International Journal of Medical, Medicine and Health Sciences
ISSN: 2517-9969
Vol:6, No:7, 2012

Identification of Differentially Expressed Gene (DEG) in Atherosclerotic Lesion by Annealing Control Primer (ACP)-Based Genefishing™ PCR

M. Maimunah, G.A. Froemming, H. Nawawi, M.I. Nafeeza, O. Effat, M.Y. Rosmadi, M.S. Mohamed Saifulaman

***Abstract*—**Atherosclerosis was identified as a chronic inflammatory process resulting from interactions between plasma lipoproteins, cellular components (monocyte, macrophages, T lymphocytes, endothelial cells and smooth muscle cells) and the extracellular matrix of the arterial wall. Several types of genes were known to express during formation of atherosclerosis. This study is carried out to identify unknown differentially expressed gene (DEG) in atherogenesis. Rabbit's aorta tissues were stained by H&E for histomorphology. GeneFishing™ PCR analysis was performed from total RNA extracted from the aorta tissues. The DNA fragment from DEG was cloned, sequenced and validated by Real-time PCR. Histomorphology showed intimal thickening in the aorta. DEG detected from ACP-41 was identified as cathepsin B gene and showed upregulation at week-8 and week-12 of atherogenesis. Therefore, ACP-based GeneFishing™ PCR facilitated identification of cathepsin B gene which was differentially expressed during development of atherosclerosis.

***Keywords*—**Atherosclerosis, GeneFishing™ PCR, cathepsin B gene

I. Introduction

ATHEROSCLEROSIS is an arterial disease that affects the large and medium sized arteries causing reduction of blood flow due to thickening and hardening of the arterial wall. Consequence to the narrowed arterial lumen, several clinical syndromes is manifested including ischemic heart disease, peripheral vascular disease, cerebrovascular disease, thrombosis, plaque hemorrhage and aneurysm [1].

Many studies on arterial biology have demonstrated pathways of atherogenesis revealed the association of endothelial dysfunction and inflammatory processes in the arterial wall [2-6]. Inflammation was indicated as a key role in the initiation, progression and rupture of the atherosclerotic plaque [7]. Furthermore, alteration of arterial wall permeability marked dysfunction of the endothelium causing the arterial wall to be more susceptible particularly to pro-atherogenic component such as low-density lipoprotein [8]. Accumulation of low-density lipoprotein (LDL) in the vessel lumen stimulated atherogenesis which strongly related the blood cholesterol and atherosclerosis [9-11].Atherosclerosis involved expression of associated genes that are differentially expressed throughout various stages of its development [12-14]. Expression profiles of the genes can be characterized by Genefishing™ PCR technique which facilitates detection of unknown gene in atherogenesis [15]. This system used annealing control primers (ACPs) that possessed a unique tripartite structure consist of 3'-end and 5'-end distinct portion separated by a regulator. There are two sets of ACPs that play important roles in amplification of a specific target gene. The first primer, dT-ACP1, reverse transcribed the RNA templates to produce first cDNA strand. The procedure is achieved by hybridizing the 3'-end core portion of dT-ACP1with a sequence complementary to the polyA region of mRNA transcripts. The first-strand cDNA produced at the first stage possessed the universal sequence of dT-ACP1 at its 5'-end. The second primers consist of combination of arbitrary ACPs (forward primers) and dT-ACP2 (reverse primers), possessed a hybridising sequence complementary to a region on the first strand cDNA, and are participating in synthesizing the second strand cDNA by PCR. This ACP-based system allows amplification of specific target gene by stringent hybridization of the ACPs with the intended template. Therefore, gene expression profiles can be characterized with elimination of false positive result. The present study is carried out to detect unknown differentially expressed genes (DEGs) underlying development of atherosclerosis by GeneFishing™ PCR.

II. Materials And Methods

All chemicals, reagents and solvents used were of Analar Grade or of the highest grade commercially available, obtained from Sigma Chemical Co., U.S.A., Merck, Germany, BDH Chemicals Ltd., England, Ambion, Inc., USA and Oxoid Ltd., England. Reagent kits for RNA extraction, reverse transcription and cloning were obtained from Qiagen U.S.A. Microchips and reagents for RNA and DNA quantification were obtained from Agilent Technology Inc, Germany. DEG

M. Maimunah, is with the Faculty of Health Sciences, Universiti Teknologi Mara Puncak Alam Campus, 42300 Bandar Puncak Alam, Selangor, Malaysia. (phone: 603-32584423; fax: 603-32584599; e-mail: maimunah@ puncakalam.uitm.edu.my).

G.A. Froemming, is with the Institute of Medical Molecular Biotechnology, Universiti Teknologi Mara Sungai Buloh Campus, Jalan Hospital, 47000 Sungai Buloh, Selangor, Malaysia (e-mail: gabriele@salam.uitm.edu.my).

H. Nawawi, is with the Faculty of Medicine, Universiti Teknologi MARA, Selayang Campus, 68100 Batu Caves, Selangor, Malaysia.
(e-mail: Hapizah@salam.uitm.edu.my).

M.I. Nafeeza, is with the Faculty of Medicine, Universiti Teknologi MARA, Selayang Campus, 68100 Batu Caves, Selangor, Malaysia.
(e-mail: nafeeza@salam.uitm.edu.my).

O. Effat, is with the Faculty of Medicine, Universiti Teknologi Mara, Selayang Campus, 68100 Batu Caves, Selangor, Malaysia.
(e-mail: effat@salam.uitm.edu.my).

M.Y. Rosmadi, is with the Faculty of Pharmacy, Universiti Teknologi MARA Puncak Alam Campus, 42300 Bandar Puncak Alam, Selangor, Malaysia. (e-mail: rosmadi365@ puncakalam.uitm.edu.my).

M.S. Mohamed Saifulaman, is with the Faculty of Applied Sciences, Universiti Teknologi Mara, 40450 Shah Alam, Selangor, Malaysia.
(e-mail:drsaifulaman@yahoo.com)

Premix Kits for GeneFishing™ was obtained from Seegene Inc., Korea. ECOS™ Competence cells was obtained from Yeastern Biotech Co., Ltd, Taiwan. PCR-gel extraction kit was obtained from Promega Corporation, MD, U.S.A. EvaGreen® qPCR mix plus for Real-time PCR assay was obtained from Solis BioDyne, Estonia.

A. Induction of Atherosclerosis in New Zealand White Rabbits by Cholesterol Diet

The protocol of animal study was approved and endorsed by Animal Ethics Committee, Universiti Teknologi MARA (ACUC/CA/07(03)-UiTM). A total of thirty six young adult male New Zealand White rabbits, weighing 2.0 to 2.5kg were used in this study. The rabbits were housed individually in metal cages and subjected to adaptation to the animal facility for not less than 1-week during which they were supplied with normal rabbit pellet. They were assigned into two feeding group i.e normal-fed and 1% cholesterol-fed. Upon commencement of the atherosclerosis study, animals were fed 100g per day rabbit pellet with or without incorporation of cholesterol diet. Water was given ad libitum. Preparation of cholesterol-fed diet was followed according to Campbell *et al*., (2001). Cholesterol was added to the rabbit pellet as diethyl ether solution and was dried of the solvent at 40^{o}C overnight before being used. Rabbits were euthanized after an overnight food deprivation, with an intravenous injection of lethal dose of sodium pentobarbital (120mg/kg). The studies were carried out at 1, 2, 4, 6, 8 and 12 weeks of cholesterol feeding.

B. Preparation of Tissue Samples

After euthanizing, the thoracic cavity was dissected longitudinally; other organs were removed to expose the aorta. The ascending aorta extending to iliac bifurcation was excised. The samples were cleaned of adhering fat and connective tissues and perfused with cold phosphate-buffered saline. Aortic tissues from the arch segment were selected to be used in all experimental protocols in this study as described by Yu *et al*., 2008. Samples were snap-frozen in liquid nitrogen and stored at –80oC for further analyses. Aortic tissues for histo-morphology were fixed in 10% buffered formaldehyde.

C. Hematoxylin and Eosin (H & E) Staining

Fixed aortic tissue was processed and prepared for paraffin-block at tissue embedding centre (Shandon) and sectioned at 5 µm thickness using a rotary microtome (Microme). Sectioned tissue was placed onto a glass slide and allowed to dry and prepared for staining with Haematoxylin & Eosin. H&E slides were examined and photographed using Olympus Microscope equipped with analysisLS professional software.

D. Total RNA Extraction and Purification

All protocols in this experiment were conducted in DNase and RNase free condition. Aortic tissues (50-100mg) were ground to a powder in liquid nitrogen using mortar and pestle. Total RNA from the aortic segments was extracted using TRI Reagent® (Ambion) as described by Yasuda *et al*., 2002, followed by purification using RNeasy column (Qiagen) according to the manufacturer's instruction.

E. Evaluation of RNA Concentration and Integrity

Extracted total RNA was assessed for concentration and integrity using ND-1000 Spectrophotometer (Nanodrop Technologies, Inc., USA). Further quantification of RNA yield and purity was carried out using RNA 6000 Nano Assay kit (Agilent Technologies, Inc.). RNA concentration and integrity were measured using the 2100 expert software.

F. GeneFishing™ PCR

Differentially expressed genes were screened using 120 arbitrary annealing control primers (ACPs) from GeneFishing™ kits. First strand cDNA was prepared using Sensiscript® Reverse Transcription (Qiagen). 50 ng of template RNA from control, 2-week, 4-week, 6-week, 8-week and 12-week was added into each reaction tube containing 1 µM dT-ACP1 primer (Seegene), 1X buffer RT, 0.5mM dNTP mix, 1.0 µl Sensiscript reverse transcription , 10U/ µl RNase inhibitor and RNase-free water. A total volume of 20 µl reaction mixtures for each sample were briefly centrifuged followed by incubation at 37^{o}C for 1 hour.

GeneFishing™ PCR was prepared in a 20 µl reaction volume consisting of first-strand cDNA (~50 ng), 5 µM arbitrary ACP (one of the arbitrary ACPs), 10 µM dT-ACP2 and 2X SeeAmp™ ACP™ Master Mix. Positive control was carried out using the control cDNAs (kidney and liver) provided in the DEG Premix kits. PCR reaction was performed at 94^{o}C (5 min), 50^{o}C (3 min), 72^{o}C (1 min), 40 cycles of 94^{o}C (40 sec), 65^{o}C (40 sec), 72^{o} C (40 sec) and final extension at 72^{o}C (5 min). 2 µl of PCR products were electrophoresed on a 2% agarose gel containing Ethidium Bromide. Differentially expressed bands from the agarose gel were extracted using Wizard® SV Gel and PCR Clean-Up System (Promega).

G. Cloning and Sequencing of GeneFishing™ PCR Products

GeneFishing™ PCR products which showed distinct bands from week-0 to week-12 were cloned according to the protocol provided by Qiagen PCR cloning kit. DNA purification was performed according to the protocol provided by GeneJET™ Plasmid Miniprep Kit (Fermentas). The sequence of the clones was determined by ABI 3730XL genetic analyzer.

H. Primer Sequence

The DNA sequence of the unknown gene was analyzed by searching for similarities using the BLAST search program at the National Center for Biotechnology Information (NCBI) GenBank. The primer sequence of the gene was designed using Primer3Plus program and synthesized by AITBiotech Company.

I. Validation of Differentially Expressed Gene by Real-time PCR Assay

Real-time PCR (RT-PCR) was performed using Rotor-gene 6000 System (Corbett Research). The reaction solution was assembled in a volume of 20µl, which comprised of EvaGreen® qPCR Mix Plus (Solis Biodyne), forward and

reverse primers (final concentration 200nm each) and 50ng cDNA template.

A total of 50ng of template was subjected to RT-PCR with cycling condition as follows: 95°C for 5 minutes for activation of Taq DNA Polymerase followed by 40 cycles of denaturation, annealing and extension at 95°C for 10 seconds, 60°C for 30 seconds and 72°C for 30 seconds respectively. All samples were normalized to glyceraldehydes-3-phosphate dehydrogenase (GAPDH) which gave constant Ct values in all studied samples. The normalized value for each target cDNA reflects the expression level of the corresponding gene in a test sample relative to the standard tissue.

J. Data Analysis

Data obtained from differentially expressed gene by GeneFishing™ was compared with the registered sequence in the GenBank using BLAST algorithm [16]. Real-time PCR analysis was performed by delta delta Ct generated using Rotor-Gene 6000 Software 1.7.87 (Corbett Research).

III. Results

A. Total RNA Integrity

The integrity of total RNA used for GeneFishing™ PCR assay were in the range of 6.7 to 8.3 of the RNA integrity number (RIN) indicating good quality of intact RNA were used in all experiments (Fig. 1).

B. Morphological Changes of Aorta Tissue in Development of Atherosclerosis

H&E staining of cross section tissues from 1, 2, 4, 6, 8 and 12 weeks of cholesterol diet-animals were presented in Fig. 2A - 2F. Evident morphological changes of atherogenesis exhibited by increased intima areas commenced from 6 week and continuously progress to 12 week cholesterol-diet (Fig. 2D - 2F).

C. Detection of Differentially Expressed Genes in Atherogenesis

A total of 120 ACPs were screened from total RNA isolated from aortic tissues of 1, 2, 4, 6, 8 and 12 weeks of cholesterol diet animals. Of the 120 ACPs screening, DEG fragments from ACP41 at week-12 showed intense band compared to other weeks studied (Fig. 3). An analysis using BLAST (NCBI GenBank), DEG sequence derived from ACP41 (week-12) showed that this sequence has 100% homology to a published mRNA sequence coding for *Oryctologus cuniculus* cathepsin B. Fig. 4A displayed nucleotide sequence of the 250bp PCR fragment from ACP41 was presented as the upper sequence (query) and cathepsin B gene as the lower sequence (sbjct). Complete sequence of XM_002709436 is presented in Fig. 4B.

Real-time PCR assay using primer sets, Cathepsin B: Forward 5'- CACGTGAATGTGGAGGTGTC–3'and Reverse 5' – CCAGAAGTTCCAAGCTCCAG – 3' exhibited differentially expressed gene at week-8 and week-12 (Fig. 5). Cathepsin B gene was upregulated at 1.5-fold and 1.3-fold corresponding with thickening of the intimal area as detected by histomorphology at week-8 and week-12 respectively.

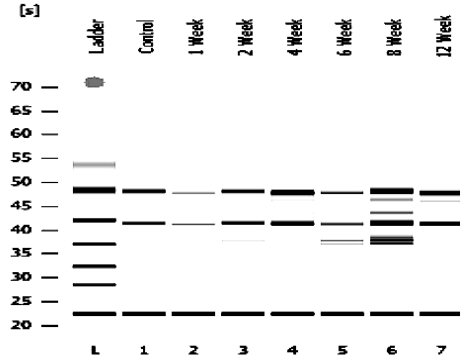
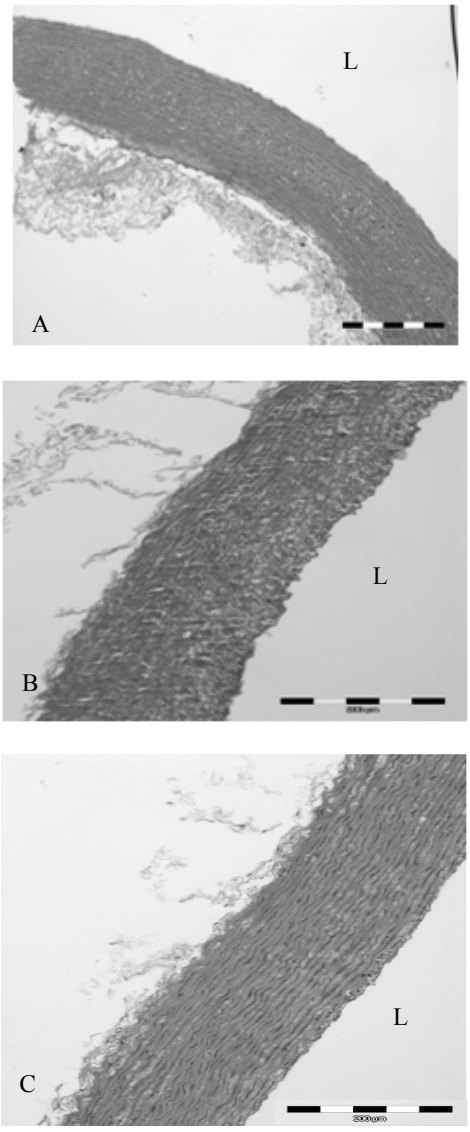


Fig. 1 Gel Picture of Agilent 2100 Bioanalyser for Total RNA Extracted from Aorta Tissue Samples. Lane 1, control tissue from normal-diet rabbit; lanes 2 – 7, cholesterol-diet rabbits from 1 to 12 weeks respectively



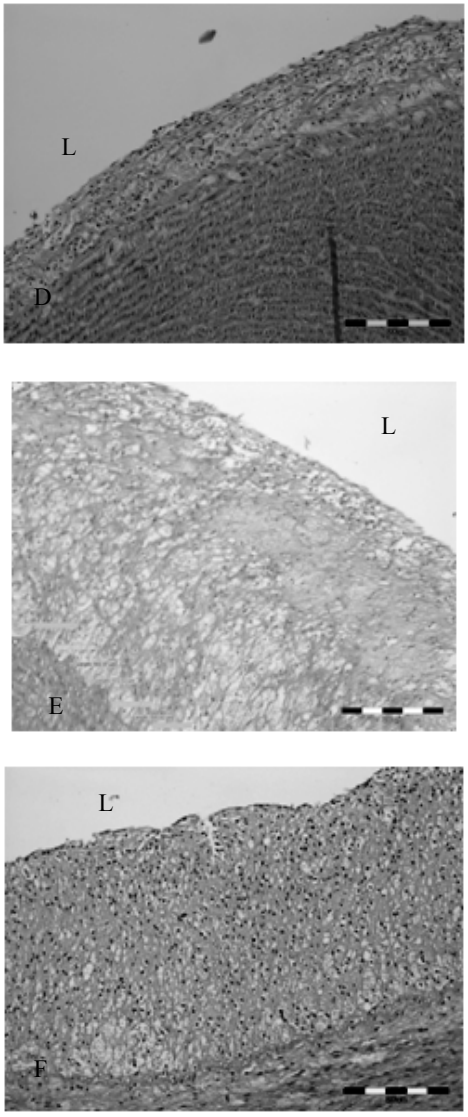


Fig. 2 H&E stain of aorta tissues from 1 to 12-week cholesterol-diet rabbits. 1-week (A), 2-week (B), 4-week (C), 6-week (D), 8-week (E), and 12-week (F). L = lumen, I = intima. Bar represent 200µm

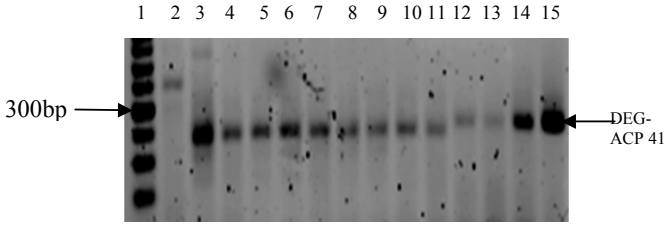


Fig. 3 2% Agarose gel electrophoresis of ACP 41 and ACP 79 viewed by Typhoon™ 9410 (GE). Lanes 1, 50bp DNA ladder; 2, control DEG kidney; 3, control DEG liver, 4, ACP79-1 week; 5, ACP79-2 week; 6, ACP79-4 week; 7, ACP79-6 week; 8, ACP79-8 week; 9, ACP79-12 week; 10, ACP41-1 week; 11, ACP41-2 week; 12, ACP41-4 week;13, ACP41-6 week; 14, ACP41-8 week; 15, ACP41-12 week. The arrow indicates DEG-ACP 41 exhibiting more intense fragment

```
Query  157  TAGAAGGAGAAACCTTTTATTGGCACAGTCATTCCTCATTAAATGGACAGGGTGAAGCAG  216
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1882 TAGAAGGAGAAACCTTTTATTGGCACAGTCATTCCTCATTAAATGGACAGGGTGAAGCAG 1823

Query  217  TGATTTTTAGAAAACAGTAAAAGCTGGTTTCTCCCACACTAGTCCTGCCAGGTGCCATGT  276
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1822 TGATTTTTAGAAAACAGTAAAAGCTGGTTTCTCCCACACTAGTCCTGCCAGGTGCCATGT 1763

Query  277  CAGGAGACAGTCAGCAAATCCAGACCACCAGGTTTTGTTTCACGCCGCGCCATTGGGA  334
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1762 CAGGAGACAGTCAGCAAATCCAGACCACCAGGTTTTGTTTCACGCCGCGCCATTGGGA 1705
```

Fig. 4A Nucleotide sequence of DEG-ACP 41(Query) and the comparison with cathepsin B gene from *Oryctolagus cuniculus* (Sbjct), Genbank accession # XM_002709436

```
   1 atgtggcgac tcctgtcccc tctctgctgc ctgctggcac tgaccagcgc ctggagcgac
  61 tcccatctgc acccgctgtc ggatgagctg gtcaacttca tcaacaagca gaacacgacg
 121 tggcaggccg ggcacaactt cttcaacgtg gaggtgagct acctcaagaa gctgtgcggc
 181 accttcctgg gagggcccaa gctgccccgc agagttgagt tcgctgatga cataaaactg
 241 cctgagagct tcgacgcgcg ggaacagtgg ccgaactgtc caaccatcaa ggagatcaga
 301 gaccaggggt cctgcggctc ttgctgggct ttcggggccg tggaagccat ctccgaccgg
 361 atctgcatcc acaccaacgg gcacgtgaat gtggaggtgt cggcggagga catgctcacc
 421 tgctgcggcg gccagtgtgg agacggctgt aatggcggct acccctctgg agcttggaac
 481 ttctggacca aaaaaggcct cgtgtccggg ggcctctatg actcccacgt aggctgcaaa
 541 ccctactcca tccctccgtg cgagcaccac gtcaacggct cccggcccgc gtgcacgggg
 601 gagggagaca cgcccaggtg cagcaagacc tgcgagcccg gctacagccc gtcctacaag
 661 gaggacaagc actatggata cagttcctac agcgtctcca gtgacgagaa tgagatcaag
 721 gcagagatct acaaaaacgg cccggtggag ggcgccttca ccgtgtactc cgacttcctg
 781 atgtacaagt caggagtgta ccagcacaca actggcgaca tcatgggagg ccacgccatc
 841 cgcatcctgg gctggggcga ggagaacggc gtcccctact ggctggtggc caactcgtgg
 901 aacaccgact ggggtgacaa gggcttcttt aaaatcctca gaggacagga tcactgtgga
 961 atcgaatccg aaattgtggc cggaatcccg cgcactgacc agtactggcg acagatctaa
1021 cccgctgtgg gctggccccc tcccccgccg tgggggaggc gcccctaaat gtatcggatt
1081 gggctgggga ggagatgaga tgggggttggg gtaggtagga atggctttcc acacaagatg
1141 atacgggagg ttttagacag ggcctgaagg actggactgg gccacgcttg cgtccgcccc
1201 agagctgtct tccaaggaga cgcatccatg gctgcccccc ccaagcctgc tcagtggacg
1261 ctggtcagga ggcaacgggc cgcgccggcc actgctgtga gcgcaggagg ctcctcctcc
1321 actaggcacc tgctgctccc gccccagctg caaccaccgc acccctcctc tctccggaac
1381 ctccaagagc aggacagacc tgtcggcgtg ggcagcaggc gttccgaggc attggcgagt
1441 ggcgttcctg ctgacgccgc cagcaagtcg ccttctagac ctgcaccagg ttccgaggag
1501 agatggcggc aactctctgg aagacgccac ctccccccag ggcccctgca cctgccaagc
1561 ttcgcagtgt cgcaaacccc ctccggtctt gtcctcggca tggttccttt ctaatagaag
1621 ttgtattttt ttgcatcctt gagctgatca cgtgaccaaa caggtctaat agttggaaga
1681 gcggcactcc ctttccagat tgtctcccaa tggcgcggcg tgaaacaaaa cctggtggtc
1741 tggatttgct gactgtctcc tgacatggca cctggcagga ctagtgtggg agaaaccagc
1801 ttttactgtt ttctaaaaat cactgcttca ccctgtccat ttaatgagga atgactgtgc
1861 caataaaagg tttctccttc ta
```

Fig. 4B Complete sequence of XM_002709436 of *Oryctolagus cuniculus* cathepsin B with 100% (178/178) homology to DEG ACP41 transcript showed by underlined sequence from 1705 to 1882

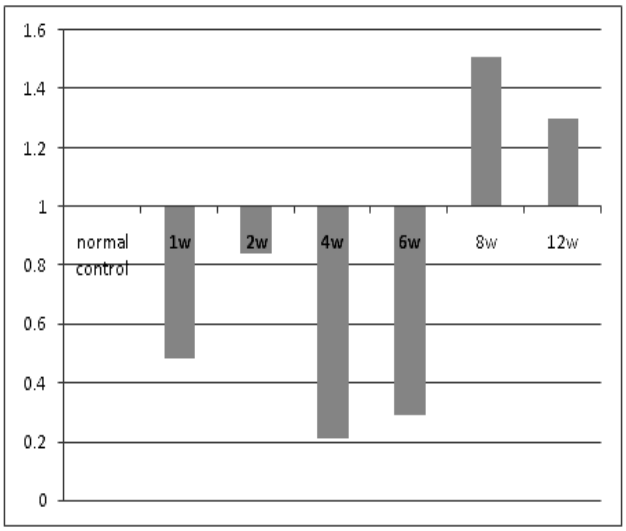


Fig. 5 Expression profiles of Cathepsin B gene from 1- week to 12-week atherogenesis. Increased expressions exhibited in 8 and 12-week atherogenesis

IV. Discussion

Atherosclerosis involved expression of associated genes that are differentially expressed throughout various stages of its development [12-14]. Therefore, this study was performed to identify any unknown gene(s) profiling associated with atherogenesis. Expression profiles of the genes were evaluated by Genefishing™ PCR technique which facilitates detection of unknown gene(s) in atherogenesis. This technique was firstly introduced by Hwang and co-workers in 2003 in an attempt to improve specificity of conventional PCR primer during annealing process and eliminating false positive PCR products from subsequent amplification process. The novel oligonucleotide primer designated 'Annealing Control Primer' (ACP™) provides a high annealing specificity to the target sequences and allows only genuine products to be amplified [17]. ACP™ technology is developed based on its unique tripartite structure and utilization of precise annealing temperature which has the capability to minimize mismatched or non-specific hybridization of primer-template. The unique tripartite structure of ACP™ consisted of (i) a 3′ end region with a target core nucleotide sequence contains 10 nucleotides (nts) complementary to the template nucleic acid for hybridization; (ii) a 5′ end region with a universal nucleotide sequence (22 nts); and (iii) a polydeoxyinosine [poly (dI), 5 nts] linkerbridging the 3′ and 5′ end sequences [17]. The poly (dI) linker generates a region with a lower melting temperature (Tm) via the formation of a bubble-like structure at specific temperatures. The poly (dI) linker in the bubble-like structure regulates the annealing of each 3' end region and 5' end region at distinct annealing temperature during PCR reactions. Therefore, the ACP linker acts as a regulator for a discrete interaction of nucleotide sequences of 5′ and 3′ region with the target template. Due its high annealing specificity, the application of the ACP for identification of differently expressed gene (DEG) generates reproducible, accurate, and PCR products ranging from 100 bp to 2 kb that are detectable on agarose gels [18].

GeneFishing PCR™ assay was developed specifically to be employed in screening of differentially expressed gene (DEG) in living cells under various normal physiological stages and pathological conditions [15]. Often, under certain biological stages, genes are expressed at low level which creates difficulties for their identification. Therefore, GeneFishing PCR assay resolve the difficulties in 'fishing out' the low concentrations of DEG transcripts.

In the present study, Genefishing™ PCR demonstrated the ability to detect the unknown genes in atherosclerosis by using 120 arbitrary ACPs. The unknown gene was then identified as Cathepsin B, a cysteine protease localized in lysosomes and endosomes of inflammatory cells. This cystein protease was found to be associated with degradation of intracellular and endocytosed proteins.

Cathepsin B, expressed by macrophages play an important role in atherogenesis by accelerating degradation of the vascular extracellular matrix [19].

The pro-atherogenic property of Cathepsin B was characterized by its high mRNA expression in atherosclerotic lesion developed in apoE-deficient (apo-E-/-) mice following a 20-week high fat diet [20]. The present study also demonstrated Cathepsin B as pro-atherogenic by investigating its mRNA expression profiles in atherogenesis from 1-week to 12-week cholesterol-diet rabbit. High expression level of Cathepsin B at 12-week atherogenesis could be associated with ultrastructural changes of the endothelial surface (data not shown). Cathepsin B may provoke loss of endothelial surface integrity by degrading native and modified LDL in the vessel wall and promote macrophage foam cell formation. Lutgens *et al*., (2007) stated that inhibition of Cathepsin B impede its role as LDL degrading agent.

The present study also identified accumulation of macrophages characterised by increased intima region in the blood vessel. The phenotypic changes was apparent from 6-week to 12-week atherogenesis. Atherosclerosis-stimulating role of Cathepsin B may contribute to the morphological changes of the intimal area due to its capability to degrade ECM of the blood vessel. Accumulation of macrophages increased Cathepsin B expression that eventually promoting ECM degradation.

Acknowledgment

This work was supported by Fundamental Research Grant Scheme (FRGS) no. 600 IRDC/ST/FRGS.5/3/1337 from the Ministry of Higher Education Malaysia.

References

[1] Newby, A. C., George, S.J., Ismail, Y., Johnson, J.L., Sala-Newby, G.B. and Thomas, A.C. "Vulnerable atherosclerotic plaque metalloproteinases and foam cell phenotypes". *Thromb Haemost*, vol. 101(6): pp. 1006-11, 2009.

[2] Newby, A.C. "Matrix metalloproteinases regulate migration, proliferation, and death of vascular smooth muscle cells by degrading

matrix and non-matrix substrates". *Cardiovasc Res*, vol. 69(3): pp. 614-24, 2006.

[3] Matsumoto, S., Kobayashi, T., Katoh, M., Saito, S., Ikeda, Y., Kobori, M., Masuho, Y. and Watanabe, T. "Expression and localization of matrix metalloproteinase-12 in the aorta of cholesterol-fed rabbits: relationship to lesion development". *Am J Pathol*, vol. 153(1): pp. 109-19, 1998.

[4] Watanabe, N. and Ikeda, U., Matrix metalloproteinases and atherosclerosis. *Curr Atheroscler Rep*, 6(2): p. 112-20, 2004.

[5] Newby, A.C. and Zaltsman, A.B. Fibrous cap formation or destruction--the critical importance of vascular smooth muscle cell proliferation, migration and matrix formation. *Cardiovasc Res*, 41(2): p. 345-60, 1999.

[6] Blake, G.J. and Ridker, P.M. Inflammatory bio-markers and cardiovascular risk prediction. *J Intern Med*, 252(4): p. 283-94, 2002.

[7] Beaudeux, J. L., Giral, P., Bruckert, E., Foglietti, M. J., & Chapman, M. J. Matrix metalloproteinases, inflammation and atherosclerosis: therapeutic perspectives. *Clin Chem Lab Med*, 42(2): p. 121-31, 2004.

[8] Koga, T.,Kwan, P., Zubik, L., Ameho, C., Smith, D.and Meydani, M. Vitamin E supplementation suppresses macrophage accumulation and endothelial cell expression of adhesion molecules in the aorta of hypercholesterolemic rabbits. *Atherosclerosis*, 176(2): p. 265-72, 2004.

[9] Bobryshev, Y.V. Monocyte recruitment and foam cell formation in atherosclerosis. *Micron*, 37(3): p. 208-22, 2006.

[10] Aikawa, M. and Libby, P. Lipid lowering therapy in atherosclerosis. *Semin Vasc Med*, 4(4): p. 357-66, 2004.

[11] Aikawa, M. and Libby, P. Lipid lowering reduces proteolytic and prothrombotic potential in rabbit atheroma. *Ann N Y Acad Sci*, 902: p. 140-52, 2000.

[12] Kang, J. G., Patino, W. D., Matoba, S., & Hwang, P. M. Genomic analysis of circulating cells: a window into atherosclerosis. *Trends in cardiovascular medicine*, 16(5): p. 163-8, 2006.

[13] Satterthwaite G, Francis SE, Suvarna K, Blakemore S, Ward C, Wallace D, Braddock M, and Crossman D. Differential gene expression in coronary arteries from patients presenting with ischemic heart disease: Further evidence for the inflammatory basis of atherosclerosis. *American heart journal*, 150(3): p. 488-499, 2005.

[14] Sinnaeve, P. R., Donahue, M. P., Grass, P., Seo, D., Vonderscher, J., Chibout, S. D., Kraus, W.E., Michael Sketch, M.J., Nelson, C., Ginsburg, G.S., Goldschmidt-Clermont, P.J., Granger, C.B. Gene expression patterns in peripheral blood correlate with the extent of coronary artery disease. PLoS One, 4(9): p. e7037, 2009.

[15] Kim, Y. J., Kwak, C. I., Gu, Y. Y., Hwang, I. T., & Chun, J. Y. Annealing control primer system for identification of differentially expressed genes on agarose gels. *Biotechniques*, 36(3): p. 424-6, 2004.

[16] Ryu, H. H., Jung, S., Sun, H. S., Jung, T. Y., Jin, S. G., Jin, Y. H., Kim, I.Y., Jeong, Y.I1 and Kang, S.S. Screening for motility-associated genes in malignant astrocytoma cell lines. *J Neurooncol*, 82(2): p. 125-31, 2007.

[17] Hwang, I.-T.K., Y-J; Kim, S-H; Kwak, Cll. and a.J.-Y.C. Young-Yun Gu, Annealing control primer system for improving specificity of PCR amplification. *BioTechniques*, 35(6): p. 1180-1184, 2003.

[18] Kim, Y. S., Hwan, J. D., Bae, S., Bae, D. H., & Shick, W. A. Identification of differentially expressed genes using an annealing control primer system in stage III serous ovarian carcinoma. *BMC Cancer*, 10: p. 576, 2010.

[19] Lutgens, S. P., Cleutjens, K. B., Daemen, M. J., & Heeneman, S. Cathepsin cysteine proteases in cardiovascular disease. *Faseb J*, 21(12): p. 3029-41, 2007.

[20] Ma, Y., Malbon, C.C., Williams, D.L. and Thorngate, F.E. Altered gene expression in early atherosclerosis is blocked by low level apolipoprotein E. *PLoS One*, 3(6): p. e2503, 2008.