense, deletions and insertions, and splice-site mutations. The results of these gene mutations is the decrease or total loss of expression of the BSEP protein on the hepatic canalicular membrane \(^\text{[7]}\).

Thirty three Egyptian subjects were enrolled in our study; twenty two control subject (22/33), eleven subjects (50%) were males and 11/22 (50%) were females, and eleven suspected PFIC-2 patients (11/33), their age ranged from 1 to 6 years. All patients were subjected to full history taking including: age at presentation, familial pedigree, familial illness, current age, clinical features including: continuous jaundice or intermittent, pruritis and its severity, hepatomegaly, splenomegaly, diarrhea, hearing loss, manifestation of vitamins (A,D,E,K) deficiency, results of GGT, AST, ALT, Bilirubin, serum bile acids, radiological, histological examination and laboratory investigation included DNA sequencing of ABCB11 gene (exon 8&9) for patients and controls.

We investigated the possible association of mutations in exons 8&9 which is considered site for common mutations of ABCB11 gene as a cause of ABCB11 transporter functional deficiency that leads to PFIC among studied Egyptian children and our study revealed no mutations or variations in the sequencing results involving exons 8&9 of the ABCB11 gene of studied PFIC-2 patients and phenotypically healthy subjects

Our results were similar to the results of Lang et al. \(^\text{[8]}\), who studied genetic variability and haplotype structures of ABCB11 in 292 healthy populations of different ethnic backgrounds in which they detected 28 genetic variants in 27 coding exons and 1 noncoding exon; among these were 10 missense mutations, 17 silent mutations and 1 mutation in the un translated exon, with the two Cacasian-specific variants in two exons not including exon 8 or exon 9. The two Cacasian-specific variants were in exon 13 (c.1331T>C; 59.4%) and exon 17 (c.2029A>G; 4.2%) coded for amino acid substitutions p.V444A and p.M677V respectively. All amino acid polymorphisms of ABCB11 were predicted to be located in the extracellular region.

On the contrary, many collaborative studies have identified mutations in exon 8 &9 of ABCB11 producing marked BSEP protein deficiency \(^\text{[1, 7, 12]}\).

Lam et al. \(^\text{[7]}\), made a molecular model of BSEP illustrating some of the most common BSEP gene mutations that may result in a clinical phenotype of PFIC2 with marked protein deficiency; p.G238V (located in TMD4 & encoded by exon 8), p.E297G (located in intracellular span between TMD4 and TMD5 & encoded by exon 9), p.D482G (located in NBD1 and encoded by exon 14), p.G982R (located at TMD11 & encoded by exon 23), and p.R1268Q (located in NBD2 at conserved signature C motif & encoded by exon 28).

Strautnieks et al. \(^\text{[12]}\), identified Eighty-two different mutations (52 novel) (9 nonsense mutations, 10 small insertions and deletions, 15 splice-site changes, 3 whole-gene deletions, 45 missense changes); p.G238V (located in TMD4 & encoded by exon 8) were included in missense mutation results.

In addition, Byrne et al. \(^\text{[1]}\), classified the majority (63) of known ABCB11 missense mutations and 21 single-nucleotide polymorphisms (SNPs), e.g. (p.G260D, p.E297K, p.D482G
Gene Mutation in PFIC and BSEP in Egyptian Children

R832C, p.A1028A, p.S1144R, and p.R1153H) and they found reduced wild-type splicing for 20 mutations/SNPs, with normal mRNA levels reduced to 5% or less. The common ABCB11 missense mutation encoding p.D482G enhanced aberrant splicing, whereas the common SNP p.A1028A promoted exon skipping. Addition of exogenous splicing factors modulated several splicing defects of the mutants expressed in vitro, most appeared to be retained in the endoplasmic reticulum and degraded. All these mutations can result in truncated or misfolded BSEP proteins. Thus, primary defects at either the protein or the mRNA level (or both) contribute significantly to BSEP deficiency. These results will help to develop mutation-specific therapies for children and adults suffering from intrahepatic cholestasis due to BSEP deficiency.

Conclusion: This work detected no mutations or variations involving exon 8& 9 of ABCB11 gene in studied Egyptian patients with PFIC-2 as a cause of severe BSEP deficiency, so further investigation of other exons of the gene are necessary in order to confirm diagnosis of PFIC 2 and to prove that in spite of considering exon 8&9 mutations are common worldwide, but these mutations are not that common among Egyptian ethnic population, however larger sample size is needed.

REFERENCES
يعتبر مرض احتباس العصارة الصفراوية الوراثي المتصاعد الكبدي من النوع الثاني من الاضطرابات الوراثية التي تؤدي إلى اضطرابات في تكوين الصفراء وتكون أعراضه ركوداً قومياً (PFIC2) بسبب طفرة في الوراثة الخاصة بـABCB11 ( PFIC2 ) وهو الجين الذي يحول الرسالة (ABCB11) بسبب طفرة في الجين ATP الخاصة بـPFIC2 ( المعتمد على مضخة الأحماض الصفراوية للكلب البشري ) والذي يعد المصدر الأول للأحماض الصفراوية في مواجهة الدرجات العالية من التركيز. وحدوث الاضطراب في هذه المضخة هو المسؤول الأول عن نقص إفراز أملاح العصارة الصفراوية والذي يحدث بشكل واضح في المرضى الذين يتأثرون بهذا المرض النادر، والذي يؤدي بدوره إلى نقص تدفق العصارة الصفراوية وتراكم الأملاح الصفراوية داخل خلايا الكبد. الهدف من الدراسة: الكشف عن الاضطرابات التي تحدث في ABCB11 في مرضى LFIC2 من النوع PFIC2 (PFIC2) للجين ( ABBC11 ) في عدد من المرضى الذين خضعوا لهذه الدراسة وقد تم دراسة الاضطرابات في PFIC2 من التشخيص للمريض حيث أنه هو السبب في النقص الحاد في البروتين في مضخة الأحماض الصفراوية للكلب البشري. طريقة البحث: خضع حوالي 33 شخص للفحوصات المخبرية بما فيها تتابع PFIC2 وفقاً للنتائج، لم تظهر الدراسة أي اختلافات أو الاختلافات في النتائج الجينية للمرضى المصريين فيما يخص PAN8&9 و( PFIC2 ) (gene) (gene)