UNIVERSITI PUTRA MALAYSIA

IDENTIFICATION OF CANDIDATE GENES EXPRESSIONS AND POLYMORPHISMS ASSOCIATED WITH ATHEROSCLEROSIS FROM POSTMORTEM CASES IN HOSPITAL KUALA LUMPUR AND HOSPITAL SERDANG, MALAYSIA

JOAN ANAK BLIN

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MASTER OF SCIENCE
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By

JOAN ANAK BLIN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

September, 2013
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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

IDENTIFICATION OF CANDIDATE GENES EXPRESSIONS AND POLYMORPHISMS ASSOCIATED WITH ATHEROSCLEROSIS FROM POSTMORTEM CASES IN HOSPITAL KUALA LUMPUR AND HOSPITAL SERDANG, MALAYSIA

By

JOAN ANAK BLIN

September 2013

Chairperson: Zalinah bte Ahmad, PhD

Faculty: Medicine and Health Sciences

Atherosclerosis as presented by coronary artery disease is a slow progressing, pathological process of plaque formations within arteries. Differential expression of susceptibility genes plays important role in the pathogenesis of atherosclerosis. This study aimed to determine differential expression of 11 candidate genes and polymorphisms associated with atherosclerosis from postmortem cases in Hospital Kuala Lumpur and Hospital Serdang. Seventy six atherosclerotic coronary artery tissues (ACAT) (cases) and 149 non-atherosclerotic coronary artery tissues (NCAT) (healthy controls) were gathered from the Forensic Department of Hospital Kuala Lumpur and Hospital Serdang based on predetermined inclusion and exclusion criteria. Gross examination findings showed the presence of mild to severe atheroma in ACAT samples, whereas NCAT samples showed clean tissues. Microscopic findings of ACAT samples showed presence of both stable (80.3%) and unstable atheroma (19.7%) respectively, with 11.8% of the samples were superimposed with thrombosis. Six RNA samples of each ACAT and NCAT were analyzed using
GenomeLab Genetic Analysis System (GeXP). Student’s *t*-test showed significant increase in expression levels of low density lipoprotein receptor (*LDLR*), tumor protein 53 (*TP53*), and matrix metalloproteinase 9 (*MMP9*) genes in ACAT samples (*p* < 0.05). No significant differences in the expression levels were obtained for the remaining eight candidate genes. Restriction fragment length polymorphism (RFLP) analysis was performed on 76 ACAT and 149 NCAT samples to investigate the variation in *LDLR* (C88S), *TP53* (*TP53* codon 72), and *MMP9* (*MMP9*-1562C>T) genotypes. Descriptive statistics were used to determine genotypes distribution of each polymorphism in both groups. The Chi-square (*χ*²) test revealed significant differences in CT genotype and T allele of *MMP9*-1562C>T polymorphism when compared between the ACAT and NCAT groups (CT: *χ*² = 19.758, *df* = 1, *p* = 0.000 and T allele: *χ*² = 21.109, *df* = 1, *p* = 0.000). The CT genotype and the T allele were significantly higher in ACAT samples (CT: 57.9% in ACAT vs. 27.5% in NCAT and T allele: 77.9% in ACAT vs. 48.3% in NCAT). There were positive associations of CT genotype and T allele with atherosclerosis (CT: OR = 3.622, 95% CI = 2.028 – 6.470 and T allele: OR = 3.780, 95% CI = 0.2115 – 6.755). There were no significant differences of CT genotype and T allele distribution among the three major ethnicities and between genders of the healthy population. No significant associations were identified in the C88S and *TP53* codon 72 polymorphisms with regard to atherosclerosis risk. In conclusion, CT genotype and T allele of the *MMP9*-1562C>T polymorphism displayed associations with the risk of developing atherosclerosis from postmortem cases in Hospital Kuala Lumpur and Hospital Serdang.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGENALPASTIAN EKSPRESI CALON GEN DAN POLIMORFISME BERKAITAN DENGAN ATEROSKLEROSIS DARIPADA KES-KES BEDAH SIASAT DI HOSPITAL KUALA LUMPUR DAN HOSPITAL SERDANG, MALAYSIA

Oleh

JOAN ANAK BLIN

September 2013

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Fakulti: Perubatan dan Sains Kesihatan

Aterosklerosis yang ditunjukkan melalui penyakit arteri koronari adalah proses patologi pembentukan plak secara perlahan di dalam arteri. Pengekspresan berbeza oleh gen-gen berkaitan memainkan peranan penting dalam patogenesis aterosklerosis. Kajian ini bertujuan untuk menentukan ekspresi 11 calon gen dan polimorfisme-polimorfisme yang berkaitan dengan risiko aterosklerosis daripada kes-kes bedah siasat di Hospital Kuala Lumpur dan Hospital Serdang. Tujuh puluh enam tisu arteri koronari aterosklerotik (ACAT) (kes) dan 149 tisu arteri koronari bukan aterosklerotik (NCAT) (kawalan sihat) telah dikumpul dari Jabatan Forensik Hospital Kuala Lumpur dan Hospital Serdang berdasarkan kriteria-kriteria penglibatan dan pengecualian yang telah ditetapkan. Penemuan pemeriksaan “mata kasar” menunjukkan kehadiran ateroma daripada sederhana kepada teruk dalam sampel ACAT, sementara sampel NCAT menunjukkan tisu-tisu bersih. Penemuan mikroskopik terhadap sampel ACAT menunjukkan kehadiran kedua-dua ateroma stabil (80%) dan tidak stabil (19.7%), dengan 11.8% daripada sampel tersebut
menunjukkan thrombosis yang bertindih. Enam sampel RNA daripada setiap ACAT dan NCAT telah dianalisis menggunakan Sistem Analisis Genetik GenomeLab (GeXP). Ujian-t menunjukkan peningkatan yang signifikan dalam tahap ekspresi gen-gen reseptor lipoprotein ketumpatan rendah (LDLR), protein tumor 53 (TP53), dan metalloproteinase matriks 9 (MMP9) di dalam sampel ACAT ($p< 0.05$). Tiada perbezaan yang signifikan dalam tahap ekspresi yang telah diperoleh untuk kelapan-lapan baki gen lain. Analisis polimorfisme panjang serpihan penyekatan (RFLP) telah dilakukan ke atas 76 sampel ACAT dan 149 sampel NCAT untuk menyiasat variasi dalam genotip-genotip LDLR (C88S), TP53 (TP53 codon 72), dan MMP9 (MMP9-1562C>T). Statistik deskriptif telah digunakan untuk menentukan taburan genotip setiap polimorfisme di dalam kedua-dua kumpulan. Ujian Chi-square ($\chi^2$) telah menunjukkan perbezaan yang signifikan terhadap genotip CT dan alel T dalam polimorfisme MMP9-1562C>T semasa perbandingan di antara kumpulan ACAT dan NCAT (CT: $\chi^2 = 19.758$, $df = 1$, $p = 0.000$ dan alel T: $\chi^2 = 21.109$, $df = 1$, $p=0.000$). Genotip CT dan alel T secara signifikan lebih tinggi di dalam sampel ACAT (CT: 57.9% di dalam ACAT vs. 27.5% di dalam NCAT dan alel T: 77.9% di dalam ACAT vs. 48.3% di dalam NCAT). Terdapat perkaitan yang positif bagi genotip CT dan alel T dengan aterosklerosis (CT: OR= 3.622, 95% CI= 2.028 – 6.470 dan alel T: OR= 3.780, 95% CI= 0.2115 – 6.755). Walau bagaimanapun, tidak terdapat perbezaan yang ketara pada taburan genotip CT dan alel T di kalangan tiga etnik utama dan antara jantina populasi yang sihat. Tiada perkaitan yang signifikan dikenalpasti di dalam polimorfisme C88S dan TP53 codon 72 ke atas risiko aterosklerosis. Kesimpulannya, genotip CT dan alel T bagi polimorfisme MMP9-1562C>T telah menunjukkan perkaitan dengan risiko mendapat aterosklerosis daripada kes-kes bedah siasat di Hospital Kuala Lumpur dan Hospital Serdang.
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I certify that a Thesis Examination Committee has met on 27th September 2013 to conduct the final examination of Joan Anak Blin on his thesis entitled "Identification of Candidate Genes Expressions and Polymorphisms Associated with Atherosclerosis from Postmortem Cases in Hospital Kuala Lumpur and Hospital Serdang, Malaysia " in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

JOAN ANAK BLIN

Date: 27th September 2013
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Introduction</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Background of study</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Research Objectives</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Hypothesis</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>Literature Review</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>Materials and Methods</td>
<td>35</td>
</tr>
</tbody>
</table>
3.5.3 Sample collection in hospitals 42
3.5.4 Microscopic examination of coronary artery tissues 43
3.5.5 RNA extraction 44
3.5.6 DNA extraction 46
3.5.7 Differential expressions of candidate genes analysis
  3.5.7.1 Multiplex primer design 50
  3.5.7.2 Preparation of multiplex primers 52
  3.5.7.3 Complimentary DNA (cDNA) synthesis using reverse transcription polymerase chain reaction (RT-PCR) 52
  3.5.7.4 Amplification of single stranded cDNA using PCR 54
  3.5.7.5 Separation of PCR products using GeXP system 55
3.5.8 Single nucleotide polymorphism (SNP) analysis
  3.5.8.1 PCR amplification of targeted gene sequences 58
  3.5.8.2 Digestion of PCR products with restriction enzymes 61
3.5.9 DNA sequencing analysis 62
3.6 Variables 63
  3.6.1 Dependent variables 63
  3.6.2 Independent variables 63
3.7 Analysis of data 64

4 RESULTS
4.0 Characteristics of subjects 65
4.1 Characteristic of ACAT and NCAT samples by gross examinations 67
4.2 Morphology of ACAT samples by microscopic examination 69
4.3 Candidate genes expressions profiles of ACAT and NCAT samples 75
4.4 Differential expressions of candidate genes associated with the risk of developing atherosclerosis
  4.4.1 Differential expressions in cholesterol transport-related candidate genes between ACAT and NCAT samples 77
  4.4.2 Differential expressions in coagulation cascade-related candidate genes between ACAT and NCAT samples 78
  4.4.3 Differential expressions in inflammatory-related candidate genes between ACAT and NCAT samples 78
  4.4.4 Differential expressions in enzyme 79
activity-related candidate genes between ACAT and NCAT samples

4.5 Polymorphisms of candidate genes associated with risk of developing atherosclerosis

4.5.1 PCR-RFLP analysis of C88S polymorphism in LDLR gene

4.5.2 Genotypes distribution of C88S polymorphism in ACAT and NCAT samples

4.5.3 DNA sequencing analysis of C88S polymorphism in LDLR gene

4.5.4 PCR-RFLP analysis of TP53 codon 72 polymorphism in TP53 gene

4.5.5 Genotypes distribution of TP53 codon 72 polymorphism in ACAT and NCAT samples

4.5.6 DNA sequencing analysis of TP53 codon 72 polymorphism in TP53 gene

4.5.7 PCR-RFLP analysis of MMP9-1562C>T polymorphism in MMP9 gene

4.5.8 Genotypes distribution of MMP9-1562C>T polymorphism in ACAT and NCAT samples

4.5.9 Genotypes distribution of MMP9-1562C>T polymorphism associated with atherosclerosis risk in different gender of healthy population

4.5.10 Genotypes distribution of MMP9-1562C>T polymorphism associated with atherosclerosis risk in different ethnicity of healthy population

4.5.11 DNA sequencing analysis of MMP9-1562C>T polymorphism in MMP9 gene

5 DISCUSSION

5.0 Characteristics of subjects

5.1 Characteristics of RNA and DNA samples

5.2 Gross and microscopic findings of coronary artery tissues

5.3 Differential expressions of candidate genes in ACAT and NCAT samples

5.4 Polymorphism of LDLR, TP53 and MMP9 gene in ACAT and NCAT samples

6 CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH

6.0 Conclusion

6.1 Limitation of study

6.2 Recommendation for future research

6.2.1 Findings
6.2.2 Sample size 121
6.2.3 Study design 122
6.2.4 Improvement in the sample collection method 122
6.2.5 The use of microarray method for genes expression analysis 122

REFERENCES 123
APPENDICES 144
BIODATA OF STUDENT 170
LIST OF PUBLICATIONS 171
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Summary table of sample size calculation</td>
<td>38</td>
</tr>
<tr>
<td>3.2</td>
<td>Summary of candidate genes for gene expression analysis using GeXP method</td>
<td>49</td>
</tr>
<tr>
<td>3.3</td>
<td>Summary of reverse and forward primers sequences used in GeXP analysis</td>
<td>51</td>
</tr>
<tr>
<td>3.4</td>
<td>Reaction mixture of reverse transcription for PCR template generation</td>
<td>53</td>
</tr>
<tr>
<td>3.5</td>
<td>Thermal-cycler program for reverse transcription protocol</td>
<td>54</td>
</tr>
<tr>
<td>3.6</td>
<td>PCR reaction mixture setup for one sample run</td>
<td>54</td>
</tr>
<tr>
<td>3.7</td>
<td>Thermal-cycler program for PCR protocol</td>
<td>55</td>
</tr>
<tr>
<td>3.8</td>
<td>Summary of polymorphisms associated with risk of developing atherosclerosis</td>
<td>57</td>
</tr>
<tr>
<td>3.9</td>
<td>Summary of primer sets used and PCR product sizes generated during PCR amplification</td>
<td>59</td>
</tr>
<tr>
<td>3.10</td>
<td>PCR reaction mixture setup for one sample</td>
<td>59</td>
</tr>
<tr>
<td>3.11</td>
<td>Summary of PCR amplification programmes for different polymorphisms</td>
<td>60</td>
</tr>
<tr>
<td>3.12</td>
<td>Reaction setup for digestion of PCR products</td>
<td>61</td>
</tr>
<tr>
<td>3.13</td>
<td>Summary of digestion protocol for different polymorphisms</td>
<td>62</td>
</tr>
<tr>
<td>4.1</td>
<td>Descriptive statistics concerning distribution of case and control subjects according to age, ethnicity and gender</td>
<td>66</td>
</tr>
<tr>
<td>4.2</td>
<td>Summary table describing the morphology of atheroma tissues</td>
<td>69</td>
</tr>
<tr>
<td>4.3</td>
<td>Summary table describing clinical complications and evidence of thrombosis in stable and unstable atheroma</td>
<td>74</td>
</tr>
<tr>
<td>4.4</td>
<td>Summary table of differential expressions of candidate genes in ACAT and NCAT samples</td>
<td>80</td>
</tr>
</tbody>
</table>
4.5 Genotypes distribution of C88S polymorphism in ACAT and NCAT samples

4.6 Genotypes distribution and allele frequency of TP53 codon 72 polymorphism in ACAT and NCAT samples

4.7 Genotypes distribution and allele frequency of MMP9-1562C>T polymorphism in ACAT and NCAT samples

4.8 MMP9-1562C>T genotypes distribution and allele frequency associated with atherosclerosis risk in different gender of healthy population

4.9 MMP9-1562C>T genotypes distribution and allele frequency in Malay, Chinese, and Indian of healthy population

C1 Summary of RNA purity, concentration and RNA integrity number (RIN)

D1 Summary of purity and yield of ACAT samples

D2 Summary of purity and yield of NCAT samples
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Pathogenesis of atherosclerosis.</td>
<td>14</td>
</tr>
<tr>
<td>2.2</td>
<td>High power images of (A) acute plaque rupture in thin cap fibroatheroma and (B) plaque erosion of an inflamed fibrous cap.</td>
<td>16</td>
</tr>
<tr>
<td>2.3</td>
<td>Thin cap fibroatheroma showing a relatively large necrotic core (A) with the presence of numerous macrophages (B), cholesterol clefts (C), and less smooth muscle cells (SMCs) within the fibrous cap (D).</td>
<td>17</td>
</tr>
<tr>
<td>4.1</td>
<td>Gross examinations of left coronary artery tissues obtained from deceased subjects.</td>
<td>68</td>
</tr>
<tr>
<td>4.2</td>
<td>Microscopic examination of ACAT samples showing stable atheroma (A &amp; B) and unstable atheroma (C &amp; D). Note: lumen (lu), atheroma plaque (P), intima layer (I), media layer (M), adventitia layer (A), arrow indicating the thickness of atheroma (Actual magnification x 40).</td>
<td>71</td>
</tr>
<tr>
<td>4.3</td>
<td>Sections of coronary artery demonstrate (A) cholesterol clefts (cc), (B) thrombosis (t), (C) calcification (ca), (D) foam cells (fc). Note: (ic) inflammatory cells; lumen (lu); Actual magnification x 40 (C); Actual magnification x 100 (A &amp; D); Actual magnification x 200 (B).</td>
<td>73</td>
</tr>
<tr>
<td>4.4</td>
<td>Representative electropherograms showing quantitative multiplex GeXP of candidate genes related to atherosclerosis using custom design GeXP multiplex assay. Housekeeping genes (a: Ezrin; j: ATP5O; n: RPLP0). Candidate genes (b: TP53; c: TGFBR2; d: CBS; e: IRS1; f: LPL; g: MTHFR; h: THBD; i: LCAT; k: MMP9; l: LDLR; o: ALOX5AP). Internal control gene (m: Knar).</td>
<td>76</td>
</tr>
<tr>
<td>4.5</td>
<td>Differential expressions of 11 candidate genes assessed using GeXP analysis. Each value was represented as Mean ± SD as described in Table 4.4. * indicates significance at p&lt;0.05.</td>
<td>81</td>
</tr>
<tr>
<td>4.6</td>
<td>A. Amplified PCR products of LDLR gene electrophoresed through a 3.0% agarose gel. B. PCR products after digestion with AatII restriction enzyme. Note: M: 25-700 bp DNA marker.</td>
<td>83</td>
</tr>
<tr>
<td>4.7</td>
<td>DNA sequencing analysis of C88S polymorphism in LDLR gene showing GG genotype.</td>
<td>86</td>
</tr>
<tr>
<td>4.8</td>
<td>A. Amplified PCR products of TP53 codon 72 polymorphism electrophoresed through a 3.0% agarose gel. B. PCR products after digestion with BsrUI restriction enzyme. Note: M: 25-700 bp DNA</td>
<td>88</td>
</tr>
</tbody>
</table>
marker.

4.9 DNA sequencing analysis of TP53 codon 72 polymorphism showing (A) PP genotype, (B) PR genotype, and (C) RR genotype.

4.10 A. Amplified PCR products of MMP9-1562C>T polymorphism of MMP9 gene electrophoresed through a 3.0% agarose gel. B. PCR products after digestion with SphI restriction enzyme. Note: M: 25-700 bp DNA marker.

4.11 DNA sequencing analysis of MMP9-1562C>T polymorphism showing (A) CC genotype, (B) CT genotype, and (C) TT genotype.

C2 RNA integrity numbers of ACAT and NCAT samples assessed using Agilent 2100 Bioanalyzer.

E1 Multiplex panels of candidate genes expressions in ACAT sample 1.

E2 Multiplex panels of candidate genes expressions in ACAT sample 2.

E3 Multiplex panels of candidate genes expressions in ACAT sample 3.

E4 Multiplex panels of candidate genes expressions in ACAT sample 4.

E5 Multiplex panels of candidate genes expressions in ACAT sample 5.

E6 Multiplex panels of candidate genes expressions in ACAT sample 6.

E7 Multiplex panels of candidate genes expressions in NCAT sample 1.

E8 Multiplex panels of candidate genes expressions in NCAT sample 2.

E9 Multiplex panels of candidate genes expressions in NCAT sample 3.

E10 Multiplex panels of candidate genes expressions in NCAT sample 4.
LIST OF ABBREVIATIONS

%  Percentage
° C  Degree of Celsius
α  Alpha
β  Beta
μ  Micro
μg  Microgram
μl  Microliter
μm  Micrometre
μM  Micromolar
χ²  Chi-square test
ABCA1  ATP-binding cassette, subfamily A, member 1
ACAT  Atherosclerotic coronary artery tissue
ALOX5AP  Arachidonate 5-lipoxygenase-activating protein
ANGPTL3  Angiopoietin-like 3
Apo A-I  Apolipoprotein A-I
APOA3  Apolipoprotein A3
APOA4  Apolipoprotein A4
APOA5  Apolipoprotein A5
APOB  Apolipoprotein B
APOB100  Apolipoprotein B-100
APOC  Apolipoprotein C
ApoE  Apolipoprotein E
ApoE ε2  Apolipoprotein E ε2 allele
ApoE ε3  Apolipoprotein E ε3 allele
ApoE ε4  Apolipoprotein E ε4 allele
ASP-PCR  Allele-specific primer polymerase chain reaction
ATP5O  ATP synthase, H+ transporting, mitochondrial F1 complex, O
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC</td>
<td>Area-under-the-curve</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>CABG</td>
<td>Coronary artery bypass grafting</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CBS</td>
<td>Cystathionine-beta-synthase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic acid</td>
</tr>
<tr>
<td>CEA</td>
<td>Carotid endarterectomy</td>
</tr>
<tr>
<td>CETP</td>
<td>Cholesteryl ester transfer protein</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>df</td>
<td>Degree of freedom</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>Anti-Deoxyribonuclease</td>
</tr>
<tr>
<td>EST</td>
<td>expression sequence tag</td>
</tr>
<tr>
<td>FH</td>
<td>Familial hypercholesterolemia</td>
</tr>
<tr>
<td>g</td>
<td>Centrifugation force</td>
</tr>
<tr>
<td>GALNT2</td>
<td>UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2</td>
</tr>
<tr>
<td>GCKR</td>
<td>Glucokinase regulatory protein</td>
</tr>
<tr>
<td>GeXP</td>
<td>GenomeLab Gene Expression Profiler</td>
</tr>
<tr>
<td>H &amp; E</td>
<td>Haematoxylin and eosin staining</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors</td>
</tr>
<tr>
<td>IHD</td>
<td>Ischemic heart disease</td>
</tr>
<tr>
<td>IRS1</td>
<td>Insulin receptor substrate 1</td>
</tr>
<tr>
<td>Knar</td>
<td>Kanamycin gene</td>
</tr>
<tr>
<td>LAD</td>
<td>Left anterior descending</td>
</tr>
</tbody>
</table>
LCAT  Lecithin-cholesterol acyltransferase
LDL  Low density lipoprotein
LDL-C  Low density lipoprotein cholesterol
LDLR  Low density lipoprotein receptor
LIPG  Endothelial lipase gene
LMNA  Lamin A/C gene
Lp(a)  Lipoprotein (a)
LPL  Lipoprotein lipase
MALDI-TOF-MS  Matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MCP-1  Monocyte chemoattractant protein-1
mg  Milligram
MgCl₂  Magnesium chloride
MLXIPL  MLX interacting protein-like
mM  Millimolar
MMAB  Methylmalonic aciduria (cobalamin deficiency) cblB type
MMP9  Matrix metalloproteinase 9
MMPs  Matrix metalloproteinases
mRNA  messenger Ribonucleic acid
MTHFR  5,10-methylenetetrahydrofolate reductase
MVK  Mevalonate kinase
n  Sample size
NADPH  Nicotinamide adenine dinucleotide phosphate
NCAN  Chondroitin sulfate proteoglycan 3 (neurocan)
NCAT  Non-atherosclerotic coronary artery tissue
ng  Nanogram
NO  Nitric oxide
OR  Odds ratio

\( p \)  Significant alpha value
Estimate exposure rate (proportion exposed) in cases

PCSK9 Proprotein convertase subtilisin/kexin type 9

Estimated exposure rate (proportion exposed) in controls

Relative risk

Restriction Fragment Length Polymorphisms

RNA integrity number

Ribonucleic acid

Reactive oxygen species

Ribosomal protein, large, P0

Reverse transcription

Reverse Transcription-Polymerase Chain Reaction

Serial analysis of gene expression

Standard deviation

Smooth muscle cells

Single nucleotide polymorphisms

Sortilin 1

Statistical Package for Social Sciences

Thrombin-activatable fibrinolysis inhibitor

Tris-Borate-EDTA

Tris (2-carboxyethyl) phosphine

Thin cap fibroatheroma

Transforming growth factor, beta receptor II

Thrombomodulin

Tumor protein p53

Tribbles homolog 1 (Drosophila)

Vascular cell adhesion protein 1

Very low density lipoprotein
CHAPTER 1

INTRODUCTION

1.0 Background of study

Cardiovascular disease (CVD) is a leading cause of death in Malaysia for the last 45 years and remains as a major public health problem globally (Rampal et al., 2008). Cardiac related mortality statistics have shown increasing trends in many developing countries, including India, China, Korea and other Asian countries (Cheng et al., 2005). Although a lot of effort has been invested for interventions, prevention, and treatments of the disease, cardiac related mortality remains high. Unsurprisingly, this CVD burden in most Asian populations is expected to double over the next decade (Tai et al., 2009).

Atherosclerosis as presented by coronary artery disease (CAD) is one of the major contributors of CVD. Atherosclerosis is a slow progressing, pathological process of plaque formations within the intima of coronary arteries. It develops asymptotically for decades, and usually is advanced by the time symptoms occur. This phenomenon poses a big challenge for treatments of atherosclerotic vascular disease (Graham and O’Callaghan, 2003).
One of the strategies for better management of the disease is through personalized medicine approach. Personalized medicine is particularly based on the science of pharmacogenomics, a study on how individual’s genetic inheritance affects the body’s response to drugs. Pharmacogenomics is a combination of traditional pharmacoceutical sciences with knowledge of genes, proteins, and single nucleotide polymorphisms. It is more precise and predictable and may lead us to more accurate diagnoses, safer drug prescribing, and more effective treatment. Personalized medicine approach depends mainly on a comprehensive understanding of the genes and gene variants that contribute to disease susceptibility and progression (Seo et al., 2004). Thus, a better understanding on the molecular mechanisms of atherosclerosis is crucial as a fundamental initial step in this long journey.

Candidate gene association study is the most widely used to identify susceptibility genes for coronary artery disease. The approach seeks for associations between phenotype and genotype of the candidate genes (Franchini et al., 2008). It is a branch of genome-wide association studies, which has been attempted to assess the role of genes that are already implicated in the pathophysiology of the disease (Lee et al., 2009). Candidate genes are predetermined genes located within regions of interest and are identified based on prior knowledge of their function. These candidate genes are suspected of being involved in the expression of a trait that contributes to the disease development. In atherosclerosis, up- or down-regulation pattern of candidate genes have been thought to alter the microenvironment that may protect or worsen the lesion progression (Fortunato and Taranto, 2007). Besides, polymorphisms within susceptibility genes in lipid metabolism, inflammation, and thrombogenesis
are all responsible for atherosclerotic vascular disease susceptibility (Chen et al., 2007).

Association studies are designed to compare statistical difference in genotype frequencies between case and control groups, which may offer evidence that a genotype is associated with the trait (Arnett, 2007). A problem with many published association studies is that a positive association observed in one report is often not reproducible in subsequent studies. This scenario is due to inconsistently defined phenotypes, variability in sample size, as well as different populations and ethnic groups under study. Indeed, in single nucleotide polymorphisms (SNPs) analysis, allele frequencies may differ widely in different populations (Gibbons et al., 2004). Thus, data collection of genetic variation associated with the risk of developing atherosclerosis in Malaysian population which is also multiracial is very important. This baseline data could lead towards early detection of CVD risk in Malaysia.

Research concerning CAD in Malaysian population is still limited, although its molecular basis has been widely studied in the western world (Abdullah et al., 2012). Without proper prevention and managements of CVD, the increasing burden of CVD will directly affect the economic status of the country. More extensive effort is required in molecular aspects so that thorough understanding of the disease is acquired. Therefore, there is an urgent need for a comprehensive study concerning the genetics basis of atherosclerotic vascular disease in Malaysia. The outcome of these fundamental efforts is hoped to facilitate the development of early detection
technique which in turn may contribute to a better prevention action of CVD in Malaysia.

1.1 Research Objectives

General Objective:

1. To study the association between the differential expression of candidate genes and polymorphisms with the risk of developing atherosclerosis from postmortem cases in Hospital Kuala Lumpur and Hospital Serdang, Malaysia.

Specific Objectives:

1. To determine the morphology of atherosclerotic coronary artery tissue using haematoxylin and eosin (H & E) staining.

2. To determine list of candidate genes associated with risk of developing atherosclerosis from postmortem cases in Hospital Kuala Lumpur and Hospital Serdang, Malaysia using GenomeLab Gene Expression Profiler (GeXP) genetic analysis system.

3. To determine the polymorphisms and distribution of genotypes and alleles frequency of candidate genes associated with risk of developing atherosclerosis from postmortem cases in Hospital Kuala Lumpur and Hospital Serdang, Malaysia.
1.2 Hypothesis

There are associations of differential expressions and polymorphisms of candidate genes with the risk of developing atherosclerosis from postmortem cases in Hospital Kuala Lumpur and Hospital Serdang, Malaysia.
REFERENCES


130


