EXPRESSION OF *STK15*, *CENPW* AND *TIMP1* GENES IN BREAST AND COLORECTAL CANCERS FROM HUMAN BLOOD SAMPLES

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DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, equations, summaries and references, which have been duly acknowledged.

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ABSTRACT

Cancer is one of the highest incidence and mortality worldwide. Despite the great information on cancer, particularly in breast cancer (BC) and colorectal cancer (CRC), the rate of BC and CRC incidence and deaths remains alarming. This pilot case-control study is conducted to further understand the biology of cancer especially in mRNA/gene expression aspect using whole blood system by analyzing the differential gene expression of three potential BC and CRC related genes which were *STK15, CENPW*, and *TIMP1*. Whole blood samples were collected from 31 healthy individuals and 36 cancer patients (14 BC and 22 CRC) followed by total RNA isolation using Tempus™ Blood RNA System. The RNA integrity number (RIN) of extracted RNA was determined using bioanalyzer. Gene expression analysis was performed with 5'-hydrolysis probes in triplicates using real-time PCR. Additionally, influence of other factors such as age, gender, and gene polymorphism to gene expression were also evaluated in this study. Statistical Mann-Whitney *U*-test was applied to determine the differential expressions of *STK15*, *CENPW* and *TIMP1* normalized to two reference genes (*GAPDH* and *HPRT1*) in BC and CRC patients, with $p<0.05$ as statistical significant. This study showed that the extracted total RNA was highly intact with RIN>7.0 for reliable gene expression analysis. *STK15* expression was significantly ($p<0.05$) downregulated in CRC regardless of both age and gender. Interestingly, a significant ($p<0.05$) reduction of *STK15* expression of BC were found in age ≥50 but not in age <50 suggesting an age related factor in BC and not in CRC. No significant difference was observed in the downregulation of *CENPW* and *TIMP1* gene expression in BC and CRC. Further investigation based on genotyping of the *STK15* Phe31Ile polymorphism showed that *STK15* expression in BC was non-significantly ($p>0.05$) downregulated after samples were grouped according to genotypes (*Phe/Phe*, *Phe/Ile*, and *Ile/Ile*), but significant ($p<0.05$) downregulation was observed in CRC patients carrying homozygous wild-type (*Phe/Phe*) or homozygous variant (*Ile/Ile*) genotypes. This indicated *Phe/Phe* or *Ile/Ile* genotype in *STK15* Phe31Ile polymorphism associated to reduce *STK15* expression in CRC but not in BC. In summary, the reduced *STK15* expressions of this study provide an initial insight of using blood-based biomarker for BC and CRC.

ABSTRAK

EXPRESI GEN STK15, CENPW DAN TIMP1 DALAM KANSER PAYU DARA DAN KOLOREKTAL DARIPADA SAMPEL DARAH MANUSIA

Kanser adalah salah satu factor kejadian teringgi dan kematian di dunia. Walaupun kemajuan dalam ilmu terhadap kanser ,terutamanys dalam kanser payu dara dan kolorektal, kadar kejadian dan kematian kanser masih meningkat masih meningkat setiap tahun termasuk Malaysia. Kajian kes-kawalan ini dijalankan untuk lebih mamahami kanser biologi terutamanya expresi mRNA/gen dalam darah, dengan menganalisa kelainan tiga kanser-kaitan expresi gen termasuk STK15, CENPW, dan TIMP1 dalam pesakit kanser payu dara dan kolorektal. Sampel darah telah dikumpul daripada 31 sukarelawan sihat dan 36 pesakit kanser (14 kanser payu dara dan 22 pesakit kolorektal) diikuti dengan isolasi RNA menggunakan Tempus™ Blood RNA System. RNA integrity number (RIN) RNA yang telah diestrak telah ditentukan dengan menggunakan bioanalyzer. Expresi gen telah dilaksanakan dengan 5' probe hydrolysis dalan tiga replika menggunakan real-time PCR. Selain itu, pengaruh factor lain seperti, umur, jantina, dan polimofisme gen terhadap expresi gen telah dinilai dalam kajian ini. Analisis statistik dilakukan dengan menggunakan Mann-Whitney U-tests dalam SPSS V17.0 untuk menentukan kelainan gen expresi STK15, CENPW dan TIMP1 dinormalisasi kepada gen rujukan di pra-tentukan (GAPDH dan HPRT1) dalam kanser payu dara dan kolorektal apabila dibandingkan dengan kawalan, dengan $p<0.05$ sebagai signifikan secara statistik. Kajian ini menununjukkan RNA yang diestrak adalah sangat utuh dangan RIN > 7.0 untuk kebergantungan analisa expresi gen. Expresi STK15 adalah berkurangan secara ketara ($p<0.05$) dalam kolorektal, tanpa mengambil mengira perbezaan dalam kumpulan umur dan jantina. Menariknya, kekurangan signifikan ($p<0.05$) expresi STK15 untuk kanser payu dara dalam umur ≥50 tetapi bukan dalam <50 mencadangkan faktor kaitan umur dalam kanser payu dara dan bukan kolorektal. Tiada bukti signifikan telah ditunjukkan dalam kekurangan regulasi gen CENPW dan TIMP1 menyumbang kepada kanser payu dara dan kolorektal dalam kajian ini. Siasatan lanjut bergantung pada STK15 Phe31Ile genotip menunjukkan bahawa expresi STK15 dalam kanser payudara adalah berkurangangan seara tidak signifikan ($p>0.05$) selepas disusun mengikut setiap genotip masing (Phe/Phe, Phe/Ile, dan Ile/Ile) tetapi adalah berkurangan secara signifikan ($p<0.05$) dalam kolorektal dalam genotip Phe/Phe atau Ile/Ile apabila dibandingkan dengan kawalan masing-masing. Ini menunjukkan Phe/Phe atau Ile/Ile genotip dalam STK15 Phe31Ile polimorfsime berhubung kait dalam kekurangan espresi STK15 dalam kanser kolorektal tetapi tidak dalam kanser payudara. Secara rumusan, kekurangan expresi STK15 dalam kajian ini memberi pandangan awal untuk menggunakan darah sebagai biopenanda bagi kanser payu dara dan kolorektal.

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LIST OF SYMBOLS, UNITS, AND ABBREVIATIONS

APC	-	Adenomatous Polyposis Coli
AMP	**-**	Autocrine motility factor
BC	**-**	Breast Cancer
CCAN	-	Constitutive Centromere-associated Network
CENPW	**-**	Centromere Protein W
CG	**-**	Control Gene
Cq	**-**	Quantification Cycle
CRC	**-**	Colorectal Cancer
CUG2	**-**	Cancer-upregulated Gene 2
CV	**-**	Coefficient of Variation
EAC	**-**	Europe against Cancer
FAK	**-**	Focal adhesion kinase
FAP	-	Familial Adenomatous Polyposis
FISH	-	Fluorescence *in situ* hybridization
FTase	**-**	Farnesyl Transferase
gDNA	-	Genomic DNA
HNPCC	-	Hereditary Non-polyposis CRC Syndromes
IHC	-	Immunohistochemistry
KRAS	**-**	Kristen Rat Sarcoma Viral Oncogene
MGB-NFQ	**-**	Minor Groove Binder-Non Fluorescence Quencher
MMP	**-**	Matrix metalloproteinases
MRD	-	Minimal Residual Disease
PBS	**-**	Phosphate Buffer Saline
PCR-RFLP	-	Polymerase Chain Reaction-Restriction Fragment Length Polymorphism
PGI	-	Phosphoglucose isomerise
qPCR	-	Quantitative Real-Time PCR
R^2	-	Correlation
RIN	-	RNA Integrity Number
RT	-	Reverse Transcription
SD	-	Standard Deviation

SMAD2	-	Mothers Against Decapentaplegic 2
SMAD4	-	Mothers Against Decapentaplegic 4
SNP	**-**	Single Nucleotide Polymorphism
STK15	**-**	Serine/Threonine Kinase 15
TGF-β	-	Transforming Growth Factor Beta
TIMP1	**-**	Tissue inhibitor of metalloproteinases 1
%	**-**	Percentage
μg	-	Micrograms
s	-	Seconds
mL	-	Millilitre
mM	-	Milli Molar
ng	-	Nanogram
μL	-	Microliter
μM	-	Micro Mole
xg	-	Relative Centrifugal Force
rpm	-	Rotation Per Minute
kb	-	Kilobase
bp	**-**	Basepair

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CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Cancer is one of the primary causative of mortality worldwide where lung cancer was leading, followed by breast cancer (BC) and colorectal cancer (CRC) in year 2012. In Malaysia, CRC is the second most prevalent among Malaysian men (14.1%) and women (10.2%) after lung cancer (Ferlay *et al.*, 2012).

BC has been reported to be more prevalent in women and is the most common cancer in Malaysian woman that accounts for 28% of total cancer incidence. Approximately, 5-10% cancer was caused by genetic inheritance (Rajnish *et al.*, 2012) indicated the importance of genetic in BC carcinogenesis. BC is highly heterogeneous due to its composition of different cell types (Kim *et al.*, 2005). Generally, BC is divided into invasive and non-invasive. However, there are subtypes of BC among these two groups. Moreover, all BC cases are tested for estrogen receptor (ER), progesterone receptor (PR) and HER2 protein expressions for prognosis prediction.

CRC develops through multiple steps and complex process. Its progression from normal epithelium to cancerous cells involved sequential acquire of multiple genomic abnormalities. Common mutated genes such as *APC*, *KRAS*, *Smad*, *p53* and other genetic mutations. The knowledge and understanding on the progression and development of CRC are well understood. However, the incidence and deaths caused by CRC remain significantly high. The socio-economic impact of cancer indicates that there is a need for more understanding of cancer especially BC and CRC.

The genetic cause of cancer, especially BC and CRC were vastly studies in genomics and gene expression studies. So far, the main approach of gene expression studies uses invasive primary biopsy sampling as the main target. However, tumour does no manifest the disease by itself but affect the whole body especially whole blood. Previous studies have showed that cancer cells (Twine *et al.*, 2003; Burczynski *et al.*, 2005), including BC (Sharma *et al.*, 2005; Aaroe *et al.*, 2010) and CRC (Nichita *et al.*, 2014; Xu *et al.*, 2014) caused the changes in the blood environment, and thus evoke response in blood cells. Whole blood collection is minimal invasive and represents the physiological state of the body. Hence, whole blood presents as an alternative sample to invasive sampling of biopsy.

The aberrant expression of genes causes the development of various cancers such as BC and CRC (Pihan *et al.*, 1998). Moreover, the uncontrolled expression of *STK15, CENPW* and *TIMP1* has been linked to the incidence of cancers. *STK15* and *CENPW* genes were involved in the cell division whereas *TIMP1* is involved in the inhibition of *MMP*. However, their roles remain ambiguous as reported by previous studies. To date, expression of *STK15*, *CENPW*, and *TIMP1* in whole blood of BC and CRC patients is still unknown. Therefore, we hypothesized that the expression of *STK15*, *CENPW*, and *TIMP1* in human blood samples would exhibit overexpression in BC and CRC patients when compared with healthy individual.

1.2 Objectives

The main objectives of the study are:

(a) To extract and quantify high quality of RNA from human blood.
(b) To determine the most stable reference genes (*ACTB*, *GAPDH*, and *HPRT1*) for BC and CRC.
(c) To analyze the mRNA expression of *STK15*, *CENPW* and *TIMP1* in whole blood of healthy controls and BC patients as well as CRC patients.
(d) To determine the association between the genotype of *STK15* polymorphism and the expression of *STK15*.

CHAPTER 2

LITERATURE REVIEW

2.1 An Overview in Global Cancer Statistics

Cancer is one of the leading causes of death worldwide. Cancer has accounted for approximately 14.1 million of new cases and 8.2 million of deaths in 2012 (Table 2.1) (Ferlay *et al.*, 2012). This indicated that the role of prevention, detection and treatment for cancer were crucial. In addition, cancer incidence and deaths also differ by geographical means. In 2012, less developed regions showed a higher number of new cases (58.8%) and deaths (64.9%) caused by cancer when compared with developed regions (Table 2.2). The population residing in developed regions could be aware of cancer development and preventive measure towards cancers was taken more seriously as compared to less developed regions.

The most common causative of cancer mortality were cancers of the lung (1.6 million, 19.4%), liver (0.8 million, 9.1%), and stomach (0.7 million, 8.8%). Furthermore, incidence rate in men is roughly 25% higher than in women and the most frequent number of cases differ in both genders (Ferlay *et al.*, 2012).

In year 2012 it was reported that, women have the highest incidence and mortality rates due to BC which was 25.2% and 14.7%, respectively (Ferlay *et al.*, 2012). However, there was no BC incidence and mortality reported in male. In the same year, Malaysia recorded that BC was also one of the most common cancer incidence (5410 cases, 28.0%) and death (2572 cases, 11.9%) in women.

In the same year, CRC was the third most common cancer incidence in men and second in women that comprised of 10.0% and 9.2%, respectively

(Ferlay *et al.*, 2012). Moreover, the mortality rate of CRC for men and women were 8.0% and 9.0%, respectively (Ferlay *et al.*, 2012). In Malaysia, CRC was the second most common in men (2563 cases, 14.1%) and women (1976 cases, 10.2%).

Mammogram is usually conducted as screening strategy for BC, often it requires skilled persons to handle the procedure and produces false positive that induced costs (Christiansen *et al.*, 2000). It would require a biopsy tissue to confirm the presence of cancerous cell which is highly invasive and time consuming. BC treatment involves chemotherapy, radiotherapy, or mastectomy depends on the stages of cancer. Compared to the diagnosis of BC, CRC screening and diagnosis is comparably invasive and time consuming as the gold standard of procedure is colonoscopy and confirmed by biopsy tissues. CRC treatment is similar with BC where chemotherapy, radiotherapy, or resection of certain part of the colon/rectal based on the advance of CRC.

Despite various screening and treatment strategies have been employed in the current population the incidence and mortality of BC and CRC is still alarming. Various genetic approaches have been used to study BC and CRC. However, most approach involves biopsy samples which are highly invasive procedure. Hence, this study is conducted to study the gene expression of blood obtained from BC and CRC patients, and its potential to serve as a non-invasive molecular marker in the future.

Table 2.1: Summary of cancer cases and deaths worldwide

WORLD	Male	Female	Both sexes
Population (thousands)	3557717	3496728	7054446
Number of new cancer cases (thousands)	7427.1	6663.0	14090.1
Age-standardised rate (W)	205.4	165.3	182.3
Risk of getting cancer before age 75 (%)	21.0	16.4	18.5
Number of cancer deaths (thousands)	4653.1	3547.9	8201.0
Age-standardised rate (W)	126.3	82.9	102.4
Risk of dying from cancer before age 75 (%)	12.7	8.4	10.4
5-year prevalent cases, adult population (thousands)	15362.3	17182.3	32544.6
Proportion (per 100,000)	592.0	661.4	626.7
5 most frequent cancers (ranking defined by total number of cases)			
	Lung	Breast	Lung
	Prostate	Colorectum	Breast
	Colorectum	Lung	Colorectum
	Stomach	Cervix uteri	Prostate
	Liver	Stomach	Stomach

Source: Ferlay *et al.*, 2012

Table 2.2: Summary of cancer cases and deaths in developed and un-developed regions

Estimated numbers (thousands)	Men		Women		Both gender	
	Cases	Deaths	Cases	Deaths	Cases	Deaths
More developed regions	3244	1591	2832	1287	6076	2878
Less developed regions	4184	3062	3831	2261	8014	5323

Source: Ferlay *et al.*, 2012

2.1.1 Breast Cancer (BC)

BC is highly heterogeneous in terms of biological and clinical. The tumour itself is composed of cells from multiple origins, such as fibroblast, endothelial, myoepithelial, immune cells and the extracellular matrix (Kim *et al.*, 2005). These complexicity provide a vast challenge to characterize BC tumours. In general, there are two form of BC, non-invasive and invasive. Non-invasive cancer localizes in the mild ducts in the breast and do not grow into normal tissues beyond the breast.

They are also referred as *in situ* or pre-cancers. On the contrary, most cases of BC incidence are invasive form that has grown into normal tissues (Figure 2.1).

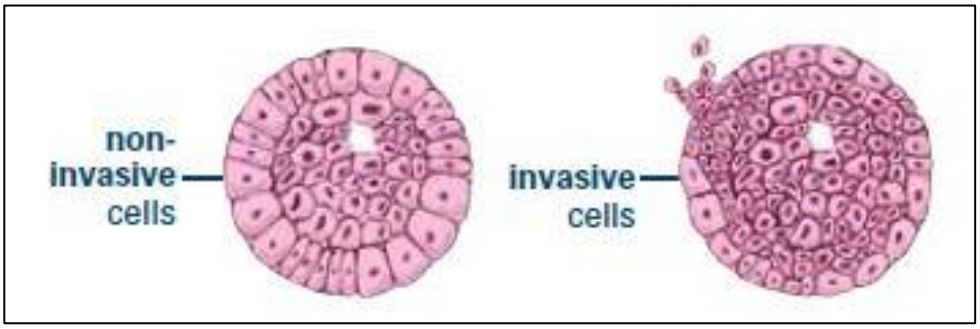

Figure 2.1 : Forms of Breast Cancer.

Source : breastcancer.org, Accessed on 30/2/2015

2.1.2 Colorectal Cancer (CRC)

CRC development and progression is well studied and develops through multiple steps and complex process. Its progression from normal epithelium to cancerous cells involved sequential acquire of multiple genomic abnormalities. As shown in Figure 2.2. CRC occur in three specific settings: (a) over 85% of all cases were accounted of sporadic form, familial form that constitutes approximately 10%, which include (b) familial adenomatous polyposis (FAP), and (c) hereditary non-polyposis CRC syndromes (HNPCC) (De La Chapelle, 2004). Even though the progression of CRC tumours is well studied, the incidence of CRC remains alarming.