Association of Genetic Polymorphisms in Vitamin K Epoxide Reductase and GAS6 haplotypes with Recurrent Pregnancy Loss among Palestinian Women

Anthar Saqer Mohammad Darwish

M.Sc Thesis

Jerusalem- Palestine

2010
Association of Genetic Polymorphisms in Vitamin K Epoxide Reductase and GAS6 haplotypes with Recurrent Pregnancy Loss among Palestinian Women

Prepared By:
Anthar Saqer Mohammad Darwish

Supervisor: Professor Hisham Darwish

A thesis submitted in partial fulfilment of requirements for the degree of Master of Science in Biochemistry and Molecular Biology
Department of Biochemistry- Faculty of Medicine- AL-Quds University

1431/2010
AL-Quds University
Deanship of Graduate Studies
Biochemistry and Molecular Biology/ Faculty of Medicine

Thesis Approval

Association of Genetic Polymorphisms in Vitamin K Epoxide Reductase and GAS6 haplotypes with Recurrent Pregnancy Loss among Palestinian Women

Prepared By: Anthar Saqer Mohammad Darwish
Student Number: 20812081

Supervisor: Professor Hisham Darwish

Master thesis submitted and accepted, Date:

The names and signatures of the examining committee members are as follows:

Head of Committee: Signature
Prof. Hisham Darwish

Internal Examiner: Signature
Dr. Imad Maatouq

External Examiner: Signature
Dr. May Maghathi
Declaration:

I certify that this thesis submitted for the degree Master of Science is the result of my own research, except where otherwise acknowledged, and that this thesis (or part of the same) has not been submitted for a higher degree to any other University or Institution.

Signed

Anthar Saqer Mohammad Darwish

Date
Dedication

To those who taught me the beauty of life

& the joy of science.........

Mom and Dad
Acknowledgements

This research could not have been possible without the guidance and support of all the members in Prof. Darwish’s Lab.

My respect and gratitude to my thesis advisor, for granting me the opportunity to work with him and his countless efforts to teach me.

I extend a warm thank you to Dina Ali, from the Biomedical Research Centre at Al-Quds University, for her constant willingness to help.

My appreciation to Suheir Eriqqat and Dr. Khaldoun Bader for showing me how to use SPSS.

We thank Alaa’ Darwish from Al-Najah University for providing the DNA samples for the breast cancer patients.

I would also like to thank Ula Abu Hilal for her assistance in providing the epidemiological data on the RPL study.
Abstract

Understanding the relationship of Single Nucleotide Polymorphisms (SNPs) with the incidence of disease is a step towards individualized medicine. The main objective of this study is to explore the potential association of SNPs in Vitamin K epoxide reductase complex subunit 1 (VKORC1) gene and Growth Arrest- Specific 6 (GAS6) gene with the occurrence of unexplained Recurrent Pregnancy Loss (RPL) and Breast Cancer.

Variants in VKORC1 gene have been found to affect the amount of reduced vitamin K (VK), a cofactor for γ-carboxylation of vitamin K–dependent proteins. The C allele of the VKORC1 +2255 T/C SNP is associated with higher activity at this locus and has been linked to increased vascular events. VK is important for post-modification of clotting factors involved in the coagulation cascade and Vitamin K₂ (VK₂) exerts an apoptotic effect on cancer cells. Studying a polymorphism which affects the status of VK in the body may be significant for a thrombophilia approach to unexplained RPL and Breast Cancer incidence.

Gas6 (Growth Arrest- Specific 6) is a Vitamin K dependent protein that exerts an anti-apoptotic effect by interacting with receptor tyrosine kinase, TAM family; Tyro3, Axl and MerTK. GAS6 expression has been found to be up-regulated in several types of cancer; this protein is also involved in clot stability. The A allele of the GAS6 polymorphism c.834+7G>A may have a protective role against thrombophilia. This allele is possibly linked with a decrease in expression. The GAS6 c.834+7G>A SNP was explored for a potential protective role in cancer and RPL.

The same SNPs were screened in both Breast Cancer and RPL cases, but were considered for different prospects and each was approached as a separate case-control type study. Genotyping was performed by using NcoI and AlwN I restriction enzymes for VKORC1 and GAS6 SNPs, respectively.
In the RPL study, 45 patients and 77 age matched controls were screened at the loci in question, no significant difference in haplotype distribution was observed for either $\text{GAS6}$ c.834+7G>A ($P=0.83$) or $\text{VKORC1}$ +2255 T/C ($P=0.20$) among the groups. This polymorphism maybe thrombophilia unrelated in the unexplained RPL cases. Further research is recommended to explore the significance of these SNPs in the Palestinian population.

In the Breast Cancer study, 81 patients and 84 controls were analysed for the indicative haplotypes. No significant difference was observed in the allele distribution for the $\text{GAS6}$ c.834+7G>A SNP among all participants ($P=0.32$). However, a significant difference in haplotype distribution for the $\text{VKORC1}$ +2255T/C SNP was observed ($P=0.02$). The TT haplotype was found in 32% of the Breast Cancer patients, and in only 16.7% of the control group. The CC and CT haplotypes were found in 83.3% of controls and 67.9% of patients. The T allele conferred a more than 2 fold increased risk for developing Breast Cancer OR 2.36, 95% CI (1.13 - 4.95).

Further work is needed to explain the association of the T allele of the $\text{VKORC1}$ gene with Breast Cancer and other factors which may affect VK status in the body.
# Table of Contents

Dedication ............................................................................................................................................. i  
Acknowledgements ................................................................................................................................ ii 
Abstract .................................................................................................................................................. iii 
Table of Contents ..................................................................................................................................... v  
Index of Tables .......................................................................................................................................... vii 
Index of Figures ......................................................................................................................................... viii  
Index of Abbreviations .......................................................................................................................... ix 

**Chapter I**  
Introduction ............................................................................................................................................... 1  
1.1.1 Vitamin K ..................................................................................................................................... 1  
1.1.2 Vitamin K dependent proteins ........................................................................................................ 2  
1.1.3 The Vitamin K cycle ....................................................................................................................... 3  
1.1.4 Vitamin K epoxide reductase complex subunit 1 (VKORC1) Gene ................................................ 3  
1.1.5 VKORC1 Polymorphisms ................................................................................................................ 4  
1.1.6 Vitamin K2 and Apoptosis ............................................................................................................ 5  
1.2. Growth Arrest Specific 6 (GAS6) ..................................................................................................... 6  
1.2.1 GAS6 and Hemostasis .................................................................................................................... 6  
1.2.2 Anti-apoptotic effect of Gas6 ........................................................................................................ 7  
1.3 Recurrent Pregnancy Loss ................................................................................................................ 8  
1.3.1 Thrombophilia ................................................................................................................................ 8  
1.3.2 Thrombophilia and Recurrent pregnancy loss .............................................................................. 9  
1.4 Breast Cancer ..................................................................................................................................... 10  
1.5 Hypothesis and Objectives ................................................................................................................ 11  

**Chapter II**  
Experimental Approach .......................................................................................................................... 12  
2.1 Participant selection ............................................................................................................................. 12  
2.1.1 RPL - Control study ...................................................................................................................... 12  
2.1.2 Breast Cancer – Control study ...................................................................................................... 13  
2.2 DNA Extraction .................................................................................................................................. 13  
2.3 Polymerase Chain Reaction (PCR) .................................................................................................... 13  
2.3.1 PCR amplification for VKORC1 +2255 T/C ................................................................................. 14
2.3.2 PCR Amplification for GAS6 SNP 843+7 G>A.................................................................15
2.4 Restriction Fragment Length Polymorphisms (RFLP).................................................16
  2.4.1 Genotyping VKORC1 +2255 T/C polymorphism......................................................16
  2.4.2 Genotyping GAS6 843+7 G>A polymorphism.......................................................16
2.5 Gel electrophoresis.........................................................................................................17
2.6 Statistical analysis..........................................................................................................17

Chapter III
Results.....................................................................................................................................18
  3.1 General Characteristics.................................................................................................18
  3.2 Genotyping of VKORC1 SNP +2255 T/C......................................................................18
  3.3 Genotyping of GAS6 SNP 843+7 G>A........................................................................20
  3.4 Hardy-Weinberg equilibrium........................................................................................22
    3.4.1 VKORC1 +2255 T/C Haplotype frequencies in RPL patients and Controls............23
    3.4.2 GAS6 c.843+7 G>A Haplotype frequencies in RPL patients and Controls..........24
  3.5.1 VKORC1 +2255 T/C Haplotype frequencies in Breast Cancer patients and Controls......25
  3.5.2 GAS6 c.843+7 G>A Haplotype frequencies in Breast Cancer patients and Controls ......26

Chapter IV
Discussion............................................................................................................................27

Chapter V
Recommendations...............................................................................................................32
Bibliography.......................................................................................................................33
Index of Tables

3.1 Participant Distribution..................................................................................................................................................18

3.2 Hardy-Weinberg equilibrium for VKORC1 +2255 C/T allele..........................................................................................22

3.3 Hardy-Weinberg equilibrium for GAS6 SNP c.843+7 G>A...............................................................................................22

3.4 Risk assessment of VKORC1 SNP + 2255 with RPL............................................................................................................23

3.5 Risk assessment of GAS6 SNP c.843+7 G>A with RPL........................................................................................................24

3.6 Risk assessment of VKORC1 SNP + 2255 with Breast Cancer..........................................................................................25

3.7 Risk assessment of GAS6 SNP c.843+7 G>A with Breast Cancer.....................................................................................26
Index of Figures

1.1 Vitamin K structures.........................................................................................................................2

1.2 Vitamin K-dependent γ-carboxylation and VK cycle..............................................................................3

3.1 Representative Agarose Gel for VKORC1 PCR product.................................................................19

3.2 Representative Agarose Gel for VKORC1 PCR product after digestion with NcoI, restriction enzyme.................................................................................................................................19

3.3 Representative Agarose Gel for GAS6 PCR product........................................................................20

3.4 Representative Agarose Gel for GAS6 PCR product after digestion with AlwNI, restriction enzyme...................................................................................................................................................21

3.5 VKORC1 +2255 T/C Haplotype distribution in RPL patients and Controls.................................23

3.6 GAS6 c.843+7 G>A Haplotype distribution in RPL patients and Controls...............................24

3.7 VKORC1 +2255 T/C Haplotype distribution in Breast Cancer patients and Controls........25

3.8 GAS6 c.843+7 G>A Haplotype distribution in Breast Cancer patients and Controls.........................26
# Index of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>BC</td>
<td>Breast Cancer</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence Interval</td>
</tr>
<tr>
<td>df</td>
<td>degrees of freedom</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleotide Triphosphates</td>
</tr>
<tr>
<td>G</td>
<td>Guanine</td>
</tr>
<tr>
<td>Gla</td>
<td>γ-carboxyglutamic acid</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilo base pairs</td>
</tr>
<tr>
<td>OR</td>
<td>Odds Ratio</td>
</tr>
<tr>
<td>P</td>
<td>Probability</td>
</tr>
<tr>
<td>P&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Primer 1</td>
</tr>
<tr>
<td>P&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Primer 2</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>RPL</td>
<td>Recurrent Pregnancy Loss</td>
</tr>
<tr>
<td>RTKs</td>
<td>receptor tyrosine kinases</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphisms</td>
</tr>
<tr>
<td>T</td>
<td>Thymine</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>VK&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Vitamin K&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>VK&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Vitamin K&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>$X^2$-test</td>
<td>Chi-Square Test</td>
</tr>
</tbody>
</table>
Chapter I
Introduction

Single nucleotide polymorphisms (SNPs) are the most common genetic variations among humans; it is a change in a single nucleotide at any location in the genome. Studying the pattern among SNPs in specific targeted genes and the incidence of a particular disease or response to a medical treatment, is one of the first steps towards a more individualized and prophylactic approach to medicine. Case-control SNP association studies are increasingly popular, populations may differ drastically in haplotype frequencies, therefore the findings of one study does not necessarily apply to all. It is important for research in this area to be conducted in the Palestinian population, in order to help transform our current health system to a more personalized approach. This thesis will explore the potential association between SNPs in the VKORC1 and GAS6 genes with the occurrences of Breast Cancer and Recurrent Pregnancy Loss (RPL). The background information for the rationale of this study is provided in the sections of this chapter.

1.1.1 Vitamin K

Vitamin K is a fat-soluble vitamin discovered in the 1930s, during cholesterol experiments in chickens (Dam and Schonheyder, 1934). Vitamin K (VK) is a collective term for several related chemical compounds, they all share a 2-methyl-1, 4 naphthoquinone backbone structure, but differ in the composition of the side chain at position C-3 (Oldenburg et al., 2008). Vitamin K1 (VK1), also known as phylloquinone, is present in cyanobacteria and plants; it possesses a mostly saturated C-20 phytol side chain. Vitamin K2 (VK2) is produced by microbial organisms and is characterized by a partly unsaturated, predominantly C-40 side chain (menaquinone). In organisms that produce VK, both types are involved in electron transport processes (Oldenburg et al., 2008).
1.1.2 Vitamin K dependent proteins

Vitamin K is an essential participant in posttranscriptional modification of proteins involved in coagulation, calcium metabolism, and other physiological processes (Oldenburg et al., 2008). All of these proteins are modified by carboxylation of glutamic acid residues to form γ-carboxyglutamic acid (Gla), in the absence of vitamin K the carboxylation does not occur and the proteins are biologically inactive (Oldenburg et al., 2008). VK dependent proteins in the coagulation cascade include the clotting factors II, VII, IX, X, and anticoagulant proteins C, S and Z which are integral to regulating hemostasis (Martinez and Barsigian, 1998). Each of these proteins is γ-carboxylated at several amino terminal glutamyl residues, which enables Ca^{2+} binding (Garcia and Reitsma, 2008 and Oldenburg et al., 2008).

Osteocalcin and matrix Gla-protein are γ-carboxylated proteins involved in bone metabolism. Gas6 is another VK dependent protein; some of its functions include cell growth and survival. In addition, four presumed carboxylated transmembrane proteins (abbreviated as PRGP1, PRGP2, TmG3, and TmG4) are predicted to be VK dependent although their biological function remains to be identified (Kulman et al., 2001 and Oldenburg et al., 2006).