THE EGYPTIAN JOURNAL OF LABORATORY MEDICINE
“DAR EL HEKMA”, 42, Kasr El-Eini Street, Cairo, Egypt
PUBLISHED BY
THE EGYPTIAN SOCIETY OF LABORATORY MEDICINE
(ESLM)

Editor in Chief:  Ali Ahmed Shams El-Din
Editor:  Naguib Zoheir Mostafa
e-mail: ejlm@hotmail.com
Assistant Editors:  Sahar Kamal
                  Mervat Mamdooh Khorshied
                  Heba Mahmoud Gouda

ADVISORY BOARD
Alphabetical Order

Clinical Chemistry
Ahmed Abdel Samie Omran
Fatma El Mogui
Omar Ali Alroubi
Omneya Youssef
Ousama Bakr Seddik
Sawsan Housny
Mona Salem
Mouna Sedrak
Mohga Zewar
Nabiela Thabet
Naila Omran
Nevine Kassem
Samir Sahlab
Sherif Ahmed Ali
Sourya Badawy

Clinical Microbiology
Amany El Kholy
Ragaa Lashin
Soheir Helal
Walaa Gad
Abdullah Khalil
Eleya Ishaak

Immunology
Aida Abd El Azim Abd El Salam
Aisha Abdel Ghaaffar
Azza Aboul Enein
Azza Kamel
Farha El Shennawy
Mervat El-Ansary
Moemen Abdel Wahab Kamel
Mona Rafik
Nawal Afifi
Safaa El Karaksy
Taghreerd Gaafar

Hematology
Azza Ahmed Mohamed
Azza Mostafa
Fadila Sabri
Hala Farawella
Hala Gabr
Laila Hegazy
Loutfi Abdul Nabui
Azza Kamel
Farha El Shennawy
Mervat El-Ansary
Moemen Abdel Wahab Kamel
Mona Rafik
Nawal Afifi
Safaa El Karaksy
Taghreerd Gaafar
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Articles to be published should not be published elsewhere, and should be accepted by a referee of the advisory board.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.
CONTENTS

1. IMPLICATION OF TLR-3 AND TLR-9 SINGLE NUCLEOTIDE POLYMORPHISMS ON HCV INFECTION, RESPONSE TO THERAPY AND FIBROSIS PROGRESSION AMONG EGYPTIAN PATIENTS
   Rania A. Zayed, Dalia Omran, Zinab Zakaria, Sameera Ezzat, Salwa Tawfeek and Hossam El-Sweesy .......................... 1

2. ROLE OF PLACENTAL PROTEIN 13 AS A BIOMARKER FOR EARLY DETECTION OF PRE- ECLAMPSIA, METHYLENETETRAHYDROFOLATE REDUCTASE GENE POLYMORPHISM AND RELATION TO DISEASE RISK, AN EGYPTIAN STUDY
   Engy El Khateeb and Sherif Dahab.................................................................................................................. 13

3. CORD BLOOD Copeptin AND CALPROTECTIN AS POTENTIAL BIOMARKERS OF EARLY-ONSET NEONATAL SEPSIS
   Manal Mohsen, Wafaa K.Zaki, Rania Ismail, Nehal El-Raggal and Mohamed A. Abd El-Wahed......................... 21

4. THE STUDY OF BCR-ABL TRANSSCRIPT VARIANTS (B2A2 AND B3A2) RELATION TO DIFFERENT RISK SCORES
   Hisham Abdelaziz, Ahmed Omar, Ali Alshemary and Mohamed Keshk.................................................................... 31

5. CRP AND SERUM AMYLOID A AS MARKERS FOR THE AGGRESSIVENESS OF BREAST CANCER
   Rania Elhelely, Rasha Elzehery, Mohammed AF Hegazy and Ramy Faris.............................................................. 39

6. EXPRESSION OF CD69 IN CHRONIC LYMPHOCYTIC LEUKEMIA; RELATION TO PROGNOSIS AND DISEASE PROGRESSION
   Mahira I Elmougy and Ghada M Elgohary.............................................................................................................. 47

7. COMBINED ANALYSIS OF LEVELS OF SERUM B-CELL ACTIVATING FACTOR AND A PROLIFERATION INDUCING LIGAND IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA; CORRELATION WITH CLINICAL FEATURES AND DISEASE PROGRESSION
   Mahira I Elmougy and Ghada M. Elgohary............................................................................................................ 57

8. IN VITRO APOPTOTIC EFFECT OF METFORMIN ON HUMAN CERVICAL CANCER CELLS
   Dina Sabry, Sahar H. Ahmed and Mohamed A. S. Al-Ghussein........................................................................... 65

9. ROLE OF INTERCELLULAR ADHESION MOLECULE-1 (ICAM-1) G241R AND K469E GENE POLYMORPHISM AND SOLUBLE ICAM-1 SERUM LEVELS IN THE DEVELOPMENT OF ISCHEMIC STROKE IN EGYPTIAN PATIENTS
   Abeer Mohamed Mohy, Ahmed Abd Allah Hassan Ali and Heba Omar............................................................. 73

10. γ- GLOBIN GENE XMN1 POLYMORPHISM-158 AND ITS CORRELATION TO THE RESPONSE TO HYDROXYUREA IN βTHALASSEMA MAJOR PATIENTS IN BENISUEF GOVERNORATE(EGYPT)
    Abdel Meged A. Abdel Meged, Dalia G. Amin, Dalia S. Morgan and Safwat L. Shaker................................. 81
IMPLICATION OF TLR-3 AND TLR-9 SINGLE NUCLEOTIDE POLYMORPHISMS ON HCV INFECTION, RESPONSE TO THERAPY AND FIBROSIS PROGRESSION AMONG EGYPTIAN PATIENTS


ABSTRACT

Toll-like receptors (TLRs) are recognized as fundamental contributors to the immune system function against infections. We studied the association of genetic variation in TLRs and HCV infection susceptibility, response to therapy and fibrosis progression. Frequency of TLR-3 (r7 C/A [rs3775296]), TLR-3 (c.1377C/T [rs3775290]) and TLR-9 (1237T/C [rs5743836]) single nucleotide polymorphisms (SNPs) was studied in 100 chronic HCV-positive Egyptian patients and 100 healthy controls. Frequency of SNP in TLR-3 (r7 C/A), TLR-3 (c.1377C/T) and TLR-9 (1237T/C) were not significantly different between studied HCV-positive patients and controls with p-value 0.121, 0.112, 0.683 respectively. TLR-3 c.1377 T- allele was associated with failure of end of treatment response (p= 0.006), failure to achieve SVR (p= 0.006) and was found to be related to advanced stage of fibrosis (p= 0.003). In conclusion, TLR-3 c.1377 T- allele is associated with impaired response to therapy and fibrosis progression in HCV infected Egyptian patients.

Key words: TLR-3; TLR-9; HCV; Fibrosis; IFN; Egypt

INTRODUCTION

Hepatitis C virus (HCV) infection is a multifactorial disease representing a major health problem in Egypt where the prevalence is almost 10-fold higher than that in other countries. The immune system is an essential determinant of viral infection outcome. Interactions between the viruses, hepatocytes, and the host immune systems may determine viral persistence and disease progression.

Toll-like receptors (TLRs) are fundamental component of the innate immune system and key regulators of acquired immunity. In humans, 10 TLRs proteins (TLR1–10) have been identified, that possess different subcellular localization depending on the specific pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) they recognize, thus called pattern recognition receptors. TLR1, 2, 4, 5, 6, and 10 are found on the extracellular surface of cells, while TLR3, 7, 8, and 9, are nucleic-acid sensors located within the endoplasmic reticulum and endosomes. TLRs 3 and 9 recognize microbial nucleic acid molecular patterns, TLR-3 identifies viral RNA, while TLR-9 is specific for unmethylated cytosine–phosphate–guanine (CpG) dinucleotide motifs in pathogen DNA.

TLRs have an important role in pathogen recognition and subsequently immune system activation. They trigger inflammatory cytokines production through nuclear factor-κB–dependent or interferon (IFN) regulatory factor–dependent signaling pathways. Activation of TLR by pathogen binding stimulates inflammatory cytokines production that triggers induction of type I IFNs. Type I IFNs (IFN-α, IFN-β) have potent antiviral properties as they interfere with virus replication and possess immunomodulatory activities by promoting multiple immune functions. Type I IFNs promote CD8+ T cell effector functions, survival and memory, polarization of CD4+ T cells by Th1, NK cell activation, differentiation and maturation of DCs. Thus, Type I IFNs derive its name from a function to “interfere” in viral replication.

Several data supports the role of SNPs in TLR genes in modulating the risk of viral and bacterial infections. SNP may alter promoter activity affecting gene expression, mRNA conformation and stability or protein structure and function.

HCV infection adds great financial burden on the health sector especially in Egypt, having the highest prevalence of HCV worldwide. “Know your epidemic, know your response” concept,
necessitates the study of every aspect of the disease that may help in controlling disease dissemination in the community or disease progression which compromises the life quality of chronic HCV-infected patients. The treatment regimen for HCV-4 is composed of IFN-α2b plus ribavirin for 48 weeks. As TLRs play a role in type I IFN production, this implies they having antiviral potencies against HCV.

The genetic make-up of the host plays an important role in susceptibility and response to infections. In the present work, we studied the frequencies of TLR-3 (c.1377C/T [rs3775290]), TLR-3 (c.7C/A [rs3775296]) and TLR-9 (1237T/C [rs5743836]) SNPs in chronic HCV-positive Egyptian patients in order to clarify the role of TLR-3 and TLR-9 polymorphism in HCV infection and response to therapy.

SUBJECTS AND METHODS

Two hundred participants were included in the study; one hundred naïve chronic HCV-positive patients and one hundred age and sex matched normal healthy individuals as control group. Informed consent was obtained from all participants before enrollment, the study was performed in accordance with the Helsinki Declaration, and the protocols were approved by Faculty of Medicine, Cairo University Ethics Committee.

HCV infection was diagnosed by anti-HCV antibodies testing and detection of HCV-RNA (Applied biosystems). All patients had no coinfection with hepatitis B virus and no other causes of chronic liver disease.

Pretreatment Liver biopsy, Clinical and Laboratory evaluation

Liver biopsy specimens were obtained from all patients included in the study within 3 months prior to start of treatment regimen. Histopathologic features of fibrosis and activity were scored according to the Metavir scoring system. Fibrosis was staged on a scale of 0–4.

Abdominal ultrasound, echocardiography, fundus examination and body mass index (BMI) calculation were done for all patients before start of therapy and on regular basis every month during treatment. Body mass index (BMI) was calculated as weight divided by the square of the height (kg/m²).

Hematological tests and blood chemistry were analyzed before therapy and at least once every month during treatment, laboratory investigations included complete blood count, blood glucose level, liver function tests (ALT, AST, Alkaline phosphatase, total bilirubin, Albumin), prothrombin time and concentration, alfa-fetoprotein, creatinine, blood glucose and quantitative HCV-RNA by PCR.

Treatment regimen and Evaluation of Treatment outcomes

Although, first and second generations of protease and polymerase inhibitors are used for treatment of HCV in West Europe and USA, they are not readily available in developing countries. Pegylated interferon (PEG-IFN) combined with daily oral ribavirin were the only drugs used for treatment of HCV in Egypt until recently.

In the current study, all patients received complete course of IFN-α2b (1.5 μg/kg/week) plus ribavirin (1000-1200 mg/day) therapy for duration of 48 weeks.

Quantitative HCV-RNA by real-time PCR was done for all patients after complete course of treatment for 48 weeks and six month later to detect sustained virologic response (SVR). SVR was defined as undetectable HCV RNA in serum 24 weeks after cessation of therapy. Patients with reappearance of HCV RNA after complete course of treatment or serum HCV RNA positivity during therapy were considered as non-responders (NR).

Polymorphism analysis of TLR-3 c.1377C/T (rs3775290), TLR-3 c.7C/A (rs3775296) and TLR-9 1237T/C (rs5743836)

Genomic DNA extraction was done from peripheral blood samples using Gene JET Whole Blood Genomic DNA purification Mini kit (Thermo Scientific) according to the manufacturer’s instructions. Isolated DNA was stored at -20°C until used for PCR amplification. Polymorphism analysis was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. All PCR reactions were performed in a total volume of 25 μl contain-
ing 150-ng genomic DNA, 2X Taq Green PCR Master Mix, 25 pM of each forward and reverse primers (Biosearch technologies). PCR amplification was carried out in the DNA thermal cycler (PTC programmable thermal controller, MJ Research, Watertown, MA). Amplification conditions were initial denaturation at 95°C for 5 min followed by 35 cycles of; 95°C for 45 s, * for 45 s, and 72°C for 30 s, with final extension for 7 min at 72°C (* 55°C for TLR-3 c.1377C/T and TLR-9 1237T/C and 59°C for TLR-3 _7 C/A). The amplified PCR products were visualized by 2% agarose gel electrophoresis under UV light. Primers sequences used and amplified product size are shown in table 1.

Digestion of the amplified product by specific restriction enzyme for each polymorphism was done as follows; 10 µl of the amplified product were mixed with 1 µl restriction enzyme (Thermo Scientific) and the mixture was incubated 10 minutes (at 37°C for MboII and BstNI and at 65°C for TaqI). The product was analyzed by gel electrophoresis using 4% agarose gel (Promega). The separated fragments were stained with ethidium bromide and visualized along with 50 bp ladder (MBI Fermentas, Vilnius, Lithuania) as a size marker using transilluminator (Bio-Rad, USA). Restriction enzyme used and the resulting bp length are shown in table 1.

**Statistical analysis:**

Data were analyzed using SPSS (Statistical Package for Social Science) program for statistical analysis, (version 20; Inc., Chicago, IL). Chi-square or fisher exact (when expected counted in 25% of the cell or more is 5) were used to compare qualitative variable. Logistic regression analysis was used to calculate odds ratios (OR) and 95% confidence intervals (CI) for risk estimation. P value less than 0.05 was considered significant.

**RESULTS**

Baseline demographic, clinical and laboratory data of the studied patients are shown in table 2.

The frequency of the studied genetic polymorphisms in HCV patients and controls is presented in table 3. There was no statistically significant difference noticed in the distribution of TLR-3 _7 C/A, TLR-3 c.1377C/T and TLR-9 1237T/C genotypes between HCV patients and controls. Also, combined genotypes analysis of the studied genetic polymorphisms showed that co-inheritance of the genetic polymorphism in the three studied genes didn’t confer increased risk to HCV infection.

HCV Patients were stratified according to hepatic fibrosis stage, group with mild fibrosis (F1) and another group with advanced fibrosis (F2, F3). Different parameters that may relate to fibrosis progression were studied. Patients with advanced hepatic fibrosis have significantly elevated AFP serum levels; 5.82 ± 6.07 ng/ml in advanced fibrosis versus 3.29 ± 2.39 ng/ml in mild fibrosis (p = 0.000). Otherwise, there were no statistically significant differences noticed between the two patients’ groups regarding age, gender, baseline laboratory data (data not shown). No symptomatic significance was found as regards

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Primer Sequence (5' –3')</th>
<th>Length (bp)</th>
<th>Restriction enzyme</th>
<th>Fragments size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR-3 c.1377C/T (rs3775290)</td>
<td>CCGGCCCAAAAAGCAATATGGGAACAGGGGAAGGAGTTCT</td>
<td>337</td>
<td>TaqI</td>
<td>CC: 274, 63 CT: 337, 274, 63 TT: 337</td>
<td>(13)</td>
</tr>
<tr>
<td>TLR-3 _7 C/A (rs3775296)</td>
<td>GCATTGGAAAGCCATCCTGCTAAGTGCCGGCTGGTAATCT</td>
<td>279</td>
<td>MboII</td>
<td>AA: 257, 17 AC: 279, 257, 17 CC: 279</td>
<td>(13)</td>
</tr>
</tbody>
</table>
the distribution of TLR-3 _7 C/A, TLR-9 1237T/C across the two patients’ groups, but as regards TLR-3 c.1377C/T, the T- allele was found to be associated was advanced stage of fibrosis (p= 0.003), (Table 4).

After complete course of combined interferon and ribavirin therapy for 48 weeks, 86% of the patients tested negative for HCV-RNA by real time PCR while 14% were positive. Ninety three patients were followed up six months later, 72/93 (77.4%) achieved SVR where 21/93 (22.6%) tested positive for HCV-RNA.

TLR-3 c.1377 T/T genotype was associated with failure to achieve end of treatment response as well as SVR (p= 0.004 and 0.008 respectively). No association was found between TLR-3 _7 C/A and TLR-9 1237T/C different genotypes and response to treatment or SVR, (Tables 5, 6).

Age, gender, baseline laboratory data and degree of fibrosis were studied among responders and patients’ group who failed to achieve SVR, no statistically significant difference was found between the two groups (data not shown).

Table 3: The Distribution of TLR-3 _7 C/A, TLR-3 c.1377C/T and TLR-9 1237T/C genotypes in HCV Patients and Controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Patients (100)</th>
<th>Control (100)</th>
<th>P value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR3 _7 C/A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>2</td>
<td>3</td>
<td>1.000</td>
<td>0.748 (.122-4.599)</td>
</tr>
<tr>
<td>AC</td>
<td>24</td>
<td>14</td>
<td>0.076</td>
<td>1.923 (.927-3.989)</td>
</tr>
<tr>
<td>CC</td>
<td>74</td>
<td>83</td>
<td>REF</td>
<td></td>
</tr>
<tr>
<td>AA+AC</td>
<td>26</td>
<td>17</td>
<td>0.121</td>
<td>1.715 (.863-3.410)</td>
</tr>
<tr>
<td>Allele A</td>
<td>28</td>
<td>20</td>
<td>0.218</td>
<td>1.468 (.796-2.698)</td>
</tr>
<tr>
<td>Allele C</td>
<td>172</td>
<td>180</td>
<td>0.763</td>
<td>0.833 (.254-2.731)</td>
</tr>
<tr>
<td>TLR-1377 C/T</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>6</td>
<td>6</td>
<td>0.094</td>
<td>0.598 (.327-1.094)</td>
</tr>
<tr>
<td>CT</td>
<td>28</td>
<td>39</td>
<td>0.049</td>
<td>0.792 (.437-1.419)</td>
</tr>
<tr>
<td>CC</td>
<td>66</td>
<td>55</td>
<td>0.112</td>
<td>0.630 (.356-1.115)</td>
</tr>
<tr>
<td>TT+CT</td>
<td>34</td>
<td>45</td>
<td>0.190</td>
<td>0.730 (.456-1.619)</td>
</tr>
<tr>
<td>Allele T</td>
<td>49</td>
<td>51</td>
<td>0.745</td>
<td></td>
</tr>
<tr>
<td>Allele C</td>
<td>160</td>
<td>149</td>
<td>0.745</td>
<td></td>
</tr>
<tr>
<td>TLR-9 1237T/C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>3</td>
<td>3</td>
<td>1.060</td>
<td>0.474 (.019-2.464)</td>
</tr>
<tr>
<td>TC</td>
<td>19</td>
<td>10</td>
<td>0.813</td>
<td>0.901 (.381-2.130)</td>
</tr>
<tr>
<td>TT</td>
<td>78</td>
<td>37</td>
<td>0.683</td>
<td>0.803 (.365-1.768)</td>
</tr>
<tr>
<td>CC+TC</td>
<td>22</td>
<td>13</td>
<td>0.405</td>
<td>0.750 (.380-1.479)</td>
</tr>
<tr>
<td>Allele C</td>
<td>25</td>
<td>16</td>
<td>0.745</td>
<td></td>
</tr>
<tr>
<td>Allele T</td>
<td>175</td>
<td>84</td>
<td>0.745</td>
<td></td>
</tr>
</tbody>
</table>

OR: Odds ratio, CI: Confidence Interval, p-value <0.05 is considered significant.
### Table 4: Association of gene polymorphism and degree of liver fibrosis

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Degree of liver fibrosis</th>
<th></th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mild fibrosis F1 (n=65)</td>
<td>Advanced fibrosis F2, F3 (n=35)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Count</td>
<td>%</td>
<td>Count</td>
</tr>
<tr>
<td>TLR3 7C/A</td>
<td>AA</td>
<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td></td>
<td>AC</td>
<td>17</td>
<td>26.2%</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>48</td>
<td>73.8%</td>
</tr>
<tr>
<td></td>
<td>AA+AC</td>
<td>17</td>
<td>26.2%</td>
</tr>
<tr>
<td></td>
<td>Allele A</td>
<td>17</td>
<td>13%</td>
</tr>
<tr>
<td></td>
<td>Allele C</td>
<td>113</td>
<td>87%</td>
</tr>
<tr>
<td>TLR3-1377 C/T</td>
<td>TT</td>
<td>4</td>
<td>6.2%</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>10</td>
<td>15.4%</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>51</td>
<td>78.5%</td>
</tr>
<tr>
<td></td>
<td>TT+CT</td>
<td>14</td>
<td>21.5%</td>
</tr>
<tr>
<td></td>
<td>Allele T</td>
<td>18</td>
<td>14%</td>
</tr>
<tr>
<td></td>
<td>Allele C</td>
<td>112</td>
<td>86%</td>
</tr>
<tr>
<td>TLR-9 1237T/C</td>
<td>CC</td>
<td>1</td>
<td>1.5%</td>
</tr>
<tr>
<td></td>
<td>TC</td>
<td>10</td>
<td>15.4%</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>54</td>
<td>83.1%</td>
</tr>
<tr>
<td></td>
<td>CC+TC</td>
<td>11</td>
<td>16.9%</td>
</tr>
<tr>
<td></td>
<td>Allele C</td>
<td>12</td>
<td>9%</td>
</tr>
<tr>
<td></td>
<td>Allele T</td>
<td>118</td>
<td>91%</td>
</tr>
</tbody>
</table>

### Table 5: Association of gene polymorphism and response to treatment

<table>
<thead>
<tr>
<th>Genotype</th>
<th>HCV-RNA at Week 48 (End of treatment)</th>
<th></th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative (n=86)</td>
<td>Positive (n=14)</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td></td>
<td>Count</td>
<td>%</td>
<td>Count</td>
</tr>
<tr>
<td>TLR3 7C/A</td>
<td>AA</td>
<td>1</td>
<td>1.2%</td>
</tr>
<tr>
<td></td>
<td>AC</td>
<td>22</td>
<td>25.6%</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>63</td>
<td>73.3%</td>
</tr>
<tr>
<td></td>
<td>AA+AC</td>
<td>23</td>
<td>26.7%</td>
</tr>
<tr>
<td></td>
<td>Allele A</td>
<td>24</td>
<td>14%</td>
</tr>
<tr>
<td></td>
<td>Allele C</td>
<td>148</td>
<td>86%</td>
</tr>
<tr>
<td>TLR3-1377 C/T</td>
<td>TT</td>
<td>2</td>
<td>2.3%</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>25</td>
<td>29.1%</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>59</td>
<td>68.6%</td>
</tr>
<tr>
<td></td>
<td>TT+CT</td>
<td>27</td>
<td>31.4%</td>
</tr>
<tr>
<td></td>
<td>Allele T</td>
<td>29</td>
<td>17%</td>
</tr>
<tr>
<td></td>
<td>Allele C</td>
<td>143</td>
<td>83%</td>
</tr>
<tr>
<td>TLR-9 1237T/C</td>
<td>CC</td>
<td>2</td>
<td>2.3%</td>
</tr>
<tr>
<td></td>
<td>TC</td>
<td>17</td>
<td>19.8%</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>67</td>
<td>77.9%</td>
</tr>
<tr>
<td></td>
<td>CC+TC</td>
<td>19</td>
<td>22.1%</td>
</tr>
<tr>
<td></td>
<td>Allele C</td>
<td>21</td>
<td>12.2%</td>
</tr>
<tr>
<td></td>
<td>Allele T</td>
<td>151</td>
<td>87.8%</td>
</tr>
</tbody>
</table>
Table 6: Association of gene polymorphism and sustained virological response (SVR)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>HCV-RNA at Week 72 (SVR)</th>
<th>P value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative (n=72)</td>
<td>Positive (n=21)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Count</td>
<td>%</td>
<td>Count</td>
</tr>
<tr>
<td>TLR3_7C/A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>1</td>
<td>1.4%</td>
<td>1</td>
</tr>
<tr>
<td>AC</td>
<td>17</td>
<td>23.6%</td>
<td>5</td>
</tr>
<tr>
<td>CC</td>
<td>54</td>
<td>75.0%</td>
<td>15</td>
</tr>
<tr>
<td>AA+AC</td>
<td>18</td>
<td>25.0%</td>
<td>6</td>
</tr>
<tr>
<td>Allele A</td>
<td>19</td>
<td>13.2%</td>
<td>7</td>
</tr>
<tr>
<td>Allele C</td>
<td>125</td>
<td>86.8%</td>
<td>35</td>
</tr>
<tr>
<td>TLR3-1377 C/T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>1</td>
<td>1.4%</td>
<td>4</td>
</tr>
<tr>
<td>CT</td>
<td>20</td>
<td>27.8%</td>
<td>6</td>
</tr>
<tr>
<td>CC</td>
<td>51</td>
<td>70.8%</td>
<td>11</td>
</tr>
<tr>
<td>TT+CT</td>
<td>21</td>
<td>29.2%</td>
<td>10</td>
</tr>
<tr>
<td>Allele T</td>
<td>22</td>
<td>15.3%</td>
<td>14</td>
</tr>
<tr>
<td>Allele C</td>
<td>122</td>
<td>84.7%</td>
<td>28</td>
</tr>
<tr>
<td>TLR-9 1237T/C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>1</td>
<td>1.4%</td>
<td>1</td>
</tr>
<tr>
<td>TC</td>
<td>13</td>
<td>18.1%</td>
<td>4</td>
</tr>
<tr>
<td>TT</td>
<td>58</td>
<td>80.6%</td>
<td>16</td>
</tr>
<tr>
<td>CC+TC</td>
<td>14</td>
<td>19.4%</td>
<td>5</td>
</tr>
<tr>
<td>Allele C</td>
<td>15</td>
<td>10.4%</td>
<td>6</td>
</tr>
<tr>
<td>Allele T</td>
<td>129</td>
<td>89.5%</td>
<td>36</td>
</tr>
</tbody>
</table>

OR: Odds ratio, CI: Confidence Interval, p-value <0.05 is considered significant

Figure (1): Characterization of the TLR3_7 C/A polymorphism using MboII restriction enzyme. Ethidium bromide stained 4% agarose gel.

Cases 1,4,7 show Homozygous CC (wild genotype): 1 band 279 was detected.
Cases 2, 3, 5 show Heterozygous AC genotype: 3 bands 279, 257, 17 were detected.
Case 6 shows Homozygous AA genotype: 2 bands 257, 17 was detected.
M: PCR marker (50-100-150-200-250-300-350-400 bp etc)
N.B. The 17 bp band cannot be visualized in the horizontal gel electrophoresis.
Figure (2): Characterization of the TLR3 c.1377C/T polymorphism using Taq I restriction enzyme. Ethidium bromide stained 4% agarose gel.

Cases 1, 2, 4, 6, 8 show Homozygous CC (wild genotype): 2 bands 274 and 63 bp were detected.
Cases 3, 7 show Heterozygous CT genotype: 3 bands 337, 274 and 63 bp were detected.
Case 5 shows Homozygous TT genotype: 1 band 337 was detected.
M: PCR marker (50-100-150-200-250-300-350-400 bp etc).

Figure (3): Characterization of the TLR-9 (-1237 T/C) Polymorphism using BstNI restriction enzyme. Ethidium bromide stained 4% agarose gel.

Cases 1, 2, 3, 5 show Homozygous TT (wild genotype): 2 bands 108 and 27 bp were detected.
Case 6 shows Homozygous CC genotype: 3 bands 60, 48 and 27 bp were detected.
Cases 4, 7, 8 show Heterozygous TC genotype: 4 bands 108, 60, 48 and 27 bp were detected.
M: PCR marker (50-100-150-200-250-300-350-400 bp etc).
N.B. The 27 bp band cannot be visualized in the horizontal gel electrophoresis.
DISCUSSION

Knowing that, genetic variations in the TLR-signaling pathway contributed in either susceptibility or resistance to several infectious diseases,[56,58] we studied the impact of TLR-3 (c.1377T/C [rs3775290]), TLR-3 (c.7 C/A [rs3775296]) and TLR-9 (1237T/C [rs5743836]) SNPs on HCV infection.

The TLR-9 gene is located on chromosome 3p21.3 and spans approximately 5 kb, TLR-9 gene has a major coding region exon in one of its two exons.[56] TLR-9 has been demonstrated to bind only to DNA virus, so the binding of HCV which is single-stranded RNA (ssRNA) to TLR-9 is assumed unlikely.[49] However, studies demonstrated that TLR-9 mRNA and protein are down-regulated in peripheral blood mononuclear cells of HCV-infected patients compared to normal controls, and are negatively correlated with serum viral copies.[62]. In addition, TLR-9 stimulation showed antiviral effects in HCV-infected individuals,[62] and was found to participate in the early immune response against HCV infection of the central nervous system.[23]. On the other hand, some other studies showed that TLR-9 level was present at higher levels in HCV patients compared to healthy controls.[61]. Although data are controversial, it points that TLR-9 plays a role during HCV infection. In our study, the presence of TLR-9 1237T/C SNP is not associated with susceptibility to HCV infection or response to antiviral therapy. Similarly, Wei et al.[58] showed no significant association in regards to TLR9 (rs187084) genotype and allele frequency between chronic HCV patients and subjects who spontaneously cleared the virus.

In human, TLR-3-promoter region is responsible for maintenance of promoter integrity and promoter specific virus responsive element.[59]. In our study, the TLR-3 (c.7 C/A) SNP within the promoter region is not associated with HCV infection, and our results are comparable to results in other ethnicities.[7,13,40]. On the other hand, TLR-3 (c.1377 T/T) SNP was associated with failure of end of treatment response (p = 0.006) and failure to achieve SVR (p = 0.006). This can be attributed to unsuccessful host defense against viral infections which relies on early production of type I IFN and subsequent activation of a cellular cytotoxic response. TLR3, TLR7, or TLR8 recognize PAMP thus allow virus linkages with dendritic cell and subsequent activation and stimulation of dendritic cells, TLRs-SNPs result in disruption of PAMP recognition mechanism.[27,40]. In a recent study by Firdaus et al.[19] TLR-3 expression was found upregulated in patients who successfully cleared HCV infection, further supporting the antiviral role of TLR-3 in HCV infection.

Previous studies stated the association between TLR-9 and hepatic failure, where, TLR-9 signals caused hepatic failure by promoting TNF-α production.[15]. However, in our study no symptomatic significance was found as regards the distribution of TLR-9 1237T/C, TLR-3 (c.7 C/A in patients with mild hepatic fibrosis or advanced fibrosis. However, interestingly enough, TLR-3 c.1377 (T) - allele was found to be associated with advanced stage of fibrosis (p= 0.003), because significant hepatic fibrosis is closely related to failure of response to PEG- INF/ ribavirin therapy as well[44,49] and this matches with our results. Also, in the current study, patients with advanced hepatic fibrosis had significantly elevated AFP serum levels. This goes with the results of Hu et al.[26] who found that chronic hepatitis C patients had elevated serum AFP that was independently associated with stage III/IV hepatic fibrosis. Patients with elevated AFP serum levels are less likely to respond to therapy[3,13,19]. In our cohort of patients, non-responders were found to have elevated serum AFP levels however these levels did not reach statistical significance.

We assumed that both TLR-3 and TLR-9 signaling mechanisms work in synergy to establish an antiviral state against HCV infection, so we analyzed whether combined genetic variants in TLRs act as a potential indicator for Egyptian host susceptibility to HCV infection, but co-inheritance of the genetic polymorphism in the three studied genes didn’t confer increased risk to HCV infection.

In conclusion, we aimed to demonstrate associations between TLR-3 and TLR-9 SNPs and;
susceptibility, therapy outcome and effect on fibrosis progression in HCV-infected Egyptian patients; as in the recent years, TLRs are gaining increased importance due to their role in influencing host immunity. It has been suggested to use TLRs as biomarkers for HCV pathogenesis\(^\text{19}\). Also, TLRs use as agonists in antiviral therapy has been studied\(^\text{33}\). In our study, TLR-3 (c.1377 (T) – allele was found to be associated with failure of response to therapy and advanced fibrosis stage, however, as regards TLR-3 – allele was found to be associated with failure of response to therapy and advanced fibrosis stage, however, as regards TLR-9 1237T/C, TLR-3_7 C/A, no significant association was found and the results are comparable between the patients and control groups. Our study has certain points of strength as well as certain limitations; the most important issue is that most of Egyptian patients are infected with HCV genotype-4 and the predominant subtype is HCV-4d\(^\text{47}\), thus the obtained results were not fragmented due to inclusion of various HCV-genotypes as the pathogenesis of infection and IFN responsiveness vary according to genotype\(^\text{16,39,59,69}\). On the other hand, the study encompassed a limited number of cases. Further studies with larger samples of patients are required to further add to the validity of our results, and also studies including other members of TLR family identified in humans are required.

REFERENCES:


TLRs-SNP and HCV: Response to Therapy
تأثير التعدد الشكلي لجين مستقبلات شبيه التول -9 و 3 على الإصابة بفيروس الكبد الوبائي- سي، الاستجابة للعلاج و درجة التلف الكبدي بين المرضى المصريين

رانيا زايد – دalia عمران – زينب زكرياء - سهيلة غزت - سلوى توفيق - حسام السويسى

المصابة بفيروس الكبد الوبائي، سي يعتبر وسيلة مستمر في مصر. عرفت مستقبلات شبيه التول بالقيام بدور مهم في تثبيز الجهاز المناعي لمقاومة الأمراض المعدية. في هذا البحث قمنا بدراسة تأثير التعدد الشكلي لجين مستقبلات شبيه التول -9 و 3 على الإصابة بفيروس الكبد الوبائي، سي، الاستجابة للعلاج و درجة التلف الكبدي بين المرضى المصريين. شمل البحث 100 مريض مصاب بفيروس الكبد الوبائي، سي و 100 شخص من الأصحاء و المتطابقين في العمر والجنس كعينة ضابطة. تم دراسة التعدد الشكلي الجيني لكل TLR-3 (_7 C/A [rs3775296] تLR-3 (c.1377C/T [rs3775290] (1237T/C [rs5743836 من: 36 إظهار البحت النتائج التالية: لا يوجد اختلاف بين المرضى و العينة الضابطة من الأصحاء في تمثيل التعدد الشكلي لجين مستقبلات شبيه التول (TLR-3 c.1377T) و (TLR-3 [c.1377C/A), TLR-3 (c.1377C/T) and TLR-9 (1237T/C التول النتاج). كان مرتبطة بعدم الاستجابة للعلاج وله تأثير على تقدم درجة التليف الكبدى في المرضى المصريين المصابين بفيروس الكبد الوبائي، سي.
ROLE OF PLACENTAL PROTEIN 13 AS A BIOMARKER FOR EARLY DETECTION OF PRE-ECLAMPSIA. METHYLENETETRAHYDRO FOLATE REDUCTASE GENE POLYMORPHISM AND RELATION TO DISEASE RISK, AN EGYPTIAN STUDY

Engy El Khateeb* and Sherif Dahab**

ABSTRACT

Objective: to investigate the value of maternal serum Placental Protein 13 (PP13) measurement and uterine artery indices (Resistance Index (RI) and Pulsatility Index (PI) ) Doppler during first trimester screening as the prediction of early pre-eclampsia with genotyping for detection of Methylenetetrahydrofolate reductase gene (MTHFR) polymorphisms (MTHFR677 C/T). Subjects and Methods: Fifty pregnant females were divided into two groups, twenty five as a control group and twenty five as high risk group; they were subjected to uterine artery Doppler, measurement of maternal serum (pp-13) and detection of (MTHFR) gene polymorphisms in first trimester at 11 to 14 weeks of gestation, all pregnancies were followed until 40 weeks for development of pre-eclampsia. Results: In the cases that developed pre-eclampsia- in both groups- in comparison with un-affected pregnancies it was noticed that they had low serum level of (PP-13), detection of (MTHFR) gene polymorphisms and higher (PI and RI) which were of statistically significant difference. Conclusion: Lower level of maternal serum PP-13 in first trimester, high prevalence of (MTHFR) gene polymorphisms and abnormal increase in uterine artery indices (PI and RI) are associated with developing pre-eclampsia several months later in pregnancy so they can be used for early prediction of pre-eclampsia in first trimester.

INTRODUCTION

Preeclampsia (PE) is a pregnancy-specific disease defined by new-onset hypertension and proteinuria after 20 weeks of gestation. It is present in around 5–10% of all pregnant women worldwide(5) and is associated with an increased risk of maternal and fetal morbidity and mortality, and recently it has been associated with an increased risk of later-life death due to cardiovascular disease for both mother and child(2, 10,12).

Several factors have been suggested to be involved in the etiology of PE, but there is consensus that the first step in the pathogenesis of this disease is a defective trophoblastic invasion early in pregnancy(18), which leads to reduced placental perfusion and hypoxia(13).

Placental protein 13 (PP-13, galectin-13) was first isolated in 1983(3,25). It is a homodimer which is linked by disulfide bonds and has special hemostatic and immunobiological functions at the feto-maternal interface or a developmental role in the placenta(24).

Placental protein 13 (PP-13) is a member of the galectin family, predominantly expressed by the placenta, specifically by the syncytiotrophoblast in which it is localized on the brush border membrane at the maternal fetal interface, recently maternal serum (PP13) concentrations were found to be significantly reduced during the first trimester among women who subsequently developed pre-eclampsia(24).

Intracellular folate hemostasis depends on the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene, which is located at position 36 on the short arm of chromosome 1(27). This gene codes for the enzyme MTHFR, which catalyses the irreversible conversion of 5,10-MTHFR to 5-metlytetrahydrofolate, a substrate for methylation of homocysteine to methionine. 5,10-MTHFR C677T is located in the catalytic N-terminal domain of the enzyme, while 5,10-MTHFR A1298C is located in the regulatory domain of the enzyme(7).

Biochemically, the 5,10-MTHFR C677T polymorphism is associated with thermolability and reduced enzyme activity. The metabolic consequences are folate deficiency and mild hyperhomocysteinemia, a risk factor for thrombotic vascular diseases. It has been sug-
gested that oxidative stress, platelet aggregation, and endothelial cell dysfunction contribute to the vasculotoxicity of homocysteine, and 5,10-MTHFR polymorphisms have been widely investigated in relation to a spectrum of several disease outcomes. Specifically, several studies have identified maternal 5,10-MTHFR C677T polymorphisms as obstetric genetic risk factors for spina bifida, placenta-related vasculopathies, spontaneous fetal loss, preterm delivery (PTD), low birth weight (LBW), small for gestational age (SGA), neuro developmental delays, and other congenital anomalies.

Doppler ultrasonography is a non-invasive method for studying the uteroplacental circulation, provides the capability to qualitatively evaluate blood flow in small branches of the uterine arteries, in normal pregnancy, impedance to flow in the uterine arteries decreases with gestation, however in cases of impairment of trophoblastic invasion, doppler studies showed increased impedance to flow in the uterine arteries as they failed to develop into low resistance vessels.

The aim of this work was to assess uterine artery Doppler and maternal serum placental protein 13 (PP-13) indices as early predictors for pregnancy induced hypertension of occurrence of pre-eclampsia and detection of MTHFR gene polymorphisms in those patients.

SUBJECTS AND METHODS.

This prospective observational case-control study was performed in Obstetrics and Gynecology Department Kasr El Aini Maternity Hospital (Cairo University). It was conducted on 50 pregnant females who were recruited from the Antenatal Care Clinic in Kasr El Aini Maternity Hospital.

Fifty pregnant females were divided into two groups, twenty five as a control group and twenty five as high risk group; they were subjected to uterine artery Doppler, measurement of maternal serum (PP-13) and detection of (MTHFR) gene polymorphisms in first trimester at 11 to 14 weeks of gestation, all pregnancies were followed until 40 weeks for development of pre-eclampsia.

PP13 ELISA Assay:

- Blood samples were collected with all aseptic precautions from all study subjects. Samples were centrifuged and serum was separated and stored at -20 degrees Celsius until analysis.

- The blood samples were cooled and centrifuged immediately after collection and the erythrocytes removed. Serum PP-13 level was determined by enzyme linked immune sorbent assay (ELISA) kit (Cusabio, USA).

- Detection of (MTHFR677 C/T) polymorphisms by PCR-RFLP:

- Three ml of blood were withdrawn from all the subjects included in the study in a sterile ethylenediaminetetraacetic acid (EDTA) vacutainer. DNA was extracted from the whole blood using DNA extraction kit. (QIAamp Blood Kit (Cat. No. 51106; Qiagen Inc., Valencia, CA) according to the manufacturer’s instructions.

- The method described by Frosst et al.1995, was used for detection of the 677 C→T polymorphism. A length of 198 base pairs on exon 4 of the MTHFR gene was amplified using (5’ TGA AGG AGA AGG TGT CTG CGG GA 3’) as the forward primer and (5’ AGG ACG GTG CGG TGA GAG TG 3’) as the reverse primer.

- The C to T polymorphism at codon 677 introduces a restriction site for enzyme Hinf 1. The PCR was carried out in a total volume of 25 µL containing about 200 ng/ml DNA, 2 µl of 2.5 mM of each of the dNTP’s, 0.5 µl of 25 mM MgCl₂, 10 pmol of both primers and 0.5 U Taq polymerase. PCR cycling conditions were: an initial denaturation step at 94°C for 5 minutes, and 30 cycles of the following: 94°C for 1 minute, 57°C for 1 minute, and 72°C for 15 seconds. This was followed by a 10 minute extension at 72°C. Restriction digestion with Hinf 1 (Fermentas, INC, USA) was carried out on 2 µl buffer, 1 µl Hinf 1, and 8 µl of PCR amplicons incubated at 37°C for 4 to 6 hours. The digested fragments were separated on 2.5% agarose gel electrophoresis stained with ethidium bromide. The electrophoretic pattern was visualized un-
under UV light then photographed using a Polaroid camera with a red orange filter. Wild type (677CC) showed a single band at 198 bp. The presence of the ‘T’ allele introduces a cut among heterozygous (677 CT) and three bands of 198 bp, 175 bp and 23 bp were seen. The homozygous (677 TT) had two bands of 175 bp and 23 bp. The size of the amplified product was read with the use of a DNA ladder of different molecular weights (fermentas, No Limits™ 100 bp DNA Fragment, catalogue number SM1441).

Ultrasonography assessment:
- The ultrasound equipment used for both vaginal sonography and colour Doppler technique was Elegra (Siemen), trans-vaginal 6.5 MHZ ultrasound with pulsed and colour Doppler. The high pass filter was set at 50-100 HZ and the pulse repetition frequency ranged between 1000-2000 HZ.
- Each woman underwent a trans-vaginal ultrasonography examination including colour Doppler techniques to assess the following data:
  - Gestational age was determined from the onset of the normal period, measurement of the crown–rump length(CRL) was done to confirm the fetal gestational age, fetal viability and careful search for any fetal abnormalities beside to the resistance index (RI) and pulsative index (PI) in the uterine arteries both side right and left.
  - All the scans were performed by the same ultrasound machine by senior staff that has extensive experience in early first trimester scans.
  - Uterine arteries were seen as aliasing vessels at the level of the cervical-corporeal junction with the colour Doppler technique, and blood velocity waveform were obtained by placing the Doppler gate over the coloured areas and activating the pulsed Doppler function. Angel correction was then applied and the signal updated until at least four consecutive flow velocity wave form of good quality were obtained then the RI and PI of the right and left arteries was calculated to determine the vascular resistance. The presence or absence of an early diastolic notch in the flow profile of uterine arteries was noted and determined if unilateral or bilateral was recorded. All cases were followed until termination of pregnancy to detect the development of hypertensive disorders of pregnancy by serial clinical examination, blood pressure measurement and, urinary proteins.

Statistical Analysis
- Data were statistically described in term of range, mean and standard deviation. Numerical data were analysed by using unpaired student’s t test. Non parametric data were analysed using (Mann Whitney test) (for independent samples). P value less than 0.05 was considered statistically significant, Receiver Operator Characteristic (ROC) analysis was used to determine the optimum cut-off value for the studied diagnostic markers.

RESULTS
The current study was carried out on 50 pregnant females divided into two groups, twenty five as a control group and twenty five as high risk group.

The high risk group age ranged between 23 to 35 years with a mean value of 28.5±3.58. Control group age ranged between 21 and 39 years with a mean of 28.4±4.4. The gestational age of high risk group ranged between 11-13.5 weeks with a mean of 12.0±1.01. Control group gestational age ranged between 10-14 weeks with a mean of 11.9±0.79. There were no statistically significant differences between the 2 groups as regards age (p value >0.05) or gestational age (p value >0.05).

Two cases out of the 25 control cases developed pre eclampsia (PE) while 23 cases didn’t develop PE.

Four cases out of the 25 high risk group cases developed PE while 21 cases didn’t develop PE.

As regards the PP13 results of control subjects, in the PE group the PP13 index was (27.5±3.535) Pg/ml and in the non PE group it was (231.13±67.1) Pg/ml, by comparing the two groups P value was < 0.0001 which is of highly significant difference.
Table (1): Comparison between Control subjects and high risk subjects regarding Doppler ultrasonography results

<table>
<thead>
<tr>
<th></th>
<th>Control Subjects</th>
<th>High Risk Subjects</th>
<th>P-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PE (n=2) Range (mean±SD)</td>
<td>NO – PE (n=23) Range (mean±SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right mean uterine artery RI</td>
<td>0.79-0.81 (0.8±0.01)</td>
<td>0.6-0.79 (0.72±0.04)</td>
<td>0.0109</td>
<td></td>
</tr>
<tr>
<td>Left mean uterine artery RI</td>
<td>0.85-0.9 (0.87±0.03)</td>
<td>0.6-0.88 (0.72±0.08)</td>
<td>0.0163</td>
<td></td>
</tr>
<tr>
<td>Right mean uterine artery PI</td>
<td>2.32-2.34 (2.33±0.01)</td>
<td>0.96-2.2 (1.44±0.3)</td>
<td>0.0004</td>
<td></td>
</tr>
<tr>
<td>Left mean uterine artery PI</td>
<td>2.32-2.34 (2.33±0.01)</td>
<td>0.96-1.6 (1.37±0.17)</td>
<td>0.0001</td>
<td></td>
</tr>
</tbody>
</table>

R value < 0.05 is considered significant
PE: Pre-eclampsia
RI: Resistance Index
PI: Pulsatility Index

Table (2): Genotype and allele frequencies in high risk and control groups for MTHFR 677C→T and MTHFR 1298 A→C polymorphisms

<table>
<thead>
<tr>
<th>Genotypes, alleles</th>
<th>High risk group (n=25)</th>
<th>Control group (n=25)</th>
<th>OR(95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR677</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-CC</td>
<td>15(60%)</td>
<td>21(84%)</td>
<td>0.154(0.034-0.546)</td>
<td>0.002[S]</td>
</tr>
<tr>
<td>-CT</td>
<td>5(20%)</td>
<td>3(12%)</td>
<td>2.4(1.00-13.5)</td>
<td>0.032[S]</td>
</tr>
<tr>
<td>-TT</td>
<td>5(20%)</td>
<td>1(4%)</td>
<td>6.3(1.15-8.76)</td>
<td>0.028[S]</td>
</tr>
<tr>
<td>-C allele</td>
<td>35(46.6%)</td>
<td>91(60%)</td>
<td></td>
<td>0.024[S]</td>
</tr>
<tr>
<td>-T allele</td>
<td>15(20%)</td>
<td>5(6.6%)</td>
<td></td>
<td>→0.000[HS]</td>
</tr>
</tbody>
</table>
As regards the PP13 results of high risk group cases, in the PE group the PP13 index was (25.2 ± 4.20) pg./ml in comparison with non-PE group PP13 level which was (140.4 ± 57.09) pg./ml with p value < 0.0035 which was of significant difference.

The results of Doppler assessment regarding high risk group and control cases were summarized in the tables from Table 1.

The P values of comparing the right and left uterine arteries PI & RI indices in high risk group and control group were <0.05 with highly significant differences.

The results of genotype and allele frequencies in high risk and control groups for MTHFR 677C→T and MTHFR 1298 A→C polymorphisms are summarized in Table 2.

The results showed significant difference between high risk and control groups in CC, CT and, TT alleles of MTHFR gene (p values 0.002, 0.032 and 0.028 respectively).

The results showed also significant difference and highly significant difference between high risk and control groups in frequencies of C allele and T allele in MTHFR gene (p values 0.024 and 0.0001 respectively).

**DISCUSSION**

Pre-eclampsia is a disorder of pregnancy characterized by high blood pressure and large amounts of protein in the urine. Though present in the majority of cases, protein in the urine need not be present to make the diagnosis of preeclampsia(16,6).

There are a host of contributing and related factors that complicate finding a precise mechanism for preeclampsia(22). These include immunologic, hematologic, genetic, and environmental factors. Central to the effects of preeclampsia are the resulting presence of uteroplacental hypoxia, an imbalance in angiogenic and antiangiogenic proteins, oxidative stress, maternal endothelial dysfunction, and elevated systemic inflammation(22,1).

We are concerned in our study with serum level of PP13 and uterine arteries Doppler evaluation as first trimester marker for predicting the occurrence of preeclampsia and screening of genotype and allele frequencies in patients and control groups for MTHFR 677C→T and MTHFR 1298 A→C polymorphisms.

In our study we had two groups: control group (n=25) and high risk group (n=25) in control group two patients (n=2) developed pre-eclampsia (8%) and other patients didn’t develop it; serum PP13 level showed highly statistically significant difference p<0.0001.

In high risk group comparing serum PP13 level in patients who developed preeclampsia (n=4), (16%) and patients who didn’t develop it (n=21) there was highly statistically significant difference between both of them p<0.003. So it was observed that serum level of PP13 was low in patients developed preeclampsia comparing with other patients who didn’t develop it in both groups (control group and high risk group).

A prospective observational study was done by maternal blood testing at 6-10 gestational weeks demonstrated significantly decreased serum level PP13 in women who developed preeclampsia several months. Later, with sensitivity 80% and P<0.001 and it is possible that if combined with other variables such as BP, maternal history, and uterine artery Doppler flow velocity may have prediction in low risk population(19).

Another study showed that PP13 declined with maternal weight and was lower in in vitro fertilization. Levels were converted into multiples of the median (MoMs) accordingly. In twins, the median was 1.74 MoM (n=76) vs. 1.00 in singletons (n=676, P<0.0001). Among twins with severe PE (n=10), the median was 1.53 MoM vs. 1.74 in unaffected twins (P=0.10), and 2.26 (n=6) for mild PE (P=0.30). Among singletons with severe PE, the median was 0.44 MoM (n=26, P<0.0001), and for mild PE 0.62 (n=17, P<0.001)(23).

In our study, Doppler of uterine arteries were chosen as they were the most common arteries used to evaluate utero-placental circulation in pregnancy through analysis of uterine artery flow velocity wave form for prediction of preeclampsia.

In control group it showed that right and left
uterine arteries RI were higher in women who developed pre-eclampsia than those with normal outcome (P = 0.0109 and 0.0163 respectively) which is statistically significant.

As for high risk group also right and left uterine arteries RI were high in women developed pre-eclampsia in comparison with women who did not develop it (P=0.027 and 0.0190 respectively) which were found to be highly statistically significant.

In control group there was highly statistically significant difference between pre-eclampsia group (who developed pre-eclampsia) and normal group (who did not develop it) regarding to right and left uterine arteries PI (P=0.0004 and 0.0001 respectively).

Examination of 3058 patient, using T.V.S uterine artery Doppler at 11-14 weeks with singleton pregnancies was done, this study has found that presence of pre-eclampsia because of high prevalence of this finding in normal pregnancies(45%) with low specificity (55%), sensitivity (55%)(11) and this finding is consistent with pervious study which concluded that early diastolic notch was unlikely to be useful screening for pregnancy complications(8,9).

In our study early diastolic notch was not recorded except in two patient in high risk group which was statistically insignificant as P value was <0.05, with specificity 35% and sensitivity 22%, we have no clear explanation for this finding but may be our sample size was limited in comparing with other studies numbers (11).

More recent studies showed comparable results, they had larger studies including 1091, 384, 1123 singleton pregnancies for routine prenatal ultrasound examination at 11-14 weeks of gestation, as regarding the prognostic value of unilateral / bilateral uterine artery notching in predicting of pre-eclampsia(14).

In our study we revealed that significant associations were detected between MTHFR C677T polymorphism and risk of PE in patients vs. controls for CC (OR = 0.154, 95% CI:0.034-0.546), CT (OR = 2.4, 95% CI: (1.00-13.5)), and TT genetic model (OR = 6.3, 95% CI: 1.15-8.76).

A recent study revealed that significant associations were detected between MTHFR C677T polymorphism and risk of PE in the overall population for TT vs. CC (OR = 1.280, 95% CI: 1.074-1.525), recessive model (OR = 1.264, 95% CI: 1.067-1.303), and dominant genetic model (OR = 1.174, 95% CI: 1.057-1.303); in Caucasian population for dominant model (OR = 1.136, 95% CI: 1.022-1.263), and in East Asia population for TT vs. CC (OR = 2.199, 95% CI: 1.366-3.924) CT vs. CC (OR = 1.453, 95% CI: 1.001-2.109), recessive model (OR = 1.742, 95% CI: 1.202-2.525), and dominant model (OR = 1.783, 95% CI: 1.271-2.501). Conversely, no associations were detected in Latin America, South Asia, and Africa populations(26).

Xia et al., 2013 discussed a number of studies that investigated the association between the methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism and the risk of pre-eclampsia (PE) in various populations and have delivered inconsistent results. Therefore, this meta-analysis of 36 case-control studies, comprising 4253 PE cases and 4950 controls, were assessed to evaluate a possible association. The pooled results showed that the MTHFR C677T polymorphism was significantly associated with PE (P=0.03, odds ratio (OR)=1.25, 95% confidence interval (CI)=1.02-1.54, for the additive comparison; P=0.04, OR=1.14, 95% CI=1.01-1.29, for the dominant genetic model). The results of the subgroup analysis showed that MTHFR 677T had the effect of increasing the PE risk for the recessive genetic model (P<0.0001, OR=1.76, 95% CI=1.33-2.33, P(heterogeneity)=0.28), the additive comparison (P=0.002, OR=2.09, 95% CI=1.31-3.31, P(heterogeneity)=0.08) and allele contrasts (P=0.03, OR=1.42, 95% CI=1.04-1.95, P(heterogeneity)=0.0001) in the Asians, while no evidence of an association between MTHFR C677T polymorphisms and PE was observed in the Caucasians. This meta-analysis suggests that the MTHFR C677T polymorphism is capable of causing PE susceptibility in the Asians but not in the Caucasians(28).

Another study revealed that the frequency of combined genotypes of MTHFR CT and TT (CT+TT) and T allele tended to be higher in se-
PP-13 MTHFR Gene Polymorphism and Pre-eclampsia

were preeclamptic women compared to controls. A significantly higher level of triglycerides was observed in the presence of combined genotypes of MTHFR CT and TT in preeclamptic women compared to controls with the same genotype.17

Finally in our study we confirmed the potential role of serum Placental Protein 13 (PP-13) in combination with uterine arteries Doppler indices (PI and RI), as early predictors of pre-eclampsia in first trimester of pregnancy and that there is an association between the MTHFR C677T polymorphism and susceptibility to preeclampsia.

REFERENCES

16. Pregnancy, developed by the Task Force on Hypertension in (2013). Hypertension in pregnancy..


دور البروتين المشيمي 13 في الاكتشاف المبكر لتسام الحمل وعلاقة تعدد الشكل الجيني

المثيلينهيديروفلوتات ريديكترز وعلاجه بخطر حدوث المرض (دراسة مصرية)

النجي الخطيب - شريف دهش

يهدف البحث لفحص قيمة البروتين المشيمي 13 في دم الأم (PP-13) وقياس مؤشرات الشريان الرحمي (Pulsatility (PI) ومؤشر المقاومة (RI)). (MTHFR677 C / T) المبكر بما قبل تسام الحمل مع التنبؤ الجيني للمثيلين تراثيين. وتتم تقسيم الأمهات إلى مجموعتين، خمسة وعشرين كمجموعة مراقبة وخمسة وعشرين مجموعة الخبراء؛ وقد تم عمل الدور بالشرج الرحمي، وقياس (بروتين 13) والكشف عن الأشكال الجينية لـ (MTHFR) في الأمهات الثلاثة لأولياها، بين 11 إلى 14 أسبوعًا من الحمل، وتم متابعة جميع حالات الحمل حتى 40 أسبوعًا لتأكيد تسام الحمل، وتم الحصول على أنه في الحالات التي أُصيبت بـ eclampsia-pre-PP-13، وفي الأشكال الجينية لـ (MTHFR) (PP-13) وارتفاع (RI) وارتفاع (MTHFR) (PP-13) والأشكال الجينية لـ (MTHFR) (PP-13) وارتفاع (RI) وارتفاع (PI) والمجموعة بالمقارنة مع الحالات التي لم تصابا. وظلت نسبًا أدنى ونسبةً أعلى في النسبية.(PP-13) وزيادة غير طبيعية في الـ (MTHFR) في الأشهر الثلاثة الأولى، وارتفاع معدل انتشار الأشكال الجينية لـ (MTHFR) (PP-13) مع حدوث ما قبل تسام الحمل بعد عدة أشهر في الحمل حتى أنها يمكن أن تستخدم للتنبؤ المبكر للتسام الحملي في الثالث من الحمل.
CORD BLOOD COPEPTIN AND CALPROTECTIN AS POTENTIAL BIOMARKERS OF EARLY-ONSET NEONATAL SEPSIS
Manal Mohsen*, Wafaa K.Zaki**, Rania Ismail***, Nehal El-Raggal*** and Mohamed A. Abd El-Wahed***

ABSTRACT

Background: The correct diagnosis of neonatal sepsis is a relevant problem because sepsis is one of the most important causes of neonatal morbidity, mortality, and prolonged hospital stay. Therefore, early diagnosis and treatment of neonates with suspected infection is crucial to prevent life threatening complications. New infection markers may potentially improve guidance of therapeutic decisions. Copeptin and Calprotectin are two markers which have been recently demonstrated to be strongly elevated in samples from adult patients with sepsis. However a few reports are available in neonates and pediatric patients. Objective: The aim of this study was to investigate the clinical utility of assaying cord blood copeptin and calprotectin as markers of early-onset neonatal sepsis. Subjects and Methods: This case control study comprised 138 newborn infants with gestational age of 28-40 weeks and birth weights between 1500 and 4100 grams, delivered between February 2014 and January 2015 at the Maternity Departments of Manshiet El-Bakry and Ain Shams University Hospitals, Cairo, Egypt. They were transferred to the Neonatal Intensive Care Unit (NICU) of the hospital because of having at least one risk factor for suspicion of neonatal sepsis. On the basis of clinical observation over their first 5 postnatal days and sepsis work-up results, into 2 groups: Early onset neonatal sepsis (EOS) group (n=78) and non-septic control group (n=60). For all included population, two cord blood samples were collected for blood culture, and assay for Copeptin and Calprotectin by competitive and sandwich enzyme immunoassays (EIA), respectively. Results: Results of both inflammatory markers were significantly higher in septic neonates than controls [median copeptin concentration in cord blood was 212 (IQR 95 – 537) vs. 82(60 – 125) pg/mL, and median calprotectin concentration was 4.6(4.0-9.1) vs. 0.93(0.7-2.0) ng/mL for calprotectin, respectively; p<0.001 for both]. Levels of copeptin and calprotectin did not associate with CRP results, only calprotectin levels associated with culture positivity, however, both tests significantly correlated with Rodwell’s score indicating that they are good diagnostic tests for early-onset septicemia. Moreover, copeptin and calprotectin levels were significantly associated with patients’ outcome. Receiver-operating-characteristic (ROC) curve analysis showed that copeptin cord blood concentrations were strongly associated with EOS: sensitivity of 73.3%, a specificity of 100%, positive predictive value (PPV) of 100%, negative predictive value (NPV) of 87.5% and efficacy 81%. ROC curve for calprotectin showed 100%, sensitivity and 97.5%, specificity. PPV is 98%, NPV is 100% and efficacy is 98.9%. Conclusion: the current study reveals that copeptin and calprotectin may be considered two promising sensitive and specific predictors for EOS. Keywords: neonatal sepsis, neonatal infection, EOS, copeptin, calprotectin.

INTRODUCTION

Sepsis is one of the most important causes of neonatal morbidity, mortality, and prolonged hospital stay, particularly in preterm, due to their immature immune response and exposure to infectious risks in neonatal intensive care units (NICUs) (11,5). In this issue, an early diagnosis becomes crucial as signs of sepsis in the newborn are nonspecific(15,19).

Blood culture remains the gold standard test for diagnosis of neonatal sepsis. However, the results of blood culture are not available before 24–48h and there are possible false negative responses in many instances. For this reason, a broad spectrum of inflammatory markers has been proposed for the diagnosis of neonatal sepsis (10).

Copeptin is a novel neuroendocrine peptide. It is 39 amino acids glycopeptides co-synthesized with arginine vasopressin (AVP) and released together in stoichiometric pattern from the hypothalamus upon stimulation of AVP release. Due to difficulties of AVP assay, copeptin largely replaced it in clinical assay as surrogate biomarker because copeptin has easier and more valid measurement methods. In acute stress condition, copeptin rises and reflects stress level exactly like AVP which was largely known as a mediator of non-specific stress conditions (1).

Calprotectin is antimicrobial, calcium and zinc binding heterocomplex protein. It is contained in the cytosol fraction of innate immunity cells and released immediately after host-pathogen interaction. For this it
has been used as a nonspecific marker for activation of granulocytes and mononuclear phagocytes\(^{(27,20)}\). In addition to protecting cells against microorganisms, calprotectin regulates adhesion of leukocytes to the endothelium and extracellular matrix during the inflammatory process\(^{(28)}\). So calprotectin has been proposed for the diagnosis of inflammatory conditions.

**Aim of the Work**

The aim of this study was to investigate the clinical utility of assaying cord blood copeptin and calprotectin as markers of early-onset neonatal sepsis.

**SUBJECTS AND METHODS**

**I. Subjects:**

The study series comprised 138 newborn infants with gestational age of 28-40 weeks and birth weights between 1500 and 4100 grams, delivered between February 2014 and January 2015 at the Maternity Departments of Manshiet El-Bakry and Ain Shams University Hospitals, Cairo, Egypt. They were transferred to the Neonatal Intensive Care Unit (NICU) of the hospital because of having at least one risk factor for suspicion of neonatal sepsis (prolonged premature rupture of membranes > 24 hr, premature onset of labour, chorioamnionitis or peripartum maternal fever). EOS cases were defined as neonates who presented with sepsis within the first 72 hours of life as defined by the following criteria\(^{(25)}\): i) at least two clinical signs of sepsis (temperature instability, irritability or apathy, feeding difficulties, poor capillary refill >2 seconds, apnea, tachycardia and/or tachypnea); ii) elevated C-reactive protein >20 mg/L, iii) decision of the attending physician to treat for at least 7 days with intravenous antibiotics and iv) recovery of bacterial pathogens in blood-culture. In infants with negative blood cultures but clinical diagnosis of EOS, all first three criteria mentioned above were required to be present.

**Exclusion criteria:**

- Maternal diabetes or pre-eclampsia.
- Chromosomal abnormalities or major congenital malformations.
- Inborn error of metabolism.

Cord blood samples were withdrawn immediately at birth for determination of:

a. Complete blood counts (CBC) with differential smear: using ABX Micros 60 cell counter (ABX DIAGNOSTICS, Rue du caducée, ParcEuromédecine, Montpellier Cedex, France)

b. Semiquantitative measurement of serum C-reactive protein (CRP) concentration, using latex agglutination test using the AVITEX CRP commercial kit (Omega Diagnostics Ltd, NOVA Century Scientific, South Service Road, Burlington, Ontario)

c. Blood culture for isolation of pathogenic microorganisms.

d. Measurement of copeptin and calprotectin concentrations by enzyme immunoassays using commercially available copeptin (Human) EIA Kit (Phoenix Pharmaceuticals, Inc.Beach Rd., Burlingame, California, USA) and Calprotectin ELISA Kit (MRP 8/14) (Immundiagnostik AG Stubenwald-Allee, Bensheim, Germany), respectively.

The neonates were assigned retrospectively, on the basis of clinical observation over their first 5 postnatal days and sepsis work-up results, into 2 groups:

1. **Early Onset Neonatal Sepsis group (EOS) (n=78):**

   Cases diagnosed in the first 72 hours of life according to the clinical score of Tollner, and hematological score of Rodwell. They were 31 (39.8%) males and 47 (60.2%) females; 19 (24.4%) were delivered vaginally and 59 (75.6%) by cesarian section. They had a mean gestational age of 32.0 ±3.6 weeks, and a mean birth weight of 2845±766 grams.

2. **No infection, (non-septic) Control group (n=60):**

   Infection was ruled out on both clinical and laboratory basis. They were 22 (36.7%) males and 38 (63.3%) females; 16 (26.7%) were delivered vaginally and 44 (73.3%) by cesarian section. They had a mean gestational age of 36.5±3.88 weeks, and a mean birth weight of 3217±569 grams. They served as a control group.
**Procedure of blood sampling:**

An informed written consent was taken from the parents before enrollment of neonates in this study. Cord blood samples were collected under complete aseptic conditions immediately after birth. The collected blood was divided among a blood culture bottle, an EDTA tube for complete blood counts, and a plain test tube for serum separation. Blood withdrawn into the plain tubes were allowed to clot for 30 minutes before centrifugation for 15 minutes at approximately 1000xg. Serum was separated, for immediate assessment of C-reactive protein and stored at -20°C for the assay of copeptin and calprotectin. Hemolysed samples were discarded, repeated freezing and thawing was avoided.

**Procedure of blood culture:**

Using a signal blood culture system (oxoid), the formulation of medium enhances the growth of aerobic, anaerobic, micro-aerophilic and fungi. Two sets of blood culture were drawn from each patient under complete aseptic condition. The medium were designed to create pressure in selected bottle when organisms are growing causing displacement of blood/broth mixture into the chamber as assign of microbial activity. Examination of blood culture bottle was done every other day, any bacterial growth was identified by subculture on solid blood agar, Mac-Conkey agar and Sabouraud’s dextrose agar (SDA). isolates were identified on the basis of conventional identification methods described in Colle et al (1996) based on colonial morphology, microscopic examination of Gram stained films and biological activity of isolated strain.

**Principle of Copeptin and Calprotectin enzyme immunoassays (EIA):**

The assay of copeptin is based on a competitive immunoassay technique. In this technique, the immunoplate is pre-coated with secondary antibody and the nonspecific binding sites are blocked. The secondary antibody can bind to the Fc fragment of the primary antibody (peptide antibody) whose Fab fragment would be competitively bound by both biotinylated peptide and peptide standard or targeted peptide in samples. The biotinylated peptide interacts with streptavidin – horseradish peroxidase (SA-HRP) which catalyzes the substrate solution. The intensity of the yellow is directly proportional to the amount of the biotinylated peptide-SA-HRP complex but inversely proportional to the amount of the peptide in the standard solutions or samples. This is due to the competitive binding or samples to the peptide antibody (primary antibody).

The assay of calprotectin utilizes the two-site “sandwich” technique. Standards, controls and prediluted (1:50) serum samples are added to the wells of a microplate coated with a high affinity monoclonal anti-human calprotectin antibody. During the first incubation step, Calprotectin in the samples is bound by the immobilized antibody. Then a peroxidase labeled conjugate is added to each well and the following complex is formed: capture antibody - human calprotectin – Peroxidase conjugate. Tetramethyl-benzidine (TMB) is used as a peroxidase substrate. Finally, an acidic stop solution was added to terminate the reaction. The color changes from blue to yellow. The intensity of the yellow color is directly proportional to the calprotectin concentration of sample.

Accordingly, standard curves of known concentration were established from which unknown concentration of both assayed markers were determined.

**Statistical Analysis:**

IBM SPSS statistics (V. 23.0, IBM Corp., USA, 2015) was used for data analysis. Data were expressed as mean and standard deviation (Mean±SD) for quantitative parametric measures in addition to Median and interquartile range (IQR) for quantitative non-parametric measures and both number and percentage for categorized data. Comparative statistics was done by Wilcoxon’s rank sum in case of non-parametric data. Correlation analysis was performed by Spearman’s rank correlation (rs). P values<0.05 were considered significant, whereas values<0.01 were considered highly significant. Receiver operating characteristic curve (ROC) analysis was applied to assess the overall diagnostic performance of each test.
RESULTS

Demographic and clinical characteristics of the studied groups show no significant difference between sepsis and control group in terms of sex distribution and delivery modalities (p>0.05). Septic neonates, however, were found to have a significantly lower mean gestational age (p<0.01) and mean birth weight (p<0.05) as compared to the control group.

Compared to controls, septic newborns demonstrated significantly lower 1&5 minutes Apgar scores (p<0.001). In the same group, 93% had hematological scores ≥3 over the study period. Most of cases (92.3%) had positive CRP (>6mg/L). Positive blood cultures for bacteria were encountered in 30 (38%) cases. Positive blood cultures demonstrated the presence of Klebsiella pneumoniae (14%), Coagulase negative Staph. (8%), Acinetobacter (6%) and E.coli (5%) (Figure 1).

When we searched for risk factors for early-onset neonatal sepsis (EOS) in septic cases, results showed 34(43.6%) cases were due to prolonged premature rupture of membranes (PROM)> 24 hr, 25(32%) due to preterm labor, 16(20.6%) due to chorioamnionitis and 3(3.8%) due to peripartum maternal fever.

Table 1 shows a highly statistical significant increase in the level of copeptin and calprotectin in septic than control group.

Table 2 demonstrates comparison between the levels of copeptin and calprotectin in septic group according in relation to CRP, blood culture results and patients’ outcome. Fifty seven (73.1%) cases improved while 21(26.9%) died.

Gestational age (using Ballard score), birth weight and mode of delivery did not correlate with copeptin (rs= 0.195, 0.261 and 0.045, respectively) and calprotectin levels (rs= 0.256, 0.313 and 0.60, respectively); for all p>0.05.

Levels of assayed cord blood copeptin(pg/mL) and calprotectin(nɡ/mL) correlated to some hematological tests in the septic newborns (Table 3). However, both markers’ levels showed significant correlation with Rodwell’s Sepsis score [median score=5 (3.25-5.3), with copeptin: rs=0.471; p=0.021 and with calprotectin rs=0.704; p=0].

Diagnostic performance test results for determination of the best cut-off value of copeptin and calprotectin for prediction of early-onset neonatal sepsis are shown in table 4 and figure 2.

![Figure 1: Blood culture results of sepsis group.](image-url)
Table 2: Comparison between levels of copeptin and calprotectin in septic group according to CRP, blood culture results and patients’ outcome

<table>
<thead>
<tr>
<th>CRP</th>
<th>Blood culture</th>
<th>Patients’ outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>n=5</td>
<td>n=72</td>
<td>n=48</td>
</tr>
<tr>
<td>Copeptin (pg/mL)</td>
<td>Median (IQR)</td>
<td>Z</td>
</tr>
<tr>
<td>236(184-462)</td>
<td>294(237-520)</td>
<td>0.683</td>
</tr>
<tr>
<td>Calprotectin (ng/mL)</td>
<td>Median (IQR)</td>
<td>7.6(4-13.4)</td>
</tr>
<tr>
<td>Z</td>
<td>0.371</td>
<td>3.409</td>
</tr>
<tr>
<td>P</td>
<td>0.707</td>
<td>0.026</td>
</tr>
<tr>
<td>significance</td>
<td>NS</td>
<td>S</td>
</tr>
</tbody>
</table>

Table 3: Correlation of cord blood Copeptin(pg/mL) and Calprotectin(ng/mL) to other laboratory parameters in the septic newborns:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Median (IQR)</th>
<th>rs</th>
<th>p value</th>
<th>Significance</th>
<th>rs</th>
<th>p value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLC (x10³/mm³)</td>
<td>13.1(4.6-26.8)</td>
<td>0.437</td>
<td>0.016</td>
<td>S</td>
<td>0.374</td>
<td>0.044</td>
<td>S</td>
</tr>
<tr>
<td>Platelet count</td>
<td>99 (54-166)</td>
<td>-0.424</td>
<td>0.020</td>
<td>S</td>
<td>-0.195</td>
<td>0.819</td>
<td>NS</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>6.8 (1.9-17.4)</td>
<td>0.319</td>
<td>0.002</td>
<td>S</td>
<td>0.261</td>
<td>0.051</td>
<td>NS</td>
</tr>
<tr>
<td>I/T ratio</td>
<td>0.3 (0.2-0.4)</td>
<td>0.654</td>
<td>&lt;0.001</td>
<td>HS</td>
<td>0.352</td>
<td>0.019</td>
<td>S</td>
</tr>
</tbody>
</table>

TLC= total leucocytic count, I/T ratio= ratio of immature to mature granulocytes.

Table 4: Diagnostic performance test results of copeptin and calprotectin for prediction of early-onset neonatal sepsis:

<table>
<thead>
<tr>
<th></th>
<th>Cord blood Copeptin (pg/mL)</th>
<th>Cord blood Calprotectin (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Best cut-off value</td>
<td>85</td>
<td>2.25</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>73.3</td>
<td>100</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>100</td>
<td>97.5</td>
</tr>
<tr>
<td>Positive predictive value (%)</td>
<td>100</td>
<td>98</td>
</tr>
<tr>
<td>Negative predictive value (%)</td>
<td>87.5</td>
<td>100</td>
</tr>
<tr>
<td>Efficacy (%)</td>
<td>81</td>
<td>98.9</td>
</tr>
</tbody>
</table>
DISCUSSION

Prompt diagnosis of sepsis is crucial to establishing treatment and modifying neonatal prognosis. Laboratory sepsis markers represent a helpful tool in the evaluation of a neonate with a potential infection. Neonatal sepsicaemia is associated with hyper-inflammatory host responses that subtend activation of immune system including innate immunity which is fully developed in the first weeks of life\(^{(19,21)}\).

Copeptin concentrations were strongly elevated in samples from adult patients with sepsis\(^{(26,17,23)}\) and high copeptin levels were predictive of mortality\(^{(26,17,23)}\). Many studies in adults have shown that copeptin is a valuable biomarker of infection in patients with community-acquired and ventilator-associated pneumonia\(^{(18)}\).

Calprotectin, a major product of innate immunity cells, is an antimicrobial that protects cells against invasive microorganisms and regulates adhesion of leukocytes to the endothelium and extracellular matrix during the inflammatory process\(^{(27)}\). It has been proposed for the diagnosis of many inflammatory conditions; however, its use in the diagnosis of neonatal sepsis remains a matter of research.

Therefore, we aimed in this study to investigate the clinical utility of assaying cord blood copeptin and calprotectin as markers of early-onset neonatal sepsis. We performed a case control study that comprised 138 newborn infants with gestational age of 28-40 weeks and birth weights between 1500 and 4100 grams who were transferred to the NICU because of having a risk factor of neonatal sepsis. On the basis of clinical observation over their first 5 postnatal days and sepsis work-up results, into 2 groups: Early onset neonatal sepsis group (EOS) (n=78) and non-septic control group (n=60).

Our findings revealed no significant difference between sepsis and control group in terms of sex distribution and delivery modalities. Chaco and Sohi (2005) found that the rates of infection were similar in males and females\(^{(6)}\). In Werner et al., 2012, there was no significant difference in sepsis between cesarean and vaginal delivery groups\(^{(29)}\). In the current study, Septic neonates, however, were found to have a significantly lower mean gestational age and mean birth weight.
as compared to the controls. This goes in agreement with the study of Abdelmaaboud et al., and Hornik et al., who observed that very low birth weight (VLBW) infants are at high risk for both early- and late-onset neonatal sepsis (2,13).

Compared to controls, septic newborns demonstrated significantly lower 1 & 5 minutes Apgar scores. Unfortunately, low Apgar score usually necessitates more prolonged and aggressive resuscitation which is a known risk factor for sepsis (12).

Concerning risk factors for EOS in septic cases, prolonged PROM> 24 hr was found in 34 cases (43.6%), preterm labour in 25(32%), chorioamnionitis in 16(20.6%) and peripartum maternal fever in 3(3.8%).

For all infants included in this work, cord blood copeptin and calprotectin were assayed by competitive and sandwich enzyme immunoassays (EIA), respectively. Results of both inflammatory markers were significantly higher in septic neonates than controls [212 (95 – 537) vs. 82(60 – 125) pg/mL for copeptin; and 4.6(4.0-9.1) vs. 0.93(0.7-2.0) ng/mL for calprotectin, respectively; p<0.001 for both]. Our results are in accordance with previous demonstrations as those of Benzing et al., Morgenthaler et al. and Abdelmaaboud et al. (3,17,2).

Gestational age (Using Ballard score), birth weight and mode of delivery did not correlate with copeptin (rs= 0.195, 0.261 and 0.045, respectively) and calprotectin levels (rs = 0.256, 0.313 and 0.60, respectively); for all p>0.05.

Our data are in contrast with reports from Schlapbach et al who stated that infants after vaginal delivery compared to cesarean delivery had significantly higher copeptin levels, even when adjusting for gestational age indicating that the vasopressin system in the neonate is strongly activated upon perinatal stress (24). However, our explanation is that most of septic babies, in our study, were born by cesarian section 59 (75.6%) as their mothers had had risk factors which necessitated cesarean section.

In the study by Abdelmaaboud and co-workers, they found significant negative correlations between calprotectin and both of gestational ages and weights (2). They attributed their results to the fact that sepsis is more severe in neonates with younger ages and lower weights resulting in higher levels of these inflammatory markers. However, their reports depended on cases with late onset sepsis (more than 3 and less than 30 days) rather than ours of EOS.

Most of our patients; 72 (92.3%) had positive CRP (>6mg/L). Comparison of copeptin and calprotectin in neonates with positive and negative CRP was non-significant. Our findings are in accordance with those of Schlapbach et al. and Decembrino et al. (24,10). In fact, the utility of CRP for the diagnosis of neonatal infection has been the subject of controversy because of its unsatisfactory sensitivity. The CRP concentration increases physiologically in newborns within the first days after birth. This dynamic behavior may in part account for the low diagnostic accuracy of CRP measurements in neonatal infection, particularly when measured shortly after birth (24).

Positive blood cultures for bacteria were encountered in 30 (38%) cases. A recent study by Decembrino et al., showed positive culture in 8/41 neonates (19.5%) with sepsis (10). This is explained by that the sensitivity of blood culture for diagnosis of bloodstream infection is still dependent upon the volume of blood used to inoculate the culture medium (4,8). Moreover, low level of bacteremia is possible in the NICU where infants are often exposed to multiple courses of empirical antibiotics, including in utero antibiotics.

Fifteen percent of positive blood culture cases were due to Klebsiella pneumoniae (14%), coagulase negative staph (8%) Acinetobacter (6%) and E.coli (5%). Our findings are consistent with those of Stoll et al., 2005 and Klinger et al., 2009, who found that EOS was caused mainly by gram negative bacteria; while Abdelmaaboud reported that nosocomial sepsis caused by coagulase negative staphylococcal (CoNS) infections continues to be an important cause of morbidity in NICUs (25,14).

When serum copeptin and calprotectin were assayed in septic neonates with positive and negative blood culture results, higher levels of both markers were obtained in septic neonates with
positive blood culture. These high levels were significant in calprotectin, however, they did not reach a significant level in copeptin. Our results were in accordance with those previously obtained by Abdelmaaboud et al. and Decembrino et al.\(^\text{(2,10)}\).

Of the 78 septic patients, 57(73.1\%) infants improved, while 21(26.9\%) died. These results significantly associated with copeptin and calprotectin initial cord blood values; denoting that both are early predictor markers of survival in neonatal septicemia. Morgenthaler and colleagues, in their study, had also elevated copeptin concentrations are elevated in hemorrhagic and septic shock and that copeptin was higher on admission in non-survivors as compared with survivors, suggesting copeptin as a prognostic marker in sepsis\(^\text{(17)}\). Moreover, reports of Abdelmaaboud and colleagues showed that patients with positive blood cultures, and/or those who died within one week after diagnosis of sepsis had significantly higher levels of calprotectin than cases that were negative for blood cultures or those who survived beyond the first week after diagnosis of sepsis\(^\text{(2)}\). These findings may reflect the reliability of serum calprotectin level as a marker of the severity and outcome of cases with sepsis.

Correlation analysis of cord blood copeptin and calprotectin to hematological tests was performed. Copeptin significantly correlated with total leucocytic count (TLC), granulocytes, platelet count and I/T ratio, while calprotectin showed significant correlation with TLC and I/T ratio. Thrombocytopenia is one of the most common complications of neonatal sepsis and is considered one of the hematological parameters of severity of neonatal sepsis\(^\text{(22,16,2)}\). The significant negative correlation of copeptin with platelet counts indicate that it may be used as parameters of the severity of sepsis.

Rodwell score is a hematologic scoring system that uses parameters of complete blood count to diagnose sepsis; in which score above 3 raises the possibility of sepsis. Both markers significantly correlated with Rodwell’s score indicating that they are good diagnostic tests for EOS. Such assumptions are strengthened by studying the diagnostic performance of the two tests for prediction of EOS.

Receiver-operating-characteristic (ROC) curve analysis showed that copeptin cord blood concentrations at a cut-off of 35pg/mL could discriminate between neonates with and without septicemia with a sensitivity of 73.3\%, a specificity of 100\%, positive predictive value (PPV) of 100\%, negative predictive value (NPV) of 87.5\% and efficacy 81\%.

Regarding the discriminating ability of calprotectin between healthy neonates and those having EOS, even though their blood culture is negative, our ROC curve at a best cut-off value of 2.25ng/mL had sensitivity of 100\%, specificity of 97.5\%, PPV of 98\%, NPV of 100\% and efficacy is 98.9\%.

**Conclusion:**

As diagnosis and early treatment of EOS is crucial to decrease neonatal morbidity and mortality, our study reveals that copeptin and calprotectin may be considered two promising sensitive and specific predictors for early onset neonatal sepsis.

**REFERENCES**

8. Chu A, Hageman JR, Schreiber MD (2012): Antimicro-
Cord Blood Copeptin and Calprotectin in Neonatal Sepsis

قياس معدل الكاليبروتاين الموجود في دم الحبل السري كمؤشر حيوي لتشخيص الاسم الدهم المبكر في الأطفال

مل محسن - فؤاد خليل زكي - رانيا إسماعيل - نهال الرجال - محمد عبد الوهاب

يعتبر الاسم الدهم عند الأطفال حديثي الولادة من المشاكل الأساسية التي تسبب في ارتفاع معدل الولادات والوفيات عند هؤلاء الأطفال إلى حد كبير ولذلك فإن تشخيص المبكر وتوفير العلاج المناسب أمر بالغ الأهمية ويعتبر سببًا في إنقاذ حياة هؤلاء الأطفال، وعلي الرغم من أن مزعة الدم هو اختبار حيوي لتشخيص الاسم الدهم في حديثي الولادة فإن نتائجه لا تكون مثالية قبل 24-48 ساعة وقد تكون سلبية في كثير من الأحيان. بمعنى أن غالبية الاسم الدهم عند حديثي الولادة ينفع النتيجة المفتوحة مع المضيف والتي تقابل بتشخيص الجهاز المناعي، ولذلك تم إجراء مجموعة واسعة من الدراسات الاستقصائية لتشخيص الاسم الدهم في حديثي الولادة.

وقد قمنا في دراستنا هذه بقياس التوسط من العوامل الديوية والذي تم التعريف به بعد كلا الكاليبروتاين والكاهتروتاكين بغض معرفة القيم التشخيصية لما كي الديوية لتشخيص حالات الاسم الدهم المبكر عند الأطفال حديثي الولادة. تم تنفيذ هذه الدراسة في وحدة الدراسة الحالية لحديثي الولادة حيث تم تقسيم الأطفال إلى مجموعتين: المجموعة الأولى: وتشمل مجانين من سويين دم مبكر وعددهم 78 طفلاً والمجموعة الثانية: وتشمل مجانين إطار آخر وعددهم 60 طفلاً. وقد أظهرت النتائج البحث وجود ارتفاع ذو دالة إحصائية في متوسط الكاليبروتاين والكاهتروتاكين في مصل الأطفال حديثي الولادة المصابة بالاسم الدهم المبكر مقارنة بالأطفال الآجري.

في المجموعة الضيقة، كما أظهرت الدراسة ارتفاع معدل إكيشان سويين الدهم المبكر في الحالات الدهم المصابة بعزل ميكروب من مزارع الديم ولم يتم تسجيل ذلك مع سويين دم مبكر والذي لم يسجل ارتفاع بشكل ملحوظ مع حالات المزارع الإيجابية. وكذلك أظهرت الدراسة وجود علاقة وثيقة بين ارتفاع سويين الديم والكاهتروتاكين والكاهتروتاكين ومقياس سوء في حالة الولادة مما يدل على إمكانية استخدام الدهم المبكر كمقياس هام لتشخيص الاسم الدهم المبكر لدى هؤلاء الأطفال. كما أظهرت مسارات الديم والكاهتروتاكين ارتفاع ذو دالة إحصائية مع حالات الدهم المبكر الشديد الذي قد يؤدي إلى الوفاة، وقياس الدالة الإحصائية لذين العاملين الحيويين أشارت النتائج إلى وجود هماسة وخصوصية عالية في تشخيص حالات الدهم المبكر عند الأطفال حديثي الولادة، وعما سبق نستنتج أن قياس سويين الديم والكاهتروتاكين يعتبر معياراً حيوي جداً وحساساً لتشخيص حالات الدهم المبكر لدى الأطفال حديثي الولادة خاصة في الحالات التي يتعرض تشتريها بواسطة نتائج مزارع الدم المستخدمة لعزل الديم المبكر.

المسبب للمرض.
THE STUDY OF BCR-ABL TRANSCRIPT VARIANTS (B2A2 AND B3A2) RELATION TO DIFFERENT RISK SCORES

ABSTRACT

Background: The exact role of the different transcript variants of BCR-ABL in the pathogenesis of chronic myeloid leukemia (CML) and their impact on prognosis is yet to be definitely enumerated. Aims: In this study, we have tried to correlate the presenting features, risk scores and treatment response with the BCR-ABL variants detected in our patients. Settings and Design: A cross-sectional unicentric hospital-based study on 80 patients in Dammam University Hospital, Saudi Arabia, diagnosed to have CML by bone marrow cytogenetics and confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR). Materials and Methods: RT-PCR for BCR-ABL was performed on consecutive patients with CML attending the CML clinic from January 2010 to December 2010. The medical charts of these patients were analyzed after a follow-up of 18 months in a retrospective manner. Statistical Analysis: Box plot and histogram were used to see the distribution of variables. t-test was performed to enumerate the difference between risk scores in two populations of patients carrying two different BCR-ABL transcript variants. Results: Nearly 56.25% of patients had b3a2 (e14a2) while 41.25% of patients showed b2a2 (e13a2) transcripts. The rest 2.5% (two patients) expressed the rare e19b2 variant. Patients with b2a2 presented with higher Sokal, Hasford and European Treatment and Outcomes Study score than their b3a2 counterpart. Different parameters such as the platelet count, leukocyte count, hemoglobin and splenomegaly showed a minor difference between the groups. More patients in the b2a2 group achieved complete hematologic response at 3 months, but it was not significant. Conclusions: Patients with b2a2 variant CML tend to present with higher risk score, but do not behave in a vastly different manner than their b3a2 counterparts. Key words: BCR-ABL transcript variants, chronic myeloid leukemia, chronic myeloid leukemia risk scores, imatinib mesylate

INTRODUCTION

Philadelphia chromosome i.e., the chromosome originating from the reciprocal translocation between long arms of chromosome 9 and chromosome 22 t(9;22)(q34;q11), along with its product BCR-ABL oncogene is perhaps the most extensively studied oncprotein. Since its discovery in 1960(13), its different variants in different types of leukemia has been recorded and analyzed. In chronic myeloid leukemia (CML) BCR-ABL fusion oncogene translates chimeric protein p210, which exerts its oncogenic role by activating the tyrosine kinase, which does not block differentiation but leads to the development of malignancy by enhancing proliferation of myeloid cell line(15). In CML, most of the cases of p210 constitutes principally of two variants: b3a2 or e14a2 and b2a2 or e13a2(4,11). The b3a2 variant is produced by the fusion of exon 14 of BCR gene with exon 2 of ABL gene while the other variant, i.e., b2a2 is produced by fusion of exon 13 of BCR and exon 2 of ABL(18). These transcripts variants are produced as the result of alternate splicing. Many studies had been performed to find interrelation between the particular variant of p210 BCR-ABL and the etiology, pathogenesis, prognosis of the disease. What our study constitutes is the endeavor to find the interrelation between these two principal transcript variants of p210 and its association with different aspects of the disease such as white blood cell (WBC) count, platelet count, organomegaly, prognostic markers of the CML (Sokal score(19), Hasford/ Euro score(8), European Treatment and Outcomes Study (EUTOS) score(7)) along with the response to the modern day mainstay therapy of CML through Imatinib mesylate. Reverse transcriptase polymerase chain reaction (RT-PCR) chosen as the method of choice to determine the transcript variant of BCR-ABL, is one of the most sensitive methods for this purpose.

MATERIALS AND METHODS

The study was approved by the ethics committee of the Dammam University Hospital.

Departments of Hematology*, National Cancer Institute, University of Cairo, Egypt, Clinical Hematology**, University of Dammam, KSA and Molecular Biology***, University of Dammam, KSA.
Patients

A total of 80 consecutive patients who were diagnosed to be suffering from CML in our clinic over the period of January 2010 to December 2010 were selected for the study and followed up for 18 months.

Sample collection

Blood samples were collected from 80 patients from the Out-Patient Clinic (3 ml for each patient), in a proper aseptic manner, into properly labeled ethylene diaminetetraacetic acid vacuum tubes. Processing of the collected blood, i.e., beginning of ribonucleic acid (RNA) isolation from the collected blood sample, was started immediately, thus evading any chance of messenger RNA degradation. Proper consent from each patient was taken at the time of sample collection.

RNA isolation

Total RNA was isolated from a blood sample by using the QIAGEN RNA isolation kit (QIAGEN, Germany) by following the protocol provided by the manufacturer. RNA quantity was determined by spectrophotometry.

Complementary DNA (cDNA) synthesis

RNA was reverse transcribed into cDNA for using as template in PCR reaction. RT reaction was performed by using Transcriptor First Strand cDNA Synthesis Kit, Roche applied science, USA.

Six microliter of RNA (~2.4 µg RNA) was added to 14 µL of mix containing 2 µL of 10x RT reaction buffer (40 mM Tris-HCL, 100 mM KCl, 20 mM DTT), 10 mM dNTP, 10 mM oligo-dT, 20 units of RNase inhibitor and 40 units of Reverse Transcriptase. Next the mix was incubated in 42°C for 45 min, 99°C for 3 min and finally held at 4°C for 20 min. Manufacturer provided RNA is used as a positive control.

PCR amplification

The primers of standardized PCR protocol of BIOMED1(Europe against Cancer)\(^{21}\) were used to amplify the cDNA (Except the shifted primer ABL-a3-E3'). The sequences of the primers are given as follows:

- BCR-b1-A 3086 (22): GAAGTGTTTCAGAGCTTCTCC
- ABL-a3-B 458 (21): GTTTGGGCTTCAACACCATTCCT
- BCR-b2-C 3126 (21): CAGATGCTGACCAACTGCTGT
- ABL-a3-D 441 (23): TTCCCCATTGTTGATTATGCCTA.

The cDNA product of the former reaction was amplified with PCR reaction in two rounds (nested PCR), 150 mM primers, 1 U/ml Taq Polymerase, 10 mM dNTP mix and \(\times 10\) PCR buffer with 25 mM MgCl\(_2\) was used in each round. Initial denaturation was performed at 95°C for 3 min followed by 35 cycles of denaturation at 94°C for 20 s; annealing at 55°C for 15 s; extension at 72°C for 15 s with a total 35 cycles using thermocycler. Final extension was performed at 72°C for 5 min. After performing the PCR reaction, the products were checked through agarose gel electrophoresis in 2% of agarose gel medium.

RESULTS

The b3a2 transcript variant was recognized as 417 base pair long product and b2a2 was recognized as 342 base pair long product when primers A and B were used [Figure 1] and as 360 and 285 bp products when primers C and D were used.

The baseline characteristics of patients are shown in table 1. Amongst 80 patients (including 24 females) 45 (56.25%) of patients showed the presence of b3a2 and 33 (41.25%) of patients showed the presence of b2a2 transcript variant. The rest 2.5% (numerically, 2 cases) showed the expression of rare e19b2 variety, recognized as a 243 base pair long product in agarose gel electrophoresis. However, there was no case of b3a2 and b2a2 co-expression in any patient. Bone marrow cytogenetics did not reveal any abnormality other than the presence of Philadelphia chromosome in any patient.

Qualitative nested PCR were performed on the patients at the end of the follow-up period as it was done at the beginning of the study. All the patients, including two patients who underwent complete molecular response, did show qualita-
BCR-ABL Variants Relation to Different Scores

tive nested PCR value as positive at the end of the follow-up period (which is a common occurrence).

Hence the first round PCR (at the time of diagnosis) and the second round PCR (at the end of follow-up) did not discriminate the patient population. From the database of history of the patients, we had calculated the Sokal score, Hasford score and EUTOS score of all patients at the time of diagnosis and correlated them with the respective transcript variants of the patients is given in figure 2.

From figure 2, it is apparent that, according the Sokal, Hasford and EUTOS score, the patients with b2a2 transcript variants are presenting with higher scores than the patients having b3a2 variants. Hence we have performed t-test to test if the risk scores are significantly higher in patients with b2a2 variants and it was found that the P < 0.05 in both Sokal and EUTOS score, but not in Hasford score (P = 0.03 in case of Sokal score, 0.027 in EUTOS score and 0.24 in Hasford score).

We analyzed the correlation between different transcript variants and the progression of disease among the patients. We tabulated the transcript variants of the patients against their stage of the disease i.e., if they stayed in chronic phase (CP) all along or progressed to accelerated phase or blast crisis in tables 2. We have also tabulated the Platelet count, organomegaly and other hematological features against the transcript variants in table 3, as there are earlier reports on the correlation between these two in the form of higher platelet count found in b3a2 variety.\(^{14,22,5,3}\) However, we found higher platelet counts in patients with b2a2 variety of BCR-ABL than in b3a2.

We also studied and tabulated our patients’ response to the first generation tyrosine kinase inhibitor Imatinib mesylate and any possible relation with the transcript variants of the patients in table 4. For this purpose, we had only considered the data of the patients who presented to us in the CP of the disease (1st CP of CMLCP) and are currently being treated by Imatinib mesylate (at the standard dose of 400 mg/day) for at least a period of 6 months. In our population, we found 30 such cases of b3a2 and 23 such cases of b2a2. For assessment of the Imatinib response, we had to depend mostly on the hematological response as most of the patients could not afford the recommended bone marrow cytogenetics or quantitative. RT-PCR (qRT-PCR). Cytogenetic response (partial, major or complete) and molecular response was assessed for BCRABL in a few cases where the patient could afford the test.

**Table 1: Baseline characteristics of patients**

<table>
<thead>
<tr>
<th>Parameters at diagnosis</th>
<th>b3a2</th>
<th>b2a2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>Interquartile Range</td>
</tr>
<tr>
<td>Platelet count (10^3/µl)</td>
<td>206</td>
<td>310</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>10</td>
<td>2.8</td>
</tr>
<tr>
<td>WBC count (10^3/µl)</td>
<td>37.7</td>
<td>98.3</td>
</tr>
<tr>
<td>Splenomegaly (cm)</td>
<td>3.5</td>
<td>6</td>
</tr>
<tr>
<td>Hepatomegaly (cm)</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

WBC – White blood cell

**Table 2: Disease stage**

<table>
<thead>
<tr>
<th>b2a2 b3a2</th>
<th>CML-CP</th>
<th>CML-AP</th>
<th>CML-BC</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients having b3a2 variant</td>
<td>32</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>No. of patients having b2a2 variant</td>
<td>23</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

CML – Chronic myeloid leukemia; CP – Chronic phase; AP – Accelerated phase; BC – Blast crisis
Table 3: Clinical features of patients with transcript variants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>b2a2</th>
<th>b2a2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>42</td>
<td>35</td>
</tr>
<tr>
<td>Gender</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>Hemogram on diagnosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Leukocyte count (X10^9/μl)</td>
<td>112.66</td>
<td>263.6</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>740</td>
<td>210</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>38</td>
<td>40</td>
</tr>
<tr>
<td>Basophil</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Blast</td>
<td>18</td>
<td>5</td>
</tr>
<tr>
<td>Hemogram on test day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Leukocytic count</td>
<td>4.1</td>
<td>6.35</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>741</td>
<td>100</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>32</td>
<td>44</td>
</tr>
<tr>
<td>Basophil</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>Blast</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>Organomegaly on diagnosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver (cm)</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Spleen (cm)</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>Sokal score</td>
<td>1.17 (int.)</td>
<td>1.22 (high)</td>
</tr>
<tr>
<td>Hasford score</td>
<td>777.3 (high)</td>
<td>111.3 (mL)</td>
</tr>
<tr>
<td>EUTOS score</td>
<td>81 (low)</td>
<td>63 (low)</td>
</tr>
</tbody>
</table>

Table 4: Response to treatment

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>b3a2</th>
<th>b2a2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of patient receiving IM for at least 18 Months</td>
<td>30</td>
<td>23</td>
</tr>
<tr>
<td><strong>Hematological Response</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHR at 3 months**</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td>Incomplete Hematological Response</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td><strong>Cyto genetic response</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Major (1-35%)</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Minor (36-65%)</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Minimal (66-95%)</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Complete</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Not complied</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td><strong>Molecular response</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Major</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Could not afford</td>
<td>26</td>
<td>21</td>
</tr>
</tbody>
</table>

IM – Imatinib mesylate; **CHR – Complete hematological remission i.e., resolution of splenomegaly (if previously present), restoration of normal blood counts and loss of marrow hypercellularity (Platelet <450×10^9/L; WBC <10×10^9/L; No immature granulocyte and basophil <5%)^(1)^

Figure 1: Reverse transcriptase-polymerase chain reaction of BCR-ABL transcript variants in 2% agarose gel. Lane 1: 100bp ladder; Lane 2: Positive control of b3a2 (417bp); Lane 3: Positive control of b2a2 (342 bp); Lane 4 and 5: Patient sample showing b3a2 variant; Lane 6: Patient sample showing b2a2 variant; Lane 7: Negative control
DISCUSSION

In our study, the frequency of b3a2 and b2a2 was found to be 56.25% and 41.25% respectively which is quite closer to the data derived in the similar studies performed in the Caucasian population\(^{(22)}\). No co-expression of these two variants was found in any of the patients. In other countries, the distribution was also similar to our findings\(^{(6,19)}\). In all the aforementioned studies, the ratio was roughly calculated to be b3a2:b2a2 = 3:2. However, the role of BCR-ABL transcripts variants in the prognosis had always been a controversial issue. Although some authors reported no such role on the part of the transcript variants, others found data referring to the transcript variants to have prognostic values\(^{(5,16,20)}\). In the Middle East population, this is perhaps the first study on the transcript variants and their possible role on the presenting relative risks (measured by Sokal, Hasford and EUTOS score) and Imatinib response. Our study does not reveal a significant difference of treatment response in the two patient populations. The patients with b2a2 variants of our population seem to be presenting with higher relative risk (according to Sokal and EUTOS score).

In the study by Martínez-Mancilla et al., a higher leukocyte count in b2a2 patients was reported\(^{(12)}\). The presenting features of the pa-
tients of these two sub-populations are not starkly different either. Although some of the studies failed to show any correlation between platelet count and BCR-ABL transcript variants\(^{(17)}\), others recognized higher platelet count in patients carrying b3a2\(^{(14,22,9,3)}\). However almost all the reported studies correlating these two, related higher platelet count in the b3a2 variety our study had revealed otherwise [Table 4]. The other presenting features as Total WBC count, Hemoglobin, hepatomegaly and splenomegaly are within moderate range of discrimination showing the b2a2 population presenting with relative organomegaly and slightly lower hemoglobin as well as lower WBC count.

Due to the issue of affordability of our patients, the world-wide recommended follow-up protocol through qRT-PCR to analyze transcript load of BCR-ABL every 3 months interval\(^{(2,1)}\) could not be performed. Rather the common practice is to follow-up patients through checking for hematological remission. In addition in case of Imatinib failure, switching over to second generation tyrosine kinase inhibitor is impossible for the same reason of affordability.

Conclusion

According to the criterion of achieving and maintenance of hematological remission, b2a2 patients seem to respond well to the Imatinib therapy. Patients with b2a2 variant CML tend to present with higher risk score, but do not behave in a vastly different manner than their b3a2 counterparts. Still keeping the small sample size in mind, it is better to say that further investigation is required to conclude this aspect as well as to investigate the association between transcript variants and treatment response in terms of cytogenetic and molecular response.

REFERENCES

11. Lucas CM, Harris RJ, Giannoudis A, et al. 2009 Chronic myeloid leukemia patients with the e13a2 BCR-ABL fusion transcript have inferior responses to imatinib compared to patients with the e14a2 transcript. Haematologica ; 94:1362.
BCR-ABL Variants: Relation to Different Scores

A study correlating BCR-ABL mutation with clinical and laboratory parameters in patients with chronic myeloid leukemia.


A study correlating BCR-ABL (b2a2 and b3a2) with clinical and laboratory parameters in patients with chronic myeloid leukemia.

Dr. Eliezer Y. Boonmoh
CRP AND SERUM AMYLOID A AS MARKERS FOR THE AGGRESSIVENESS OF BREAST CANCER
Rania Elhelely*, Rasha Elzehery*, Mohammed AF Hegazy** and Ramy Faris**

ABSTRACT

Introduction: Chronic inflammation is included in the development and progression of breast cancer. Serum C - reactive protein (CRP) and serum amyloid A (SAA) are measures of low-grade chronic inflammation. Aim: the present study investigated the possibility to use CRP and SAA as predictors for the aggressiveness of breast cancer. Subjects and methods: serum CRP and SAA levels were measured in 181 female patients (24-78 years old) with breast cancer and 60 healthy age-matched control females (33-67 years old) using Enzyme-linked Immunosorbent Assay (ELISA) kit. Results: serum CRP levels were higher in malignant and benign breast lesions than control group but with no statistical significant difference among diseased groups (P < 0.001). SAA levels were statistically higher in malignant breast lesions than benign and control group (P< 0.001), but there was no statistical significant difference in its levels between FA and control group (P= 0.57). CRP levels showed no statistical significant difference between benign breast tumor and stage I cancer breast (P= 0.97). SAA and CRP concentrations were correlated positively with tumor stages [(r=0.861, P< 0.001) and (r=0.680, P< 0.001)] respectively and with each other (r=0.628, P< 0.001). Conclusion: SAA can be used as a predictor marker than CRP for malignant breast lesions. Key Words: Breast Cancer; Serum Amyloid A; C - reactive protein.

INTRODUCTION

The International Agency for Research on Cancer (GLOBOCAN 2012) found that breast cancer (BC) is the world’s most common cancer among women, and the most likely cause of death among them (12). BC is reported as the most frequent cancer among women in 140 of 184 countries worldwide including China the world’s most populous country (5). After 1990 the mortality rate of BC has decreased, due to widespread BC screening and the general use of anticancer agents (33). The 5-year survival rates have improved to 98% for early stage and 39% for late stage BC (28). Thus, early detection is vital to improve the prognosis of cancer patients and this requires non-invasive and specific biomarkers; among them, blood markers may offer a good surrogate selection.

Chronic inflammation is a key contributor to cancer development and progression. Cancer survivors with chronic inflammation may have an elevated risk of recurrence as a result of the effects of inflammatory processes on cell growth or the presence of cancer cells that induce inflammation (8).

Clinical and experimental data suggest that chronic inflammation promotes mammary tumor development through mechanisms involving chronic activation of humoral immunity and infiltration of Th2 cells and polarized innate inflammatory cells (10).

Moreover; inflammation associated oxidative damage could initiate carcinogenesis by causing inactivating mutations in tumor-suppressor genes or post-translational modifications in proteins involved in DNA repair or apoptotic control. In addition; inflammatory cytokine signaling via intracellular enzymes and transcription factors may inhibit apoptosis and promote the growth and proliferation of cancer cells. Moreover; activation of inflammatory pathways might enhance tumor progression by promoting cell motility, vascular permeability and angiogenesis (8,10,16).

Acute-phase proteins (APPs) generally have a nonspecific rapid response to such processes as inflammation/infections, tissue damage, surgery, myocardial infarction or the presence of tumors. The relationship between the APPs and cancer has been well documented in the literature with numerous investigations reporting an altered levels of various APPs with different types of cancers and evidence that many APPs are actually produced directly by tumor tissue (34).

It has been suggested that APPs were not likely to be specific for any type of cancer and would be expected to be elevated in all malignancies.
and in inflammatory diseases. Moreover, APPs were thought unlikely to be tumor-derived and thus to represent cancer epiphenomena rather than direct tumor-derived proteins. Proteomics studies, which profiled the serum proteins of patients with cancer and those of normal individuals, proved that the altered expression of APPs was different for various types, subtypes, and even stages of cancer (23).

It is likely that panels of biomarkers in the future will be comprised of biomarkers that reflect tumor-specific proteins together with proteins from the tumor microenvironment (25).

Serum amyloid A (SAA) is a 12-kD acute-phase protein which can be induced up to 1000-fold (22). HDL is the main carrier of SAA in human(4).

C-reactive protein (CRP) and serum amyloid A (SAA) are nonspecific, acute-phase, hepatic proteins secreted in response to cytokines including interleukin-1, interleukin-6 and tumor necrosis factor-α (19).

Previous epidemiologic studies have reported that elevated CRP levels may be associated with poor prognosis of several types of solid cancers(1), as endometrial(10), cervical (27), colorectal, pancreatic, hepatocellular, esophageal, renal cell, bladder, prostate, ovarian, and non-small-cell lung cancer (24,29).

Up-regulation of SAA was found in a wide range of malignancies as lung, pancreatic, prostate, colon and gastric cancer(11,19,18,9,7). Previously; it was considered that the elevation of SAA in the serum of cancer patients is of liver origin rather than from tumor cells. However, other researches indicated that some tumor cells (such as endometrial and colon cancer cells) also synthesize and secrete SAA(7,6). SAA is an inflammatory modulator that is involved in cholesterol metabolism and transport and is important in cancer progression (21).

The aim of the present study was to determine whether circulating markers of inflammation (CRP and SAA) could be used as predictors for the aggressiveness of breast cancer.

MATERIALS AND METHODS

Materials:

This study was conducted on 181 female patients (24-78 years old) diagnosed as cancer breast admitted at Mansoura Oncology Center. 60 healthy age- matched females (33-67) were taken as a control group. Subjects with a history of any cancer and those with associated diseases that might raise serum CRP or SAA (such as infectious diseases, autoimmune conditions, asthma, and osteoarthritis) were excluded.

All studied patients were subjected to full history taking and thorough clinical examination. Fine needle aspiration biopsy from breast lesion was taken for histopathological examination.

Sampling: 5 ml venous blood was obtained from breast cancer patients and the healthy controls. Serum samples were frozen and maintained at -80°C until the assay was conducted. All 120 breast cancer patients and healthy volunteers signed informed consent forms for sample collection. The study was conducted in accordance with the guidelines of the Helsinki Declaration.

Methodology: The following investigations were done to both patients and controls:

* Serum SAA concentrations were determined using Enzyme-linked immunosorbent assay (ELISA) kit supplied by Assay pro (3400 Harry S Truman Blvd St. Charles, MO 63301, USA).

* Serum CRP concentrations were determined using commercially available ELISA kit supplied by Abcam (1 Kendall Square, Suite B2304 Cambridge, MA 02139-1517 USA).

Statistical Analysis: All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) version 17.0 software. Data were first tested by Kolmogorov-Smirnov test for distribution of data. Description was done in the form of median and IQR for numerical non-parametric data, and number and percentage for categorical data. Kurskal Wallis test was used for comparison of the studied groups in all variables followed by comparisons using the Mann Whitney U test. Spearman’s rank correlation was also used to check the correlation between CRP and SAA concentrations and stages of breast cancer.
For all tests, p<0.05 was considered to indicate a statistically significant difference.

RESULTS

According to histopathological examination of fine needle aspiration biopsy obtained from different breast lesions included in our study; we found 44 patients with invasive ductal carcinoma (IDC), 43 patients with invasive lobular carcinoma (ILC), 42 patients with Ductal carcinoma in situ (DCIS), 32 patients with Mucinous adenocarcinoma (MC) and 20 patients with Fibroadenoma (FA).

Our study found that median CRP levels were 9, 8.3, 7.8, 8.55 and 6.45pg/ml in patients with IDC, ILC, DCIS, MC and FA respectively vs 3.4 pg/ml in control group (P< 0.001) (Table 1). When Mann-Whitney test was performed; serum CRP levels were higher in malignant and benign breast lesions than control group but with no statistical significant difference among diseased groups (Figure 1).

As regards SAA; it was found that its levels were statistically higher in malignant breast lesions than benign and control group. Median SAA levels were (50, 27, 17.3, 6.95 μg/ml in patients with IDC, ILC, DCIS and MC vs FA (2.15 μg/ml) and control group (1.9 μg/ml) (P< 0.001) (Table 1). By Mann-Whitney test; there was a statistical significant difference among malignant and benign control groups (P ≤ 0.002). In contrast; there was no statistical significant difference in SAA levels between FA and control group (P= 0.57). Moreover; SAA levels were directly proportional with aggressiveness of cancer breast (Figure 1).

According to different stages of cancer breast; our study included 22 patients in stage I, 52 patients in stage II, 58 patients in stage III and 29 patients in stage IV. The results of the statistical assessment showed the median serum CRP and SAA concentrations were 6.3 pg/ml and 11μg/ml in stage I, 8.4 pg/ml and 19.7 μg/ml in stage II, 11.2 pg/ml and 31 μg/ ml in stage III and 13 pg/ml and 63.1 μg/ml in stage IV respectively (P< 0.001) when compared to control group (Table 2 & Figure 2).

Using Mann-Whitney test; a statistical significant difference in CRP levels between FA and control group (P = 0.002) was found, while there was no statistical significant difference in SAA between the two groups (P= 0.57). On the other hand; there were statistical significant differentes as regards SAA levels between benign breast tumor and cancer breast (P< 0.001). As regards CRP, there was no statistical significant difference between benign breast tumor and stage I cancer breast (P= 0.97), while there were statistical significant differences among higher stages of cancer breast (P< 0.006).

Moreover; SAA and CRP concentrations were correlated positively with tumor stages [(r=0.861, P<0.001) and (r=0.680, P< 0.001)] respectively (Figure 3&4). CRP levels were correlated positively with SAA levels (r=0.628, P< 0.001).

Table (1): Serum levels of SAA and CRP among the studied groups as regard histological type:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>IDC (n = 44)</th>
<th>ILC (n = 43)</th>
<th>DC IS (n = 42)</th>
<th>MC (n=32)</th>
<th>FA (n = 20)</th>
<th>Control (n = 60)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAA (μg/ml) (median &amp; Range)</td>
<td>50 (13–77)</td>
<td>27 (12-59)</td>
<td>17.3 (8.6-31)</td>
<td>6.95 (3.9-10.5)</td>
<td>2.15 (1.2-3.6)</td>
<td>1.9 (0.9-3.6)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>CRP (pg/ml) (median &amp; Range)</td>
<td>9 (3.1-15.3)</td>
<td>8.3 (4.5-18.2)</td>
<td>7.8 (3.2-14.5)</td>
<td>8.55 (4.5-12)</td>
<td>6.45 (2.4-11)</td>
<td>3.4 (1.1-6.5)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>
Table (2): Serum levels of SAA and CRP among different stages of cancer breast:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>IDC (n = 44)</th>
<th>ILC (n = 43)</th>
<th>DCIS (n = 42)</th>
<th>MC (n = 32)</th>
<th>FA (n = 20)</th>
<th>Control (n = 60)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAA (μg/ml)</td>
<td>50 (13–77)</td>
<td>27 (12-59)</td>
<td>17.3 (8.6-31)</td>
<td>6.95 (3.9-10.5)</td>
<td>2.15 (1.2-3.6)</td>
<td>1.9 (0.9-3.6)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>CRP (pg/ml)</td>
<td>9 (3.1-15.3)</td>
<td>8.3 (4.5-18.2)</td>
<td>7.8 (3.2-14.5)</td>
<td>8.55 (4.5-12)</td>
<td>6.45 (2.4-11)</td>
<td>3.4 (1.1-6.5)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Figure (1): Serum levels of CRP and SAA among different histological types of cancer breast.

Figure (2): Serum levels of CRP and SAA among different stages of cancer breast.

Figure (3): Concentrations of SAA in controls, benign breast disease (FA) and breast cancer patients at various stages of the disease.

Figure (4): Concentrations of CRP in controls, benign breast disease (FA) and breast cancer patients at various stages of the disease.
DISCUSSION

Although there have been significant advances in imaging technology, many breast lesions remain indeterminate following conventional evaluation. Genomic and proteomic high-throughput approaches may help in identifying new blood biomarkers, which provide a convenient, relatively noninvasive approach for diagnosis and monitoring patients.

Besides the potential of SAA as a cancer biomarker, the role of SAA in the progression of neoplastic diseases is of current interest. An acute immune response may cause an increased risk of peripheral metastases\(^{17}\). SAA protein stimulates the production of various cytokines by macrophages and can play an important role in acute immune response, stimulating tumor metastasis indirectly. It stimulates matrix metalloproteinase-9 (MMP-9) production by macrophages\(^{20}\). These metalloproteinases (MMPs) are a family of extracellular matrix degrading proteases that are zinc-dependent and associated with invasion and metastasis during tumor progression\(^{31}\).

In the present study; patients with breast tumors were classified according to both histopathological examination and clinical staging. CRP and SAA levels were measured at time of diagnosis of breast tumor.

According to histopathological classification; serum CRP levels were higher in malignant and benign breast lesions than control group but with no statistical significant difference among diseased groups. These results are not in agreement with Allin et al\(^{2}\) who reported that elevated CRP levels were indeed associated with larger tumor size, presence of distant metastases, and lower tumor grade.

Two hypotheses have been proposed to explain the relationship between CRP level and cancer. First; it has been suggested that elevated CRP levels are a result of an underlying cancer. Second; chronic inflammation and elevated CRP might have a causal role in carcinogenesis\(^{19}\). SAA levels showed statistical significant difference among malignant groups and between malignant and both benign and control groups. In contrast; there was no statistical significant difference in SAA levels between FA and control group. Moreover; SAA level was directly proportional with aggressiveness of cancer breast. Sura et al demonstrated a highly significant increase of SAA concentration in benign and malignant breast tumors compared to control group \(p<0.001\)\(^{32}\). Chronic inflammation may promote carcinogenesis through complex processes such as polarization of M2 tumor-associated macrophages via cytokines and the subsequent production of tumor growth factors or promotion of angiogenesis\(^{10}\).

Solid tumours typically trigger inflammatory responses that result in the formation of a pro-tumourigenic and pro-angiogenic microenvironment around the tumour. Immune and inflammatory cells in the tumour microenvironment interact with malignant cells in a complicated fashion, resulting in stimulation of tumour growth, invasion, and metastasis\(^{19}\).

Our study investigated both CRP and SAA levels in different stages of cancer breast. There was a statistical significant difference in CRP levels between FA and control group, while there was no statistical significant difference between FA and stage I cancer breast, but statistical significant differences appeared in higher stages of cancer breast.

As regards SAA; there was no statistical significant difference between FA and control group, but there were statistical significant differences between FA and stage I cancer breast and the difference increased in higher stages. These results are nearly similar to a recent study done by Zhang et al\(^{35}\) who found no difference in SAA concentrations among the controls, benign breast lesion and stage I breast cancer patients, but there was a gradual increase of SAA concentrations in increasing stages of breast cancer patients.

Spearman’s correlation showed that SAA and CRP concentrations were correlated positively with tumor stages and with each other.

Other studies have demonstrated that higher concentrations of SAA were associated with increasing concentrations of CRP, and that el-
evated SAA and CRP were associated with reduced disease-free survival and reduced overall survival in breast cancer patients, regardless of adjustment for age, tumor stage, race and body mass index.

So, inflammation is closely linked to tumor progression since inflammation in the tumor microenvironment stimulates tumor growth, invasion, and metastasis, inflammation seems to favors invasion and metastasis more than to mount an effective host anti-tumour response.

So, inflammatory status may be an important prognostic factor for breast cancer. Additionally, reduction of the inflammatory reaction by improving diet quality may benefit breast cancer survivors.

Conclusions:

Our results indicated that inflammation plays a key role in pathogenesis and aggressiveness of breast cancer. SAA can be used as a predictor marker than CRP for malignant breast lesions.


استخدام بروتين α سي ر ب و بروتين الأميلويد A كعلامات لتقديم سرطان الثدي
رانيا الهلالى - رشة الزهري - محمد عبد الفتاح حجازي - و رامي فارس

الالتهاب المزمن يعد من الإسباب المؤدية لحدوث وتقدم سرطان الثدي. البروتين التفاعلي (CRP) وبروتين الأميلويد في الدم يمكن استخدامهما كمقياس لدرجة الالتهاب المزمن. تهدف هذه الدراسة إلى إمكانية استخدام CRP و SAA لدرجة تقدم سرطان الثدي. تم في هذه الدراسة قياس مستوى CRP و SAA في الدم من 181 مريضة (78-24 سنة) و 60 من الإناث الطبيعيه كمجموعة ضابطة (ELISA) باستخدام طريقة الإيلايزا (ELISA).

النتائج: كانت مستويات CRP أعلى في المرضى الذين لديهم سرطان الثدي وكذلك المرضى أصحاب الأورام الحميدة في الثدي من المجموعة الضابطة ولكن مع عدم وجود فروق ذات دلالة إحصائية بين المجموعات المصاحبة (P>0.001). وكانت مستويات SAA أعلى إحصائيا في المرضى أصحاب سرطان الثدي من المرضى أصحاب الأورام الحميدة والمجموعة الضابطة (P<0.001). ولكن لم يكن هناك فروق ذات دلالة إحصائية في مستويات بين المرضى أصحاب الأورام الحميدة والمجموعة الأخرى (P<0.57. أظهرت مستويات بروتين سي التفاعلي (CRP) أنه لا يوجد فرق إحصائيا كبير بين المرضى ذوي الأورام الحميدة في الثديا و المرضى ذوي سرطان الثدي مرحلة أولى (P=0.97). كما أثبتت الدراسة أن هناك علاقة إيجابية طردية إحصائية بين تراكمات CRP و SAA مع مراحل أورام مختلفة (ص = 0.861, P < 0.001). 

الخلاصة: يمكن استخدام كلا من CRP و SAA كمؤشر لدرجة تقدم سرطان الثدي خاصة.
EXPRESSION OF CD69 IN CHRONIC LYMPHOCYTIC LEUKEMIA; RELATION TO PROGNOSIS AND DISEASE PROGRESSION

Mahira I Elmougy* and Ghada M Elgohary**

ABSTRACT
Background: Chronic lymphocytic leukemia (CLL) is a disease characterized by extensive clinical heterogeneity despite a common diagnostic immunophenotype. CD69 is an integral membrane protein that is expressed in CLL as an early activation marker. Aim of the work: to evaluate the expression of CD69 in CLL and correlate it with clinical and cytogenetic prognostic markers. Subjects and Methods: the present work was carried on 55 patients diagnosed as CLL in Ain Shams University Hospitals, 20 hematological matched age and sex normal subjects served as control group. Expression of CD69 was detected by flow cytometric immunophenotyping. Results: Regarding CD69 expression, there was a significant difference in expression between patients and control group. 40 patients (73%) out of 55 had positive CD69 expression (≥30%) while 15 patients (27%) had negative CD69 expression (<30%). Comparative study between positive versus negative expression groups of patients revealed a significant difference in expression as regarding hemoglobin level, LDH level, CD38 expression, β2 microglobulin level, advanced disease stage as well as immunoglobulin heavy chain gene mutation. CD69 was significantly associated with splenomegaly, advanced disease stage, absence of immunoglobulin heavy chain gene mutation as well as increased β2 microglobulin level, high CD38 expression and low hemoglobin level. Patients with positive CD69 expression had a significantly shorter progression free survival than those with negative CD69 expression. Conclusion: CD69 could be considered as a novel independent prognostic marker which is significantly correlated with poor clinical and biological factors in B-CLL as well as disease progression. In addition, antiCD69 could be a suitable target for immunotherapeutic intervention.

INTRODUCTION
B-cell chronic lymphocytic leukemia (B-CLL) is a neoplasm of mature-appearing monoclonal B-lymphocytes, it is an incurable disease characterized by extensive clinical heterogeneity despite a common diagnostic immunophenotype (Small mature B cells display CD19+, CD20+, CD5+, CD23 markers) (32).

Whether defects in the apoptotic pathway are frequently encountered in a variety of cancers, B-CLL represents a paradigm of the tumors that arise as a consequence of alterations in the processes leading to programmed cell death (14). Indeed, B-CLL cells display multiple intrinsic defects in their apoptotic machinery and dysregulated production of survival signals from their microenvironment (4).

The clinical course of patients with B-CLL is quite variable (33) and the two major clinical staging systems are unable to prospectively discriminate an indolent or aggressive course within the low and intermediate risk categories (1,25). For this reason, several biological parameters have been added to the staging systems to differentiate prognostic subsets (8,9,12,17,22,27).

CD69, also known as activation inducer molecule (AIM), early activation antigen (EA-1), is a type II integral membrane protein with an extracellular C-type lectin domain. CD69 is not detectable on normal peripheral blood lymphocytes, but cell surface expression is upregulated in response to a wide variety of stimuli (20). CD69 is constitutively expressed on monocytes, platelets, Langerhans cells and a small percentage of resident lymphocytes in the thymus. In addition CD69 expression is induced very early upon activation of T and B lymphocytes, NK cells, macrophages, neutrophils (23). Activation-induced CD69 expression precedes the expression of other cell activation markers, such as CD25 (26). Therefore CD69 represents the first cell surface glycoprotein detected after lymphocytes activation. Therefore, the aim of this study is to investigate the potential merit of detection of CD69 expression by flow cytometry in B-CLL as an early activation marker and to correlate it with other biological and cytogenetic parameters as well as its independent prognostic value on disease progression.
SUBJECTS AND METHODS

Subjects:

The present study was conducted on 55 newly diagnosed B-CLL patients; who attended to Ain Shams University Hospitals, during the period from February 2012 till February 2013. Patients were 36 males and 19 females with a male to female ratio of 1.9:1. Their ages ranged from 53-71 years, with a mean of 62±12.73 years. In addition, twenty hematologically matched age and sex normal subjects were included as a control group which included 14 males and 6 females with a male to female ratio 2.3:1, their ages ranged from 52-69, with a mean of 60.5±12.02 years. A written consent was obtained from each patient and control before participation in the study. Patients were followed up for 30 months.

Methods:

All the patients were subjected to the following:

I- Full history and thorough clinical examination, laying stress on lymphadenopathy, splenomegaly and hepatomegaly, Rai staging according to disease burden and the degree of bone marrow involvement. II- Abdominal ultrasonography for spleen, liver and lymph node enlargement.

II- Laboratory investigations:

1- Complete blood count (CBC) with examination of peripheral blood (PB) smears stained with Leishman stain for lymphocyte count and morphology (done for both patients and control). 2- Bone marrow (BM) examination with morphological examination of Leishman stained smears laying stress on BM lymphocytes %, 3- immunophenotyping (IPT) of bone marrow or whole peripheral blood using Coulter EPICS-XL flow cytometer, USA. The following panel of monoclonal antibodies was used: CD20, CD79b, FMC7, s.IgM, CD5/CD19, CD10, CD103, CD123, CD23 and CD38) as well as κ and λ light chains labeled with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE) labeled supplied by coulter electronics. Negative isotypic control for determining the non-specific binding of the MoAbs.

Interpretation:

Positivity threshold for CD69 was defined as expression of ≥ 30% of lymphocytes for the marker.7

Statistical Analysis:

The processing of data was computed using SPSS (version 15) IBM compatible PC. Data were described in the form of number, percentage, range and median. Student-t test was used to compare two groups regarding quantitative variables. Receiver operating characteristic (ROC) curve was used to determine the best cut-off value. Progression-free survival (PFS) was calculated from the date of diagnosis to the date of progression.15 PFS curves were calculated by the Kaplan-Meier method and comparison between groups was performed by the Log rank test. P values of ≤ 0.05 and ≤ 0.01 were considered significant and highly significant respectively, in all analyses.

RESULTS

The clinical and laboratory characteristics of patients are listed in table (1)

Expression of CD69:
Expression Of CD69 in CLL: Relation To Prognosis

CD69 was expressed in all B-CLL patients with a percentage of expression ranging from 4 - 86% with a mean of 44.5±17.5%, in the control group CD69% expression ranged from 1% - 3% with a mean of 2.0±1.0% revealing a highly significant difference between patients and control groups (P < 0.001).

A cut off value at 30% was established to allow the most significant separation and differentiation between B-CLL cases with positive and negative expression. According to this cut off value, 40 cases (73%) were ≥ 30% for CD69 expression and 15 cases (27%) were < 30% for CD69 expression.

Comparative study between patients with positive versus negative CD69 expression: (Table 2)

A significant difference was detected between both groups (positive versus negative expression groups) regarding hemoglobin level (P< 0.001), LDH level (P= 0.001), CD38% expression (P= 0.022), β2M (P= 0.002), advanced disease stage (P< 0.001), level as well as IgVH mutation (P< 0.001) while no significant difference could be detected between both groups regarding other clinical and laboratory parameters.

Correlation between CD69 expression and laboratory data among patients (Table 3)

A highly significant positive correlation was detected between CD69 expression and each of β2 microglobulin (P=0.013) and CD38 expression (P= 0.033).

On the other hand, a significantly inverse correlation was detected between CD69 and Hb level (p= 0.004).

Relation between CD69 expression and clinical data as well as Rai stage and IgVH mutation among patients (Table 4)

A significant relation was detected between CD69 expression and each of splenomegaly, advanced Rai stage and absence of IgVH mutation (P< 0.001, < 0.001, < 0.001, respectively).

Relation between CD69 expression and disease progression

Kaplan Meier survival analysis revealed that patients with positive CD69 expression had a significantly shorter progression free survival (PFS) than those with negative CD69 expression (Log rank 38.860; P< 0.001) (Figure 1).

Combination analysis of CD69 expression with tumor burden markers (β2M and Rai stage) revealed that cases with high tumor burden (β2M > 2 mg/dl, Rai stage III-IV) and negative CD69 expression had significantly longer PFS compared to those with high tumor burden and positive CD69 expression (Log rank 16.728, P< 0.001) (Figure 2).

Table (1): Clinical and laboratory characteristics of studied group of patients

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>55</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>(53-71), median 60</td>
</tr>
<tr>
<td>Males, n(%)</td>
<td>36(65%)</td>
</tr>
<tr>
<td>Splenomegaly, n(%)</td>
<td>32(58%)</td>
</tr>
<tr>
<td>Lymphadenopathy, n(%)</td>
<td>20(36%)</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>11.095±1.76</td>
</tr>
<tr>
<td>Total leucocytic count TLC(x109/L)</td>
<td>90.65±25.5</td>
</tr>
<tr>
<td>Platelet count (x109/L)</td>
<td>196.7±56.55</td>
</tr>
<tr>
<td>Absolute peripheral blood lymphocyte count (x109/L)</td>
<td>55.975±20.375</td>
</tr>
<tr>
<td>B2-microglobulin (β2M) (mg/dl)</td>
<td>3.725±2.15</td>
</tr>
<tr>
<td>Lactate dehydrogenase (LDH) (1u/L)</td>
<td>401.95±253.15</td>
</tr>
<tr>
<td>CD38%</td>
<td>50.05±24.75</td>
</tr>
<tr>
<td>IGVH mutation</td>
<td>17(31%)</td>
</tr>
<tr>
<td>Rai stage (III-IV)</td>
<td>33(60%)</td>
</tr>
</tbody>
</table>
Table (2): Comparison between patients with positive versus negative CD69 expression regarding clinical and laboratory data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Negave CD69</th>
<th>Positive CD69</th>
<th>Independent t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 15</td>
<td>N = 40</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin (g/dl) Mean±SD</td>
<td>12.72±2.60</td>
<td>9.47±0.92</td>
<td>6.917</td>
</tr>
<tr>
<td>TLC (x10⁹/L) Mean±SD</td>
<td>83.80±20.5</td>
<td>97.5±30.5</td>
<td>1.604</td>
</tr>
<tr>
<td>Platelet count (x10⁹/L) Mean±SD</td>
<td>192.6±54.6</td>
<td>200.8±58.5</td>
<td>0.471</td>
</tr>
<tr>
<td>Peripheral bl. Lymphocyte count (x10⁹/L) Mean±SD</td>
<td>57.56±21.15</td>
<td>54.39±19.6</td>
<td>0.523</td>
</tr>
<tr>
<td>LDH (iu/L) Mean±SD</td>
<td>271.9±256.3</td>
<td>532±250</td>
<td>3.413</td>
</tr>
<tr>
<td>CD 23% Mean±SD</td>
<td>72.5±16.4</td>
<td>69.3±19.5</td>
<td>0.564</td>
</tr>
<tr>
<td>CD38% Mean±SD</td>
<td>41.6±27.2</td>
<td>58.5±22.3</td>
<td>2.356</td>
</tr>
<tr>
<td>β2M (mg/dl) Mean±SD</td>
<td>2.3±1.1</td>
<td>5.15±3.2</td>
<td>3.359</td>
</tr>
<tr>
<td>Rai stage</td>
<td></td>
<td></td>
<td>42.132</td>
</tr>
<tr>
<td>I</td>
<td>12 (80.0%)</td>
<td>0 (0.0%)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>2 (13.33%)</td>
<td>8 (20.0%)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>1 (6.67%)</td>
<td>20 (50.0%)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>0 (0.0%)</td>
<td>12 (30.0%)</td>
<td></td>
</tr>
<tr>
<td>IgVH mutation</td>
<td></td>
<td></td>
<td>23.275</td>
</tr>
<tr>
<td>Present</td>
<td>12 (80.0%)</td>
<td>5 (12.5%)</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>3 (20.0%)</td>
<td>35 (87.5%)</td>
<td></td>
</tr>
</tbody>
</table>

Table (3): Correlation between CD69 expression and laboratory data among patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CD69 expression</th>
<th>R</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLC (x10⁹/L)</td>
<td></td>
<td>0.054</td>
<td>0.609</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td></td>
<td>-0.559</td>
<td>0.004</td>
</tr>
<tr>
<td>Platelet count (x10⁹/L)</td>
<td></td>
<td>0.244</td>
<td>0.316</td>
</tr>
<tr>
<td>Peripheral bl. Lymph (x10⁹/L)</td>
<td></td>
<td>0.211</td>
<td>0.311</td>
</tr>
<tr>
<td>β2M (mg/dl)</td>
<td></td>
<td>0.491</td>
<td>0.013</td>
</tr>
<tr>
<td>CD23</td>
<td></td>
<td>0.391</td>
<td>0.53</td>
</tr>
<tr>
<td>CD38</td>
<td></td>
<td>0.428</td>
<td>0.033</td>
</tr>
</tbody>
</table>

Table (4): Relation between CD69 expression and clinical data as well as Rai stage and IgVH mutation among patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>No.</th>
<th>% of CD69 (Mean±SD)</th>
<th>T-test</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Splenomegaly</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>23</td>
<td>18±5.4</td>
<td>27.810</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Positive</td>
<td>32</td>
<td>69±7.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphadenopathy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>35</td>
<td>42±6.9</td>
<td>1.624</td>
<td>0.110</td>
</tr>
<tr>
<td>Positive</td>
<td>20</td>
<td>45±6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rai stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I, II</td>
<td>22</td>
<td>22±4.3</td>
<td>21.669</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>III, IV</td>
<td>33</td>
<td>65±8.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgVH mutation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>38</td>
<td>69±6.6</td>
<td>26.854</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Positive</td>
<td>17</td>
<td>18±6.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Expression Of CD69 in CLL; Relation To Prognosis

**Fig. (1):** Kaplan Meier survival curve showing PFS of CD69 (positive and negative expression)

<table>
<thead>
<tr>
<th></th>
<th>Median</th>
<th>SE</th>
<th>Log rank</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative CD69</td>
<td>24.0</td>
<td>0.9</td>
<td></td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Positive CD69</td>
<td>10.0</td>
<td>0.3</td>
<td>38.860</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

**Fig. (2):** Kaplan Meier survival curve showing PFS of CD69 (positive and negative expression) in combination with tumor burden markers (β2M and Rai stage)

<table>
<thead>
<tr>
<th></th>
<th>Median</th>
<th>SE</th>
<th>Log rank</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative CD69</td>
<td>18.00</td>
<td>1.89</td>
<td></td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Positive CD69</td>
<td>9.00</td>
<td>0.38</td>
<td>16.728</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>


**DISCUSSION**

In the present work, regarding CD69 expression, the threshold of positivity was set at 30% to identify patients with positive and negative CD69 expression. Mean CD69% was significantly higher in B-CLL patients compared to the control group. This comes in accordance with a study done by Del Poeta et al.\(^{(7)}\) who stated that the optimal cut off for CD69 expression yielding the best separation of CLL patients into 2 subsets with significantly different prognosis was fixed at 30% of positive cells \(^{(3,7,12,19)}\). The same comes in accordance with Gattei et al.\(^{(12)}\), Krober et al.\(^{(19)}\) and Bomben et al.\(^{(3)}\).

In our study 40(73%) patients out of 55 patients had CD69% expression ≥ 30% and were identified to have a positive expression, while 15 (27%) patients had < 30% CD69 expression and were identified as negative expression group.

Similarly, Smilveska et al.\(^{(30)}\) showed that 64.3% of their CLL cases were CD69 positive.

In this work, comparative study between patients with negative versus positive CD69 expression revealed a significant difference detected among them regarding hemoglobin level, LDH level, CD38 expression, β2M level, IgVH mutation as well as advanced disease stage. As patients with positive CD69 expression were associated with a lower hemoglobin, higher LDH, β2M levels, CD38 expression, absence of IgVH mutation as well as advanced disease stage compared to those with negative CD69 expression. While no significant difference could be detected regarding other parameters.

Damle et al.\(^{(5)}\) and Smilveska et al.\(^{(30)}\) stated in their studies that advanced disease stage is significantly associated with positive CD69 expression.

In our study, a highly significant association was detected between CD69 expression and splenomegaly. This comes in accordance with Del Poeta et al.\(^{(7)}\) who found that CD69 expression is significantly associated with splenomegaly.

Also, significant association was detected between CD69 expression and each of CD38 expression and unmutated IgVH, this was similarly detected by Del Poeta et al.\(^{(7)}\) who reported that CD69 expression was significantly related with CD38, CD49d, ZAP-70 and unmutated IgVH.

In our work, the significant association between positive CD69 expression and many of the poor clinical and biological standard prognostic factors as splenomegaly, high CD38 expression increased β2M level, advanced disease stage, low Hb level as well as unmutated IgVH strongly suggests its beneficial utility as an independent poor prognostic factor in B-CLL. In agreement with our work, Grund et al.\(^{(13)}\) showed in their study that negative CD69 expression was associated with mutated IgVH. The concordance between CD69 positivity and mutation status was 96%, since it is well known that patients with unmutated IgVH genes have a worst prognosis and decreased survival compared to those with mutated IgVH genes.

Herishanu et al.\(^{(16)}\) and Walsby et al.\(^{(31)}\) stated that CD69 has been found to be stronger predictor of CLL prognosis. In our study, patients with positive CD69 expression had a significantly shorter PFS than those with negative CD69 expression, this was in agreement with Del Poeta et al.\(^{(7)}\) who demonstrated that CD69 expression was an independent risk factor for PFS and OS in their large series of CLL patients. CD69 was found to be upregulated on cells in the tissue microenviroment, both in bone marrow (BM) and lymph nodes (LN). Interestingly, stronger upregulation of CD69 was also found in LN-resident compared with BM-resident CLL cells and LN-derived cells showed an increase in BCR signature genes compared with those from the BM and the P.B\(^{(16)}\). Moreover, because CD69 is involved in retaining lymphocytes at the site of stimulation \(^{(18,28,29)}\), the levels of this molecule on circulating B-CLL cells might be less than those in the solid tissue (BM and LN) and, therefore, might indicate that B-CLL cells expressing CD69 are recent emigrants from such sites. Tumor proliferation, quantified by the expression of E2F and c-MYC target genes and verified with Ki67 staining by flow cytometry was highest in the LN and was correlated with clinical disease progression \(^{(3)}\). Thus CD69 appear to offer an easy to perform and reliable marker both of progression and poor
Expression Of CD69 in CLL; Relation To Prognosis

outcome in CLL. In the present work, combined analysis of CD69 expression with tumor burden markers (ß2M and Rai stage) revealed that cases with high tumor burden (ß2M > 2 mg/dl, Rai stage III-IV) and negative CD69 expression had significantly longer PFS compared to those with high tumor burden and positive CD69 expression (Log rank 16.728, P< 0.001). This comes in agreement with Del Poeta et al.\textsuperscript{17} who had done a multivariate analysis of PFS and OS, in which entered Rai stages, ZAP-70 beta-2 microglobulin, CD38 and CD69 resulted to be independent prognostic factors.

Interestingly, Gattei et al.\textsuperscript{12} made a combined analysis of CD69 with CD38 or CD49d or ZAP-70 where they demonstrated that CD69 expression had true additive properties, allowing us to identify CLL subsets (CD69-CD38-;CD69-CD49d-, CD69-ZAP-70; CD69-M IgVH) presenting a very good outcome with regard to PFS and OS. Conversely, double positive (CD69+ CD38+, CD69+ CD49d+, CD69+ ZAP-70+ subsets) and CD69 positive UM IgVH patients showed the worst outcome. Moreover, CD69 was also necessary to correctly prognosticate the active/progressive disease status than CD38 and ZAP-70. Furthermore, they stated that CD69 is easily analyzed with flow cytometry and well suited for routine analysis.

Significant progress is being made in augmenting specific immune effector functions in experimental tumor therapy, particularly using monoclonal antibodies (mAbs)\textsuperscript{24}.

Mechanisms of NK-cell cytotoxicity activation by costimulatory molecules have been described and new studies reveal novel roles for regulatory molecules on NK cells. Similarly, mAb CD69 promotes NK cytolytic activity in the presence or absence of the tumor microenvironment. Consequently, in vivo treatment with anti-CD69 mAbs, in either preventative or therapeutic settings, is efficient in promoting NK-cell-dependent tumor elimination. Importantly, this occurs in the face of significant levels of TGF-ß made by the tumors themselves \textsuperscript{21}.

Furthermore, CD69 targeting by a non-depleting anti-CD69 antibody similarly increases anti-tumor responses by enhancing NK cell activity, and treatment of NK cells with this antibody results in increased cytotoxic activity and IFN-γ production, CD69 thus regulates anti-tumor immune responses by modulating the expression of various cytokines, including TGF-ß and IFN-γ \textsuperscript{13}.

In conclusion, CD69 is significantly correlated with poor clinical and biological factors in B-CLL as well as disease progression which could be considered as a novel independent prognostic marker determined by flowcytometry. This supports its utility in routine laboratory assessment and, possibly, in a prognostic scoring system for B-CLL. Moreover, anti-CD69 could be a suitable target for immunotherapeutic intervention.

REFERENCES


ظرور بروتينين في سرطان الدم الليمفاوي المتقدم وعلاقته بالتنبؤ بمسار وتقدم المرض

في التدفقات الموجبة - غاد الجوهر

يعتبر سرطان الدم الليمفاوي المتقدم أحد الأمراض الإكلينيكية على الرغم من اشتراعها جماعيا في نظام ظهاري مناعي. واحد من التشخيصات والعيوب في حالات سرطان الدم الليمفاوي متقدم علاجه علاج التعرف على تعريف الهدف من التدفقات في حالات سرطان الدم الليمفاوي المتقدم GLL وربط ذلك بمعنويات التنبؤ الالكليبيكية والوراثية الخلوية. للحالات وطرق البحث: تم إجراء الدراسة الحالية على 55 مريضا من أك التحكم اصابتهم بسرطان الدم الليمفاوي المزمن بمستشفيات جامعتين سيسما بالإضافه الي 20 خصام من الأشخاص الأصصاء المتواقيين في العمر والنوع.

لكشفه عن ظهور بروتين CD69 بواسطة النمط الظاهري للتدفقات الخلوى المأذية.

النتائج: كشفت الدراسة عن ظهور بروتين CD69 في جميع مرضى سرطان الدم الليمفاوي المتقدم، وكان هناك اختلاف كبير في درجة الظهور بين المرضى المجموعه الضامنة. حيث كانت نسبة الظهور بروتين CD69 مرتفعة (≥) 30% في مجموعتين من بين 50 مريضا (76%) في حين أن الظهور الضعيف (≤) 30% كشفت المقارنة بين مجموعتي الظهور المنخفض والمرتفع اختلاف كبير في مستوي الظهور بما يتعلق بمستوي الهيموجلوبين ومستوي النيتروجين الهيدروجين (LDH) وبروتين CD38 ومستوي الميكروجولوبولين (P<) ومرحلة تقدم المرض وكذلك الطفرات الجينية لجولوبولين المعتاد تقل السلسلة. واظهرت الدراسة ارتباط موجب بين بروتين CD69 في حالات سرطان الدم الليمفاوي المتقدم.

ينبغي أن يتحرك الحالات والتشخيصات المتقدمة للمرض في الحين كان هناك ارتباط إيجابي مع مستوي الهيموجلوبين وكذلك النتروجين الهيدروجين وظروف الجينية للجولوبولين المناعي. تقل السلسلة. واظهرت الدراسة قصر قوة البقاء على قيد الحياة مع عدم تقدم المرض لدى المرضى الذين يعانون من ارتفاع ظهور بروتين CD69 علاجه بدلا من مستقات جنسيات والتي ترتبط بشكل كبير مع العوامل الإكلينيكية والبيولوجية مشابهة في حالات سرطان الدم الليمفاوي بي (B-CLL) وحلقة من تشخيص المرض وبالإضافه الي ذلك يمكن أن اعتبار مضادات بروتين CD69 Hegan لناحية للتلقيح بالعلاج المناحال
COMBINED ANALYSIS OF LEVELS OF SERUM B-CELL ACTIVATING FACTOR AND A PROLIFERATION INDUCING LIGAND IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA; CORRELATION WITH CLINICAL FEATURES AND DISEASE PROGRESSION

Mahira I Elmougy* and Ghada M. Elgohary**

ABSTRACT

Background: B-cell activating factor (BAFF) and a proliferation inducing ligand (APRIL) are involved in normal B-cell survival and differentiation. Aim of the work: to evaluate the role of BAFF and APRIL in B-CLL and their prognostic relevance.

Subjects and Methods: 50 patients diagnosed as CLL and 30 age and sex-matched healthy controls were enrolled into this study for assessment of serum BAFF and APRIL levels by enzyme linked Immunosorbent assay (ELISA). Results: In CLL-patients, median serum BAFF level was significantly lower than in healthy controls, whereas serum APRIL was significantly higher than in healthy controls. BAFF was significantly correlated to platelet count, CD5 expression, peripheral blood lymphocyte count and bone marrow infiltration. Moreover BAFF level was significantly lower in patients with stage C Binet staging system than those in group B and group A. APRIL serum level was significantly correlated with CD38 expression as well as advanced clinical stage. Combined analysis of BAFF and APRIL serum levels emerged as an independent predictor of disease progression.

Conclusion: Our results suggest that the combined analysis of serum BAFF and APRIL seems to be a reliable predictor of B-CLL, and highlights its importance in identifying patients at a higher risk of progression.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is characterized by the progressive accumulation of monoclonal B-cells with a distinctive immunophenotype (i.e. CD5+ CD19+, CD20 dim, CD23+, sm Ig dim) in peripheral blood, bone marrow, and the lymphoid tissues[14]. This accumulation is mainly due to a defective regulation of programmed cell death[5]. CLL cells express high levels of antiapoptotic proteins such as Bcl-2 and Bcl-xL[28]. However, CLL cells undergo spontaneous apoptosis in vitro, which reflects the requirement of external factors (i.e. microenvironment signals for survival of the CLL clone[16].

Currently, most patients with CLL are diagnosed in early, stable phases of their disease, but all of them eventually progress. Factors that predict disease progression not only are clinically useful but also pinpoint biological mechanisms that underlie the disease process and which may be amenable to therapeutic intervention.

BAFF (B cell activating factor of the TNF family) /BlyS (B lymphocyte stimulator) and APRIL (a proliferation-inducing ligand) belong to the TNF ligand superfamily and share several biological characteristics and functions[2,18]. Both BAFF and APRIL bind with high affinity two members of TNF-receptor superfamily, BCMA (B-cell maturation antigen) and TACI (Transmembrane activator and calcium modulator and cyclophilin ligand interactor), BAFF, but not APRIL, interacts with the third receptors named BAFF receptor (BAFF-R/BR3)[30]. BAFF, APRIL and their receptors are crucial for the survival of normal B lymphocytes. Both factors play an important role in the control of their maturation and differentiation into Ig-secreting cells. Defects in the synthesis of these molecules or expression of their receptors have been associated with various B cell malignancies[18,30]. BAFF and APRIL were found to protect B-CLL cells from spontaneous and drug-induced apoptosis and to enhance cell survival[16]. These findings suggest that BAFF and APRIL may be involved in the pathogenesis of B-cell chronic lymphocytic leukemia (B-CLL).

The aim of our study was to investigate the role of BAFF and APRIL in the pathogenesis and prognosis of B-CLL by assessment of correlation between their expression and clinical and laboratory parameters.
Moreover, we have been interested to study the relationship between BAFF, APRIL and some prognostic factors as CD38 expression which is a known poor prognostic factor in B-CLL (7).

SUBJECTS AND METHODS

Fifty patients with B-CLL attending the clinical hematology and oncology unit, Ain Shams University Hospital, from August 2011 to February 2013, and 30 age- and sex matched healthy controls were enrolled into this study. Patients included 32 males and 18 females, with a male to female ratio of 1.8:1. Their median age at diagnosis was 71 years (range 55-86 years). The control group included 18 males and 12 females (ratio, 1.5:1) with a median age of 65 years (range 51-79 years). A written consent was obtained from each patient and control before participation in the study. All patients were subjected to:

i) Full history and physical examination.

ii) Complete blood count.

iii) Laboratory investigations including blood urea nitrogen and uric acid.

iv) Pelvi-abdominal ultrasound.

v) Immunophenotyping using flowcytometry.

vi) Bone marrow aspiration and examination.

vii) Measurement of circulating BAFF and APRIL serum levels using quantitative ELISA technique.

- Patients were followed up for 24 months

The staging of B-CLL was based according to the Binet staging system.

Methods

Sample collection: Peripheral blood and BM samples were collected on ethylene diamine tetra-acetic acid (EDTA) (1.2 mg/ml) for morphological and immunophenotypic determination. For chemical analyses and assessment of serum BAFF and APRIL levels, clotted samples were obtained. Serum was isolated and stored at -20°C till subsequent use in ELISA for BAFF and APRIL.

BAFF and APRIL serum concentrations by enzyme linked immunosorbent assay (ELISA):

Serum samples from B-CLL patients and healthy controls were analyzed by ELISA kit for human BAFF and APRIL using commercial ELISA kits: Quanticine human BAFF immunoassay (R&D systems) and human APRIL ELISA (Bender MED systems). According to manufacturer’s instructions. Human BAFF or APRIL specific-specific polyclonal antibodies were pre-coated onto 96-well plates. The human specific detection monoclonal antibodies were biotinylated. The test samples and biotinylated detection antibodies were added to the wells subsequently and then followed by washing with PBS or TBS buffer, Avidin-Biotin-Peroxidase complex was added and unbound conjugates were washed away with PBS or TBS buffer and substrate was added. The colour developed is proportioned to the amount BAFF or APRIL bound. The reaction is stopped and the intensity of the yellow color is measured Spectrophotometrically at a wave length of 450 nm. The concentration of BAFF or APRIL in the samples was then determined.

Table (1): Binet clinical stage system (15)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Clinical features</th>
<th>Overall survival years</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Lymphocytosis in peripheral blood and bone marrow and &lt; 3 lymphoid regions involved. No anemia, no thrombocytopenia</td>
<td>12</td>
</tr>
<tr>
<td>B</td>
<td>Lymphocytosis in peripheral blood and bone marrow and &gt; 3 lymphoid regions involved. With or without splenomegaly and/or hepatomegaly No anemia, no thrombocytopenia</td>
<td>7</td>
</tr>
<tr>
<td>C</td>
<td>Lymphocytosis with anemia (hemoglobin level &lt; 11 g/dL in male and &lt; 10 g/dL in female) or thrombocytopenia (platelet count &lt; 100 x 10^9/L)</td>
<td>2-4</td>
</tr>
</tbody>
</table>
by comparing the optical density of the samples to the standard curve. Results are reported in ng/ml.

Statistical analysis

The processing of data was computed using SPSS (version 15) IBM compatible PC. Data were described in the form of number, percentage, range and median. Student-t test was used to compare two groups. Receiver operating characteristic (ROC) curve was used to determine the best cut-off value. Progression-free survival (PFS) was calculated from the date of diagnosis to the date of progression (1). PFS curves were calculated by the Kaplan-Meier method and comparison between groups was performed by the Log rank test. P values of ≤ 0.05 and ≤ 0.01 were considered significant and highly significant respectively, in all analyses.

RESULTS

The clinical characteristics of patients with CLL are listed in table (2).

**BAFF and APRIL serum levels (Table 3)**

In patients with CLL, the median serum BAFF level was 1.12 ng/ml (range 0.10-2.16), significantly lower than in healthy controls [2.95 ng/ml (range 2.5-3.91); P=0.012]. Whereas serum APRIL was significantly higher than in healthy controls [11.4 ng/ml (4.8-18.5) versus. 6.54 ng/mL (2.1-10.8); P= 0.042]. The receiver operating characteristics (ROC) curve identified a cut-off value equal to 2.15 ng/ml and 4.5 ng/mL to categorize patients with low and high BAFF and APRIL levels, respectively.

**Correlation between BAFF and laboratory data among patients (Table 4)**

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>71(55-86)</td>
</tr>
<tr>
<td>Males, n(%)</td>
<td>32(64%)</td>
</tr>
<tr>
<td>Hepatomegaly, n(%)</td>
<td>39(78%)</td>
</tr>
<tr>
<td>Spleenomegaly, n(%)</td>
<td>41(82%)</td>
</tr>
<tr>
<td>Lymphadenopathy, n(%)</td>
<td>12(24%)</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>10.21±1.4</td>
</tr>
<tr>
<td>TLC (x10^9/L)</td>
<td>7.74±8.02</td>
</tr>
<tr>
<td>Absolute lymphocytic count (x10^7/L)</td>
<td>41.32±23.5</td>
</tr>
<tr>
<td>Platelet count (x10^9/L)</td>
<td>115.31±60.28</td>
</tr>
<tr>
<td>Binet stage</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>22(44%)</td>
</tr>
<tr>
<td>B</td>
<td>13(26%)</td>
</tr>
<tr>
<td>C</td>
<td>15(30%)</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>15.30±8.21</td>
</tr>
<tr>
<td>Uric acid (mg/dL)</td>
<td>6.9±3.47</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patients group</th>
<th>Control group</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAFF (ng/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>1.12</td>
<td>2.95</td>
<td>0.012</td>
</tr>
<tr>
<td>Range</td>
<td>0.10-2.16</td>
<td>2.5-3.91</td>
<td></td>
</tr>
<tr>
<td>APRIL (ng/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>11.4</td>
<td>6.54</td>
<td>0.042</td>
</tr>
<tr>
<td>Range</td>
<td>4.8-18.5</td>
<td>2.1-10.8</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patients</th>
<th>r</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g/dL)</td>
<td></td>
<td>0.088</td>
<td>0.600</td>
</tr>
<tr>
<td>Total leucocytic count (x10^9/L)</td>
<td></td>
<td>-0.126</td>
<td>0.430</td>
</tr>
<tr>
<td>Peripheral blood lymphocyte count</td>
<td></td>
<td>-0.419</td>
<td>0.009*</td>
</tr>
<tr>
<td>Platelet count (x10^9/L)</td>
<td></td>
<td>0.905</td>
<td>0.035*</td>
</tr>
<tr>
<td>BM infiltration (%)</td>
<td></td>
<td>-0.450</td>
<td>0.021*</td>
</tr>
<tr>
<td>CD19%</td>
<td></td>
<td>0.44</td>
<td>0.005*</td>
</tr>
<tr>
<td>CD23%</td>
<td></td>
<td>-0.187</td>
<td>0.370</td>
</tr>
<tr>
<td>FMC7%</td>
<td></td>
<td>-0.340</td>
<td>0.096</td>
</tr>
<tr>
<td>CD79%</td>
<td></td>
<td>0.280</td>
<td>0.175</td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td></td>
<td>0.442</td>
<td>0.457</td>
</tr>
<tr>
<td>Uric acid (mg/dL)</td>
<td></td>
<td>-0.94</td>
<td>0.398</td>
</tr>
</tbody>
</table>
There was a significant positive correlation between BAFF values and each of platelet count and CD5 expression (P=0.012 and 0.001 respectively). While there was a significant inverse correlation between BAFF values and each of peripheral blood lymphocyte count and BM infiltration (P=0.022 and 0.03 respectively). On the other hand, there was no significant correlation between BAFF and other parameters as total leucocyte count, hemoglobin level, CD23, FMC7, CD79, BUN and uric acid.

According to Binet staging system, B-CLL patients were subdivided into three subgroups (A,B,C) BAFF level was highly significantly lower in group C than group B and A as median was 0.078 in group C, 0.83 in group B and 2.1 in group A (P=0.005). This reveals that BAFF level decreased with advanced stages of CLL.

**Correlation between APRIL level and laboratory data among patients (Table 5)**

<table>
<thead>
<tr>
<th>Patients</th>
<th>APRIL</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>-0.025</td>
<td>0.0903</td>
</tr>
<tr>
<td>Total leucocytic count (x10⁹/L)</td>
<td>0.118</td>
<td>0.366</td>
</tr>
<tr>
<td>Peripheral Blood lymphocyte count</td>
<td>0.185</td>
<td>0.366</td>
</tr>
<tr>
<td>Platelet count (x10⁹/L)</td>
<td>-0.097</td>
<td>0.637</td>
</tr>
<tr>
<td>BM infiltration (%)</td>
<td>0.202</td>
<td>0.322</td>
</tr>
<tr>
<td>CD5%</td>
<td>-0.204</td>
<td>0.319</td>
</tr>
<tr>
<td>CD23%</td>
<td>0.154</td>
<td>0.804</td>
</tr>
<tr>
<td>FMC7%</td>
<td>0.136</td>
<td>0.508</td>
</tr>
<tr>
<td>CD79%</td>
<td>-0.208</td>
<td>0.737</td>
</tr>
<tr>
<td>CD38%</td>
<td>0.996</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Binet staging system (advanced stage)</td>
<td>0.421</td>
<td>0.004*</td>
</tr>
</tbody>
</table>

High serum levels of APRIL were significantly correlated with a high CD38 expression as well as advanced clinical stage (Binet staging system) (P=0.03, 0.01 respectively). While, no significant correlation was detected between APRIL level and other parameters.

The risk of progression was assessed by combing BAFF and APRIL serum levels, three different groups were identified: a low risk group (high BAFF and low APRIL), a high risk group (low BAFF and high APRIL), and an intermediate risk group (not fitting the two former categories).

Survival analysis (Figure 1) revealed that high risk group patients had a significantly shorter PFS [median 5.5 months, 95% CI 4.99-6.01] compared to those of low and intermediate risk groups [median 17.15, 10.5 months, 95% CI 14.31-19.99 and 8.22-12.78] respectively (Log rank 65.11; P < 0.001).
Despite homogenicity of B-CLL at the cellular level, it is clinically heterogeneous; some patients survive for a long time without therapy, while others progress towards more advanced stages and die despite aggressive treatment (8).

Several reliable prognostic markers were found to be capable of predicting the progression and outcome of the disease from its early stages including age, sex, lymphocyte morphology, lymphocyte doubling time, serum factors, immunophenotyping and cytogenetic finding (21).

In recent years molecular and cellular markers have helped to predict the prognosis of CLL patients. A combined panel of FISH probes are available now and can identify genomic aberration in approximately 80% of CLL cases using a 4-probe panel for the detection of deletion of 13q14, 17p13, 11q22-23 and trisomy 12 (31).

Recent years have witnessed increasing interest in BAFF and APRIL as B-cell survival factors implicated in the pathogenesis of several B-cell malignancies (10). In CLL, the biological relevance of both molecules has been demonstrated in vitro studies, which show that the addition of exogenous BAFF and APRIL protects neoplastic CLL cells from apoptosis (9, 23). Furthermore, abnormal BAFF and APRIL serum levels are found in CLL and other B-cell malignancies (10, 27). Not surprisingly, there is considerable interest in finding effective ways to target this system as a new treatment modality in B-cell lymphoproliferative disorders (4, 12).

In our study 64% of cases were males and 36% were females, this come in accordance with data shown by Okaly et al. (25) who found that males have higher incidence of CLL compared with females.

The age range at presentation in our study was 55-86 years, in contrast to Siegl et al. (29) who found that the higher incidence of CLL was seen in age range 75-84 years. This variation could have been due to the smaller number of cases that were studied or due to the ethnic differences in epidemiology.

We compared the level of BAFF and APRIL in patient’s and control’s groups by quantitative ELISA kits and we found significantly lower and higher levels of BAFF and APRIL respectively in the sera of B-CLL patients compared to healthy controls.

This was in accordance with recent observations of Planelles et al. (26), Haiat et al. (13) and Kern et al. (16) showing a decrease in circulating BAFF levels in B-CLL patients in comparison with healthy subjects. Which could be related to the absorption of BAFF by CLL cells (20). Another study showed elevated BAFF levels only in patients with familial B-CLL (24).

On the other hand, plasma APRIL levels in B-CLL patients were significantly higher as compared with healthy subjects. Our results were similar to those of Planelles et al. (26) showing an increase in circulating APRIL protein concentration compared with healthy subjects.

BAFF and APRIL are produced in excess in patients with various B-lymphoid malignancies, either by the leukemic cells themselves or by cells in their micro environment or both (17). A variety of tumoral B cells from Non-Hodgkin’s lymphoma (NHL) were found to express receptors for BAFF. Comparable levels were detected in B lymphocytes from normal individuals and from patients with diffuse large cell lymphoma (DLCL), mantle cell lymphoma (MCL) and marginal zone lymphoma, whereas those from B-CLL and follicular lymphoma displayed somewhat lower expression (3).

Regarding BAFF, there was a significant positive correlation between BAFF values and each of platelet count and CD5 expression. Bojarska et al. (1) found a positive correlation between percentage of CD5 positive B lymphocytes and BAFF concentration.

In addition there was a significant inverse correlation between BAFF values and each of peripheral blood lymphocyte count, bone marrow infiltration and advanced disease stage, which was in accordance with Ferrer et al. (11).

It was found that among patients with follicular lymphoma, those with circulating neoplastic B-cells had lower serum BAFF compared to those without detectable neoplastic cells in the peripheral blood. This suggests that serum BAFF may be a marker of tumor burden (13).
Regarding serum APRIL levels, there was a significant correlation with high CD38 expression as well as advanced clinical stage. This is in agreement with Ferrer et al.\(^{(11)}\) who found that APRIL serum levels were significantly increased in those cases with high CD38 expression. This may reflect the dependence on CD38 of the cross-talk between CLL cells and stromal or nurse-like cells which are an important source of BAFF and APRIL.\(^{(22)}\) Previous studies have suggested that either BAFF or APRIL serum levels correlate with disease progression and survival\(^{(1,19)}\).

The risk of progression was assessed by combining BAFF and APRIL serum levels, three different risk groups were identified. In our study, survival analysis revealed that high risk group patients had a significantly shorter PFS compared to those of low and intermediate risk groups. This comes in accordance with a study done by Ferrer et al.\(^{(11)}\) who demonstrated that the combined analysis of BAFF and APRIL may provide more accurate predictive information by separating patients into three risk groups. Thus subjects showing low BAFF and high APRIL serum levels had the highest risk of disease progression whereas those with high BAFF and low APRIL had a very low progression risk.

In conclusion, this study demonstrated that BAFF could be a valid marker of leukemic tumor burden and disease progression. Additionally APRIL strongly correlates with poor prognostic factors as high CD38 expression. From the clinical stand point, the new and most relevant finding of our study is that the combined analysis of serum BAFF and APRIL seems to be a better predictor of progression. However it should be validated in further studies including larger series of patients.

REFERENCES


التحليل المشترك لمستويات العامل المنشئ للخلايا بي في مصل الدم والخلايا المحفزة لانتشارها في حالات سرطان الدم الليمفاوي المزمن مع بيان الارتباط بين الظواهر الاكلينيكية وتقدم المرض

مهيره الموجي - غاده الجوهر

المقدمه: يشترك كل من عامل تنشيط الخلايا بي والجین المحفز للتسلئيحية الانتشارية في بقاء الخلايا بي العادية وتمييزها والفروض من الدراسة تقييم دور عامل تنشيط الخلايا بي والجین المحفز لانتشارية في حالات سرطان الدم الليمفاي وهمية في النتیج بمسار المرض الحالات وطرق البحث شملت الدراسة 50 مريضاً من أک بحوث مصابات بسرطان الدم الليمفاي المزمن و30 أخری من الأصحاء المقاتلين في العمر و النوع تقييم مستوي عامل تنشيط الخلايا بي ومستوى الجین المحفز لانتشارية في الدم عن طريق تقييم قياس الألزيم المناعی (الأليزا النتائج: اظهرت الدراسة اخفاق مستوى عامل تنشيط الخلايا بي في مصل الدم لدى مرضى سرطان الدم الليمفاي المزمن بصورة ملحوقة عن نظيره في المجموعة الضابط في حين كان مستوي الجین المحفز لانتشارية أعلى بكثير مما كان عليه في مجمعة الأصحاء وارتفاع مستوي عامل تنشيط الخلايا بي إلى حد كبير بعد الصفائح الدموية وظهور بروتين cd5 وعدد الخلايا الليمفايевой الطريقة في الدم وارتفاع ارتفاع العظمي وعلاو على ذلك كان مستوي ظهور عامل تنشيط الخلايا بي يبدو أقل من ذلك بكثير لدى المرضى الذين يعانون من المرحلة ج من ذلك الموجودون في المجموعة B والمجموعة ا وفقاً لطقوه القمائية بيني والارتفاع مستوي الجین المحفز لانتشارية في مصل الدم بشكل كبير يظهر بروتين 38 غضب النرقة الليمفاوية المتقدم للمرض ونورون تحليل المشترك لكل من عامل تنشيط الخلايا بي والجين المحفز لانتشارية في مصل الدم بشكل كبير يظهر تحليل المشترك لكل من عامل تنشيط الخلايا بي والجين المحفز لانتشارية في مصل الدم مستقل للفحص الخلسي: تشير النتائج إلى امكانيه الاعتماد على التحليل المشترك لعامل تنشيط الخلايا بي والجين المحفز لانتشارية كعامل متبوء موثوق فيه لحالات سرطان الدم الليمفاي مع التأكد على اهميتها في التعرف على المرضي ذوي الخطورة العالية لتقديم المرض.
IN VITRO APOPTOTIC EFFECT OF METFORMIN ON HUMAN CERVICAL CANCER CELLS
Dina Sabry*, Sahar H. Ahmed** and Mohamed A. S. Al-Ghussein***

ABSTRACT
Aim: We aimed to estimate whether metformin treatment against cervical cancer Hela cell line could affect cellular viability or not and to evaluate the genes expression of p53, caspase-3, Bcl2, COX-1 and cyclin-D1 as apoptotic and proliferation markers genes. Methods: Hela cell line was cultured and treated with different concentrations of metformin. Cells viability was investigated by MTT proliferation assay. Cells was treated by metformin for 48 h and 72 h and harvested for RNA extraction. RNA was transformed to cDNA and p53, caspase-3, Bcl2, COX-1 and cyclin-D1 genes’ expression was assessed by qPCR. Results: Metformin was cytotoxic for Hela cervix cancer cells and reduced the survival fraction in a dose response relationship. Metformin affects many genes’ expression that reflects life and death. P53 and caspase-3 genes’ expression were raised by the drug effect while Bcl-2, COX-1 and cyclin-D1 genes’ expression levels were reduced. Conclusions: Metformin showed potent apoptotic activities against cervical cancer Hela cell line through the elevation of p53 and caspase-3 and the elevation of Bcl-2, COX-1 and cyclin-D1 genes’ expression. Metformin could be the new, safe and effective treatment against cervical cancer. Keywords Hela, Cervix, p53, caspase-3, Bcl2, COX-1, cyclin-D1

INTRODUCTION
The second female cancer worldwide is the cancer of cervix as the most common malignancy in both incidence and mortality. Unfortunately, more than 80% of cervical cancer cases are found in developing countries. Many lines treatments were developed and used for cervical cancer, but each of them has apparent drawbacks. Surgical treatment line is restricted only for patients with early stage and the young patients who have lost fertility. Radiotherapy and chemotherapy are randomized, not specific to only cancer cells and often bring severe adverse effects including bone marrow suppression, nerve injury, gastrointestinal adverse reactions, renal impairment and second cancer occurrence. In spite of the advanced technology and methods, up to one third of patients will still develop persistence/recurrence/metastatic disease when the treatment results are poor.

New therapeutic strategies must be evaluated to improve survival. Thus, finding a safer and more efficient treatment remains an arduous task.

Metformin belongs to a class of compounds called biguanines that were first isolated from the plant Galega officinalis (French lilac or goat’s rue) known for its medicinal value.

It is the most widely prescribed drug for treatment of diabetes type 2 in the world. Metformin’s beneficial effects in diabetic patients have been shown to be largely through repression of hepatic gluconeogenesis, which reduces the glucose levels. In addition, it also increases insulin sensitivity and glucose uptake.

In the past decade, metformin had gained wide attention for its anticancer properties. Studies have shown that in vitro treatment with metformin inhibited the growth of myriad cancer cell lines. An early study done in breast cancer mouse model showed that metformin treatment significantly decreased the tumor accumulation of mammary adenocarcinomas accompanied by increase in the life span of mice.

Metformin is also being tested as an adjuvant cancer therapy, studies on both laboratory animals and humans, showed that metformin not only exerts a major protective effect against the development of a wide range of cancers but also improves prognosis in those found to have these cancers. It was suggested that it inhibits cancer cell proliferation, tumor growth and provokes cell-cycle arrest or apoptosis in G0–G1.

Many genes can be considered as apoptotic markers such as p53 and caspase-3. Not only
p53 can be considered a marker gene for apoptosis, but also it controls cellular apoptosis and proliferation\(^{(22)}\). P53 plays a major role in many cancer cells death specially cancer cervix\(^{(31)}\). In the same way, caspase-3 is considered a death cascade promoter for cancer cells. The High levels of this gene may demonstrate the degree of Hela cell death\(^{(37)}\).

On Contrarily, The anti-apoptotic Bcl2 gene expression reflects the cancer cells viability and vitality\(^{(29)}\). Additionally, the novel biomarker in many types of cancers is COX-1. Recently, COX-1 gene expression has a vital role as biomarker for the detection of cervical cancer development and progression\(^{(17,29)}\). Moreover, Cyclin-D1 gene was observed to be over expressed in cervical carcinoma\(^{(39)}\).

The emergence of metformin as a potential anticancer and cancer-preventive therapeutic tool is exciting. With the added benefits of being readily available, economical, and easily tolerated with good safety profile, it can be effortlessly transitioned from bench to bedside for cancer therapy\(^{(39)}\). We aimed to evaluate whether there is any effect of metformin treatment on the viability of cervical cancer Hela cell line or not and to assess the gene expression of p53, caspase-3, Bcl2, COX-1 and cyclin-D1 as apoptotic and proliferation markers genes.

**MATERIALS AND METHODS**

Dulbecco’s modified Eagle medium (DMEM), fetal calf serum, and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliunbromide (MTT) were purchased from Gibco BRL (Grand Island, NY, USA). Trypsin 2.5%, penicillin, streptomycin, metformin and all other chemicals employed in this study were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, USA).

**Cell culture and cell proliferation assay**

Metformin was dissolved in complete DMEM, the pH value adjusted to 7.2 and sterilized through a 0.2 μm filter to the desired working solutions (equivalent to 15.6–1000 μg/mL, w/v)\(^{(39)}\). Human cervical carcinoma cell line (Hela) was provided by American Type Culture Collection (ATCC, Minisota, U.S.A.). Cells were cultured in DMEM medium supplemented with 5% fetal bovine serum (FBS), 100 mg/mL streptomycin and 100 units/mL penicillin at 37°C in a humidified incubator in an atmosphere of 5% CO2. Hela cells were seeded in 96-well plates (103- 104 cells/well) for 24 h incubation, cell viability was evaluated using MTT assay as described previously\(^{(9)}\). In brief, cells were treated with metformin at a various concentration 0, 15.6, 31.3, 62.5, 125, 250, 500 and 1000 μg/ml for 48 h and untreated cells served as a control. Prior to determination, 10 μL MTT (2.5 g/L) was added to each well. After 4 h incubation, the culture media were discarded followed by addition of 100 μL of detergent reagent to each well and vibration for 10 min. The absorbance (A) in the experimental wells was measured at 570 nm with a microplate reader (ELISA reader). The absorbance in the experimental wells to that of the control wells (without test compound) is measured. The percentage of viable cells was calculated as follows: (A of experimental group/A of control group) × 100. Following this, the IC50 (cytotoxic concentration for 50% cell death) was determined from the dose-response curve.

**Total RNA isolation**

Cells were detached by trypsin (2.5%) and total RNA was isolated with RNAeasy Mini Kit (Qiagen) and further analyzed for quantity and quality with Beckman dual spectrophotometer (USA). The RNA integrity and the GAPDH-RNA (house keeping gene) ratio were used as the quality control.

**Real Time PCR (qRT-PCR) for quantitative expression of p53, Caspase-3, Bcl2, COX-1 and CyclinD1**

The mRNA expression level was quantified by qRT–PCR (Real time PCR). 1000 ng of the total RNA from each sample was used for cDNA synthesis by reverse transcription using high capacity reverse transcriptase kit (Applied Biosystem, USA). The cDNA was subsequently amplified with the Syber Green I PCR Master Kit (Fermentas) in a 48-well plate using the Step One instrument (Applied Biosystem, USA) as follows: 10 minutes at 95°C for enzyme activation followed by 40 cycles of 15 seconds at 95°C, 20 seconds at 55°C and 30 second at 72°C for the
In Vitro Apoptotic Effect Of Metformin On Human Cervical Cancer Cells

amplification step. Changes in the expression of each target gene were measured relative to the mean critical threshold (CT) values of GAPDH housekeeping gene by the ΔΔCt method. We used 1μM of both primers specific for each target gene. Primers sequence and annealing temperature specific for each gene demonstrated in table (1).

Table (1): Primer’s sequence and annealing temperature specific for each gene:

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence: 5’ 3’</th>
<th>Annealing temperature</th>
<th>Gene bank accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>P53</td>
<td>F: AGAGTCCTATAAGGCCCACCC</td>
<td>56°C</td>
<td>KJ897694.1</td>
</tr>
<tr>
<td></td>
<td>R: GCTGCCGTGGATACCTGAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspase-3</td>
<td>F: GCTATGGAGGGGCTGTG</td>
<td>58°C</td>
<td>KJ890827.1</td>
</tr>
<tr>
<td></td>
<td>R: TGTTCCCCTGAGTTTGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bcl2</td>
<td>F: CCGCTCGAGCCCTGAGCTTGAAGAAAGAC</td>
<td>60°C</td>
<td>NG_009361.1</td>
</tr>
<tr>
<td></td>
<td>R: AATGCGGGCCGGAGGGACTAACCTCAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COX-1</td>
<td>F: TTGGCAGGACCTGTATCTCTT</td>
<td>55°C</td>
<td>KM220890.1</td>
</tr>
<tr>
<td></td>
<td>R: AAGATTATTACAAATGCAGGACG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin-D1</td>
<td>F: CCTCTCCTCggGACATT</td>
<td>55°C</td>
<td>NG_007375.1</td>
</tr>
<tr>
<td></td>
<td>R: GCTCATGACGCGGTGACGGCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: CTCCCGACTGACACGTTGG</td>
<td>55°C</td>
<td>NT_009759.16</td>
</tr>
<tr>
<td></td>
<td>R: GCTCCACCTGACACGTTGG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Statistical analysis**

Results were disclosed as means ± standard deviations. One-way ANOVA and Tukey’s multiple comparison post hoc tests were performed. P<0.01 was considered significant.

**RESULTS**

**Metformin cytotoxicity and IC50 against Hela cell line**

Metformin significantly reduced Hela cell line viability in a dose response relationship after 24 h incubation period (Figure 1). Beginning with the lowest metformin concentration 15.6 μg/mL, it decreased the cancer cell line viability significantly to 95.25±2.19%; P< 0.001 against the untreated control. IC50; the cytotoxicity concentration value that fifty percent of cells were dead; was calculated and examined to equal 720.5μg/mL. The maximum cytotoxicity against Hela cell line effect was reached to 33.75%±4.40%; P<.001 against the untreated control, after the exposure to 1000 μg/mL.

**P53 and caspase-3 elevated genes expression after metformin treatment**

Both apoptotic marker genes were elevated for Hela cell line 48 h after the metformin treatment as shown in figure 2. Cells p53 expression were increased significantly (0.177±0.075; P< 0.001 compared to untreated control group 0.034±0.012) and remain unchanged after 72 h treatment. Similarly, caspase-3 expression was increased 2 fold after 48 h drug treatment (0.77±0.26;P < 0.001) and remained constant after 72 h when compared to control value (0.44±0.11).

**Bcl-2, COX-1 and Cyclin-D1 reduced genes expression after metformin treatment**

Complementary to the previous results, Bcl-2; an anti-apoptotic gene for cells viability or life; expression was significantly reduced in a time dependent to almost one half fold each time after 48 h and 72 h metformin treatment against the control (0.74±0.27 for 48 h and 0.43±0.21 for 72 h; P< 0.001 for both compared to 0.122±0.17). In the same direction, COX-1 gene expression value was reduced in a time dependent manner after 48 h and 72 h that reflect the reduction of cancerous effects (1.23 ±1.04 for 48 h and 0.95±0.64 for 72 h; P< 0.001 for both compared to control) (3.8±1.3). Parallel to these results, Cyclin-D1 gene expression was markedly decreased after 48 h and 72 h metformin treatment when compared to control (5.62±4.4 for 48 h and 2.3±1 for 72 h; P< 0.001 for both compared to 9.68±4.7).
Figure 1: Metformin cytotoxicity against Hela cell line, survival fraction percentage of Hela cells were reduced after treatment of different metformin concentrations. Data are means ± SD, (*) p value value ≤ 0.001 versus untreated control group.

Figure 2: P53 and caspase-3 elevated genes expression after metformin treatment of Hela cell line. Data are means ± SD, (*) p value value ≤ 0.001 versus untreated control group.

Figure 3: Bcl-2, COX-1 and Cyclin-D1 reduced genes expression after metformin treatment of Hela cell line. Data are means ± SD, (*) p value value ≤ 0.001 versus untreated control group.
In Vitro Apoptotic Effect Of Metformin On Human Cervical Cancer Cells

DISCUSSION

Metformin was significantly cytotoxic for Hela cervix cancer cell line in a dose dependent manner as shown in figure 1. This apoptotic effect conceded with previous anticancer, prophylactic and adjuvant activities against other types of cancers\(^{(37,3)}\). Recently, metformin was effective against pancreatic\(^{(11,32)}\), ovarian\(^{(39)}\), breast cancer\(^{(26)}\) melanoma\(^{(6)}\) and neuroblastoma\(^{(8)}\). Metformin was not so far from its anti-diabetic group in cancer treatment\(^{(21)}\).

The anticancer effect of metformin was explained through its relationship and regulation of the transcriptional certain genes (CHOP, CAV-1, HO-1, SGK-1 and Par-4) on MCF-7 cell line\(^{(29)}\). Build and complementary for this information transcriptional, regulator and apoptotic genes’ expression were examined for p53, caspase-3, Bcl-2, COX-1 and cyclin-D1 genes. Moreover, each mentioned gene affects cell proliferation or apoptosis of cancer cells specially Hela cervix cancer cells\(^{(22-39)}\).

Not only p53 gene expression was participated for cancer cervix cell death\(^{(31)}\), but also metformin affect the cancer cells susceptibility for p53 gene expression\(^{(10)}\). Even though the low concentration of metformin induces a p53-dependent senescence in hepatoma cells via activation of the AMPK pathway\(^{(39)}\). In the same direction, caspase-3 was induced in cancer by metformin\(^{(33)}\). Moreover, both p53 and caspase-3 genes were induced by the effect of metformin in prostate cancer\(^{(2)}\).

On the other hand, Metformin reduces growth of cutaneous squamous cell carcinoma by targeting mTOR signalling pathway and the reduction of Bcl-2 gene expression\(^{(7)}\). Furthermore, metformin and aspirin reduced COX-1 gene expression by targeting AMPK-mTOR and inflammation for pancreatic cancer prevention and treatment\(^{(36)}\). Confirmed with our cyclin-D1 results, metformin suppresses hepatocellular carcinoma cell growth through induction of cell cycle G1/G0 phase arrest and p21CIP and p27KIP expression and downregulation of cyclin D1 in vitro and in vivo\(^{(4)}\).

In vitro cell system analyses have demonstrated that metformin: i) inhibits the growth of various endometrial cancer cell lines; ii) attenuates the invasion and metastasis of endometrial cancer cell lines by modifying the nuclear factor-κB, matrix metalloproteinase-2/9/Akt and Erk1/2 pathways; and iii) enhances endometrial cancer cell chemosensitivity to cisplatin and paclitaxel by reducing glyoxalase I expression and regulating the mTOR signaling pathway\(^{(5)}\). At the molecular level, the fundamental activity of metformin inhibits mitochondrial oxidative phosphorylation, and may exert energy-associated stress on neoplastic cells\(^{(23)}\). This inhibition of oxidative phosphorylation reduces the production of ATP, activating the cellular energy regulator AMPK and its downstream effectors, including mTOR\(^{(14)}\). At the whole-organism level, the anti-proliferative effects of metformin may be attributed to the decrease in circulating insulin levels induced by the reduced hepatic gluconeogenesis characteristic of insulin-responsive tumors\(^{(25)}\). Metformin potently inhibits growth in a dose-dependent manner in endometrial cancer cell lines. Metformin resulted in G1 phase cell cycle arrest, induction of apoptosis and decreased human telomerase reverse transcriptase expression in endometrial cancer cells\(^{(5)}\). Treatment with metformin attenuates the estrogen-dependent proliferative expression of c-myc and c-fos in the obese rat endometrium, an effect which was accompanied by inhibition of the phosphorylation of insulin and IGF1 receptors, as well as Erk1/2\(^{(38)}\).

Precisely, metformin impairs growth of endometrial cancer cells that neighbouring cervical cancer via cell cycle arrest and concomitant autophagy and apoptosis\(^{(33)}\).

Accordingly, we recommend metformin to be a new, safe and effective therapeutic agent against cervical cancer.

Conclusion

Cervical cancer was the new type of cancer treated by metformin. Metformin has an apoptotic potent cytotoxic activities were shown by the dose response relationship against human cervical cancer Hela cells. Even if metformin Hela treatment raised p53 and caspase-3 genes’
expression as apoptotic markers, it reduced Bcl-2, COX-1 and cyclin-D1 genes’ expressions as proliferation markers.

Acknowledgement

The authors had an acknowledgement for all staff members at Medical Biochemistry and Molecular Biology Unit at the Faculty of Medicine, Cairo University.

Financial Support

No grants and no financial support.

REFERENCES


In Vitro Apoptotic Effect Of Metformin On Human Cervical Cancer Cells

تأثير المتغورمين على موت الخلايا المبرمج لسرطان عق الرحم
دينا صبري - سحر حسن أحمد - محمد الاغسين

الهدف من هذه الدراسة هو تحديد ما إذا كان العلاج بالمتغورمين ضد خط خلايا سرطان عق الرحم Hela الخلايا أم لا وتقييم التعبير الجيني لـ Cyclin D1، Caspase، P53، Cox-1، BCL2. كل من Hela ومتغورمين كانت كبيبيات دالة على موت الخلايا المبرمج والتعبير الجيني لـ P53، Caspase، BCL2، Cox-1، كل منها تؤدي إلى تآكل وانتشار الخلايا. بعد زرع خلايا Hela لقياس الكثافات، وتمت معالجة الخلايا بالمتغورمين 48 ساعة ثم الحصاد لاستخراج RNA تقييس التكلاف وتمت معالجة الخلايا بالمتغورمين 48 ساعة ثم الحصاد لاستخراج النتائج: PCR باستخدام Cyclin D1، Caspase، BCL2، Cox-1، P53 و P3، الذي تم تحويله إلى cDNA وتحليل جيني في علاقة معتمدة على الجرعات والمتغورمين. المتغورمين أثبت أنه ذو تأثير سام على خلايا سرطان عق الرحم Hela وتبديل معتمدة على الجرعات والمتغورمين. تأثير على عدد من الجينات التي تعكس حياة الخلايا أو موتها. ارتفاع التعبير الجيني باستخدام المتغورمين L-3, Caspase، P53، Cox-1، BCL2، حيث أنخفض تعبير الجيني. الخلاصة: أظهرت نتائج معالجة خلال سرطان عق الرحم Hela تأثيرات جينية على عدد من الجينات، Caspase، P53، Cox-1، BCL2، هر أي أن المتغورمين يمكن أن يكون علاج جديد وآمن وفعال ضد سرطان عق الرحم.
ROLE OF INTERCELLULAR ADHESION MOLECULE-1 (ICAM-1) G241R AND K469E GENE POLYMORPHISM AND SOLUBLE ICAM-1 SERUM LEVELS IN THE DEVELOPMENT OF ISCHEMIC STROKE IN EGYPTIAN PATIENTS

Abeer Mohamed Mohy*, Ahmed Abd Allah Hassan Ali ** and Heba Omar***

ABSTRACT

Background: Stroke is currently the third leading cause of death worldwide. Intercellular adhesion molecule-1 (ICAM-1) was involved in the pathogenic mechanisms responsible for ischemic stroke. Objectives: To determine the association of ICAM-1 G241R and K469E polymorphisms and soluble ICAM-1 level with ischemic stroke in Egyptian patients. Subjects and Methods: ICAM-1 G241R and K469E, were detected by allele specific polymerase chain reaction (ASP) and polymerase chain reaction-restriction fragment length polymorphism (PCR – RFLP) techniques respectively, in 40 Egyptian patients with ischemic stroke and 40 healthy subjects as a control group. Serum sICAM-1 was measured by enzyme linked immunosorbent assay (ELISA). Results: ICAM-1 241R allele and 469E allele were significantly associated with increased risk of ischemic stroke. The median sICAM-1 levels were significantly higher in cases than control group. Conclusion: These results suggest that ICAM-1 G241R and K469E gene polymorphism may influence the susceptibility to acquire ischemic stroke in a sample of Egyptian population

Keywords: Ischemic stroke - ICAM-1 - K469E - G241R - gene polymorphism - PCR-RFLP

INTRODUCTION

Stroke is currently the third leading cause of death and the biggest single cause of major disability worldwide. Each year more than 700 000 people experience a new or recurrent stroke and on average someone dies every 4 minutes of a stroke. Despite the diagnostic and treatment development in medicine, the recovery rate from stroke is poor (9).

The commonest type of stroke is an ischemic stroke, resulting from disruption of blood flow within the brain caused by occlusion of an artery. This deprives the brain of oxygen and nutrients and initiates a dynamic sequence of pathophysiological events (12).

Intercellular adhesion molecule-1 (ICAM-1) is a member of immunoglobulin (Ig) superfamily of cell adhesion molecules with glycoprotein structure. The ICAM-1 gene is located on chromosome 19 P13.2-13.3 and includes 7 exons that code a protein with five extracellular Ig-like domains, a transmembrane domain and a short cytoplasmic tail (1).

ICAM-1 is expressed on vascular endothelium, macrophages and activated lymphocytes and is up-regulated by inflammatory cytokines. It plays an important role in the adhesion and subsequent transendothelial migration of circulating leukocytes into the vascular endothelium which is one of the earliest events in the pathogenesis of atherosclerosis (3, 13).

Proteolytic cleavage of ICAM-1 near its membrane region leads to production of a soluble form, sICAM-1, which is partially detectable in the serum of healthy subjects, but its level is elevated in inflammatory and malignant disorders, also associated with increased risk of future ischemic stroke (1).

Two single-nucleotide polymorphisms (SNPs) in the ICAM-1 gene have been recognized, first at position +241 (+241 G/A or G241R) (rs 1799969) located in exon 4 coding Ig-like domain 3 of the ICAM-1 protein (GGG → AGG; Glycine → Arginine) and the second one at position +469 A/G or K469 E) (rs 5498) located in exon 6 coding Ig-like domain 5 of the ICAM-1 protein (AAG → GAG; lysine → Glutamic acid)(1,10).

The Ig-like domain 3 mediates binding to macrophage antigen-1 (Mac-1) and may also affect accessibility of leukocyte function associat-
ed – antigen-1 (LFA-1) binding to the Ig-like domain-1. ICAM-1 mediates adhesion interactions of circulating leukocytes to the blood vessel wall by binding to Mac-1 and LFA-1, and increased expression of ICAM-1 has been found during all phases of atherogenesis\(^{(5,14)}\).

The Ig-like domain 5 was reported to involve in cell adhesion. This region seems to be particularly important for maintaining normal protein structure, affecting the adhesion of circulating leukocytes to the activated endothelium. This domain is of crucial importance for the activity of the ICAM-1 protein because it modulates the interactions between ICAM-1 and LFA-1\(^{(8,11)}\) and the influences the adhesion of B-cells\(^{(14)}\).

These variations have been reported to be related to some inflammatory and autoimmune diseases and atherosclerosis\(^{(1,10)}\).

The purpose of the present study was to determine the association of ICAM-1 G241R and K469E polymorphisms and soluble ICAM-1 level with ischemic stroke in Egyptian patients.

**SUBJECTS AND METHODS**

**Subjects:**

The study population included 40 ischemic stroke patients (21 males and 19 females; mean age 49 ± 3.1 years) from Neurology Department, Kasr Al-Aini Hospital, Cairo University, Egypt. They were diagnosed by neurological examination and CT scan. Patients with hemorrhagic stroke, cancer, autoimmune disease were excluded from this study. As controls, 40 healthy subjects were considered, similar to the patients for age and sex distribution (20 males and 20 females; mean age 49.5 ± 4.0 years). The controls had no clinical evidence or history of neurological diseases, no history of stroke. All controls came from the same geographical area as the patients. Besides neurological history, history of hypertension, diabetes mellitus (DM) and smoking were recorded for all participants.

The study was conducted at period from September 2013 to July 2014. Patients and controls were enrolled in this study after giving informed consent to the use of part of their blood samples for an experimental study. The study was approved by the local ethical committee.

**Biochemical variables and ICAM-1 genotyping:**

Six ml venous blood samples were collected from all subjects after at least 12-hours fast. Serum total cholesterol, triglyceride (TG) levels were measured by standard enzymatic methods. Serum high-density lipoprotein cholesterol (HDL-C) level was determined by direct assay. Serum low-density lipoprotein cholesterol (LDL-C) level was calculated by Friedwald formula\(^{(6)}\). Plasma fasting glucose was measured by a glucose oxidase procedure. These biochemical parameters were measured using Dimension RXL automatic analyzer (Siemens Healthcare Diagnostics, USA). Serum sICAM-1 was assayed using enzyme-liked immunosorbt assay (ELISA) (Biovision, Egypt).

**Molecular analysis:** All kits were supplied by (Thermo-Scientific, USA).

Genomic DNA from EDTA-treated venous blood was extracted by standard techniques using a DNA spinspacecolumn extraction kit.

**G241R polymorphisms:**

To detect the G241R polymorphism, an allele-specific polymerase chain method (ASP) was performed using two sets of specific primers (G for nucleotide G, R for nucleotide A) and one common primer\(^{(1)}\). The sequence of these primers is shown in Table 1. PCR reaction was performed in a total volume of 25 µl, which included 12.5 µl 2 x PCR master mix {0.05 units/µl Taq DNA polymerase, 2 x PCR buffer, 3 mM MgCl\(_2\) and 400 µM of each dNTP (dATP, dCTP, dGTP, dTTP)}, 1 µl of either G or R primer, 1 µl common primer, 1 µl of each µ-globin primer F and R as the internal control, 6 µl nuclease free water and 2.5 µl genomic DNA.

PCR program included an initial denaturation at 95°C for 5 minutes followed by 30 cycles consisting of 95°C for 1 minute, 62°C for 1 minute, 72°C for 1 minute, and a final extension at 72°C for 10 minutes.
Role of ICAM-1 and sICAM-1 in Ischemic Stroke

Then the 137bp PCR amplification products were run electrophoretic on 2% ethidium bromide-stained agarose gel and the bands were visualized under UV light.

K469E polymorphisms:

For this polymorphism, the PCR-RFLP method was used on two forward (K) and reverse (E) primers (Table 1) and Fast Digest Bst UI restriction enzyme(1). The PCR reaction was performed in a total volume of 25 µl that contained 8 µl nuclease free water, 12.5 µl 2 x PCR mater mix, 1 µl of each primer and 2.5 µl genomic DNA. The cycling conditions consisted of an initial denaturation of 5 minutes at 94°C followed by 35 cycles of 45 seconds at 96°C, 1 minute at 59°C and 1 minute at 72°C. The reaction was terminated after a final extension of 5 minutes at 72°C.

Then 7 µl of the 223bp PCR products were then digested with 1µl (5 units) of Fast Digest Bst UI enzyme at 37°C for 15 minutes. The Bst UI restriction enzyme was able to cut the E469 allele but not K469.

**RESULTS**

**Characteristics of the subjects:**

The clinical and laboratory data of the patients and controls are presented in table (2).

**Distribution of the ICAM-1 polymorphism in patients and control group**

The ICAM-1 G241R and K469E polymorphism distributed in accordance with Hardy-Weinberg equilibrium (HWE) in both studied groups. The distribution of G241R and K469E genotypes in exons 4 and 6 of ICAM-1 gene are shown in tables 3 and 4.

**Association of genotypes with the risk of ischemic stroke:**

By logistic regression analysis adjusted for age, sex, smoking, diabetes, hypertension, fasting blood glucose, lipid profile, sICAM-1, ICAM-1 469 KE + 469 EE genotypes were sig-

---

**Table (1): The primers sequence of ICAM-1 G241R and K469E polymorphisms**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>G primer</td>
<td>GTGGTCTGTCCCTGGACG</td>
</tr>
<tr>
<td>R primer</td>
<td>GTGGTCTGTCCCTGGACA</td>
</tr>
<tr>
<td>Common primer</td>
<td>GCGGTCAACTGACTGAGGCCT</td>
</tr>
<tr>
<td>β-globin primer F</td>
<td>ACACAACTGTGTTCACTAGC</td>
</tr>
<tr>
<td>β-globin primer R</td>
<td>CAACTTCATCCACGTTACC</td>
</tr>
<tr>
<td>K primer</td>
<td>GGTGAGGATTGCATTAGGTC</td>
</tr>
<tr>
<td>E primer</td>
<td>GGAACCATTGCCCGAGC</td>
</tr>
</tbody>
</table>
Table (2): Clinical and laboratory data of ischemic stroke patients and control group

<table>
<thead>
<tr>
<th></th>
<th>Ischemic stroke (n=40)</th>
<th>Control group (n=40)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>21 (52.5%)</td>
<td>20 (50%)</td>
<td>0.823</td>
</tr>
<tr>
<td>Female</td>
<td>19 (47.5%)</td>
<td>20 (50%)</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>26 (65%)</td>
<td>22 (55%)</td>
<td>0.361</td>
</tr>
<tr>
<td>No</td>
<td>14 (35%)</td>
<td>18 (45%)</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>15 (37.5%)</td>
<td>12 (30%)</td>
<td>0.478</td>
</tr>
<tr>
<td>No</td>
<td>25 (62.5%)</td>
<td>28 (70%)</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>18 (45%)</td>
<td>11 (27.5%)</td>
<td>0.104</td>
</tr>
<tr>
<td>No</td>
<td>22 (55%)</td>
<td>29 (72.5%)</td>
<td></td>
</tr>
<tr>
<td>Fasting blood glucose*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg/dl)</td>
<td>118.8 ± 45.4</td>
<td>118.5 ± 48.6</td>
<td>0.977</td>
</tr>
<tr>
<td>Cholesterol* (mg/dl)</td>
<td>207.9 ± 60.0</td>
<td>190.9 ± 57.7</td>
<td>0.197</td>
</tr>
<tr>
<td>Triglycerides+ (mg/dl)</td>
<td>131.0 (108-178.5)</td>
<td>117.0 (100-155.2)</td>
<td>0.013**</td>
</tr>
<tr>
<td>HDL-C* (mg/dl)</td>
<td>45.8 ± 14.3</td>
<td>44.6 ± 11.5</td>
<td>0.687</td>
</tr>
<tr>
<td>LDL-C+ (mg/dl)</td>
<td>137.3 (109.5-164.7)</td>
<td>117.2 (91.1-138.7)</td>
<td>0.053</td>
</tr>
<tr>
<td>ICAM-1+ (ng/l)</td>
<td>393.2 (371.4-1180.7)</td>
<td>346.3 (310.8-431.7)</td>
<td>0.001**</td>
</tr>
</tbody>
</table>

- Qualitative data is presented as number (%), * Data presented as mean +SD
  + Data presented as median (25th – 75th percentile) ** P-value < 0.05 is statistically significant

Table (3): Frequency distribution of ICAM-1 K469E genotypes and alleles among the studied groups

<table>
<thead>
<tr>
<th>ICAM-1 K469E</th>
<th>Cases (n=40)</th>
<th>Control (n=40)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>KK</td>
<td>7 (17.5%)</td>
<td>25 (62.5%)</td>
<td>0.000</td>
</tr>
<tr>
<td>KE + EE</td>
<td>33 (82.5%)</td>
<td>15 (37.5%)</td>
<td></td>
</tr>
<tr>
<td>K allele</td>
<td>45 (56.2%)</td>
<td>65 (81.3%)</td>
<td>0.001</td>
</tr>
<tr>
<td>E allele</td>
<td>35 (43.8%)</td>
<td>15 (18.7%)</td>
<td></td>
</tr>
</tbody>
</table>
Role of ICAM-1 and sICAM-1 in Ischemic Stroke

Table (4): Frequency distribution of ICAM-1 G241R genotypes and alleles among the studied groups

<table>
<thead>
<tr>
<th>ICAM-1 G241R</th>
<th>Cases (n=40)</th>
<th>Control (n=40)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>18 (45%)</td>
<td>30 (75%)</td>
<td>0.006</td>
</tr>
<tr>
<td>GR + RR</td>
<td>22 (55%)</td>
<td>10 (25%)</td>
<td></td>
</tr>
<tr>
<td>G allele</td>
<td>52 (65%)</td>
<td>70 (87.5%)</td>
<td>0.001</td>
</tr>
<tr>
<td>R allele</td>
<td>28 (35%)</td>
<td>10 (12.5%)</td>
<td></td>
</tr>
</tbody>
</table>

Table (5): ICAM-1 genotypes and allele frequencies among males and females ischemic stroke patients

<table>
<thead>
<tr>
<th>ICAM-1 genotypes</th>
<th>Male (n=21)</th>
<th>Female (n=19)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>KK</td>
<td>6 (28.6%)</td>
<td>1 (5.3%)</td>
<td>0.06</td>
</tr>
<tr>
<td>KE</td>
<td>15 (71.4%)</td>
<td>16 (84.2%)</td>
<td></td>
</tr>
<tr>
<td>EE</td>
<td>0 (0%)</td>
<td>2 (10.5%)</td>
<td></td>
</tr>
<tr>
<td>KE + EE</td>
<td>15 (71.4%)</td>
<td>18 (94.7%)</td>
<td>0.093</td>
</tr>
<tr>
<td>K allele</td>
<td>27 (64.3%)</td>
<td>18 (47.4%)</td>
<td>0.176</td>
</tr>
<tr>
<td>E allele</td>
<td>15 (35.7%)</td>
<td>20 (52.6%)</td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>10 (47.6%)</td>
<td>8 (42.1%)</td>
<td>0.594</td>
</tr>
<tr>
<td>GR</td>
<td>7 (33.3%)</td>
<td>9 (47.4%)</td>
<td></td>
</tr>
<tr>
<td>RR</td>
<td>4 (19%)</td>
<td>2 (10.5%)</td>
<td></td>
</tr>
<tr>
<td>GR + RR</td>
<td>11 (52.3%)</td>
<td>11 (57.9%)</td>
<td>0.761</td>
</tr>
<tr>
<td>G allele</td>
<td>27 (64.3%)</td>
<td>25 (65.8%)</td>
<td>0.90</td>
</tr>
<tr>
<td>R allele</td>
<td>15 (35.7%)</td>
<td>13 (34.2%)</td>
<td></td>
</tr>
</tbody>
</table>

Significantly associated with increased risk of developing ischemic stroke (OR: 7.857, 95% CI (2.786-22.158), p= 0.000). Carriers of 469E allele or 241R allele had significantly increased risk of developing ischemic stroke (OR: 3.37, 95% CI (1.650-6.885); p= 0.001, OR= 3.769, 95% CI (1.683-8.441); p= 0.001, respectively).

In the current study it was found that the median sICAM-1 levels were significantly higher in combined 469 KE + 469 EE genotypes; 390.8 (363.2-577.2 ng/l) as compared to 469 KK genotype; 368.5 (305.02-418.8 ng/l); p=0.046.

There was no statistical significant difference between sICAM-1 levels in different G241R genotypes, p= 0.229.

Regarding sex, it was found that the frequencies of 469 KE and EE genotypes were higher in females than males, but it didn’t reach statistical significance as shown in table (5).

Haplotype analysis:

The genotype combination of K469E and G241R polymorphisms of the ICAM-1 gene revealed that the frequency of the E/R haplotype (+469 K/E and +241 G/R, respectively) significantly increased in patients compared to control group (18.8% vs 5%). Then individuals with E/R haplotype had a significantly increased risk of ischemic stroke (OR = 4.385, 95% CI = 1.942-9.898, p= 0.000).

DISCUSSION

A cross sectional study was performed to assess the role of genetic variation of ICAM-1 G241R and K469E and serum sICAM-1 levels on risk of ischemic stroke in Egyptian patients and compared the results with healthy individuals.
The 469 KE + EE genotypes and 469 E allele were more frequent in ischemic stroke patients than control. Also the mutant 469 E allele was found to be a significant risk factor for developing ischemic stroke (OR = 3.37, 95% CI (1.650-6.885; p= 0.001), it was found that 469 EE genotype was higher in females than males but it didn’t reach statistical significance (p= 0.06).

The 241 GR + RR genotypes and 241R allele were significantly increased in ischemic stroke patients than control. Also the mutant 241R allele was found to be a significant risk factor for development of ischemic stroke (OR= 3.769, 95%, CI (1.683-8.441); p=0.001. Our results also indicate that the (469 E / 241 R) haplotype was significantly higher in patients (18.8%) than in controls (5%) p= 0.000.

This agrees with the study done by Li et al. (10) which was conducted on 309 Chinese patients with ischemic stroke and 309 elderly apparently healthy subjects serving as control group, they reported that KE and EE genotype frequencies were significantly higher in patients than control, with KK genotype as a reference genotype. Also E allele had a significantly increased risk of ischemic stroke in overall population (OR= 1.79, 95% CI = 1.30-2.46; p=0.000) and in females (OR= 2.36, 95% CI= 1.41-3.96, p=0.001), but not in males (OR= 1.48, 95%, CI= 0.98-2.22; p=0.062).

Similar results were obtained by another Chinese study (17) who had found that carriers of 469E allele had a higher risk of ischemic stroke. Similarly, a German study had shown that 469E allele was a genetic factor that may determine an individual susceptibility for coronary heart disease and myocardial infarction (7).

Consistent with our results, Volcik et al. (16) showed that ICAM-1 241RR genotype was associated with significantly increased risk of ischemic stroke in both Whites (hazard rate ratios HRR=2.18 (1.01-4.68), (p= 0.05) and African Americans (HRR = 7.04 (3.7-13.3), p< 0.001), However there was no significant findings observed for the ICAM-1 K469E polymorphism and incident ischemic stroke.

The current study showed that the median sICAM-1 levels were significantly higher in ischemic stroke patients than in control group, p= 0.001. Similar to these results, the study done by Volcik et al. (10) and Tanne and Coworkers (15) who found that mean sICAM-1 concentrations were significantly higher in ischemic stroke cases (379±100 ng/l) compared to controls (350±97 ng/l), p= 0.02, a marker of inflammation, are associated with increased risk of ischemic stroke, independent of other traditional cerebrovascular factors. Also they stated that these high levels of sICAM-1 were found to significantly predict future ischemic stroke.

Our results showed that median triglyceride levels were significantly higher in patients than in the control group, p= 0.013, while other routine laboratory parameters didn’t show any significance.

These results agree with those reported by Li et al. (10) and Bansal et al. (2), who found triglycerides to be an important risk factor for ischemic stroke and also for coronary heart disease as reported by Criqui (4).

It is therefore conceivable that polymorphisms in the ICAM-1 gene might increase the risk of stroke; the observed association with stroke risk is potentially due to the functional impact on the binding affinity of ICAM-1 to both Mac-1 and LFA-1, thus possibly affecting adhesion and further interactions of circulating leukocytes to the vascular wall. Individuals carrying ICAM-1 241R allele, 469E allele and the 241R/469E haplotype might be more susceptible for developing ischemic stroke. More importantly, the linkage disequilibrium of the ICAM-1 gene with other ischemic stroke predisposing genes should be taken into accounts.

REFERENCES


Role of ICAM-1 and sICAM-1 in Ischemic Stroke


دور تعدد الأشكال لجين جزيء الالتصاق الخلوي-1 (G241R ICAM-1) ، K469E في المصل في تطوير السكتة الدماغية في المرضى المصريين

عبير محمد محي - أحمد عبد الله حسن علي - هبة عمر

خلفية: السكتة الدماغية هي حالياً ثالث سبب رئيسي للوفاة في العالم. وشارك جزيء الالتصاق الخلوي-1 (ICAM-1) في الآليات المسئولة للأمراض المسؤولة ظهور السكتة الدماغية. الأهداف: تحديد العلاقة بين تعدد الأشكال لجين جزيء الالتصاق ICAM-1، K469E ومستوي sICAM-1 في المصل في تطوير السكتة الدماغية في المرضى المصريين. 

الاستخاذ: تعدد الأشكال لجين جزيء الالتصاق في السكتة الدماغية هو حالياً ثالث سبب رئيسي للوفاة في العالم. وشارك جزيء الالتصاق ICAM-1، K469E ومستوي sICAM-1 في المصل في تطوير السكتة الدماغية في المرضى المصريين.

الخلايا: السكتة الدماغية هي حالياً ثالث سبب رئيسي للوفاة في العالم. وشارك جزيء الالتصاق ICAM-1، K469E ومستوي sICAM-1 في المصل في تطوير السكتة الدماغية في المرضى المصريين. الأهداف: تحديد العلاقة بين تعدد الأشكال لجين جزيء الالتصاق ICAM-1، K469E ومستوي sICAM-1 في المصل في تطوير السكتة الدماغية في المرضى المصريين.

النهاية: هذه النتائج تشير إلى أن تعدد الأشكال الجيني قد تؤثر على قابلية لاكتساب السكتة الدماغية في المجتمع المصري.
γ- GLOBIN GENE XMN1 POLYMORPHISM-158 AND ITS CORRELATION TO THE RESPONSE TO HYDROXYUREA IN βTHALASSEMIA MAJOR PATIENTS IN BENISUEF GOVERNORATE(EGYPT)

Abdel Meged A. Abdel Meged*, Dalia G. Amin**, Dalia S. Morgan* and Safwat L. Shaker*

ABSTRACT

β-thalassemia is an inherited disorder characterized by a deficient or absent β-globin chain production, resulting in hypochromic microcytic hemolytic anemia. Xmnl polymorphism has been reported to increase the level of hemoglobin F (HBF) and to give a better response to Hydroxyurea (HU) therapy in B-thalassemia patients. This study was conducted on 80 randomly selected cases of β-thalassemia major patients and 40 age and sex matched controls; to study the prevalence of γ-globin gene Xmnl polymorphism -158 in β-thalassemia patients by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), results were correlated with percent of HBF, response to HU, and other clinical and hematological variables. Nine out of 80 β-thalassemia major patients (11.25%) were heterozygous for Xmnl polymorphism, homozygous Xmnl polymorphism was not detected in any of the included patients. The frequency of Xmnl polymorphism in males and females showed no significant difference. Nearby frequency for heterozygous Xmnl polymorphism was found among control subjects (5 out of 40, 12.5%), none was homozygous for Xmnl polymorphism. Among the β-thalassemia major group, a significant association was found between the presence of Xmnl polymorphism and older age at first blood transfusion (p value = 0.02), higher Hb F levels (p value = 0.04), lower incidence of splenectomy (p value = 0.04) and decreased frequency of blood transfusion after HU treatment (p value = 0.03). The frequency of blood transfusion before HU treatment was lower in the presence of Xmnl polymorphic site, however, statically it was not significant (p value = 0.76). Key wards: β-Thalassemia, Xmnl polymorphic site, Hydroxyurea.

INTRODUCTION

In patients with β-thalassemia, blood transfusion at regular intervals eliminates anemia-related complications, compensatory marrow expansion and extends survival(11). Iron chelation reduces the accumulation of iron secondary to multiple transfusions, thereby dramatically improving the prognosis(12). However, adequate iron chelation is expensive; therefore alternatives to blood transfusion and chelation would be valuable to increase the safety and decrease the cost of thalassemia treatment and to provide effective treatment for patients who do not have access to blood transfusions(9).

Hydroxyurea is a pharmacological inducer of fetal hemoglobin (HbF) that significantly increases Hb levels and reduces transfusion needs in patients with thalassemia(14). Hydroxyurea induced increase in γ-chain synthesis may decrease the imbalance between α and non α chain inβ-thalassemia patients.

It has been reported that in patients with β-thalassemia the presence of Xmnl polymorphic site, is associated with higher level of HbF(6), and better effect of Hydroxyurea(14). Also, there is strong evidence that the homozygous state of Xmnl polymorphic site which is associated with increased expression of Gγ gene may play an important role among other factors in ameliorating the clinical features of homozygous β-thalassemia intermedia(14).

Aim of the Work

The aim of this study is to determine the prevalence of Xmnl polymorphic site (C/T) 5’ to the Gγ gene in position -158 in a group of Egyptian β-thalassemia major patients as well as another group of healthy subjects, also to study its co-relation to the age of receiving first blood transfusion, rate of splenectomy, level of Hb F and response to HU in β-thalassemia major patients in Egypt.

SUBJECTS AND METHODS

The present work is a prospective study conducted on 80 cases of β-thalassemia major and 40 age and sex matched control subjects; patients
were randomly selected from the Hematology Clinic of follow up and blood transfusion; Beni-Suef University Hospital in collaboration with the Clinical Pathology Department, Faculty of Medicine, Cairo University. The study was approved by the Ethics Committee of Cairo University as well as Beni-Suef University and all procedures performed in studies involving human participants were in accordance with their Ethical Standards. Informed Consent was obtained from each participant’s guardian.

Patients were subjected to full medical history and thorough physical examination stressing on the following points: age of presentation of anemia, age of first blood transfusion, frequency of blood transfusion, history of splenectomy, administration of HU, and facial bony deformities.

For both groups venous blood samples were collected in EDTA containing vacutainers to be used for performing: complete blood picture (CBC), high performance liquid chromatography (HPLC) for proper determination of Hbf percent using variant II TM Hb testing system, Bio-Rad and study of single-nucleotide Xmn1 polymorphism (C/T) in θ-globin gene position -158 in β-thalassemia patients, which was performed by polymerase chain reaction-restriction fragment length polymorphism assay (PCR-RFLP).

For PCR-RFLP; genomic DNA was extracted from peripheral blood leukocytes, and stored at -80°C. Amplification of a 650-bp fragment 5’ to the θ-globin gene that contains the Xmn1 polymorphism was done by PCR using the primers and technique that were described before (14). The 25μl reaction mixture contained 1x PCR Buffer (100 mMTris HCL, 500 mM KCl, 0.8% Nonidet P40) 1mM MgCl2, 100µM dNTPs (dATP, dGTP, dCTP, dTTP), 10 pm/μl PCR primers, 3 units/μl Taq DNA polymerase (Fermentas life Science, USA) 100 ng of genomic DNA and the final volume was made up with autoclaved MilliQ water. Working conditions were as follows: the initial denaturation at 94°C for 5 minutes, and then 30 cycles of: denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1.5 minutes followed by a final extension step at 72°C for 5 minutes.

Genotyping of wild type and mutant allele of Xmn1 was carried out using RFLP method; about 10μ of the amplified PCR product was digested with five units of Xmn1 restriction enzyme and electrophoresed on a 2% agarose gel. In the presence of T allele (mutant), two fragments of 450- and 200-bp were produced. The presence of the normal allele (C) loses cleavage site for Xmn1 and thus an intact 650-bp fragment was produced (Fig. 1).

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Primer sequence (5’ → 3’)</th>
<th>Restriction enzyme</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>θ-globin gene position -158C&gt;T XMN1 polymorphism</td>
<td>F: AAC TGT TGC TTT ATA GGA TTT T</td>
<td>Pdm1</td>
<td>Nematiet al, 2010 (14)</td>
</tr>
<tr>
<td></td>
<td>R: AGG AGC TTA TTG ATA ACT CAGAC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Statistics: The data was analyzed with the Statistical Package for Social Science (SPSS) program version 16 under windows 7 in the form of description of qualitative variables by frequency and percentage, description of quantitative variables in the form of mean and standard deviation (mean ± SD), Chi-square (χ²) test was used for comparison of qualitative variables with each other, comparison between quantitative variables was carried by using: Student unpaired t-test of two independent samples, and Significance level (p) was expressed as following: P value > 0.05 is insignificant, P value < 0.05 is significant and P value < 0.001 is highly significant.

RESULTS

This study included 80 patients with β-thalassemia major 48 (60%) were males and 32 (40%) were females. The age of the study group ranged from 3 year to 16 years, with a mean of 8.4 ±2.98. Nine out of 80 β-thalassemia major patients (11.25%) were heterozygous for Xmn1
polymorphism (+/-), homozygous Xmn1 polymorphism (+/+), was not detected in any of the included patients. Nearby frequency for heterozygous Xmn1 polymorphism was found among control subjects (5 out of 40, 12.5%), none was homozygous for Xmn1 polymorphism.

The frequency of Xmn1 polymorphic site in males and females was 10% (5 out of 48) and 12.5% (4 out of 32) respectively with no significant difference (p value = 0.589).

Pre transfusion Hb ranged from 6 g./dl to 9 g./dl. with a mean value 7.56 ± 0.52 and the age of first blood transfusion ranged from 5 months of age to 30 months old.

Although, Hb levels were higher in the group of patients with Xmn1 polymorphism compared to the group without Xmn1 polymorphism, however that difference was not statistically significant (p value = 0.78).

A statistically significant correlation was found between the presence of Xmn1 polymorphic site and the age of first blood transfusion (P = 0.02). In the presence of Xmn1 polymorphic site, the need for blood transfusion was at an older age.

The frequency of blood transfusion before HU, showed a mean value of 13.46 ± 2.53 times/year as it ranged from 8 to 20 times/year, while after HU the mean value became 3.89 ± 4.98 as it ranged from 0 to 15 times/year.

The frequency of blood transfusion before HU treatment was decreased in the presence of Xmn1 polymorphic site; however, statistically it was not significant (p value = 0.76), while after HU treatment a significant correlation was found between the presence of Xmn1 polymorphic site and the frequency of blood transfusion. The frequency of blood transfusion after HU treatment was decreased in the presence of Xmn1 polymorphic site (p value = 0.03).

A significant correlation between Xmn1 polymorphic site and Hb F level was detected. Hb F levels were higher in the presence of Xmn1 polymorphic site compared to the absence of Xmn1 polymorphic site (p value = 0.04).

A significant negative association was found between the presence of Xmn1 polymorphic site and splenectomy (p value = 0.04). Splenectomy was done for 23 patients among our studied group none of them was carrying the Xmn1 polymorphism.

No significant correlation was found between the presence of Xmn1 polymorphic site with history of consanguinity, family history of similar condition, duration of illness, or incidence of chelation therapy.

Table (2): Parameters associated with γ- globin gene -158Xmn1 polymorphism

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Xmn1 +/- (No = 9)</th>
<th>Xmn1 -/- (No = 71)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at first blood transfusion in months</td>
<td>22</td>
<td>7.27</td>
<td>0.02*</td>
</tr>
<tr>
<td>Duration of illness in years</td>
<td>9.16</td>
<td>8.29</td>
<td>0.22</td>
</tr>
<tr>
<td>Hbg./dl</td>
<td>8.60</td>
<td>7.47</td>
<td>0.78</td>
</tr>
<tr>
<td>Splenectomy (number and percent)</td>
<td>0</td>
<td>23 (32 %)</td>
<td>0.04*</td>
</tr>
<tr>
<td>Chelation therapy %</td>
<td>4 (44 %)</td>
<td>30 (42%)</td>
<td>0.9</td>
</tr>
<tr>
<td>Consanguinity%</td>
<td>7 (78 %)</td>
<td>58 (82 %)</td>
<td>0.25</td>
</tr>
<tr>
<td>Similar conditions %</td>
<td>3 (33%)</td>
<td>33 (46 %)</td>
<td>0.52</td>
</tr>
<tr>
<td>HU treatment % (number and percent)</td>
<td>4 (44 %)</td>
<td>31 (44 %)</td>
<td>0.84</td>
</tr>
<tr>
<td>Frequency of blood transfusion before HU/ per year</td>
<td>10.00</td>
<td>14.2</td>
<td>0.76</td>
</tr>
<tr>
<td>Frequency of blood transfusion after HU/ per year</td>
<td>4.19</td>
<td>8.333</td>
<td>0.03*</td>
</tr>
<tr>
<td>Hb F% after HU</td>
<td>76.38</td>
<td>26.55</td>
<td>0.04*</td>
</tr>
</tbody>
</table>

* A statistically significant difference
β-thalassemia major was found to be 11.25%, the production of HbF is able to partially compensate for the marrow’s reaches 3 to 5 years of age because the infant of age when the normal physiological anemia of the disease require a continuous and distressing treatment regimen that includes iron chelation treatment, regular medical supervision, and frequent admission to the hospital and on many occasion surgeries. The only curative treatment for this disease is bone marrow transplantation (BMT) which is expensive and has a variable success rate of 60-70%.(23).

Patients with severe β-thalassemia are usually diagnosed between 6 months and 2 years of age when the normal physiological anemia of the newborn fails to improve. Sometimes, the patients cannot be recognized until the child reaches 3 to 5 years of age because the infant is able to partially compensate for the marrow’s inability to produce hemoglobin A by prolonged production of Hb F(23).

In the present study the frequency of presence Xmn1 polymorphic site in 80 patients with β-thalassemia major was found to be 11.25%, the prevalence of Xmn1 polymorphic site 5’ to the Gγ gene is different among various populations.

Among Egyptians, a frequency of 6.25%,9%, 4%and 8.3 have been reported for Xmn1 polymorphism among Egyptians with β-thalassemia, respectively(18, 10, 21, 20).

In agreement Wong et al. (2006)(24) found that the frequency in Chinese β-thalassemia major patients was 10.3%, a nearby frequency was reported by Geoffrey etal(9), in Hong Kong who demonstrated that the frequency of Xmn1 polymorphism (at nucleotide 158 bp5’) was 13%.

On the other hand, Mehrnoosh et al(11) reported that 76% of Iranians β-thalassemia major patients had positive Xmn1 polymorphism, while a frequency of 25% for Xmn1 polymorphic site in 64 β-thalassemia patients from India has been reported(18, 24).

Regarding the current study, the most frequent genotype observed was homozgyosity for the absence of the Xmn1 site (-/-) in 88.75% of cases, heterozygosity (+/-) genotype was detected in 11.25 % of cases, while homozgyosity for the Xmn1 polymorphic site (+/+) genotype was absent.

The 158 (C-T) polymorphism of the Gγ-globin gene (Xmn1 polymorphism) is known to ameliorate the severity of the disease because of its strong association with an increased production of HbF(11).HbF production can partially compensate for the lack of adult hemoglobin (Hb A) in patients with β-thalassemia major or intermedia, and ameliorate the clinical severity of these diseases(8). The presence of Xmn1 polymorphic site 5’ to the Gγ-globin promoter region was positively correlated with elevated synthesis of fetal Hb and its Gγ-globin component in term newborn infants and is associated with delayed switch over from fetal to adult hemoglo-
γ- Globin Gene Xmn1 Polymorphism and Response to HU in βThalassemia

It is unknown how Xmn1 polymorphic site influences the expression of the Gγ-globin gene. It seems that interaction of a multi-protein transcription complex to be involved. In a genome-wide linkage study of large Asian Indian kindred, a genetic interaction between the Xmn1 polymorphic site and a locus on chromosome 8q was reported to have influence on adult F cell (FC) levels.

Analysis of β-globin gene cluster showed a strong association between the Xmn1 polymorphic site and FC levels. Unlike the rare mutations in the γ-globin promoter that are consistently associated with large discrete effects of increased Hb F levels of 10-35% in heterozygote, the so-called pan cellular hereditary persistence of fetal hemoglobin (HPFH), the change at Gγ-158 does not always raise the Hb F levels in otherwise normal individuals. The Xmn1 polymorphic site is not a recognized binding motif for any of the known transcription factors. Nonetheless, although it has little effect in normal individuals, clinical studies have shown that, under conditions of hematopoietic stress, for example in homozygous β-thalassemia and sickle cell disease, the presence of Xmn1 polymorphic site favors a higher Hb F response. This may explain why the same mutations on different β-chromosomal backgrounds (some with and others without the Xmn1 polymorphic site) are associated with different clinical severity.

However, Hb F response associated with the Xmn1 polymorphic site is usually moderate and may not be sufficient to explain the wide difference in phenotype observed in some cases.

Rahimi et al. in a study on sickle cell patients from Southwest Iran, showed that in the presence of Xmn1 polymorphic site the Hb F levels and Gγ: Aγ ratio were increased.

One more study demonstrated that in patients with hypoplastic syndromes the presence of Xmn1 polymorphic site, significantly correlated with Hb F levels.

Ballas et al., revealed that there was a significant correlation between the presence of Xmn1 polymorphic site and increased Gγ: Aγ ratio. However, the Hb F level was not significantly increased in the presence of Xmn1 polymorphic site in their study. Although Xmn1 polymorphic site maintains a Gγ: Aγ ratio typical of fetal life but does not necessarily cause elevation of Hb F. The latter seems to depend on factors other than the Xmn1 polymorphic site.

The present study demonstrated that cases with Xmn1 Gγ (+/-) showed higher Hb F, as Hb F level was 76.38% in comparison with the mean Hb F in Xmn1 Gγ (-/-) cases which was 26.55%. This agrees with the finding of Garner et al. they reported that the β-thalassemia patients who had also co-inherited the Xmn1 polymorphism in the Gγ-globin gene promoter had higher mean HbF than non carriers of this polymorphism, also Majid et al., found that C-T polymorphism at 158 upstream of the Gγ globin gene, has been shown to be responsible for high HbF levels in β-thalassemia and sickle cell disease. This was strengthened by Mehrnoosh et al. they postulated that the presence of the Xmn1 marker under the condition of hematopoietic stress might contribute to over production of Hb F causing persistence of high fetal hemoglobin HPFH. It seems that this phenomenon carries mild feature of the disease.

In the present study 29% of β-thalassemia patients had been splenectomized. All of them were negative for Xmn1 polymorphic site. We found that there is a significant negative association between the presences of Xmn1 polymorphic site and splenectomy. Splenectomy indicated when massive splenomegaly causes hypersplenism, which aggravates anemia, thrombocytopenia, and leucopenia and increases transfusion requirements. Removing of the spleen often brings the situation back to what it was earlier in life. The anemia gets better, so some people can stop transfusions again after splenectomy.

In this study the frequency of blood transfusion after HU treatment was significantly decreased in the presence of Xmn1 polymorphic site Xmn1 +/- in comparison to Xmn1 -/-, hydroxyurea enhances fetal hemoglobin production.

In agreement, Mehrnoosh et al. reported that β-thalassemia major or intermedia with Xmn1 polymorphism showed better response of
HU than Xmn1 5/- genotype. In consolidation, it is reported that treatment of β-thalassemia patients with hydroxyurea, in the presence of Xmn1 polymorphic site has affected the clinical response to hydroxyurea therapy and a better response has been achieved\(^{(16)}\).

An increase in total Hb level has been repeatedly reported during hydroxyurea treatment in patients with sickle cell disease and β-thalassemia. A marked elevation of total Hb levels with hydroxyurea can eliminate transfusion requirements in children with severe β-thalassemia\(^{(4)}\).

Majid et al.\(^{(25)}\) studied the response of hydroxyurea treatment in Iranian transfusion dependent β-thalassemia patients and found that erythroid cultures from different thalassemia patients showed different results in the presence of hydroxyurea. The number of HbF-cells and the Hb F content per cell increased in some individuals, while in others, only the HbF-cell number increased with a moderate rise in HbF content per cell, whereas in other studies only a minimal effect was observed. This variation in response to HU suggested that one or more genetic factors are involved.

The present study revealed that in the presence of Xmn1 polymorphic site, the average age at first blood transfusion was increased compared to the absence of a polymorphic site. A significant correlation was identified between Xmn1 polymorphic site and age at first blood transfusion in the present study. It seems that in the presence Xmn1 polymorphic site the need to blood transfusion is decreased.

This was in agreement with Nemati et al.\(^{(14)}\) who reported that transfusion dependency started fairly late in the presence of Xmn1 polymorphic site and thus increased the age at first blood transfusion in patients

REFERENCES

γ- Globin Gene Xmn1 Polymorphism and Response to HU in β-Thalassemia


تعد أشكال γ غلوبين جين Xmnl-158 وعلاقتها بالاستجابة لعلاج الهدروكسييوريا في مرضى الببتالاسيميا الكبرى β (في محافظة بني سويف (مصر)).

عبدالمجيد أبوالمجد - داليا جميل - داليا صابر - صفوت شاكر

البيتالاسيميا هو مرض وراثي يتميز بتخصص إنتاج أو غياب سلسلة β-غلوبين، مما يؤدي إلى فقر الدم الانحلالي صغير الكريات. تم دراسة عدد الأشكال في جين Xmnl وعلاقته بزيادة مستوى المجرامين (HBF) وانخفاض مستوى المجرامين HU في الأشخاص الذين يعانون من التشلادم الكبرى β. وقد أجريت هذه الدراسة على 80 حالة تم اختيارها عشوائيا من بين مرضى الببتالاسيميا الكبرى β و40 شخص منجامسين في العمر والنوع كمجموعة ضابطة لدراسة انتشار Xmn1 في الجين γ-غلوبين (PCR-RFLP) - بواسطة تفاعل البلمرة المتسلسل متبوعا بالتقطيع المحدد للإطارات الأنكليتيكية والعملية. تم دراسة الارتباط بينه وبين نتائج العلاج بالβ-ΗΒF، والاستجابة للعلاج بالβ-ΗΒF، الاستجابة في المرضى السالمين كمجموعة ضابطة لدراسة انتشار الأشكال، أظهر 1.5% من مرضى الببتالاسيميا الكبرى (أظهر لXmn1 مناصيل 80 مريض الببتالاسيميا الكبرى (11.25%) متخالف لـ Xmn1. عدا الأشكال في الذكور والإناث Xmnl-158. أظهر لـ Xmn1 1.5% من مرضى الببتالاسيميا الكبرى (أظهر لـ Xmn1 مناصيل 80 مريض الببتالاسيميا الكبرى (11.25%) متخالف لـ Xmn1. عدا الأشكال في الذكور والإناث Xmnl-158. أظهر لـ Xmn1 1.5% من مرضى الببتالاسيميا الكبرى (أظهر لـ Xmn1 مناصيل 80 مريض الببتالاسيميا الكبرى (11.25%) متخالف لـ Xmn1. عدا الأشكال في الذكور والإناث Xmnl-158. أظهر لـ Xmn1 1.5% من مرضى الببتالاسيميا الكبرى (أظهر لـ Xmn1 مناصيل 80 مريض الببتالاسيميا الكبرى (11.25%) متخالف لـ Xmn1. عدا الأشكال في الذكور والإناث Xmnl-158. أظهر لـ Xmn1 1.5% من مرضى الببتالاسيميا الكبرى (أظهر لـ Xmn1 مناصيل 80 مريض الببتالاسيميا الكبرى (11.25%) متخالف لـ Xmn1. عدا الأشكال في الذكور والإناث Xmnl-158. أظهر لـ Xmn1 1.5% من مرضى الببتالاسيميا الكبرى (أظهر لـ Xmn1 مناصيل 80 مريض الببتالاسيميا الكبرى (11.25%) متخالف لـ Xmn1. عدا الأشكال في الذكور والإناث Xmnl-158. أظهر لـ Xmn1 1.5% من مرضى الببتالاسيميا الكبرى (أظهر لـ Xmn1 مناصيل 80 مريض الببتالاسيميا الكبرى (11.25%) متخالف لـ Xmn1. عدا الأشكال في الذكور والإناث Xmnl-158. أظهر لـ Xmn1 1.5% من مرضى الببتالاسيميا الكبرى (أظهر لـ Xmn1 مناصيل 80 مريض الببتالاسيميا الكبرى (11.25%) متخالف لـ Xmn1. عدا الأشكال في الذكور والإناث Xmnl-158. أظهر لـ Xmn1 1.5% من مرضى الببتالاسيميا الكبرى (أظهر لـ Xmn1 مناصيل 80 مريض الببتالاسيميا الكبرى (11.25%) متخالف لـ Xmn1. عدا الأشكال في الذكور والإناث Xmnl-158. أظهر لـ Xmn1 1.5% من مرضى الببتالاسيميا الكبرى (أظهر لـ Xmn1 مناصيل 80 مريض الببتالاسيميا الكبرى (11.25%) متخالف لـ Xmn1. عدا الأشكال في الذكور والإناث Xmnl-158. أظهر لـ Xmn1 1.5% من مرضى الببتالاسيميا الكبرى (أظهر لـ Xmn1 مناصيل 80 مريض الببتالاسيميا الكبرى (11.25%) متخالف لـ Xmn1. عدا الأشكل في الذكور والإناث Xmnl-158. أظهر لـ Xmn1 1.5% من مرضى الببتالاسيميا الكبرى (أظهر لـ Xmn1 مناصيل 80 مريض الببتالاسيميا الكبرى (11.25%) متخالف لـ Xmn1. عدا الأشكل في الذكور والإناث Xmnl-158. أظهر لـ Xmn1 1.5% من مرضى الببتالاسيميا الكبرى (أظهر لـ Xmn1 مناصيل 80 مريض الببتالاسيميا الكبرى (11.25%) متخالف لـ Xmn1. عدا الأشكل في الذكر...