A STUDY OF HUMAN CHOLINE KINASE α (hCK-α) IN PATIENTS WITH ISCHEMIC HEART DISEASE (IHD) AND HEALTHY VOLUNTEERS

by

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CERTIFICATE

This is to certify that the dissertation entitled

"A Study of Human Choline Kinase α (hCK-α) in Patients with Ischemic Heart Disease (IHD) and Healthy Volunteers"

is the bonafide record of research work done by

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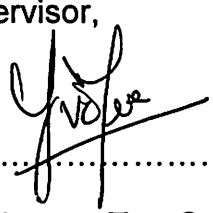
during the period from July 2008 to October 2008

under my supervision.

Supervisor,



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List of Symbols and Abbreviations

ACS: Acute coronary syndromes

ADU: Arbitrary densitometric units

AIDS/HIV: Acquired immune deficiency syndrome / human immunodeficiency virus

ATP: Adenosine triphosphate

ck-α: Choline kinase α gene

ck-β: Choline kinase β gene

CK-MB: Creatine kinase MB

CRP: C-reactive protein

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-Linked Immunosorbant Assay

hCK-α: Human choline kinase α

hCK-α1: Human choline kinase α1

hCK-α2: Human choline kinase α2

hCK-β: Human choline kinase β

hsCRP: High-sensitivity C-reactive protein

IHD: Ischemic heart disease

KCl: Potassium chloride

MOH: Ministry of Health

NC: Nitrocellulose membrane

PBST: Phosphate buffered saline detergent Tween 20

PCh: Phosphocholine

PTCA: Percutaneous transluminal coronary angioplasty

PtdCho: Phosphotidylcholine

RT-PCR: Reverse transcription polymerase chain reaction

SBP: Systolic blood pressure

DBP: Diastolic blood pressure

SDS: Sodium dodecyl sulfate

TEMED: Tetramethylethylenediamine

WHO: World Health Organization

Kajian tentang *choline kinase α* (CK-α) pada pesakit jantung iskemik dan sukarelawan sihat

Abstrak

Penyakit jantung iskemik dijangka akan menjadi penyebab kematian utama sedunia pada tahun 2030. Penyakit yang membawa maut ini tidak menunjukkan tanda-tanda pada peringkat awal. Maka, pencegahan dan pengesanan awal melalui pengenalan penanda kardiak adalah cara terbaik untuk mengatasinya. *Choline kinase α* (CK-α) telah dikaji untuk mengesan kemungkinan protein ini sebagai bakal penanda kardiak. Tujuan kajian ini ialah untuk mengkaji perbezaan taraf CK-α pada darah di antara pesakit jantung iskemik dengan sukarelawan. Sampel darah daripada sepuluh pesakit jantung iskemik dan 10 sukarelawan sihat telah dianalisis dengan menggunakan tindakbalas rantai polimerase (RT-PCR) untuk menguji kandungan mRNA bagi CK-α. *Western blot* telah dijalankan untuk mengesan kehadiran protein CK-α dalam darah. Sejurus itu, ujian ELISA juga telah dijalankan untuk menguji kepekatannya di dalam sampel darah. Protein C-reaktif (CRP) digunakan sebagai penunjuk standard untuk membezakan pesakit jantung iskemik dengan sukarelawan sihat. Analisis statistik bagi ujian parametrik dijalankan dengan Ujian t manakala ujian tidak parametrik dianalisis dengan ujian *Mann-Whitney.* Ujian statistik menunjukkan CRP mempunyai perbezaan yang ketara pada peringkat mRNA dan juga pada peringkat protein antara pesakit jantung iskemik dan sukarelawan sihat (Ujian t, $p<0.001$; ujian Mann-Whitney, $p=0.009$). Perbezaan signifikan antara peringkat mRNA bagi CK-α2 telah dikesan

dengan penurunan signifikan pada kumpulan pesakit (Ujian t, p=0.032). Namun, peringkat mRNA bagi *total CK-α* tidak menunjukkan perbezaan signifikan dalam perbandingan pada pesakit dengan sukarelawan (Mann-Whitney, p=0.705). Sementara itu, kandungan protein CK-α telah menunjukkan perbezaan yang signifikan antara kedua-dua kumpulan dengan paras yang lebih rendah dicatatkan pada pesakit (Mann-Whitney, p=0.014). Walaupun kajian ini masih pada peringkat awal, keputusan kajian ini telah menunjukkan bahawa CK-α berpotensi sebagai salah satu penanda kardiak.

A study of human choline kinase α (hCK-α) in patients with ischemic heart disease (IHD) and healthy volunteers

Abstract

Ischemic heart disease (IHD) is predicted to become the dominant cause of mortality worldwide by 2030. As a silent yet eventually fatal disease, prevention and early detection through discovery of cardiac biomarker are the best resolution for IHD. Human choline kinase α (hCK-α) was investigated for its possibility as a cardiac biomarker in this study. The aim of this study is to investigate the differences in the protein level and mRNA expression of hCK-α between IHD patients and healthy controls. Blood samples from 10 IHD patients and 10 healthy volunteers were analyzed for mRNA expression using RT-PCR. Western blot was performed as a checkpoint for detection of hCK-α protein in blood. Subsequently, ELISA test was conducted to measure the protein concentration of hCK-α protein. C-reactive protein (CRP) was used as a standard reference in this study to provide a parameter to distinguish between both study groups. Statistical analysis was performed to investigate the relationship between hCK-α with IHD. Parametric test was performed using independent t-test meanwhile non-parametric test was performed using Mann-Whitney test. As anticipated, CRP was found to be significant higher in expression both in mRNA level and protein level (Independent t-test, $p<0.001$; Mann-Whitney test, $p= 0.019$). In this study, a significant lower level of hCK-α2 mRNA expression in IHD patients was revealed (Independent t-test, $p=0.032$). However, total hCK-α mRNA expression was found to have no

significant different between IHD patients and healthy volunteers (Mann-Whitney test, p=0.705). Protein expression level of hCK-α was found to be significantly lower in IHD patients as compared to healthy volunteers (Mann-Whitney test, p=0.014). Albeit preliminary, this study found that there may be a profound relationship between hCK-α and IHD.

1.0 Introduction

Ischemic heart disease (IHD) is an intractable disease that will soon become an eminent health problem worldwide. According to World Health Statistics 2008, deaths from cardiovascular diseases (IHD and cerebrovascular disease) will rise from 17.1 million in 2004 to 23.4 million in 2030 in a projected 67 million deaths due to all causes including communicable, noncommunicable and injury (WHO, 2008). Among the 20 leading causes of deaths globally between 2002 and 2030, ischemic heart disease (IHD) has emerged as the top on the rank order of deaths, followed by cerebrovascular disease (stroke), HIV/AIDS and chronic obstructive pulmonary disease (COPD) (Fig 1.1).

Regardless of the gender and socioeconomic status, the amount of worlds populations that will succumb to IHD is predicted to be the highest among the death of all causes by the year of 2030 (Mathers and Loncar, 2006). This apprehensive projection is supported by the ongoing increase in the prevalence of cardiovascular diseases worldwide. To present this scenario, cardiovascular diseases had claimed an estimate of 30% of all deaths or about 17.5 million people in the total 58 million deaths in the world in 2005 (WHO, 2005).

LEADING CAUSES OF DEATH, 2004 AND 2030 COMPARED

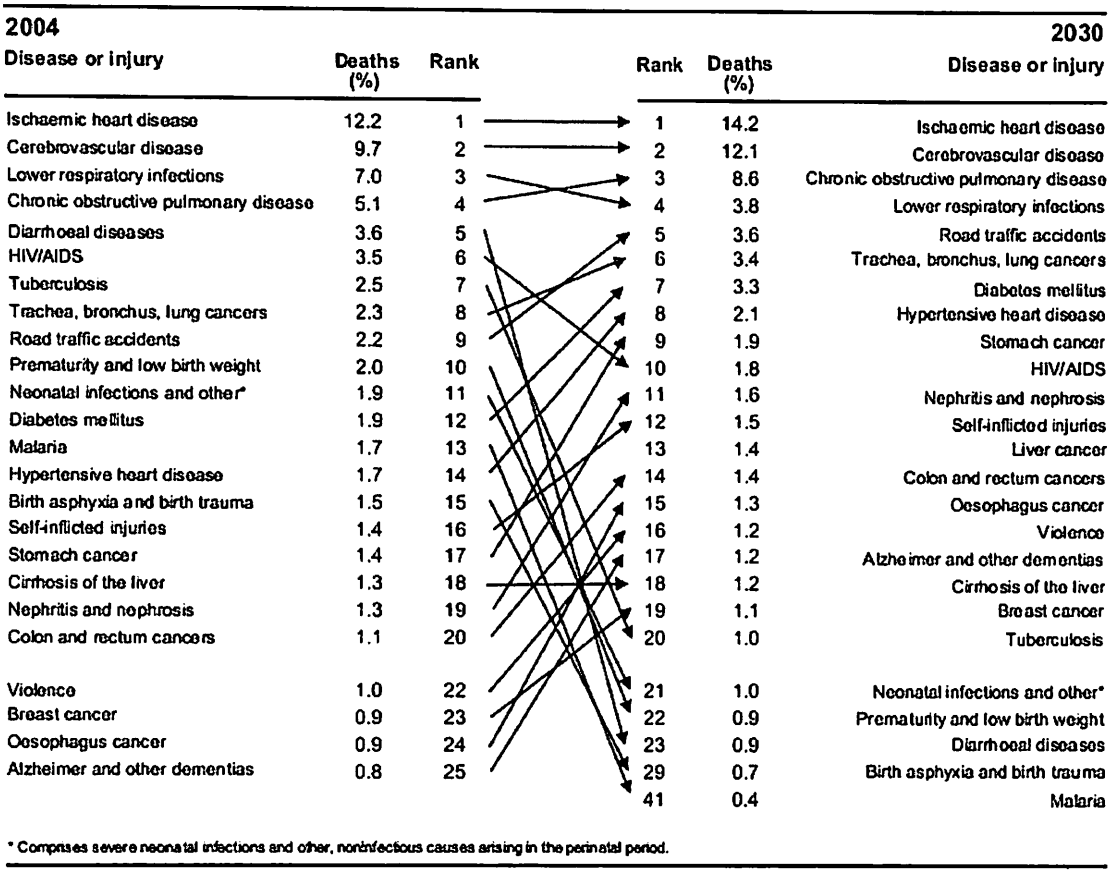

Figure 1.1. Projection on leading causes of death in 2030. Adopted from WHO, 2008.

In Malaysia, cardiovascular diseases remain the predominantly leading cause of death by a non-communicable disease from the 1970s and have remained so since except in 1980 (Khoo *et al.*, 1991). According to a Ministry of Health (MOH) Hospitals' survey in 2006, 6,372 of lives were deprived by heart diseases and diseases of pulmonary circulation (MOH[a], 2006). Cardiovascular and circulatory diseases are also the most common reasons for acute medical hospital admissions in our country. IHD is the commonest cause of cardiovascular mortality and is still

remains one of the major health problems. Nationally IHD probably accounts for 20-30% of all-cause mortality annually (MOH[b], 2006).

The true incidence of IHD and IHD-related deaths in Malaysia is unknown. All the data presented thus far relates only to the 122 MOH hospitals. Data on IHD cases and deaths at the 7 University and other non-MOH Government hospitals, and 211 private hospitals is unavailable although it is suspected the number may be double the figures from MOH hospitals only (MOH[b], 2006).

With the presented illustrious statistic on IHD worldwide and in our country, this devastating health problem is striking the alarm of our concern. Thus, there is an urgent need to find ways to resolve the crisis of IHD as it is becoming a serious health issue in the foreseeable future. Emergency attention needs to be focused on the prevention and early detection of IHD besides superior clinical decision making, disease management and therapeutic applications to safe life from being threatened by IHD.

The success of this challenge is contingent on knowledge and complete understanding of the underlying mechanisms that lead to IHD. IHD or myocardial ischemia occurred due to an imbalance between cardiac blood supply (perfusion) and myocardial oxygen demand. Reduction in coronary blood flow caused by obstructive atherosclerotic disease is the main cause of myocardial ischemia

despite increased demand (e.g., increased heart rate or hypertension), or diminished oxygen-carrying capacity (e.g., anemia, carbon monoxide poisoning) can also contribute to myocardial ischemia (Kumar *et al.*, 2007).

As a direct consequence of insufficient blood supply to the heart, the IHD manifests clinically in the basic clinical syndromes such as angina pectoris, acute myocardial infarction, chronic IHD and sudden cardiac death. However, these syndromes are all relatively late manifestations of coronary atherosclerosis. Atherosclerosis, as the predominant underlying cause of IHD, begins early in life but manifests only after occlusions reach a critical stage (dos Santos *et al.*, 2008). Acute coronary syndrome is applied to three catastrophic manifestations of IHD, namely unstable angina, acute myocardial infarction and sudden cardiac death. While ischemia to myocardium rapidly (minutes) leads to loss of function and causes necrosis after 20 to 40 minutes, gross and histological changes of infarction require hours to days to develop (Kumar *et al.*, 2007).

However, during early IHD events, no symptom is detectable. By the time for onset of symptoms, the IHD has by far reached the stage of irreversible injury and myocyte death with the predominant pattern of coagulation necrosis. The resulting massive myocardial damage and dysfunction are often fatal by ultimate culmination of myocardial infarction (Chattopadhyay and Bandyopadhyay, 2006). Currently, the diagnosis of myocardial infarction is based on symptoms, electrocardiographic changes, and measurement of serum CK-MB and troponins (Panteghini, 2004). If

the disease can be detected earlier before necrosis, this can definitely reduce the morbidity and mortality rate of IHD.

Biomarkers provide a new insight to reliably rule out myocardial damage and earlier detection of myocardial ischemia both with and without the presence of irreversible myocyte injury. The term "biochemical marker" of heart failure is used to define a biochemical substance whose plasma (or serum) levels correlate with the clinical and hemodynamic status and predict the prognosis of patients with heart failure (Apple *et al.*, 2002). Early diagnosis allows appropriate treatment in patients suspected of having IHD and the prevention of adverse outcomes. From this perspective, over the last decade, there has been an explosion of information generated on the role of inflammation, endothelial dysfunction and of specific serum biomarkers in the development of atherosclerosis. A large list of relevant cardiac biomarkers has thus been created along with strong evidence that these biomarkers act synergistically to increase an individual's risk for experiencing a heart attack (Oemrawsingh *et al.*, 2008).

Generally, all the potential cardiac biomarker are being classified into two class, known as markers of early injury/ischemia as well as markers of inflammation and coronary plaque instability and disruption. Among all these potential cardiac biomarkers, C-reactive protein (CRP) appeared to have strongest association to the predictive risk of future cardiovascular events (Kaperonis *et al.*, 2006, Rifai and

Ridker, 2001) This intriguing finding has provoke numerous epidemiological studies and clinical studies on CRP, revealing CRP higher stability and reliability as a cardiovascular events predictor when compared with other cardiac biomarkers(de Ferranti and Rifai, 2007).

However, CRP production has been related to part of the nonspecific acute-phase response. CRP is elevated to varying degrees in numerous conditions, including inflammation, infection, malignancy, tissue damage autoimmune disorder and others conditions. Thus, CRP values can never be diagnostic on their own and can only be interrelated at the bedside, in full knowledge of all other clinically and pathological results (Pepys and Hirschfield, 2003). In particular, markers of plaque destabilization and/ or markers of myocardial ischemia can added to the existing markers if shown to contribute to the independent information of IHD events. Hence, there is always an imperative need for discovering of other potential cardiac biomarkers to confront with IHD.

In this study, CRP is used as a reference to investigate the possibility of human choline kinase α (hCK-α) as a novel cardiac biomarker. hCK-α is a metabolic enzyme involved in the synthesis of phosphatidylcholine. The roles of hCK-α in cell growth, cell stress and defense mechanisms have been extensively investigated. It is also found to be overexpressed in a variety of human cancers such as mammary, lung, colorectal and prostate adenocarcinomas (Gallego-Ortega *et al.*, 2006).

However, there are no data indicated the relationship of hCK-α with IHD events. Thus, this study may serve as a basis in quest for its profound possibility as a cardiac biomarker.

2.0 Objectives

1. To optimize Enzyme-linked Immunosorbant Assay (ELISA) methods to measure CK-α level in blood.

2. To compare the CK-α protein level in blood between apparently healthy volunteers and IHD patients.

3. To measure mRNA expression of CK-α in comparison between apparently healthy volunteers and IHD patients.

3.0 Literature review

Myocardial ischemia or IHD represents a mismatch in coronary supply and myocardial demand. The various clinical symptoms caused by acute myocardial ischemia after plaque disruption are referred to as acute coronary syndromes (ACS). The fundamental role of atherosclerosis as the causative event on IHD is undisputable. Atherosclerosis, comes from Greek root words for "gruel" and "hardening" (Kumar *et al.*, 2007). For the major part of 20th Century, atherosclerosis has been known as bland lipid storage disease. Hyperlipidemia or more specifically hypercholesterolemia is the dominant theory for antherogenesis. Nonetheless, this theory of is seem to be too simplistic. In 1908, Sir William Osler first proposed the role of inflammation and infection in the pathogenesis of atherosclerosis (Osler, 1908). Since then, numerous studies on the relationship between inflammation and atherosclerosis had been done.

Atherosclerosis is characterized by intima lesions known as atherosclerotic plaques that protrude into vascular lumina. An atheromatous plaque consists of a raised lesion with soft, yellow core of lipid covered by a firm, white fibrous cap. The cellular mechanisms involved in the pathologenesis of IHD are complex and involve interaction of a number of cell types. For instances, mononuclear cells, macrophages and T-lymphocytes are prominent in atherosclerotic plaques (Navab *et al.*, 1994, Morena *et al.*, 1994). Besides obstructing the blood flow, atherosclerotic plaques can weaken the underlying media and can themselves rupture. Inflammation and immune cell activation appears to play a key role in the

loss of collagen in the fibrous cap, a prelude to fibrous cap rupture, through release of collagen degrading enzymes. Furthermore, inflammation may also play a key role in the death of collagen synthesizing smooth muscle cells which further contributes to loss of fibrous cap integrity (Kumar *et al.*, 2007).

When these atherosclerotic plaques ruptured, they exposure of the plaque core activates the clotting cascade and results in thrombosis within the plaque. The acute catastrophic vessel thrombosis is then followed by the initiation of platelet aggregation (Libby *et al.*, 2002).

Three possible mechanisms by which damage to the myocardium may occur are discussed by Collison and Gaze (2007). Firstly, intraluminal platelet aggregation may cause sufficient vascular occlusion for cardiomyocyte damage to occur. Occlusion does not have to be total to produce myonecrosis. Partial occlusion will produce a reduction in the rate of blood supply in the myocardium downstream. If there is already supply/demand mismatch in this area, the reduction in blood supply may be enough to render an area of myocardium non-viable. The tissue will then become sufficiently ischemic for necrosis to occur. This is most likely to affect small areas of myocardium at the watersheds of different branches of the vascular supply.