JOURNEY INTO GENETICS
Taking the Twists and Turns of Life

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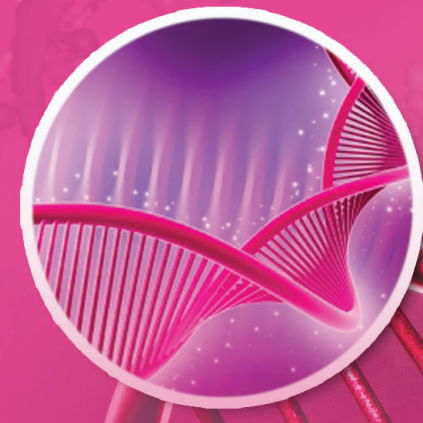

JOURNEY INTO GENETICS

Taking the Twists and Turns of Life

PROFESSOR DR. ROZITA ROSLI

JOURNEY INTO GENETICS

Taking the Twists and Turns of Life

Professor Dr. Rozita Rosli

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In Memory of

Professor Dr. Mariana Nor Shamsudin

Contents

OVERVIEW

Genetics play a major role in our lives and for the most part, we take it for granted. By definition, it is a study of heredity or how the characteristics of living things and their variations are transmitted from one generation to the next. Although genetics has a pervasive influence in every sphere of life, the focus of my research is on human and medical genetics. Almost every human trait and disease has a genetic component, whether inherited or influenced by behavioural factors such as exercise and diet. These genetic components can also modify the body's response to environmental factors such as toxins or even drugs. Understanding the underlying concepts of human genetics, especially the role of genes, is important for applying genetic and genomic information as well as technologies in healthcare. It is also important in improving disease diagnosis and treatment.

The completion of the first mapping of the Human Genome Project has opened tremendous potential for research in which genes relate to human conditions, diseases, impairment and susceptibilities. Hence, the knowledge generated through human genetics research has the potential to improve individual and community health.

The following sections will first provide some information about basic genetic concepts, the major types of genetic disease, and the impact of genetic variation. Subsequently, the selected research projects that were carried out in the application of genetics and genetic technologies in cancer, especially breast cancer, infectious diseases and therapeutics, including genetic immunisation will be chronicled and highlighted.

It is highly likely that human genetics research through the years will become an increasingly more integral component of medical research. It is hoped that the scope of genetic research illustrated by the current activities in my laboratory reflects this reality.

GENETIC NOMENCLATURES IN A "PEA POD"

Deoxyribonucleic acid or DNA contains all the instructions that are needed to direct the activities of cells, which are the fundamental structural and functional units of every known living organism. The human cell contains two sets of chromosomes, that is, one set inherited from each parent. It normally contains 23 pairs of chromosomes, which consist of 22 autosomes and a pair of sex chromosomes. The sperm and the ova, however, normally contain half as much genetic material: only one copy of each chromosome.

The human genome, which refers to the total composition of genetic material within a cell, is packaged into larger units known as chromosomes. Each chromosome contains many genes, the basic physical and functional units of heredity (Figure 1). Genes are specific sequences of bases that encode instructions to make proteins. Genes are thought to comprise only about 29 percent of the human genome; the remainder consists of non-coding regions, whose functions may include providing chromosomal structural integrity and regulating protein production. The human genome is estimated to contain approximately 20,000 to 25,000 genes (Collins *et al.*, 2004).

Although each cell contains a full complement of DNA, cells use genes selectively. For example, the genes that are active in a liver cell differ from the genes active in a brain cell because each cell performs different functions and, therefore, require different proteins. Different genes can also be activated during development or in response to environmental stimuli such as an infection or stress (Alberts *et al.*, 2002).

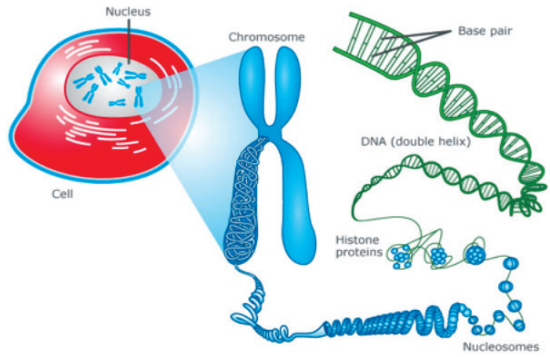

Figure 1 Our DNA is packaged neatly into chromosomes. The chromosomes are stored in the nucleus of our cells. *Source: www.sciencelearn.org.nz*

The Causes and Types of Genetic Diseases

Most diseases are caused or influenced by genetics. Genes contain the information needed to make functional molecules called proteins which are large, complex molecules that play many critical roles in the body. They are required for the structure, function, and regulation of the tissues and organs in the body. Any change in the normal protein can be harmful to the cell, and may bring about diseases.

Hence, changes in the DNA sequence of single genes, also known as mutations, can result in thousands of diseases. A gene can mutate in many ways, resulting in an altered protein product that is unable to perform its normal function. On the other hand, changes known as polymorphisms are natural variations in the DNA sequence that have no adverse effects and are simply differences among individuals in a population (Twyman, 2004). However, to be classed as a polymorphism, the least common allele must have a frequency of 1 percent or more in the population. If the frequency is lower than this, the allele is regarded as a mutation.

In addition to mutations in single genes, genetic diseases can be caused by larger mutations in chromosomes. Chromosomal abnormalities may result from either the total number of chromosomes differing from the usual amount or from the physical structure of a chromosome differing from its usual structure. The most common type of chromosomal abnormality is known as aneuploidy, an abnormal number of chromosomes due to an extra or missing chromosome. A usual karyotype (complete chromosome set) contains 46 chromosomes including an XX (female) or an XY (male) sex chromosome pair. Structural chromosomal abnormalities include deletions, duplications, insertions, inversions, or translocations of a chromosome segment (Genetic Alliance, 2010).

Multifactorial diseases are caused by a complex combination of genetic, behavioral, and environmental factors. Examples of these conditions include diabetes and cardiovascular diseases. Although multifactorial diseases can recur in families, some mutations such as cancer can be acquired throughout an individual's lifetime. All genes work in the context of environment and behavior. Alterations in behavior or the environment such as diet, exercise, exposure to toxic agents, or medications can all influence genetic traits (King *et al.*, 2002).

Disease Transmission

Single-gene diseases are usually inherited in one of several patterns, depending on the location of the gene and whether one or two normal copies of the gene are needed for normal protein activity. Five basic modes of inheritance for single-gene diseases exist: autosomal dominant, autosomal recessive, X-linked dominant, X-linked recessive, and mitochondria (Figure 2).

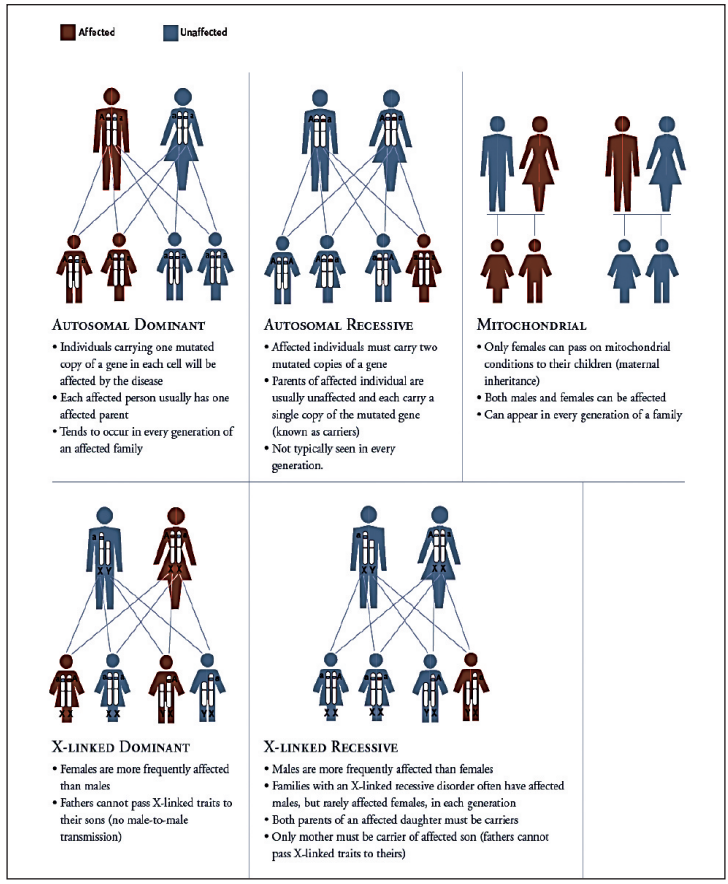

Figure 2 The basic modes of inheritance for single-gene disorders . *Source: Genetic Alliance, 2010*

Genetic Variation

In essence, all individuals are 99.9 percent the same genetically. The differences in the sequence of DNA among individuals, or genetic variation, explain some of the differences among people such as physical traits and higher or lower risk for certain diseases. Mutations and polymorphisms are forms of genetic variation. While

mutations are generally associated with disease and are relatively rare, polymorphisms are more frequent and their clinical significance is not as straightforward. Single nucleotide polymorphisms (SNPs) are DNA sequence variations that occur when a single nucleotide is altered. SNPs occur every 100 to 300 bases along the 3 billion-base human genome. Hence, a single individual may carry millions of SNPs (Twyman, 2004).

The Role of Epigenetics

Epigenetics involves genetic control by factors other than an individual's DNA sequence. These epigenetic changes can switch genes on or off. Within cells, there are three systems that can interact with each other to silence genes: DNA methylation, histone modification and RNA-associated silencing (Egger *et al.*, 2004). While these changes are required for normal development and health, they can also be responsible for some disease states. In fact, the first human disease to be linked to epigenetics was cancer. The researchers found that diseased tissue from patients with colorectal cancer had less DNA methylation compared to normal tissue from the same patients (Feinberg and Vogelstein, 1983).

Understanding the clinical significance of both genetics and epigenetics is a complicated process because of our limited knowledge of which genes are involved in a disease or condition and the multiple gene-gene and gene-behavior-environment interactions likely to be involved in complex, chronic diseases. However, new technologies are enabling faster and better understanding of diseases so that preventative measures, accurate detection and novel medical treatments can be developed.

DECONSTRUCTING CANCER

Cancer is a complex group of diseases with many possible causes and the etiology of various forms of cancer is not well known. It is known that cancer involves hyperplasia (too many cells) and/or anaplasia (abnormal, undifferentiated cells) but what exactly causes these phenotypes has yet to be discovered. It is also known, that when a cell becomes tumorigenic, three types of changes occur: immortalisation, transformation, and metastasis. Cell immortalisation takes place when cells exhibit the property of indefinite growth without any other changes in the phenotype. Transformation occurs when the transformed cells become independent of factors usually needed for cell growth and fails to observe the normal constraints of growth. Metastasis results when cancer cells become capable of invading normal tissues and form a new colony elsewhere in the body, away from the tissue of origin (Lewin, 1999).

There are several factors known to play a role in cancer development. These known factors include genetic factors (Demant, 2005), exposure to carcinogens (Calabrese and Blain, 1999), physical agents such as ionising radiation (Golubicic *et al.*, 2008) and exposure to ultra violet light and radiation (Landis *et al.*, 1998). Certain types of infections such as infection with oncogenic viruses (human immunodeficiency and hepatitis C viruses) are associated with a number of human cancers (Butel, 2000). Lifestyle (diet and physical activities) plays a major role in increasing the risk of cancer. For example, tobacco smoke is suspected to be the principle cause of lung cancer (Hecht, 1999). On the other hand, studies to date have demonstrated that consumption of certain phytochemicals found in a complex human diet, such as carotenoids (green, yellow-red, and yellow-orange vegetables), phytoestrogens (soy and some soy products), organosulfides (garlic), phenolic acids (green tea, citrus) has shown anti-mutagenic and anti-carcinogenic effects (Chatterjee *et al.*, 2012; Park *et al.*, 2013).

How is Cancer a Genetic Disease?

The cells in the human body divide and reproduce in a strictly controlled and coordinated manner. This condition keeps each tissue at the size, shape and architecture appropriate to the needs of the body. Cancer cells violate this condition and seem to have their own agenda for proliferation. Such uncontrolled reproduction leads to the formation of a tumour, of which when it becomes more advanced, develop the ability to invade other tissues or colonise other parts of the body.

This transition from a normal cell to a malignant cancer is driven by changes to the cell's DNA. Cancer forms when genes within a normal somatic cell are damaged and mutated. It is the continued cell proliferation and accumulation of the mutations as well as the altered expression of these genes that make it a genetic disease (Lewin, 1999).

When it is stated that cancer is a *genetic disease*, it is not to mean that it is a *hereditary disease*. As mentioned earlier, a hereditary disease is one that is passed from parents to a child through the inheritance of a defective gene. Although in some instances, such as retinoblastoma (a rare childhood tumour of the eye), cancer is hereditary, this is the exception rather than the rule. Most cancers are not hereditary, although for certain cancers, such as breast cancer and colon cancer, there may be a hereditary component to the disease (a susceptibility or a predisposition). However, all cancers are genetic, meaning that they result from the abnormal function of one or more genes.

In most cases, a mutation within a gene will not lead to the development of cancer. It is only when mutations occur in certain key genes that cancer develops. Hence, in cancers, multiple genes are defective. These key genes can be grouped into three classes: 1) growth promoting genes known as proto-oncogenes that normally instruct the cell when to grow and divide, 2) growth inhibiting genes or tumour suppressor genes whose normal function is to maintain

the cell in a non-dividing state, and 3) genes whose function is to repair damage to DNA called DNA repair genes (Vogelstein and Kinzler, 2004).

The process of accumulating mutations (Figure 3) in several genes normally takes many years, and this is why cancer is more frequently seen in older individuals. Anyone can be vulnerable to cancer, but research shows that age is an important contributing factor. The International Agency for Research in Cancer (IARC) estimates that 78% of all new cancer cases occur at the age of 55 or older in developed countries, the corresponding figure for the developing countries is 58%. The difference is contributed to early detection and availability of treatment in the developed countries (IARC, 2008).

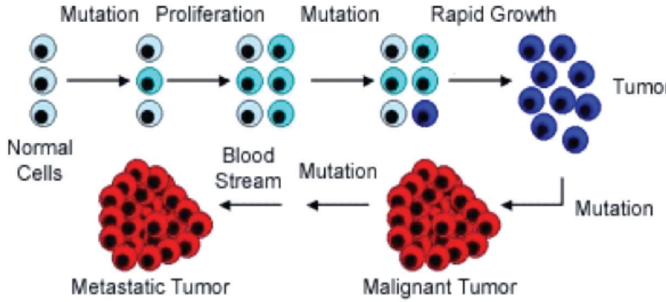

Figure 3 Several mutations must occur in the DNA of an individual cell for cancer to develop. Normal cells (light gray) may develop mutations that allow them to grow slightly faster than the cells around them. These mutated cells (light blue) must undergo further mutations before cancer can form. Cells developed from these light blue mutated cells grow slightly faster than the cells around them and they already have at least one mutation to result in cancer. When enough mutations are accumulated, cells (dark blue) form a tumour. The tumour cells will soon be malignant if left untreated. The tumour cells develop further mutations and develop into malignant cancer cells (red) in a particular organ. When additional mutations accumulate, the cancer cells will spread (metastasize) to other organs through the bloodstream. *Source*: *http://medschool.lsuhsc.edu*

Prologue

My initial exposure to cancer research was during my post-doctoral training at the Department of Medicine, Hematology/Oncology Unit, Indiana University School of Medicine in Indianapolis, Indiana under the mentorship of Professor Dr. Theodore G. Gabig. A hematologist by training, he assigned me to set-up the molecular facilities needed for the laboratory, to which I happily obliged. It was to be among the most exciting two years of my research career.

The work in the laboratory focused on identifying genes mediating programmed cell death in myeloid cells. Myeloid cells are precursor cells that include megakaryocytes, erythrocytes, mononuclear phagocytes and all of the polymorphonuclear leukocytes that function to fight infection (Kawamoto and Minato, 2004). Using interleukin-3 (IL-3) dependent murine myeloid cell line FDCP-1, a mammalian cell expression library was screened for cDNAs that would promote survival following withdrawal of IL-3. We cloned a unique 892-base pair cDNA that prevented the programmed cell death response following IL-3 deprivation by causing antisense suppression of an endogenous 2.4-kilobase (kb) mRNA. Since expression of this 2.4-kb mRNA was a prerequisite for the apoptosis response following IL-3 deprivation, the novel gene encoding it was named requiem (Gabig *et al.*, 1994). Requiem was subsequently found to encode a transcription factor required for the apoptosis response following survival factor withdrawal from myeloid cells.

In technically expanding my doctoral studies repertoire in mutational studies, oligonucleotide-directed mutagenesis was used to introduce a series of mutations into human Rap1a or Rac2 in a mammalian expression vector in another study. Compared with the parent HL60 cells, each of the stable transfected cell lines differentiated similarly into neutrophil-like cells and expressed comparable levels of NADPH oxidase components p47-phox, p67-phox and gp91-phox. Superoxide (O_2^-) production by differentiated

cell lines expressing mutated N17 Rap1a or N17 Rac2 dominant-negative proteins was inhibited, whereas a four-fold increase was observed in the subline overexpressing wild-type Rap1a. The differentiated cell line expressing GTPase-defective V12 Rap1a was also significantly inhibited, a finding that is consistent with a requirement for cycling between GDP- and GTP-bound forms of Rap1a for continuous NADPH oxidase activation in intact neutrophils. A model was proposed in which Rac2 mediates assembly of the p47 and p67 oxidase components on the cytosolic face of the plasma membrane, whereas Rap1a functions downstream as the final activation switch involving direct physical interaction with the transmembrane flavocytochrome component of the NADPH oxidase (Gabig *et al.*, 1995).

Breast Cancer

Upon returning to Malaysia, and after a brief exposure to Dengue and Human Immunodeficiency Virus research at the University of Malaya, I decided to continue with cancer research at Universiti Putra Malaysia. My work on breast cancer has become personal having lost my mother, a sister and an aunt to this disease. Breast cancer is one of the leading causes of cancer-related deaths in women worldwide. It is indeed a complex and heterogeneous disease.

Breast cancer arises from changes in gene and protein expression of a normal cell and refers to the uncontrolled growth of cells that begins in tissues of the breast, usually the ducts (tubes that carry milk to the nipple) and lobules (glands that make milk). It occurs in both men and women, although male breast cancer is rare (Beyrouti *et al.*, 2007). In the normal breast, development occurs in distinct stages throughout a woman's life, including fetal life, infancy, childhood, puberty, pregnancy, and menopause that completely take place under the effect of sex steroid hormones, peptide hormones, and growth factors (MacMahon *et al.*, 1970; Russo and Russo, 1996).

The adult human female normal breast tissue is composed of glandular, connective and fatty tissue. The tissue is clustered into lobes and each lobe has a duct that leads to the nipple (Figure 4). The milk moves through the ducts, which are tiny tubes that carry milk to the nipple (Agarwal, 2006). The breast undergoes changes through human life that affects any of these parts that cause symptoms. These breast changes can be either benign breast conditions or breast cancers. Common benign breast conditions include fibrocystic changes, benign breast tumors, and breast inflammation.

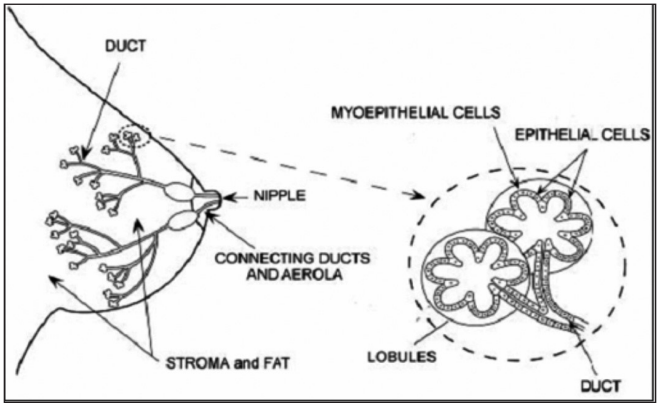

Figure 4 Anatomy of human mammary gland which shows the epithelial ducts and lobules surrounded by myoepithelial cells, stroma and connective tissues. *Source*: *Hondermarck (2003)*

The majority of breast tumours (more than 95%) are of epithelial origin and they are classified as carcinomas while sarcomas are those tumours that appear in the connective tissue and they are rarely observed. Therefore, the term breast cancer encompasses many types of tumors according to their origin and histological features (Hondermarck, 2003). Most breast lumps turn out to be benign and do not grow uncontrollably or spread, and are not life

threatening. When cancer is suspected based on clinical breast exam or breast imaging, microscopic analysis of breast tissue is necessary for a definitive diagnosis and to determine the extent of spread and characterise the pattern of the disease (Kim and Eberwine, 2010).

Incidence Rates for Breast Cancer

As mentioned earlier, breast cancer is one of the leading causes of cancer-related deaths in women and is on the rise worldwide. However, incidence rates of breast cancer vary globally, among women and men, and between different ethnicities and ages. In 2008, IARC reported a total of 1.4 million new cases, with a 50 percent split between developed and developing countries. Female breast cancer varies widely throughout the world. For example, it ranges from 8.0 cases per 100,000 in Mongolia to 109.4 per 100,000 in Belgium. Rates have reportedly been higher in North America, Australia, and Western Europe compared to Africa and Asia. However, this might be due to incomplete reporting and low screening rates. In 2012, IARC reported that 1.7 million women were diagnosed with breast cancer, which indicates that the incidence of breast cancer has increased by 20% while mortality has increased by 14%, compared to 2008. Furthermore, the report noted that breast cancer is also the most common cause of cancer death among women (522,000 deaths in 2012) and the most frequently diagnosed cancer among women in 140 of 184 countries worldwide. It now represents one in four of all cancers in women.

Incidence and Prevalence of Breast Cancer in Malaysia

In Malaysia, breast cancer is the most common cancer diagnosed in females, accounting for 26.5% in comparison to other cancers such as cervix uterine (12.6%), colorectal (9.9%), lung (5.8%) and ovary (5.4%) (Globocan Report, 2008). However, there are variations in

the incidence rates of breast cancer among the three main ethnic groups. The National Cancer Registry, Malaysia 2003, reported that among the three main ethnic groups (Malay, Chinese and Indians) in West Malaysia, the Chinese showed the highest incidence rate of breast cancer, followed by the Indians while the lowest incidence rates are reported in Malay women.

It is unclear why there are differences in the occurrence of breast cancer according to ethnicity and what causes these occurrences. Baqutayan (2012) reported the importance of several factors that might lead to and cause this disease. These factors highlight the role of diets and lifestyles, breast feeding, obesity, smoking, alcohol intake, food intake, age and family history which may be associated with breast cancer disease in Malaysia.

The latest data on breast cancer prevalence and incidence rates among Malaysian women indicates that more awareness and efforts are needed to combat breast cancer. IARC in December 2013 showed that the prevalence of breast cancer for Malaysian women was 45.7 per 100,000 females in 2012 (Table 1). This is almost three times the prevalence rate for cervix uteri (15.5), which is the cancer with the second highest prevalence rate in Malaysian women. Breast cancer is also the deadliest cancer disease among Malaysian females with an ASR mortality rate of 18.9.

Table 1 Estimated cancer incidence and prevalence of female adult population in Malaysia, 2012

Cancer type	Incidence	Prevalence		
		1-year (proportions)	3-year (proportions)	5-year (proportions)
All cancers excluding non-melanoma skin cancer	18938	12661 (123.4)	32368 (315.4)	48287 (470.5)
Lip, oral cavity	360	228 (2.2)	561 (5.5)	819 (8.0)
Nasopharynx	541	390 (3.8)	981 (9.6)	1442 (14.1)
Other pharynx	80	50 (0.5)	126 (1.2)	186 (1.8)
Oesophagus	185	64 (0.6)	132 (1.3)	172 (1.7)
Stomach	722	293 (2.9)	667 (6.5)	938 (9.1)
Colorectum	1974	1200 (11.7)	2907 (28.3)	4200 (40.9)
Liver	408	88 (0.9)	183 (1.8)	242 (2.4)
Gallbladder	136	49 (0.5)	112 (1.1)	158 (1.5)
Pancreas	248	57 (0.6)	125 (1.2)	172 (1.7)
Larynx	49	32 (0.3)	82 (0.8)	122 (1.2)
Lung	1162	360 (3.5)	745 (7.3)	986 (9.6)

Melanoma of skin	53	38	(0.4)	100	(1.0)	147	(1.4)
Kaposi sarcoma	2	1	(0.0)	3	(0.0)	4	(0.0)
Breast	5410	4691	(45.7)	12470	(121.5)	18928	(184.4)
Cervix uteri	2145	1590	(15.5)	4094	(39.9)	6130	(59.7)
Corpus uteri	710	628	(6.1)	1733	(16.9)	2694	(26.3)
Ovary	1079	762	(7.4)	1884	(18.4)	2741	(26.7)
Kidney	184	88	(0.9)	244	(2.4)	387	(3.8)
Bladder	171	116	(1.1)	297	(2.9)	442	(4.3)
Brain, nervous system	305	162	(1.6)	377	(3.7)	527	(5.1)
Thyroid	608	501	(4.9)	1438	(14.0)	2328	(22.7)
Hodgkin lymphoma	61	47	(0.5)	128	(1.2)	196	(1.9)
Non-Hodgkin lymphoma	474	230	(2.2)	553	(5.4)	798	(7.8)
Multiple myeloma	106	57	(0.6)	126	(1.2)	173	(1.7)
Leukaemia	398	147	(1.4)	329	(3.2)	452	(4.4)

Note: Proportions by 100,000 *Source*: IARC, 2014

Classification of Breast Tumour

The earliest classification of breast tumour was only based on tumour size. This classification was unable to describe the subgroups that share similar prognostic and therapeutic aspects (de Ruijter *et al.*, 2011). Breast cancer was then characterised by the histological appearance of tissue in the tumour. However, this sub-classification also failed to form homogeneous breast cancer subgroups (Weigelt *et al.*, 2008).

Currently, the combination of histo-morphological information such as histological subtype and grading as well as Tumour Node Metastasis (TNM) staging information are among the most widely used classifications of breast cancer system (Elston and Ellis, 1993). The TNM staging system, classifies tumors according to the tumour size (T), regional lymph nodes (N) and distant metastasis (M) (Edge and Compton, 2010).

Molecular Subtypes of Breast Cancer

The recent classification of breast cancer based on gene expression profiling has provided unprecedented tools for analysing and understanding cancer heterogeneity. Based on gene expression profiling, five subtypes of breast cancer can be distinguished as Luminal A and Luminal B (both estrogen receptor (ER)-positive); Basal-like (ER^-negative); $HER2^+/ER^-$; and Normal Breast-like (Perou *et al.*, 2000; Sorlie *et al.*, 2001). The most important determinants of these subtypes are the presence or absence of the Estrogen Receptor α (ERα) or the Progesterone Receptor (PR) or the amplification/over expression of the Her2/ERBB2 locus. Each subtype has been associated with different rates of mortality, diagnosis, response to therapy and clinical implications (Table 2). Therefore, understanding of each subtype would be helpful in prevention and reduction of breast cancer.

Table 2 Molecular Subtypes of Breast Cancer

Breast Cancer Subtype	Receptor Status (Characteristics)
Luminal A	ER^+/ PR^+/ $HER2^-$ (High ER levels)
Luminal B	ER^+/ PR^+/ HER2 (+/-) (Lower ER, Higher PR)
$HER2^+$	ER^-/ PR^-/ $HER2^+$ (High HER2 levels)
Basal-like (Triple-negative)	ER^-/ PR^-/ $HER2^-$
Normal-like	Low expression of luminal-type genes, high expression of basal-epithelial genes.

Source: Cheang et al., 2008; Cheang et al., 2009

Cancer Biomarker Discovery

The National Cancer Institute (NCI), USA, defines a biomarker as a biological molecule found in blood, other body fluids or tissues that is a sign of a normal or abnormal process or of a condition or disease. Biomarkers typically differentiate an affected patient from a person without the disease. Hence, in the hopes of developing new biomarkers that can be used to identify breast cancer in its early stages, we turned to proteomics, which is the study of protein structure, function, and patterns of expression, as an approach.

In a pilot study, 24 samples representing tumour and adjacent normal tissues from infiltrating ductal carcinoma (IDC) patients were tested for their proteomic profiles, in which two-dimensional gel electrophoresis (2DGE) was utilised (Figure 5). The samples represented the 4 stages of the disease and the spots of interest

were identified and subsequently analysed using MALDI-TOF mass spectrometer for protein identification (Figure 6). A number of spots of interest were determined, but one spot representing calreticulin (CRT), a calcium ion storage protein found in the endoplasmic reticulum (ER) was selected. Besides its function in the regulation of Ca^{2+} homeostasis and Ca^{2+}-dependent pathways, CRT mediates nuclear receptor export (Black *et al.,* 2001) resulting in suppression of receptor transcriptional activity. Differential calreticulin expression in breast and other epithelial cancer cells has been previously reported (Chahed et al., 2005). Calreticulin has been proposed as a potential tumour marker for bladder cancer that may have diagnostic value (Kageyama *et al.,* 2004).

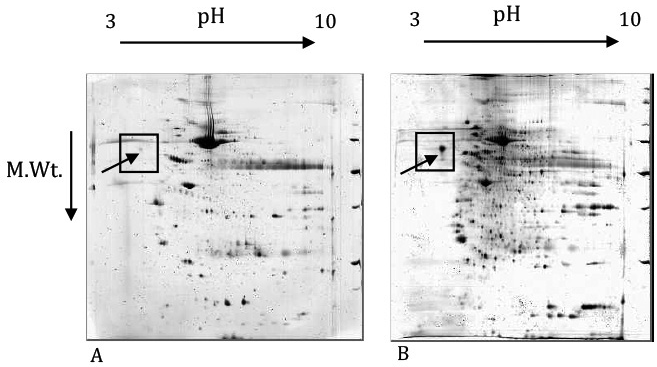

Figure 5 A representative Stage 4 IDC sample where calreticulin was over-expressed in the tumour (B), when compared to the normal adjacent tissue (A). *Source*: *Hamadneh, 2003*

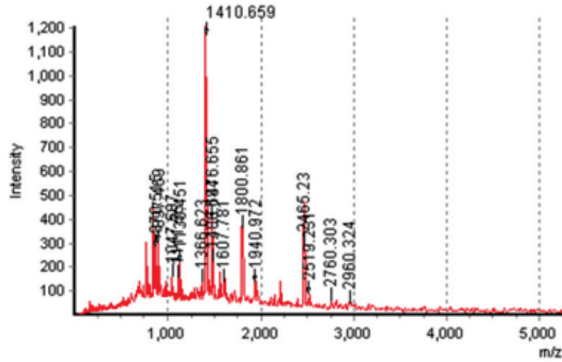

Figure 6 Ettan MALDI-TOF Mass spectrometer was used the identify the protein spot, Calreticulin which has a molecular weight of 48.12 KDa and pI of 4.3. *Source: Hamadneh, 2003*

Meta-analysis of the immunohistochemical results confirmed significantly higher expression of CRT ($p<0.05$) in the stromal compartments of malignant tissues compared to non-malignant samples. An *in vitro* CRT-knockdown model of breast cancer cells using short interfering RNA (siRNA) was used to examine the effects of CRT expression on the invasive potential of MCF7 breast cancer cells. The level of CRT expression was shown to decrease by 87% following siRNA transfection (Figure 7), which confirms the successful knockdown of CRT. This was further confirmed by western blot analysis (Figure 8). Consequently, migration and transwell invasion assays showed significant loss in the migratory and invasive potentials of CRT-deficient cells ($p<0.05$) (Figure 9). Global gene expression profiling successfully identified various gene networks involved in CRT breast cancer signaling such as p53 and MAPK pathway.

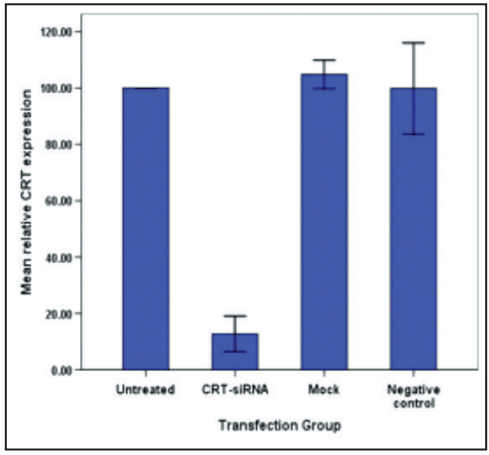

Figure 7 Relative CRT expression. CRT-siRNA transfected MCF7 cells show more than 87% decrease in expression of calreticulin. *Source: Zamanian, 2013*

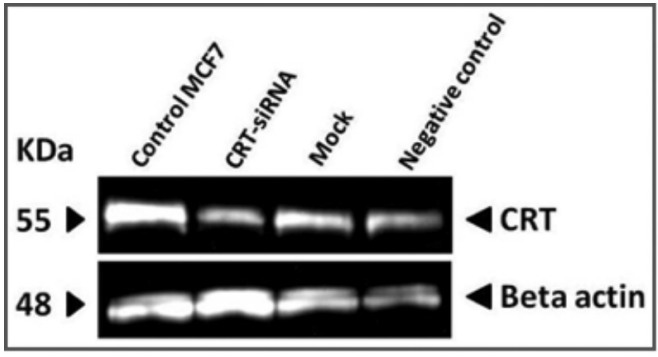

Figure 8 Western blot analysis of CRT expression post-siRNA silencing. *Source: Zamanian, 2013*

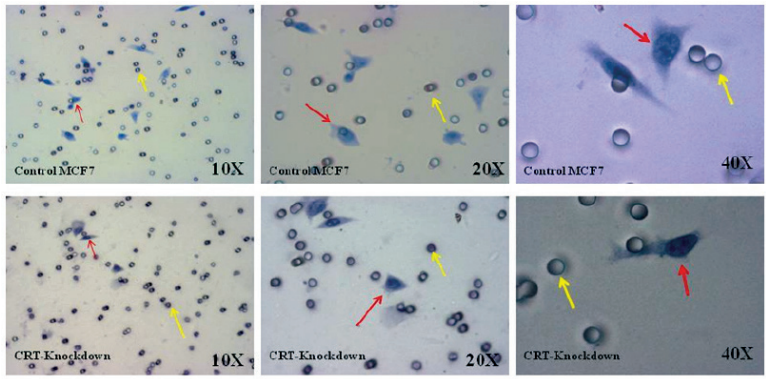

Figure 9 Matrigel invasion assay: transwell invaded cells. Upper row: Control MCF7 cells; Lower row: CRT siRNA knockdown cells. Red arrows show cells that have invaded the pores. Pores are indicated by yellow arrows. *Source: Zamanian, 2013*

CRT expression was shown to be correlated to the degree of invasiveness especially in the stroma. We concluded that CRT is able to promote both migration and invasion in breast cancer cells and can be considered a potential biomarker or target molecule in breast cancer diagnosis and treatment which will need to be characterised in future studies.

Using a similar approach, we set out to investigate the possible opposing effect of RhoGDIα on the migration and invasion of ER (+) MCF7 and the ER (-) MDA-MB-231 breast cancer cells. Rho GDP dissociation inhibitors (RhoGDIs) can inhibit cell motility, invasion, and metastasis in cancer by inactivating the RhoGTPases. A member of RhoGDI family, it has been consistently shown to interact with estrogen receptor (ER) and change its transcriptional activity. The ER is known to be inversely correlated with cell motility and invasion in breast cancer.

RhoGDIα was upregulated using GFP-tagged ORF clone of RhoGDIα (Figure 11), downregulated using short interfering RNA (siRNA) and and their ability for migration and invasion was assayed using transwell chambers. It was found that the silencing of RhoGDIα in MCF7 and MDA-MB-231 cells significantly increased migration and invasion of these cells. Overexpression of RhoGDIα in MCF7 cells suppressed their migration and invasion, but no significant effect was found on MDA-MB-231 cells. Our results indicate that the downregulation of RhoGDIα similarly affects the *in vitro* migration and invasion of ER (+) MCF7 and ER (-) MDA-MB-231 cells. However, our assays are differently affected by the upregulation of RhoGDIα in these two cell lines and this may be due to the differences in ER expression, primary invasive ability and/or other molecules between these two cell lines (Hooshmand *et al.*, 2013).

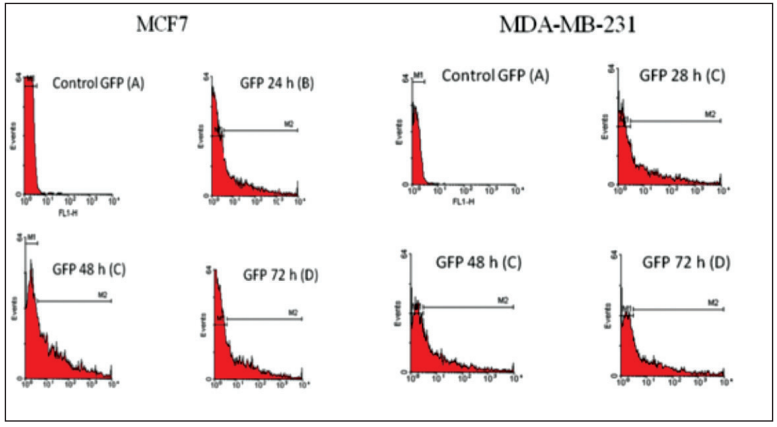

Figure 10 Maximum transfection efficiency in MCF7 and MDA-MB-231 cells for RhoGDIα upregulation was observed 48 h post infection. *Source: Hooshmand et al., 2013.*

Subsequently, the consequence of RhoGDIα activity on migration and invasion in these two cell lines was investigated. The changes in expression of RhoGDIα and other proteins interacting directly or indirectly with RhoGDIα in MCF7 and MDA-MB-231, with different metastatic potentials were of particular interest. The cell lines were subjected to two-dimensional gel electrophoresis (2-DGE) and spots of interest were identified by matrix-assisted laser desorption/ionisation time of-flight/time- of-flight (MALDI-TOF/TOF) mass spectrometry (MS) analysis after downregulation of RhoGDIα using siRNA and upregulated using GFP-tagged ORF clone of RhoGDIα .

A total of 35 proteins that were either up- or down-regulated in these cells were identified 9 and 15 proteins were differentially expressed with silencing of RhoGDIα in MCF-7 and the MDA-MB-231 cells, respectively; 10 proteins were differentially expressed in the upregulation of RhoGDIα in MCF7, while only one protein was identified in the upregulation of RhoGDIα in MDA-MB-231. Based on the biological functions of these proteins, the results revealed that proteins involved in cell migration are more strongly altered with RhoGDI-α activity. Although several of these proteins have been previously indicated in tumorigenesis and invasiveness of breast cancer cells, some have not been previously reported to be involved in breast cancer migration. Hence, these proteins may serve as useful candidate biomarkers for tumorigenesis and invasiveness of breast cancer cells. Future studies are needed to determine the mechanisms by which these proteins regulate cell migration. It is believed that the combination of RhoGDIα with other potential biomarkers may be a more promising approach in the inhibition of breast cancer cell migration. (Hooshmand *et al.*, 2014).

Breast Cancer Cell Lines as Disease Model

Although advances in genomics during the last decade have opened new avenues for translational research and allowed the direct evaluation of clinical samples, there is still a need for reliable preclinical models to test therapeutic strategies. Human cancer-derived cell lines are the most widely used models to study the biology of cancer and to test hypotheses to improve the efficacy of cancer treatment.

The first breast cancer cell line to be established was BT-20 in 1958 (Lasfargues and Ozzello, 1958). It took another 20 years, however, before establishing breast cancer cell lines became more widespread, including the establishment of the MD Anderson series (Cailleau *et al.*, 1978) and what still remains the most commonly used breast cancer cell line in the research world, MCF-7, which was established in 1973 at the Michigan Cancer Foundation (Soule *et al.*, 1973). The popularity of MCF-7 is mainly due to its characteristic hormone sensitivity through expression of estrogen receptor (ER), making it an ideal model to study hormone response (Levenson and Jordan, 1997).

In 2012, we reported what we still believe to be the first two breast cancer cell lines established from invasive ductal breast carcinoma tissues of Malaysian patients (Kamalidehghan *et al.*, 2012). The cell lines designated MBC1 and MBC2, were successfully characterised in terms of morphology analysis, population doubling time, clonogenic formation, wound healing assay, invasion assay, cell cycle analysis, DNA profiling, fluorescence immunocytochemistry, western blotting and karyotyping.

MBC1 and MBC2 exhibited adherent monolayer epithelial morphology (Figure 11) with the receptor status (ER^+, PR^+, $HER2^+$) and (ER^+, PR^-, $HER2^+$), respectively. These results were found to be discordant with the histopathological findings of the tumoral tissues, which were essentially triple negative for MBC1 and (ER^-, PR^-, $HER2^+$) for MBC2. Both cell lines were capable of growing

in soft agar culture, which suggests their metastatic potential. The MBC1 and MBC2 metaphase spreads showed abnormal karyotypes, including hyperdiploidy and complex rearrangements with modes of 52–58 chromosomes per cell.

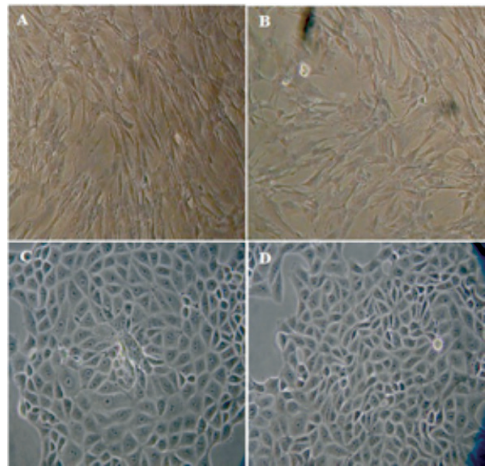

Figure 11 Morphology of the MBC1 and MBC2 Cell Lines. Panels (A) and (B) represent MBC1-PN-2 and MBC2-PN-2 cells, respectively; panels (C) and (D) represent MBC1-PN-125 and MBC2-PN-85, respectively. Magnification, 40X; PN: passage number. *Source: Kamalidehghan et al., 2012*

The loss or gain in secondary properties, deregulation and specific genetic changes possibly conferred receptor changes during the culture of tumoral cells. Thus, it was hypothesised that, among the heterogenous tumoral cells, only a small minority of $ER^+/PR^+/HER2^+$ and $ER^+/PR^-/HER2^+$ cells with lower energy metabolism were able to survive and adjust easily to *in vitro* conditions. These cell lines will hopefully pave the way for new perspectives in genetic and biological investigations, drug resistance and chemotherapy studies and would serve as prototype models in Malaysian breast carcinogenesis investigations.

Triple Negative Breast Cancer

Amongst the different subtypes of breast cancer, the triple negative breast cancer (TNBC) is of interest as TNBC is a more aggressive tumour type and associated with younger age population compared to other subtypes (Anders *et al.*, 2008; Bauer *et al.*, 2007). It does not express estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor-2 (HER-2). Although TNBC accounts for a relatively small minority of breast cancer cases (about 20% of all breast cancers), it is responsible for a disproportionate number of breast cancer deaths (Schneider *et al.*, 2008). In addition, due to the lack of hormone receptor status, it has poorer prognosis than other breast cancer subtypes and does not respond to the usual endocrine therapy or HER2-targeted therapy (Rhee *et al.*, 2008; Schneider *et al.*, 2008). Rhee *et al*. (2008) also reported that triple negative breast cancers have a high level of p53 and Ki67 expression and are generally negative for bcl-2 expression, but positive for the epidermal growth factor receptor.

Various studies have indicated that WEE1, a G2/M checkpoint regulator protein could be a potential target for cancer therapy. The tumour suppressive potential of WEE1 silencing in two different breast cancer cell lines, MCF-7 which carries the wild-type p53 and MDA-MB468, a triple negative cell line, which contains the mutant type was assessed.

Upon WEE1 knockdown with specific shRNAs, downstream effects on cell viability and cell cycle progression were determined using MTT and flow cytometry analyses, respectively. Real-time PCR and Western blotting were conducted to assess the effect of WEE1 inhibition on the expression of apoptotic (p53) and anti-apoptotic (Bcl2) factors and also a growth marker (VEGF) (Figure 12).

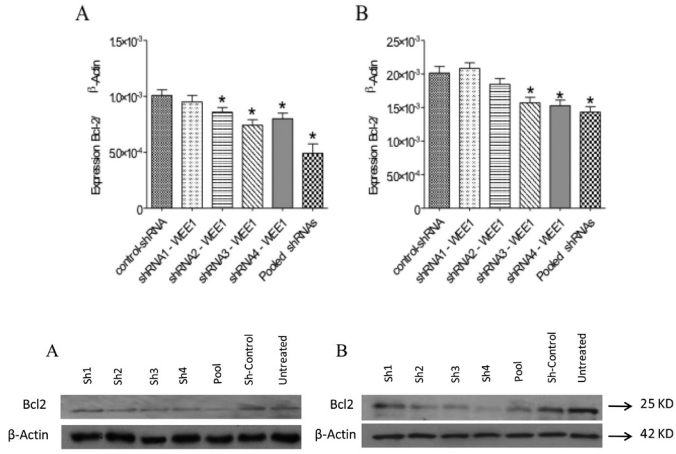

Figure 12 Real time PCR and western blot analysis show Bcl-2 decreased at both mRNA and protein levels in breast cancer cell lines, (A) MCF7 and (B) MDA-MB-468, 48 h following transfection with specific WEE1 shRNAs ($p \leq 0.05$). *Source: Ghiasi et al., 2014*

The results indicated that WEE1 inhibition could cause a significant decrease in the viability of both MCF7 and MDA-MB-468 breast cancer cell lines by more than 50%. Interestingly, DNA content assays showed a significant increase in apoptotic cells following WEE1 silencing. WEE1 inhibition also induced up-regulation of the apoptotic marker, p53, in breast cancer cells. A significant decrease in the expression of VEGF and Bcl-2 was observed following WEE1 inhibition in both cell lines.

In concordance with previous studies, our data showed that WEE1 inhibition could induce G2 arrest abrogation and consequent cell death in breast cancer cells. Moreover, the observed interactions between the pro- and anti-apoptotic proteins and decrease in the angiogenesis marker expression confirm the susceptibility to apoptosis and validate the tumour suppressive effect of WEE1

inhibition in breast cancer cells. It is noteworthy that the levels of the sensitivity to WEE1 silencing in breast cancer cells, MCF-7 and MDA-MB468, seem to be in concordance with the level of p53 expression (Ghiasi *et al.*, 2014).

We then investigated whether *in vitro* WEE1 gene silencing in MDA-MB-468 and MCF7 breast cancer cell lines could enhance the immunopotentiating effects with CD80 and 4-1BBL co-stimulation in human T cells, as an approach for potential cancer immunotherapy. The WEE1 gene was specifically silenced in the cancer cells using shRNA technology. The co-stimulatory molecules were over-expressed on the surface of the cancer cells by recombinant non-replicative adenoviruses. T cell dual co-stimulation led to a significant increase in the frequency of IFN- γ producing cells and higher percentages of degranulation in CD8 + T cells. It also resulted in higher expression levels of the cytotoxicity-related genes. WEE1 gene silencing in the target cells alone however, could not produce significant immune reactivation in the cultured T cells (Figure13). Likewise, the immune responses of T cells were neither improved nor suppressed when dually co-stimulated PBMCs were exposed to the cancer cells with silenced WEE1. Hence, in spite of antitumor effects of WEE1 silencing, the combination approach with immune co-stimulation could not boost the reactivity of cultured T cells against the breast cancer cells tested (Ghiasi *et al.*, 2013). However, other combinatory approaches need to be evaluated as potential candidates for immunotherapy.

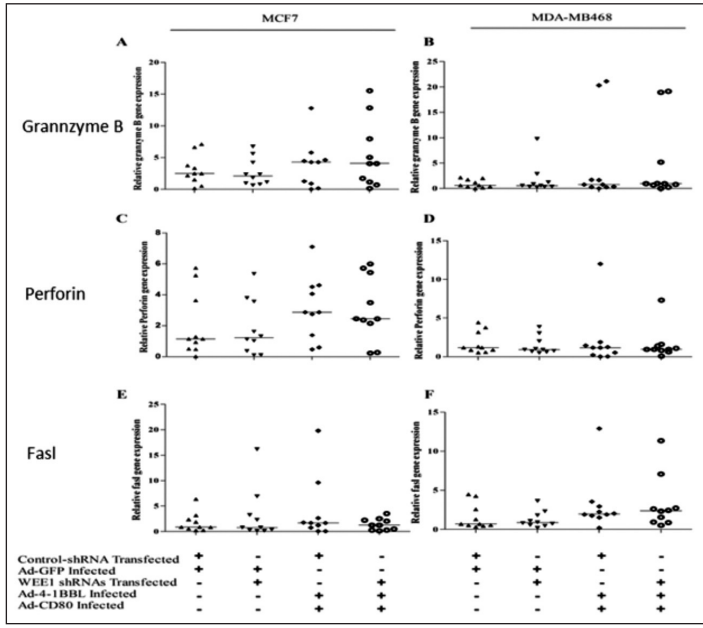

Figure 13 The effect of co-stimulation and co-culture with WEE1 silenced targets on the expression of cytotoxicity related genes in lymphocytes. *Source: Ghiasi et al., 2013*

Single Nucleotide Polymorphism (SNPs)

SNPs and Metastasis

The onset and progression of breast cancer is influenced by many factors, including single nucleotide polymorphisms. SNPs are common genetic variations that are associated with a slight increase in cancer risk. Studies using genomic-level approaches have identified genes whose expression in primary tumors are associated strongly with the likelihood of metastasis (Yau *et al.*, 2013). These findings have been useful in identifying of how, where, and when the activity of these cancer genes cause metastasis and have raised the possibility that cells with metastatic potential

may not be as rare in primary tumors as was originally believed (Bernards and Weinberg, 2002). Metastasis is a complex process which is responsible for more than 90% of cancer-associated mortality (Gupta and Massague, 2006). This reflects the most life-threatening aspects of breast cancer cells in their ability to escape from a primary site and metastasize into other parts of the body, usually by spreading through the lymphatic and blood vessels, circulating through the bloodstream and settling down into distant organs to form a secondary or metastatic tumour (Fidler, 2003). *In vitro* and *in vivo* studies have demonstrated the involvement of matrix metallopeptidase 2 (MMP2) and matrix metallopeptidase 3 (MMP3) genes (encoding enzymes that are responsible for degradation of the extracellular matrix and basement membrane) in breast cancer metastasis.

Our recent study on SNP identification in MMP2 and MMP3 genes of Malaysian breast cancer patient samples identified several coding SNPs which exert an effect on the mRNA secondary structure based on *in silico* analysis (Chan, 2012), (Figure 14). In addition, some of the SNPS were found to confer significant protective effect against breast cancer metastasis. It is possible that the structural change(s) in mRNA result in instability of the mRNA leading to a lower amount of protein synthesis (Shen *et al*., 1999; Duan *et al*., 2003) compared to the wild-type.

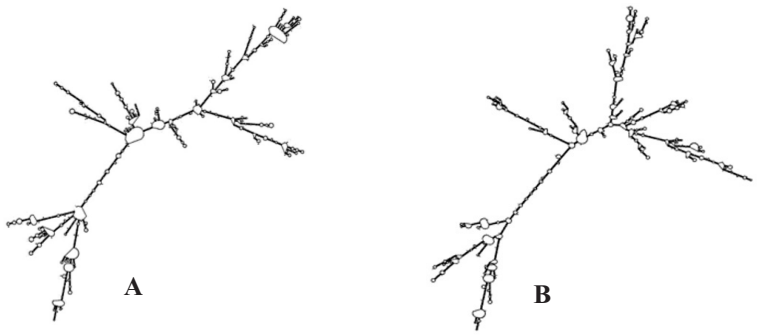

Figure 14 Secondary RNA structure of MMP-2 wild-type sequence sequence (A) as oppose to the variant (B). *Source: Chan, 2012*

This may decrease the invasiveness of cancer cells due to the insufficient quantity of protein for complete degradation of the extracellular matrix and basement membrane of the cells. Work is on-going to characterise the effects of coding SNPs on translation efficiency of normal transformed breast cell line as well as metastatic breast cancer cell lines to determine the outcome on the transcribed mRNAs and proteins.

SNPs and Drug Response

The cytochrome P450, family 2, subfamily D, polypeptide 6 (CYP2D6) is an enzyme that is predominantly involved in the metabolism of tamoxifen. Genetic polymorphisms of the CYP2D6 gene may contribute to inter-individual variability in tamoxifen metabolism, which leads to the differences in clinical response to tamoxifen among breast cancer patients. In Malaysia, the knowledge on CYP2D6 genetic polymorphisms as well as metaboliser status in Malaysian breast cancer patients, is unknown. Hence, this study aimed to comprehensively identify CYP2D6 genetic polymorphisms among 80 Malaysian breast cancer patients.

The genetic polymorphisms of all the 9 exons of CYP2D6 were identified using high resolution melting (HRM) analysis and confirmed by DNA sequencing (Table 3).

Seven CYP2D6 alleles consisting of CYP2D6*1, CYP2D6*2, CYP2D6*4, CYP2D6*10, CYP2D6*39, CYP2D6*49 and CYP2D6*75 were identified in this study. Among these alleles, CYP2D6*10 is the most common allele in both Malaysian Malay (54.8%) and Chinese (71.4%) breast cancer patients while CYP2D6*4 in Malaysian Indian (28.6%) breast cancer patients. In relation to CYP2D6 genotype, CYP2D6*10/*10 is more frequently observed in both Malaysian Malay (28.9%) and Chinese (57.1%) breast cancer patients, whereas CYP2D6*4/*10 is more frequently observed in Malaysian Indian (42.8%) breast cancer patients. In terms of the CYP2D6 phenotypes, 61.5% of Malaysian Malay breast cancer patients are predicted as extensive metabolisers in which they are most likely to respond well to tamoxifen therapy. However, 57.1% of Chinese as well as Indian breast cancer patients are predicted as intermediate metabolisers and they are less likely to gain optimal benefit from the tamoxifen therapy (Chin *et al.*, 2015).

As far as we know, this is the first report of CYP2D6 genetic polymorphisms and phenotypes in Malaysian breast cancer patients of the different ethnic groups. These data may aid clinicians in selecting an optimal drug therapy for Malaysian breast cancer patients to potentially improve their clinical outcome.

Table 3 Frequencies of CYP2D6 genotypes among different ethnic groups of 80 Malaysian breast cancer patients

CYP2D6 genotype	Predicted phenotype	Genotype frequency (%)		
		Malay	Chinese	Indian
*1/*1	EM	9.6	0.0	0.0
*1/*2	EM	1.9	9.5	14.3
*1/*4	IM	0.0	0.0	14.3
*1/*10	EM	25.0	14.3	0.0
*2/*2	EM	1.9	0.0	14.3
*2/*10	EM	17.3	14.3	0.0
*2/*39	EM	1.9	0.0	14.3
*2/*75	Unknown	0.0	4.8	0.0
*4/*10	IM	5.7	0.0	42.8
*10/*10	IM	28.9	57.1	0.0
*10/*49	IM	3.9	0.0	0.0
*39/*39	EM	3.9	0.0	0.0

Abbreviations: EM, extensive metaboliser; IM , intermediate metaboliser. *Source: Chin et al., 2015*

GENETIC APPLICATIONS IN DIAGNOSTICS AND THERAPEUTICS

Fetal DNA Testing

Current methods of fetal genetic testing typically involve obtaining samples of amniotic fluid, placenta, fetal blood or, rarely, other fetal tissues or fluids. The invasive techniques required for obtaining fetal samples (e.g. amniocentesis, chorionic villus biopsy, fetal umbilical vessel venipuncture, fetoscopy-guided biopsy) place the fetus at risk of injury or death (Shulman *et al.*, 2008). Therefore, development of accurate, safe, rapid, noninvasive tests for prenatal diagnosis is an area of active investigation.

Fetal genetic material can be found in the maternal circulation which raises the possibility of using maternal blood to diagnose fetal disease. Although intact fetal cells can be identified in maternal blood, they are not a reliable source of fetal genetic material because these cells are extremely rare (Bianchi *et al.*, 1997) and may persist for years after prior pregnancies (Bianchi *et al.*, 1996). On the other hand, fetal "cell-free (cf)" nucleic acids not contained within cell membranes (cfDNA and cfRNA) are plentiful in the maternal circulation and unique to the current pregnancy. Thus, they have great potential for use in prenatal diagnosis. It is known that fetal cells and circulating cell-free fetal DNA increases in the maternal circulation in women carrying the trisomy 21 fetus.

We attempted the use of superoxide dismutase (SOD-1) gene, which is located at the Down Syndrome Critical Region, for the prenatal screening of Down Syndrome. The prospect of using the gene using real-time quantitative polymerase chain reaction was explored. The level of SOD-1 sequences was found to be significantly elevated in the third trimester normal pregnancies (mean = 11728 copies/µl) when compared to the second trimester (mean = 5705.6 copies/µl), ($p<0.005$) and non-pregnant normal women (mean = 3580.2 copies/µl), ($p<0.0001$). Down syndrome pregnancies have the highest elevation compared to all the three trimesters of normal singleton pregnancies and twin pregnancies, $p<0.05$ (Karuppiah *et al.*, 2008) (Figure 15). These data indicate that a quantitative analysis using a gene associated with a disorder could be used in screening for prenatal diagnosis of fetal aneuploidies regardless of the sex of the fetus.

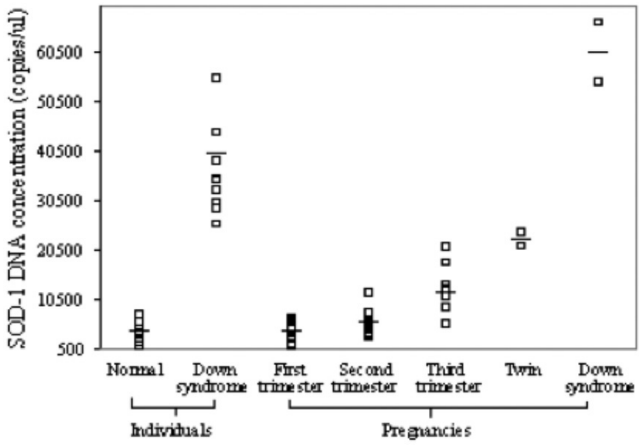

Figure 15 Levels of SOD-1 sequences as measured by real-time quantitative PCR in pregnant and non-pregnant individuals. Horizontal bars, means. *Source: Karuppiah et al., 2008*

In another exploratory study, circulating fetal DNA (fDNA) levels in the second and third trimesters of normal healthy pregnant women and those with gestational diabetes mellitus (GDM), iron deficiency anemia and gestational hypertension (GHT) were quantified using the SRY gene located on the Y chromosome as a unique fetal marker. Significant differences were only observed between the third trimester of normal and GHT pregnancy samples ($P = 0.001$). GDM and iron deficiency anemia does not affect levels of fDNA in maternal plasma while GHT significantly elevates the levels of fDNA in maternal plasma (Zamanpoor *et al.*, 2013) It is postulated that increased amount of circulating fDNA in maternal plasma could be used for early identification of adverse pregnancies where the elevated fDNA values could be used as a potential screening marker in pregnancies complicated with GHT, but not with GDM and iron deficiency anemia.

Rotavirus Genotyping

Pediatric diarrhea is often fatal since this disease results in severe dehydration (UNICEF, 2009). There are several causes of pediatric diarrhea including bacterial or viral infectious diseases. Among the pathogens that cause viral infections are rotavirus, adenovirus and norovirus (Kosek *et al.*, 2003, Wilhelmi *et al.*, 2003 and Fodha *et al.*, 2006). Rotavirus (RV) is the most commonly isolated viral cause of severe diarrhea in infants, infecting more than 111 million pediatric patients every year and causing an estimated 440,000 deaths (Parashar *et al.*, 2003; WHO, 2013). Rotavirus genetic factors, patient's immune factors, and environmental factors are associated with the incidence and severity of acute diarrhea due to RV in infants and toddlers (Listiyaningsih, 2012).

In 2005, we were roped into what was to be our first project with international collaborators and international funding from the Bill and Melinda Gates Foundation. The Asia Rotavirus Surveillance Network (ARSN) is a collaboration of investigators from medical centers and public health agencies in nine Asian countries or regions. The goal of the group is to define the epidemiology and rates of RV disease in Asia and to use these data to make informed decisions regarding the possible future use of RV vaccines. ARSN was formed in 2000 in response to a WHO report that called for expedited rotavirus vaccine evaluation and its introduction to Asia. WHO commissioned a generic protocol for sentinel hospital surveillance of RV that would allow investigators in many countries to assess, in a simple, economical, and timely fashion, the epidemiology and disease prevalence of RV (Bresee *et al.*, 2004). This protocol provides the minimum requirements for hospital-based surveillance, with attention to collecting and testing fecal samples and includes an appendix to identify the catchment population for the hospital, suggestions to assess the prevalence of fatal rotavirus disease and a discussion of the methods to characterise rotavirus strains. The first report was published in 2004 (Bresee *et al.*, 2004) and

involved Kuala Lumpur Hospital and Hospital Umum Sarawak for the Malaysian data. Figure 16 shows the number of hospitalisations for diarrhea and rotavirus-associated diarrhea from February 2001 to April 2003.

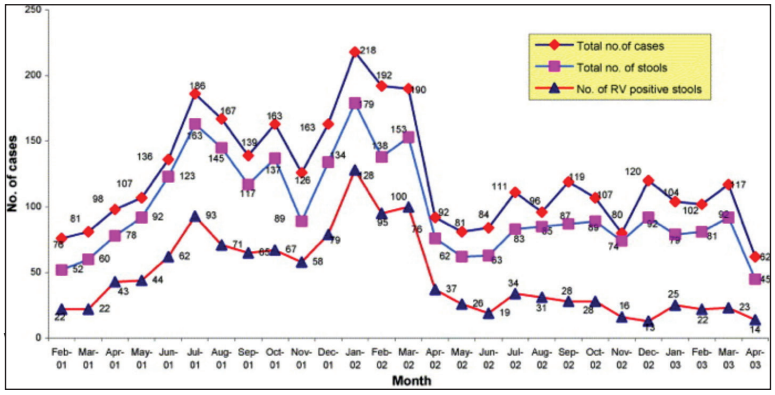

Figure 16 Total number of hospitalizations for diarrhea and rotavirus-associated diarrhea in both Kuala Lumpur Hospital and Hospital Umum Sarawak from February, 2001 to April, 2003. *Source: Bresee et al., 2004*

Rotavirus of the Reoviridae family possess a genome consisting of 11 segments of double-stranded RNA. The genome and several enzymes necessary for viral replication (VP1, VP3) are enclosed in a triple layer of proteins: the core layer (VP2), the middle layer (VP6), and the outer layer (VP7 and VP4). VP6 determines the group specificity (A - G), VP7 the G type and VP4 the P type. For G types, a complete concordance of serotypes and genotypes has been achieved, while for P types, it is not the case. Accordingly, the P serotype is indicated by a free Arabic number (e.g., P1, P2), and the P genotype is indicated by numbers in brackets (e.g., P[8], P[12]). Subsequently, we characterised the circulating rotavirus strains at the time (Hung *et al.*, 2006). RV was found to be responsible for 38% of hospitalisations for diarrhea. It was most common in the

6-17 month age group. There was no seasonality observed for RV-associated diarrhea. The most prevalent strain of RV was P[8]G9. (Table 4). The estimated incidence of RV-associated diarrhea was 27 per 10000 population under the age of 5 years per year.

Table 4 Strain characterisation of rotavirus in Malaysia by centers from August 2001 to July 2002

RV Strain	KLH (%)	HUS(%)	Total (%)
P[8]G9	80 (80.0)	35 (61.4)	115 (73.3)
P[8]G1	2 (2.0)	4 (7.0)	6 (3.8)
P[4]G2	5 (5.0)	2 (3.5)	7 (4.5)
P[4]	1 (1.0)	0 (0.0)	1 (0.6)
P[8]	7 (7.0)	4 (7.0)	11 (7.0)
G1	0 (0.0)	1 (1.8)	1 (0.6)
G2	0 (0.0)	1 (1.8)	1 (0.6)
G9	4 (4.0)	1 (1.8)	5 (3.2)
Untypeable	1 (1.0)	9 (15.7)	10 (6.4)
Total	100 (100)	57 (100)	157 (100)

Source: Hung et al., 2006

Genetic Vaccine

Cholera has affected humans for at least a millennium (Morris, 2011). Despite the available effective treatments against cholera, the disease still afflicts people, especially in poor and developing countries. Current cholera vaccines are available in the form of whole killed bacterium, attenuated live bacterium or protein subunits of the pathogen. Some of the drawbacks of these current oral cholera vaccines include the need for cold-chain transport, higher cost and need for booster immunisations due to a decline in protective efficacy after some period (Kabir, 2005). Hence, an alternative genetic immunisation strategy employing a plasmid DNA engineered to express the selected gene of the pathogen is being utilised in the current approach. DNA vaccination delivered to mucosal sites may provide the additional advantages of cheaper cost for production, for transporting at ambient temperatures, and the potential for immune modulation through plasmid vector design.

Oral delivery of the microencapsulated DNA is beneficial since it is protected from rapid degradation and the bioactivity is prolonged by controlled release. In addition, the protein expressed would lead to a stronger and persistent cell-mediated and humoral immune response compared to the current vaccines.

The choice of using the cholera toxin B (ctxB) gene is justified based on its suitability as an antigen to induce immunity against cholera since infected individuals primarily recognise the ctxB and mount an immune response, it has no enterotoxic activity on its own. It has 80 percent homology with that of the *E. coli* subunit heat-labile enterotoxin which may serve to also induce immunity against it, and that random mutations in the ctxB gene have been found to be uncommon (Olsvik *et al.*, 1993). Although effective treatments are available, they are expensive and impractical in times of disaster.

Hence, in our research, the development of a safe, relatively low cost and efficient vaccine in the form of an oral DNA vaccine

presented an attractive approach. A mammalian expression plasmid DNA vector, pVax, designed for DNA vaccine development and FDA-approved was utilised. The DNA vaccine encodes ctxB, the gene encoding the B subunit cholera toxin of the causative bacteria *Vibrio cholera*. Exploiting the expression of such an antigen coupled with an appropriate delivery system, may be more feasible and more immunogenic in eliciting long-term protection against cholera.

In vitro protein expression upon transfection in COS-7 cells of the vaccine construct pVax/ctxB was successfully achieved and verified (Syahril *et al.*, 2002). Subsequently, the construct was delivered intramuscularly in Balb/c mice and was shown to induce immune responses against ctxB (Hamadneh, 2003). However, oral regimens for cholera vaccination remain to be the most practical approach in mass immunisation strategies, especially in cases of epidemic outbreaks or in populations where cholera is endemic. Additional research focused on the concept of delivering the DNA vaccine through the oral route using microsphere carriers such as cellulose acetate phthalate (Figure 17) and alginate which protect the DNA vaccine itself from stomach acidity while releasing the vaccine within the intestinal environment (Nograles *et al.*, 2012; Hanafi *et al.*, 2013).

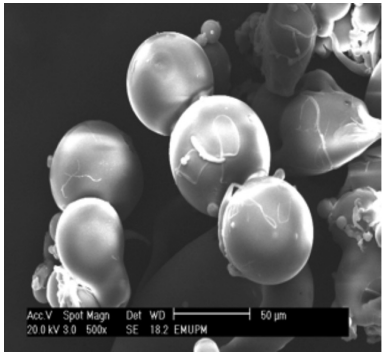

Figure 17 Scanning Electron Microscopy image of cellulose acetate phthalate microcapsules 500X magnification. *Source: Hanafi et al., 2013*

As proof-of concept *in vivo* studies involving mice, oral delivery of pVax carrying GFP reporter gene in cellulose acetate phthalate and alginate microsphere carriers showed a trend of higher percentages of GFP-expressing cells in the intestines (Rosli *et al.*, 2013). Preliminary work in a rabbit model warrants improvements in the vaccine construct with a goal to develop a prototype DNA vaccine for cholera that is efficacious, safe and optimised for production in terms of yield and quality.

In relation to this, the growing global interest in the Halal industry poses an opportunity for vaccine development in Malaysia. Since the development process of the prototype vaccine is for oral administration, the criteria for permissibility will need to be met. For example, finding an alternative source of the unlawful or forbidden product such as trypsin of porcine origin, will be required. Taken together, it is envisioned that the developed vaccine prototype will be competitive in the global market.

Natural Product

Acanthus ilicifolius and Cancer

Malaysia has a long history of using traditional medicinal plants for preventing and treating various types of diseases. *Achanthus ilicifolius* L. (Acanthaceae), known locally as *jeruju*, is often used in traditional medicine in many countries. This plant is a spiny mangrove shrub, widespread in the coastal areas, riverbanks and other places that have muddy and brackish swamps in Peninsular Malaysia. In India, *A. ilicifoulis* is used to treat rheumatism, paralysis, asthma and as an antidote for snakebite (Subudhi *et al.*, 1992). In China, it is used to treat inflammation, hepatitis-B (Wu *et al.*, 2003) and cancer (Graham *et al.*, 2000); while in Thailand, leaves mixed with pepper (*Piper ningrum* L.) are used as a tonic for longevity (Kanchanapoom *et al.*, 2001). In Malaysia, the juice from the leaves is used to treat hair loss, rheumatism, kidney stones

and neuralgia, and as an antidote for arrow wounds and snakebites. The seeds of the plant were reported to have been used in treating boils and various types of cancer (Singh *et al.*, 2009). We were interested in investigating the anti-proliferative effect of the seed extracts to identify its bioactive constituents.

Anti-proliferation assay was carried out against eight types of human cancer cell lines, namely, MCF-7, MDA-MB-231 (breast), HeLa (cervical), CaOV-3 (ovarian), Hep-G-2 (liver), CaCO-2 (colon) CEM-SS (leukemia) and MCF-10A (a normal transformed breast cell line). Seeds of *A. ilicifolius* were subjected to solvent extraction of different polarities (hexane, ethyl acetate and methanol). The anti-proliferative and apoptotic effects of the extracts were evaluated against the eight cell lines using the MTT assay (Figure 5). Bioassay-guided isolation of the active constituents was then carried out by tracing the anti-proliferative activity of the constituents in the cell lines. The structures of the isolated compounds were elucidated by spectroscopic methods and compared with published data. The cytotoxicity and apoptotic properties of the purified compounds were subsequently evaluated (Syakroni, 2007).

Table 5 Anti-proliferative activity of FE and SA extracts of *Acanthus ilicifolius* on 8 types of human cancer cells

No.	Cell Name	FW	FE
		EC_{50} (μg/ml)	EC_{50} (μg/ml)
1.	MCF-7	709 ± 71	86.30 ± 5.03
2.	MDA-MB-231	685 ± 31	94.00 ± 2.74
3.	HeLa	101 ± 15	95.00 ± 3.61
4.	CaOV-3	649 ± 13	74.00 ± 3.51
5.	Hep-G-2	618 ± 77	68.00 ± 3.51
6.	CaCO-2	343 ± 18	69.12 ± 1.41
7.	CEM-SS	237 ± 28	39.33 ± 4.16
8.	MCF-10A	NA	NA

NA – Not active

Source: Syakroni, 2007

The ethyl acetate extract (FE) of *A. illicifolius* seeds was significantly ($P<0.05$) active against all the tested cancer cell lines, except for MCF-10A. Bioassay-guided purification of the extract yielded 1) benzoxazolin-2-one, 2) N-benzoxazolol, 4-hydroxy-3H-benzoxal-2-one and 3) 2-hydroxy-2H-1,4-benzoxazin-3(4H)-one). Interestingly, N-benzoxalozolol, 4-hydroxy-3H-benzoxal-2-one and 2-hydroxy-2H-1,4-benzoxazin-3(4H)-one) showed selective anti-proliferative activity against MCF-7 cells (Table 6). These benzoxazinoids also showed suppression of c-Erb-B-2 oncogene expression (data not shown).

Table 6 Anti-proliferative activity of pure compounds isolated from FE extract of *A. ilicifolius*

Compounds	Weight (mg)	EC_{50} (μg/ml)				
		MCF-7	MDA-MB-231	HeLa	CaOV-3	MCF-10A
J1	52.60	12.00 ± 0.66	>100	>100	>100	>100
1	1.70	8.50 ± 0.21	>100	>100	>100	>100
2	5.10	7.50 ± 0.65	14.00 ± 0.32	28.30 ± 1.36	20.00 ± 0.85	>100
3	9.50	9.30 ± 3.24	Inactive	28.00 ± 1.32	>100	>100
Tamoxifen	-		21.30 ± 9.08	4.00 ± 0.73	4.39 ± 1.27	3.85 ± 1.27

J1 - 2-Benzokzazolin-2-on.

Source: Syakroni, 2007

Results from this study showed good correlation with the ethnomedicinal uses of the seed extracts of the plant. The identification of the benzoxazinoids as the bioactive constituents and their potential mechanism of action provide a better insight into the anticancer activity of the plant. The compounds will be useful as bioactive chemical markers for use in quality control and could also potentially be used as leads for the design and discovery of new anticancer agents, particularly against hormone-positive breast cancer.

Albizzia myriopylla and Diabetes

Diabetes mellitus is a metabolic disorder featured by hyperglycemia caused by insulin deficiency often combined with insulin resistance. The number of people with diabetes worldwide is increasing due to population growth, aging, urbanisation, and increasing prevalence of obesity and physical inactivity. Malaysia has the highest number of diabetics among Asean countries and is the sixth in the western pacific region. One in five adults above the age of 30 or about 3.6 million Malaysians are afflicted by this non-communicable disease (Feisul and Azmi, 2013).

Current anti-diabetic medications have been shown to be effective, but with major side effects which include hypoglycemic coma and involves hepatorenal complications (Mitka, 2007). Due to the current global interest on natural or traditional remedies, the work on *Albizzia myriophylla* (ABZ) may provide a potential alternative to the treatment of diabetes. Initially, oral glucose tolerance test was carried out in normal rats treated with 5 mg/kg, 25 mg/kg and 50 mg/kg of aqueous bark extract of ABZ, respectively. This was then followed by the administration of ABZ at doses of 5 mg/kg and 25 mg/kg, respectively, to normal and streptozotocin nicotinamide induced Type 2 diabetic rats for 4 weeks.

Subsequently, fasting blood glucose levels, levels of aspartate transaminase, alanine transaminase, urea and creatinine were determined in normal and diabetic rats. Additional histological findings of the kidney of normal and diabetic rats were also evaluated. Significant reduction of the glucose levels were seen in diabetic rats treated with ABZ at 5 mg/kg and 25 mg/kg, respectively (Table 7). The result also indicated the safety of consumption of ABZ. This is confirmed from the biochemical analysis and histological findings from the kidney, where insignificant changes were seen in the normal rats treated with ABZ at 5 mg/kg and 25 mg/kg, respectively compared to the normal control rats (Figure 18). Thus, ABZ at 5 mg/kg and 25 mg/kg, respectively show hypoglycemic activity in streptozotocin-nicotinamide induced diabetic rats with no obvious toxicological effects on the liver and kidney. In addition, ABZ at 5 mg/kg was able to ameliorate the liver and kidney damage induced by diabetes (Saat *et al.*, 2012).

Table 7 Effect of aqueous bark extract of ABZ on fasting blood glucose level (mmol/L) in normal and diabetic rats

	Fasting Blood Sugar				
Group	**Week 0**	**Week 1**	**Week 2**	**Week 3**	**Week 4**
Normal control (NC)	4.82±0.38	5.03±0.41	4.75±0.31	5.03±0.27	5.68±0.24
Normal+ABZ(5mg/kg) (NABZ1)	4.65±0.16	5.82±0.7	5.53±0.18	5.68±0.26	5.8±0.65
Normal+ABZ(25mg/kg) (NABZ2)	5.03±0.47	5.62±0.27	5.38±0.64	5.12±0.67	4.93±0.59
Diabetic control (DC)	20.12±2.97[a]	23.37±3.6[a]	18.6±3.51[a]	22.7±3.7[a]	27.57±3.36[a]
Diabetic+ABZ(5mg/kg) (DABZ1)	16.82±2.27[a]	8.57±1.11[b]	7.53±1.06[b]	7.35±1.18[b]	6.57±0.78[b]
Diabetic+ABZ(25mg/kg) (DABZ2)	17.6±1.94[a]	12.35±7.46[b]	10.18±6.65[b]	10.26±7.7[b]	9.97±7.43[b]
Diabetic+Acarbose(80mg/kg)	21.87±4.75[a]	10.52±5.26[b]	7.6±2.42[b]	6.53±0.84[b]	7.17±1.73[b]

Values are expressed as mean ± S.D (n = 6). [a] represents statistical significance versus normal control ($p<0.05$). [b] represents statistical significance versus diabetic control ($p<0.05$).
Source: Saat et al., 2012

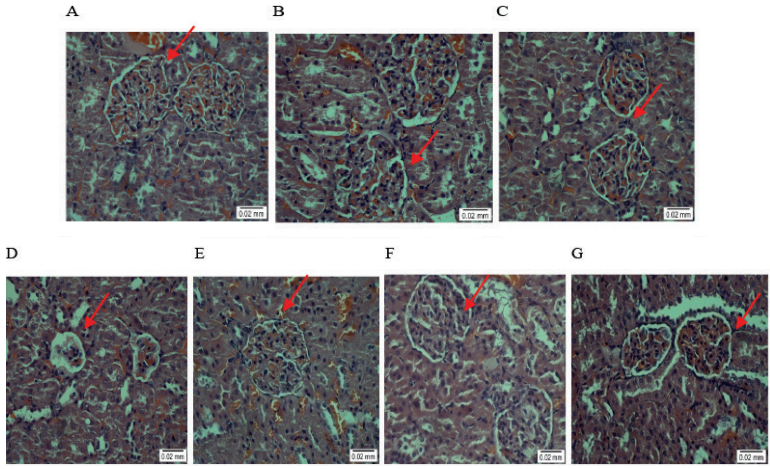

Figure 18 Histology of the kidney in normal and diabetic rats treated with ABZ, x400. (A) Normal control rats (NC) (B) Normal rats + ABZ 5 mg/kg (NABZ1) (C) Normal rats + ABZ 25 mg/kg (NABZ2) (D) Diabetic control rats (DC) (E) Diabetic rats + ABZ 5 mg/kg (DABZ1) (F) Diabetic rats + ABZ 25 mg/kg (DABZ2) (G) Diabetic rats + Acarbose 80 mg/kg. *Source: Saat et al., 2012*

The claim made by a small segment of the Malay population in Pasir Mas, Kelantan, Malaysia (Z Jusoh 2010 *pers. comm*., 10 Nov) that the bark of ABZ added to virgin coconut oil is able to reduce the sugar levels in diabetes was further investigated. Coconut oil puts less of a demand on the enzyme production of the pancreas. This lessens the stress on the pancreas during mealtime when insulin is produced most heavily, thus allowing the organ to function more efficiently. Coconut oil also helps to supply energy to cells because it is easily absorbed without the need for enzymes or insulin. It has been shown to improve insulin secretion and utilisation of blood glucose (Garfinkel, 1992).

The hypoglycaemic activity of the aqueous bark extract of ABZ and virgin coconut oil was evaluated after its oral administration

of streptozotocin-induced diabetic rats and normal rats. Rats were divided into 8 groups. Four groups were normal rats and another 4 groups were induced with diabetes. Diabetes was induced by injecting 60 mg/kg of the body weight intraperitoneally and fasting blood glucose level and body weight were monitored from day 0, 15, 30 and 45. The administration of aqueous bark extract of ABZ and virgin coconut oil simultaneously produces a significant fall in blood glucose levels to almost normal levels. This combination of treatment also brought about an increase in the body weight of diabetic rats. The combination of aqueous bark extract of ABZ and virgin coconut oil showed anti-diabetic activity as it lowers serum glucose levels in diabetic rats (Saat et al., 2013).

GENETIC LINKAGES

With the current pace of development of new technologies, it becomes increasingly difficult for individual scientists to conduct groundbreaking research on their own. This new reality not only opens up the opportunity for scientific collaboration; it necessitates it. Despite this need, effective research partnerships are often hard to come by. As technology will only continue to advance, we must actively break down barriers to forming these relationships and use collaborations to our advantage in order to bring new knowledge and therapies sooner rather than later.

Through the years, I have been fortunate to have had found colleagues who are interested in similar research questions or are able share technical expertise with the goal of enhancing the impact of each other's research. I would be remiss if I do not acknowledge them here in this section.

Fungal Genetics

Within the faculty, one of my active collaborators is Associate Prof. Dr. Chong Pei Pei from the Department of Biomedical Sciences. Her work focuses on the ubiquitous Candida spp., which represents a group of opportunistic fungal pathogens. Despite treatment with antifungal drugs, these organisms can cause fatal bloodstream infections in immunocompromised and immunodeficient persons (Lim *et al.*, 2012). My interest was piqued since understanding these infections may help cancer patients who may develop fungal infections, especially those undergoing chemotheraphy.

Studies mainly involved the pathogenesis of the systemic infections of *Candida albicans*--the most prevalent fungal pathogen in humans. Transcriptome profiling of the yeast-form and hyphae infections in an *in vitro* model indicated that many of the genes that were significantly differentially expressed were involved in apoptosis and cell death. *C. albicans* yeast-forms, at high densities, used to be dismissed as avirulent, was found to contribute to *C. albicans* pathogenesis (Lim *et al.*, 2009).

In a study to identify hotspot mutations that could be associated with drug resistance, the gene expression of the multidrug efflux transporter, CDR1 and the major drug facilitator superfamily transporter, MDR1 gene in azole drug-resistant *Candida albicans* and *Candida glabrata* clinical isolates recovered from vaginitis patients, were investigated. It was found that the expression of CaCDR1 transcript was upregulated to varying extents in the azole-resistant *C. albicans* isolates studied and also correlated with the degree of drug resistance (Looi *et al.*, 2005). Interestingly, DNA sequence analysis of the promoter region of the CaCDR1 gene revealed several point mutations in the resistant clinical isolates compared to the susceptible isolates, upstream from the ATG start codon. This finding provides new information on point mutations in the promoter region which may be responsible for the overexpression of CDR1 in drug-resistant isolates.

In other studies, new techniques have developed including an alternative method for Candida spp cell wall disruption (Lim *et al.*, 2008) and methods for localising fungal-specific gene expression in Candida-infected mice renal cells (Yong *et al.*, 2009) (Table 7 and Figure 19).

Table 7 Comparison of viable *C. tropicalis* from kidney and invasive scoring

Group	Log_{10} CFU/organ (Kidney)	Log_{10} CFU/g (Kidney)	Invasiveness scoring**
Control	0	0	0
Group I ($1X10^5$ cells)	1.522±0.008*	10.631±0.55*	0.833
Group II ($1X10^7$ cells)	3.796±0.20*	22.545±1.41*	3.167

*Invasiveness as estimated by histopathology scores of candidiasis in kidney tissues at day-7 after acute systemic candidiasis with C. tropicalis CT6338. 0 = No infected area; 1 = 1-10 organisms per HPF; 2 = 10-50 organisms per HPF; 3 = Abundant yeast and hyphae but infection is not confluent; 4: Confluent invasion of mucosal surfaces with yeast and hyphae *Source: Yong et al., 2009*

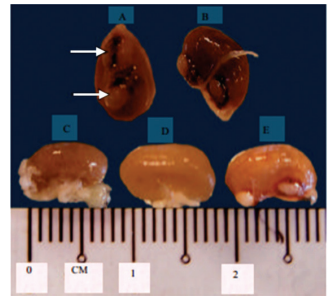

Figure 19 Gross morphology of mouse kidney at day 7 (A) Mice kidney from Group II. Note the presence of yellowish abscesses and lesion indicated by arrow, (B) Group II, (C) Control group, (D) Group I. Note the irregular and swollen kidney surface and E) Group II. *Source:Yong et al., 2012*

C. albicans is capable of undergoing yeast-hypha transition or morphology switching to attain pathogenicity in humans. The existence of quorum sensing as a basic regulatory phenomenon of the *C. albicans* population behaviour has revolutionised Candida research (Lim *et al.*, 2012). To this end, the differential expression of CaSIR2 (silent information regulator gene) via quantitative real-time PCR (qPCR), during yeast-hypha transition with and without the presence of 2-dodecanol, was investigated. The SIR2 transcript levels were found to be significantly enhanced after hyphal induction as compared to the yeast form. This study also found that 2-dodecanol is able to inhibit hyphal development and block SIR2 up-regulation, even in hyphal-inducing growth conditions. Hence, it is suggested that SIR2 may be involved in *C. albicans* quorum-sensing and serum-induced yeast-hyphae transition via the Ras1-cAMP-Efg1 signaling cascade (Lim *et al.*, 2009).

Besides *C. albicans, C. tropicalis* (Lee *et al.*, 2014a) *and C. parapsilosis* (Lee *et al.,* 2014b) were also studied as they have emerged as increasingly prevalent pathogens. The antigenic profiles in each case were identified using proteome analysis. These results may contribute in the development biomarkers of serodiagnosis or vaccines for these infections.

Anti-cancer Synthetic Compounds

Professor Dr. Karen Crouse who was at the Department of Chemistry, Faculty of Science, attracted me to her promise of synthesising "bucket loads" of compounds, and her uncanny ability to explain synthetic chemistry to a molecular biologist.

A series of synthetic compounds developed in her laboratory that have applications for treating cancer and other diseases that affect cell death were tested. An intial set of Schiff bases prepared from S-benzyldithiocarbazate with 5-fluro-, 5-chloro- and 5-bromoisatin were found to be selectively active against MCF-7 breast cancer cell line (Abdul Manan *et al.*, 2011a). The bromide

and fluoride compounds were the most active with IC_{50} values of 6.40 uM (2.6 ug/mL) and 9.26 uM (3.2 ug/mL), respectively while the chloride derivative was weakly active with an IC_{50} value of 38.69 uM (14.0 ug/mL). The cytotoxic activity of the halo substituted isatins against the breast cancer cell lines tested is in the order of Br>F>Cl.

In a subsequent study, Copper (II) complex of S-methyldithiocarbazate with isatin showed even higher cytotoxic activity against MCF-7 cell line with an IC_{50} value of 0.45 µg/mL (Abdul Manan *et al.*, 2011b). The crystal structure of this centrosymmetric Cu(SMISA)2 complex (SMISA = Schiff base formed by condensation reaction of S-methyldithiocarbazate with isatin) (Figure 20) showed that the copper atom has a distorted square-planar geometry with the Schiff base coordinated to the metal ion as a uninegatively charged bidentate ligand through the azomethine nitrogen and thiolate sulfur.

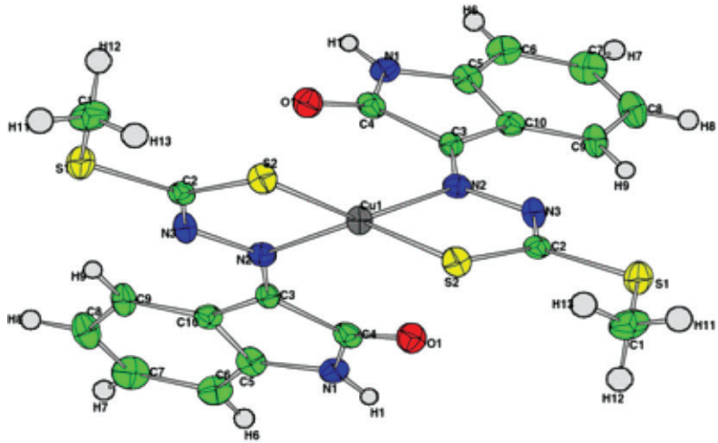

Figure 20 Crystal structure of centrosymmetric Cu(SMISA)2 complex. (SMISA = Schiff base formed by condensation reaction of S-methyldithiocarbazate with isatin). *Source: Abdul Manan et al., 2011b*

In a recent study involving synthesis of Cu(II), Zn(II) and Re(I) complexes with the Schiff base, N'-[1-(2-oxo-2H-chromen-3-yl)-ethylidene]-hydrazinecarbodithioic acid benzyl ester (SBCM-H) was prepared by condensation of S-benzyldithiocarbazate and 3-acetylcoumarin (Low *et al.*, 2015). Crystals suitable for X-ray diffractometry (XRD) were subsequently obtained from the reaction of ReCl(CO)5 with SBCM-H forming a centrosymmetric dimeric complex Re2L2(CO)6 linked by Re-S-Re bridges, where S is the thiolate sulfur of the N,S-bidentate ligand. This Re(I) complex is the first metal carbonyl complex with a bidentate dithiocarbazate ligand to have been characterised by XRD. Cytotoxicity assays revealed enhancement of the bioactivity of SBCM-H upon complexation. Both Cu(II) and Re(I) complexes were found to be active against human breast adenocarcinoma cancer cell lines MDA-MB-231 and MCF-7.

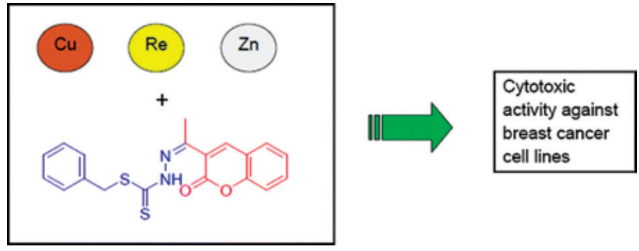

Figure 21 Cu(II), Zn(II) and Re(I) complexes with the Schiff base, N'-[1-(2-oxo-2H-chromen-3-yl)-ethylidene]-hydrazinecarbodithioic acid benzyl ester (SBCM-H) were synthesised and tested for cytotoxicity in breast cancer cell lines. *Source: Low et al., 2015*

Cancer in Engineering

A number of collaborations were initiated by the persistence of graduate students. There was one such case with a student supervised by Associate Professor Dr. Mohamad Hamiruce Marhaban from the Department of Electrical and Electronics Engineering, Faculty of Engineering who came in search of a co-supervisor. The work was to develop new parameters to be used in Microarray technology as a means to monitor the expression levels of a large number of genes simultaneously.

Constructing a classifier based on microarray data has emerged as an important problem for diseases such as cancer. The difficulty arises from the fact that the number of samples is usually less than the number of genes that may interact with one another. Hence, the selection of a small number of significant genes is fundamental to correctly analyse the samples. Gene selection is usually based on univariate or multivariate methods. Due to the inherent weaknesses in each case, a combination algorithm for gene selection integrating the univariate and multivariate approaches was created. Based on the calculated misclassification error in training independent samples of two datasets (breast cancer and leukemia), the algorithm that was developed was better able to classify the independent samples (Mahmoodian *et al.*, 2009).

In a related study, a fuzzy classifier with high performance classification that uses a subset of significant genes that were selected by different types of gene selection methods was created (Mahmoodian *et al.*, 2011). A wide variety of cancer datasets have been previously implemented by the various methods of gene selection and classification to identify the behavior of the genes in tumors and find the relationships between them and outcome of diseases. The results showed that the fuzzy classifier was able to categorize the tumors with high accuracy, while presenting the relationships between the genes using linguistic variables.

Another persistent postgraduate student was also the reason for my knowing Associate Professor Dr. Norhafizah Abdullah from the Department of Chemical and Environmental Engineering. Dr. Norhafizah's recent work focuses on the identification of probiotics with anticancer activity from dairy products. Genetic and environmental factors are known to affect the intestinal microbiome and microbial metabolome. Hence, it would be interesting if these probiotics can be developed into natural supplements or therapeutics for cancer patients.

In one study, 17 Lactobacillus strains belonging to five species (*L. delbrueckii*, *L. plantarum*, *L. rhamnosus*, *L. paracasei*, and *L. casei*) were identified from ewe dairy products of which *L. plantarum* 17C and 13C isolated from colostrum, demonstrated characteristics such as resistant to low pH and high concentrations of bile salts, susceptible to some antibiotics, and good antimicrobial activity which made them potential candidates as probiotics (Nami *et al.*, 2015). A number of strains were resistant to simulated digestion and were further investigated to evaluate their capability to adhere to human intestinal Caco-2 cells. The bioactivity assessment of *L. plantarum* 17C showed anticancer effects through the induction of apoptosis on HT-29 human colon cancer cells and negligible side effects on FHs 74 normal human epithelial cell line (Figures 23-24) The metabolites produced by this strain can potentially be used as alternative pharmaceutical compounds since they are not cytotoxic to normal mammalian cells.

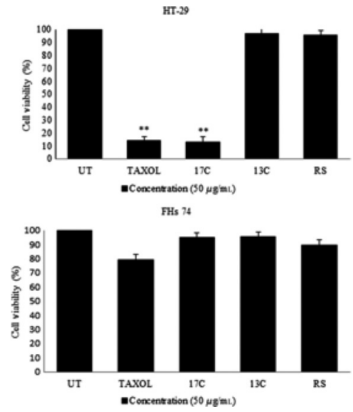

Figure 22 Effect of supernatants obtained from *Lactobacillus plantarum* 17C and 13C on the viability of HT-29 cancer cells and FHs 74 normal cells at 50 μg/mL concentration and 24 h incubation. Data are expressed as mean viability ratio ± SD. Asterisks denote statistically significant differences (**$p \leq 0.01$; *$p \leq 0.05$). All incubations were carried out in triplicate. UT = untreated and RS = reference strain, *L. plantarum* ATCC 8014. *Source: Nami et al., 2015*

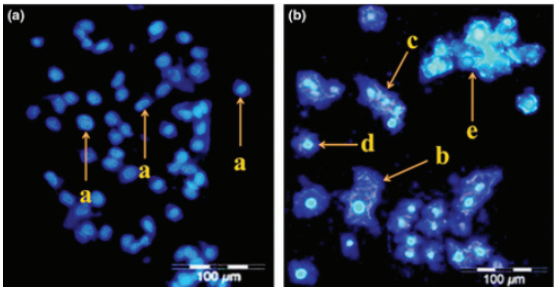

Figure 23 Detection of normal and apoptotic cells of HT-29 cancer cells without treatment with *Lactobacillus plantarum* supernatant (A) and with treatment of *L. plantarum* supernatant (B) after 24 h incubation. a: Blue intact normal cell; b: Membrane blebbing; c: Nucleus fragmentation; d: Cell shrinkage; e: Apoptotic bodies. *Source: Nami et al., 2015*

Similarly, cytotoxic properties for secreted metabolites (40 µg/ml dry weight) of *Lactococcus lactis* subsp. *Lactis 44Lac* were seen in HT29, AGS, MCF-7, and HeLa cell lines, but not in normal human cell line (HUVEC). Interestingly, a number of bacteria strains with similar secreted metabolite properties above were also identified and characterised from the vaginal microbiome (Nami *et al.*, 2014a-d, and Haghshenas *et al.*, 2014). These include *Lactococcus lactis* 2HL, *Enterococcus durans* 6HL, *Lactobacillus acidophilus* 36YL and *Lactobacillus plantarum* 5BL, *L. acidophilus* 36YL and *Enterococcus faecalis*. The strains were grown on appropriate specific culture medium, and then molecularly identified through 16S rDNA gene sequencing. Cytotoxic assessments through flow cytometry and fluorescent microscopy demonstrated that apoptosis is the main cytotoxic mechanism for the secreted metabolites of *L. lactis* subsp. *Lactis* 44Lac. However, further characterisations and both *in vitro* and *in vivo* investigations on the purified proteins need to be conducted before these metabolites can be introduced as potential anti-cancer supplements or therapeutics.

Newcastle Disease Virus

The outcome of research on the Newcastle Disease Virus (NVD) by Professor Datin Paduka Dr. Khatijah Yusoff of the Faculty of Biotechnology and Biomolecular Sciences, as well as a number of researcher groups in UPM has made significant discoveries on its pathogenesis and applications.

NDV is an avian virus that causes deadly infection in over 250 species of birds, including domestic and wild, thus resulting in substantial losses to the poultry industry worldwide (Alexander, 1999). Reports have demonstrated the oncolytic effect of NDV towards human tumour cells through the induction of apoptosis (Zolkapli *et al.*, 2003; Fauziah *et al.*, 2004 and Elankumaran *et al.*, 2006). The interesting aspect of NDV is its ability to selectively replicate in cancer cells. In fact, in the United States, some of the

studies have undergone human clinical trials (Lo, 2013). Therefore, NDV strains can potentially be a therapeutic agent in cancer therapy. However, investigation on the therapeutic perspectives of NDV is still ongoing.

In our study, the effect of a Malaysian velogenic strain of NDV, known as AF2240, on some elements of the intrinsic pathway of apoptosis was examined. It was found that NDV infection leads to a conformational change of the Bax protein (Molouki *et al.*, 2009). This is associated with the translocation of Bax from the cytoplasm to the mitochondria and the release of cytochrome c into the cytoplasm. Interestingly, the level of Bcl-2 protein was not affected by NDV treatment. To further investigate the underlying mechanisms by which NDV kills cancer cells, the clue that many viruses contain Bcl-2 homology-like domains which enabled their interaction with Bcl-2 family members, thereby accounting for their virulence and pathogenicity, was explored. Alignment of the protein sequences of AF2240, with those from members of the human Bcl-2 family showed many similar regions in which its matrix (AF2240-M) protein, large (AF2240-L) protein and fusion (AF2240-F) protein all contain BH3-like regions (Figure 24).

It was also noted that there are BH1-like domains in these proteins, where AF2240-F and Mcl-1 share 55% identity within this region. Hence, the hypothesis that the presence of the BH3-like domains in these proteins may convey cytotoxicity was investigated. AF2240-M and AF2240-F genes were cloned into pFLAG and pEGFP.N2 vectors and transfected into HeLa cells. Based on flow cytometry data, the AF2240-M protein with deleted BH3-like region showed five-fold decrease in apoptosis. Moreover, the construct containing the N-terminal of AF2240-M showed nearly the same cell death rate as to that of the full-length protein, strongly suggesting that the BH3-like domain within this protein participates in promoting cell death (Molouki *et al.*, 2011).

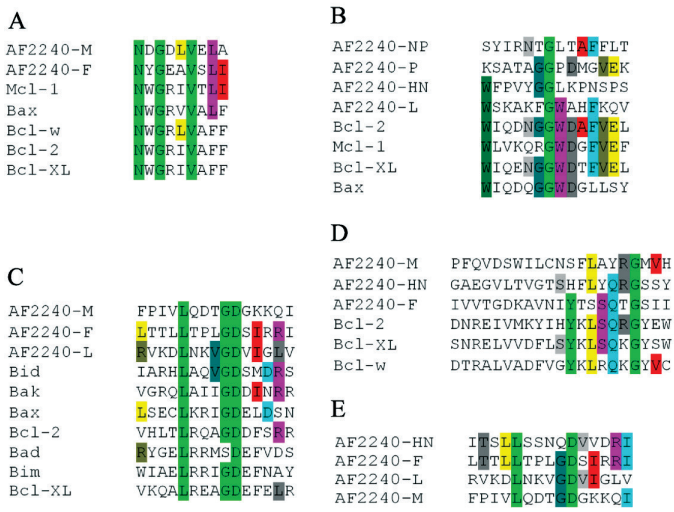

Figure 24 A comparison between Bcl-2 homology domains of Bcl-2 family members and sequences from NDV strain AF2240 viral proteins. Only identical residues between AF2240 proteins and Bcl-2 family members are highlighted. A. BH1 domain-like regions. B. BH2 domain-like regions. A few conserved amino acid residues observed. C. BH3 domain-like regions. D. BH4 domain-like regions. E. A comparison of BH3 domain-like regions between individual proteins of NDV strain AF2240 virus. *Source: Molouki et al., 2011*

As noted earlier, AF2240-M transfection promoted Bax redistribution to mitochondria. In order to determine whether there was any direct interaction between NDV viral proteins with some members of the Bcl-2 family, various constructs were co-transfected into HeLa cells (Figure 25). Co-immunoprecipitation experiments showed that the AF2240-M indeed directly interacted with Bax protein via its BH3-domain, as the mutant proteins failed to interact with Bax. AF2240-F on the other hand, failed to interact with any of the tested proteins, while Bcl-XL slowed down the rate of cell death caused by this construct by nearly five-fold. The expression

level of endogenous Bax and Bcl-2 upon infection of HeLa cells with NDV was assessed by qRT-PCR, but no statistically significant change was observed. Consequently, the Bax/Bcl-2 ratio at the mRNA level did not alter. Taken together, our study has shed some light into the mechanisms by which NDV induces apoptosis.

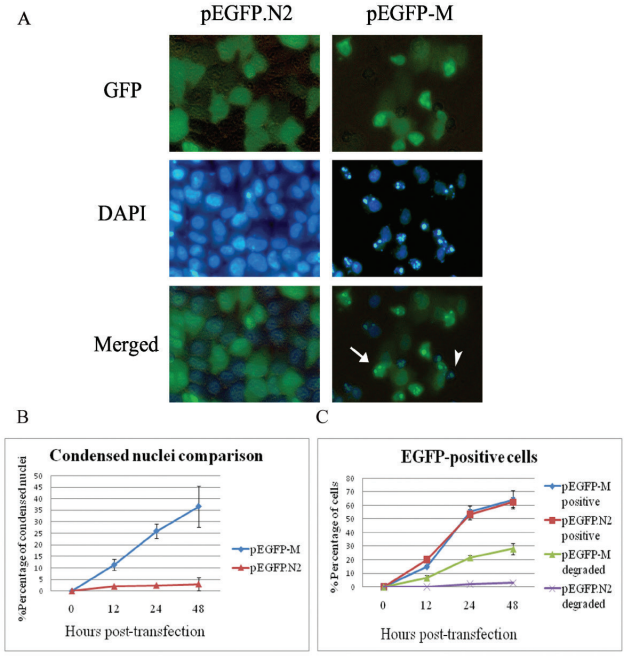

Figure 25 Transient **transfection of HeLa cells with pEGFP-M leads to apoptosis**. A. HeLa cells were transfected with either pEGFP or pEGFP-M construct. At 20 h post-transfection, cells were stained with DAPI and visualized by fluorescence microscopy. Arrow indicates a dead EGFP-M-positive cell. Arrow-head indicates a dead cell that may have lost its fluorescence due to possible de gradation of EGFP-M protein. B. Percentage of condensed nuclei as compared to control. C. Percentage of EGFP-positive cells. Each data point represents three independent measurements, and the error bars indicate the standard error of the mean. *Source: Molouki et al., 2011*

Basal-like Breast Cancer (BLBC)

My collaboration with Professor Dr. Leong Chee Onn from the International Medical University has been rather fulfilling as it thus far has resulted in further understanding the pathogenesis of breast cancer, especially that of the basal-like subtype or triple-negative breast cancer (TNBC). As has been highlighted earlier, human cancer results from genetic and epigenetic changes that affect key cellular processes. Most of these processes, including excessive cell division and attraction of blood vessels to growing tumors, are normally regulated by binding of hormones to receptors at the cell surface, called growth factors and angiogenic factors (Lodish, 2000).

Activation of the receptors by the hormones and the resulting cascade of changes inside the cells that determine whether the cells will divide or commit suicide and whether growing tumors are likely to invade other tissues and disseminate to form metastases is called signal transduction. Not surprisingly, the majority of mutations and chromosomal changes that contribute to cancer development affect these same pathways and processes. Thus, elucidation of signal transduction is important for understanding how cancer arises in each individual, and the features that distinguish benign from aggressive cancers. Most importantly, components of the cellular machinery that mediates receptor-regulated growth processes are among the best rational cancer therapeutic targets in cancer.

In addition to a lack of estrogen/progesterone receptor and Her-2/Neu amplification, these tumors have a high frequency of p53 mutation. These cancers present a clinical challenge because they do not respond to endocrine therapy or other available targeted agents (Hudisa and Gianni, 2011). Their metastatic potential is similar to that of other breast cancer subtypes, but these tumors are associated with a shorter median time to relapse and death. Hence, the identification of prognostic factors and markers that would help identify differential responsiveness to specific agents is critical.

Specific sensitivity to cisplatin has been shown, but the outcome has so far been limited due to its dose-limiting nephrotoxicity and the development of drug resistance.

A drug combination approach was utilised as a strategy to maximise the benefits associated with cisplatin therapy. Using a validated kinase inhibitor library, inhibition of the mTOR, TGFβRI, NFκB, PI3K/AKT, and MAPK pathways was shown to sensitize basal-like MDA-MB-468 cells to cisplatin treatment. The combination of the mTOR inhibitor rapamycin and cisplatin generated significant drug synergism in basal-like MDA-MB-468, MDA-MB-231, and HCC1937 cells, but not in luminal-like T47D or MCF-7 cells (Wong *et al.*, 2011). In addition, the synergistic effect of this drug combination on BLBC cells was mediated through the induction of p73 as depletion of endogenous p73 in these cells abolished the synergistic effects. In conclusion, combination therapy with mTOR inhibitors and cisplatin may be a useful therapeutic approach in the treatment of BLBC.

In a related study, knock-down of endogenous human FGFR4 (but not FGFR1-3) was shown to induce significant tumour-specific cell death in BLBC cells (Tiong *et al.*, 2013). Further analysis revealed that FGFR4 mediates the basal-like cancer cell survival through activation of AKT, as knock-down of endogenous FGFR4 in MDA-MB-468 and HCC1937 cells significantly reduced AKT phosphorylation.

It was also shown that ectopic expression of a constitutively active myristoylated AKT completely abrogates the apoptosis induced by FGFR4 knock-down. Interestingly, both MDA-MB-468 and HCC1937 also secrete fibroblast growth factor 19 (FGF19), a canonical ligand specific for FGFR4. RNAi-mediated silencing of FGF19 evidently suppresses the growth of these BLBC cells via AKT signaling as marked by diminished AKT phosphorylation following depletion of FGF19. In addition, knockdown of FGF19 triggers ERK1/2 activation to compensate for AKT signaling down-

regulation in MDA-MB-468. Together, our results demonstrated the existence of a FGFR4-FGF19 autocrine loop which could potentially be developed as yet another therapeutic target for future treatment of BLBC.

The p53 gene is the most frequently mutated tumour-suppressor gene in human cancers. These mutations mainly occur as missense mutations within the DNA-binding domain, leading to the expression of full-length mutant p53 protein. Mutant p53 proteins not only lose their tumour-suppressor function, but may also gain new oncogenic functions and promote tumourigenesis. In a recent study, it was found that silencing of endogenous p53-R273H contact mutant, but not p53-R175H conformational mutant, reduced AKT phosphorylation, induced BCL2-modifying factor (BMF) expression, sensitized BIM dissociation from BCL-XL and induced mitochondria-dependent apoptosis in cancer cells (Figure 26) (Tan *et al.*, 2015).

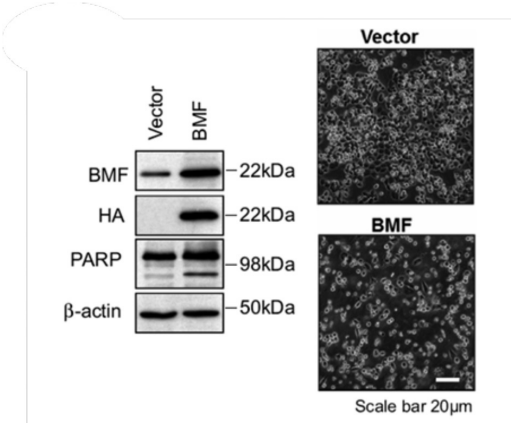

Figure 26 Ectopic expression is sufficient to induce apoptosis in MDA-MB-468 cells. *Source: Tan et al., 2015*

Cancer cells harbouring endogenous p53-R273H mutant were also found to be inherently resistant to anoikis and lack BMF induction following culture in suspension. Underlying these activities is the ability of the p53-R273H mutant to suppress BMF expression that is dependent on constitutively active PI3K/AKT signaling. These results suggest that p53-R273H can specifically drive AKT signaling and suppress BMF expression, resulting in enhanced cell survivability and anoikis resistance. Collectively, these findings open the possibility that blocking of PI3K/AKT will have therapeutic benefits in mutant p53-R273H expressing cancers.

Drug and Gene Delivery

Collaborations can also be fostered through being co-researchers on research grants. Associate Professor Dr. Ezharul Chowdhury of Monash University, Malaysia, and Professor Dr. Nashiru Billa of Nottingham University, Malaysia, have worked together with me in the area of gene and drug delivery, respectively.

It has been shown that poor survival rates in cancer patients are partly due to the development of cellular resistance and the lack of specific targeting with conventional therapies. Adverse effects on healthy cells may necessitate the lowering of the therapeutic dose with consequential lower efficacy of treatment (Bakhtiar *et al.*, 2014). Dr. Chowdhury developed and characterised carbonate apatite nanoparticles that have a range of utilities including purification of multimeric active plasmid DNA (Tee *et al.*, 2014) to carriers of chemotherapeutic agents (Tiash *et al.*, 2014).

For the rapid isolation technique of multimeric plasmids, a zwitterionic detergent and alkali were used in the protocol. Different parameters in the whole extraction process were optimised and the nanoparticles provided the eventual smooth elution of the plasmid DNA. Based on the quality assessment, the isolated multimeric DNA was found to be at least 10 times more transcriptionally active than the monomeric form isolated by the commercially available Qiagen kit.

In exploring to improve the efficacy of chemotherapeutic drugs, carbonate apatite nanoparticles and its strontium (Sr^{2+})-substituted derivative were complexed with three classical anticancer drugs: methotrexate, cyclophosphamide and 5-flurouracil. Carbonate apatite demonstrated significant binding affinity towards methotrexate and cyclophosphamide leading to more cellular toxicity than free drugs in MCF-7 and 4T1 breast cancer cells tested (Tiash *et al.*, 2014). Moreover, Sr^{2+} substitution in carbonate apatite resulted in particles less than 100 nm in diameter further promoting the binding of methotrexate to the nanocarriers. Hence, Sr^{2+}-substituted apatite nanoparticles have a better potential for loading higher amounts of anti-cancer drugs with increased therapeutic effectiveness.

Professor Dr. Nashiru Billa focuses on the formulation of nanoparticles for delivery of anti-cancer natural products. In one study, mucoadhesive nanoparticles were formulated to deliver curcumin, a well-known anticancer compound derived from turmeric, to the colon. It has low oral bioavailability because of its poor absorption, rapid metabolism and elimination. The results from an *ex-vivo* study showed that curcumin-containing chitosan nanoparticles (CUR-CS-NP) have improved mucoadhesion properties compared to unloaded chitosan nanoparticles (CS-NP), suggesting that curcumin partly contributes to the mucoadhesion process (Chuah *et al.*, 2014a). This may lead to an enhanced anticancer effect of curcumin when formulated in CUR-CS-NP. Additionally, CUR-CS-NP were taken up to a greater extent by colorectal cancer cells compared to free curcumin. The prolonged contact offered by the mucoadhesion of CUR-CS-NP onto the cells resulted in a greater reduction in percentage cell viability as well as a lower IC_{50}, indicating a potential improved treatment outcome. The formulation and free curcumin appeared to induce cell apoptosis in colorectal cancer cells by arresting the cell cycle at G2/M phase.

The superior anticancer effects exerted by CUR-CS-NP indicated that this could be a potential treatment for colorectal cancer.

In a related study, the propensity of CUR-CS-NP to muco-adhere and release curcumin under simulated colon conditions was investigated using Real-time nanoparticle tracking analysis (NTA). This novel procedure permits the correlation of physical changes to the CUR-CS-NP with the observed behaviour under simulated conditions in real-time. The CUR-CS-NP was found to form spontaneous aggregates in response to exposure to mucin (Chuah *et al.*, 2014b). This observation correlated with curcumin release from CUR-CS-NP was observed in phosphate buffer (pH 7.4) where 81% of curcumin was released within 6 hours. Atomic force microscopy imaging CUR-CS-NP exposed to mucin solution revealed a decorated surface of the CUR-CS-NP by mucin, consistent with expected electrostatic interactions between the two. The use of NTA provides a means for ascertaining the performance of the CUR-CS-NP under simulated colonic conditions and this prototype delivery system may provide a basis for an effective colon mucoadhesive drug delivery system.

The list of collaborators described above is not exhaustive since other collaborations with the members of the Medical Genetics Laboratory for instance, either individually or in groups are on-going. Recent initiated research collaborations included with Professor Dato' Dr. Lye Munn Sann (Community Health), Associate Professor Dr. Maha Abdullah (Immunology), Associate Professor Dr. Norhafizah Moktaruddin (Pathology), Dr. Kartini Farah Abdul Rahim (Dermatology) as well as Professor Dr. Rasedee Abdullah from the Faculty of Veterinary Medicine. I am extremely grateful for the camaraderie shown by them and certainly look forward to more exciting discoveries.

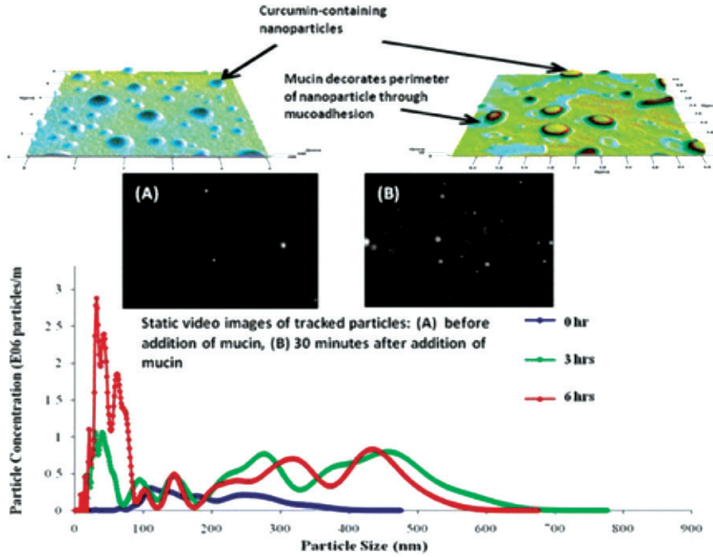

Figure 27 Real-time nanoparticle tracking analysis (NTA) was used to evaluate the propensity of curcumin-containing chitosan nanoparticles (CUR-CS-NP) to muco-adhere and release curcumin under simulated colon conditions. *Source: Chuah et al., 2014b*

LOOKING BACK AND MOVING FORWARD

As I look back on my research career, I am confronted by a number of questions. Firstly, after spending nineteen years in the States, I sometimes ask myself, "Should we have moved back to Malaysia?" Secondly, since I was attached to the oldest university in Malaysia on returning, I sometimes wonder, "Should I have stayed in UM?" Finally, with regards to my expertise, I sometimes ponder over the question, "Should I have focused on a specific area of research?" In other words, have I made the right decisions in my journey into genetics? In all honesty, I can quite confidently answer by saying, "I have no regrets."

In the case of moving back home, my two boys got the opportunity to get to know their extended families, go through the regular school system, learn their native language, and learn more about their religion. These I believe and hope has today prepared them for life on matters that are important to me.

Moving to UPM in 1997, to a newly minted medical school gave me the work experience no other established faculty could ever have provided. Though moving from UM, in retrospect, still evoke nostalgic memories, I am happy to say that the friends I made there still remain so to this day: Professor Emeritus Dato' Dr. Lam Sai Kit, Professor Dr. Sazaly Abu Bakar, and Professor Dr. Shamala Devi Sekaran are a few I can name here.

In those early years at UPM, research took a while to get started. It was then about establishing a functioning laboratory and about training human resource. In the context of our grant funding system, research was about what was relevant to society. There is certainly nothing wrong with such a view, but it took some time before fundamental research was recognised as also important. I suppose, to a certain degree, it was also about making the right connections and taking opportunities as they came along.

Research does not occur in a vacuum; hence, being involved in different areas of research today have allowed me to see the bigger picture, which I believe is key to success. Despite the constraints that still exist, I have indeed been fortunate to have had continuous research funding all these years.

So, what is next? In terms of research, there are a number of projects that I would like see to completion. For example, further work on calreticulin will need to include the mechanistic role of invasion and the characterisation of the possible calreticulin-dependent molecular targets towards *in vivo* validation. The commercialisation potential for ABZ for diabetes (*GlucoFix)* which has attracted the attention of a Russian company, and the Cholera DNA vaccine (*GeneVax)*, which successfully bid for a Prototype

Research Grant from the Ministry of Higher Education, need to be further explored. I would still continue to work on cancer, but wish to see more done for ovarian cancer, the most lethal of gynaecological malignancy.

In addition, I have always been fascinated with the brain. I am glad that Dr. Michael Ling is championing Neurogenetics at UPM. The earlier work on Down Syndrome seems to appear relevant now with the availability of a mouse model. A recent collaboration with researchers in which I am involved at Monash University, Malaysia presents another opportunity with a mouse model for epilepsy.

There is no denying that having outstanding graduate students and research assistants make a great difference to the outcome of my research. For the most part, I have been privileged to have had some of the most wonderful people under my supervision. I hope that this synergistic relationship will continue in the many years to come. They are indeed like family to me.

The world of research is full of challenges and overcoming them requires the support from many fronts. I am indeed blessed with a supportive husband, Professor Dr. Mohamed Ajmal Al-Aidrus and two wonderful children, Dr. Mohamed Shafiq and Mohamed Shabyl who are with me as I continue to complete my journey, with God's grace.

Life is after all, but a series of twists and turns that lead one to different crossroads and possibilities. To that end, I am grateful that I am here to contribute in some small way to the understanding of life.

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BIOGRAPHY

Professor Dr. Rozita Rosli was born in Batu Gajah, Perak, but was brought up in Seremban, Negeri Sembilan. She attended the Convent primary school and was subsequently selected to attend Tunku Kurshiah College. She was active in sports and received colors for basketball. She obtained her tertiary education in the United States, earning her undergraduate degree in Biology from Purdue University, her Masters and doctoral degree in 1986 and 1994, respectively from Ball State University where her laboratory skills in Molecular Biology was honed. She subsequently pursued her post-doctoral training at the Indiana University School of Medicine in the area of Hematology/Oncology where her interest in cancer research developed.

Professor Rozita currently heads the UPM-MAKNA Cancer Research Laboratory at the Institute of Bioscience, UPM, where she initiated the translational genomics research programme which focuses on the application of new molecular technologies in the identification of genetic and epigenetic alterations of various pathways of carcinogenesis or the cancer microenvironment of common cancers, especially that of the Asian-specific genetic variation. She previously served as Deputy Dean for Research and Graduate Studies (2004 – 2010) at the Faculty of Medicine and Health Sciences, UPM.

Her current research interest mainly lies in the application of genomics and proteomics as tools in understanding and combating genetic diseases, especially cancer as well as infectious diseases. Together with her graduate students, her work has led to the publication of more than a hundred research articles, the development of DNA vaccines, molecular diagnostic tools, the production of therapeutics from natural products and a number of related patents.

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My first ever sabbatical four years ago was a needed breather, but administrative work appear to be in my cards. I wish to thank our former dean, Professor Dr. Norlijah Othman, for her support on my secondment to the Institute of Bioscience, UPM. In this regard, I would also like to thank Professor Abdul Rahman Omar and team for making IBS a great place to work.

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Rozita Rosli

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5. Prof. Dr. Mohd. Ariff Hussein
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