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Effect Of Garcinol On Change In Gene Expression In Pancreatic Cancer Mouse Model

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**EFFECT OF GARCINOL ON CHANGE IN GENE EXPRESSION IN PANCREATIC
CANCER MOUSE MODEL**

by

NURUL HUDA RAZALLI

THESIS

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

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Approved By:

Advisor

Date

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DEDICATION

I would like to dedicate this work to my fellow colleagues of the Nutrition and Dietetic Program, Universiti Kebangsaan Malaysia. Thank you for the endless support, may this work be a great start to more researchers to come and may we continue to actively contribute to the quest of finding the cure to cancer and other diseases through nutrition.

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ABBREVIATIONS

Bad	B-cell lymphoma-2 associated X protein
Bax	B-cell lymphoma-2 associated death protein
BCL2	B-cell lymphoma-2
CA 19-9	Carbohydrate antigen 19-9
CDK	Cyclin-dependent kinases
cDNA	Complimentary deoxyribonucleic acid
COX2	Cyclooxygenase-2
MMP9	Matrix metalloproteinase-9
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IL8	Interleukin 8
iNOS	Inducible nitric oxide synthase
mRNA	Messenger ribonucleic acid
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated β cell
NTC	Non-template control
PDAC	Pancreatic ductal adenocarcinoma
PGE2	Prostaglandin E2
qPCR	Quantitative real-time polymerase chain reaction
TGF	Transforming growth factor
VEGF	Vascular endothelial growth factor

Chapter 1

Introduction

1.1 Cancer

Cancer is a type of disease defined by abnormal growth of cells called tumor resulting from the accumulation of gene mutations with two heritable properties; uncontrolled proliferation and colonization into other tissues. Tumors can be classified as benign or malignant. Benign tumors grow locally and remain clustered together, thus considered noncancerous, while malignant tumors have the ability of invading neighboring tissues, entering blood vessels and lymph system and metastasize to different sites. The progression of cancer can be explained by three key steps involving (a) initiation, in which normal cell is converted into initiated or abnormal cell, (b) promotion, the process of which the initiated cells is transformed into a pre-malignant cells, and (c) progression, by which the cells become neoplastic or malignant [1].

1.2 Pancreatic Cancer

Pancreatic cancer is a condition in which malignant cells are found in pancreatic tissues. The pancreas is a 6-inch organ located behind the stomach made up of both exocrine and endocrine glands. The exocrine cells of the pancreas secrete digestive enzymes which are being released into a system of small ducts that lead to the main pancreatic duct. In the digestive system, both pancreatic duct and bile duct connect together where pancreatic juice and bile will be emptied into the duodenum, aiding in the digestion of fats, proteins and carbohydrates. On the other hand, the endocrine cells of the pancreas are involved in the production of hormones, mainly insulin and glucagon. These hormones are secreted by islet cells into the bloodstream, working together in maintaining the normal level of blood glucose in the body.

Pancreatic cancer is currently ranked as the fourth leading cause of cancer-related deaths in the United States with 95% of the cases emerging from the exocrine cells, the most common type being the pancreatic ductal adenocarcinoma (PDAC), while the remaining 5% develop from the endocrine cells [2]. Although accounting for the least percentage of incidences among other types of digestive system cancers, pancreatic cancer still remains as one of the most lethal types of malignancies with the five year survival rate of less than five percent. The American Cancer Society estimated that in 2013, 45220 of new cases of pancreatic cancer would be diagnosed and 85% of death from this number is expected due to this disease [3]. Late diagnosis, early metastasis of tumors and poor response to treatments contribute to the high mortality rate [4].

It is now widely accepted that the cause for pancreatic cancer is related to the accumulation of genetic mutations and alterations which results in oncogenes being highly expressed, inactivation of tumor suppressor genes and overexpression of growth factors and their receptors [5, 6].

In pancreatic cancer, *K-ras* mutations are the most commonly seen oncogene mutations which appear in more than 95% of pancreatic cancer tissues. The normal *K-ras* proto-oncogene encodes a guanine nucleotide-binding protein which plays role in cell growth and differentiation [7, 8]. The activated *K-ras* oncogene by means of point mutations in either codon 12, 13 or 61 has an abnormal increase in membrane-bound ras proteins which appears to induce tumor development by altering the signal transduction pathway across the membrane leading to an abnormal, uncontrolled cell growth [8]. It is believed that mutations of *K-Ras* take place in the early stage of pancreatic carcinogenesis [6].

P53 is a tumor suppressor gene located on the short arm of chromosome 17 that encodes a 53kDa nuclear phosphoprotein which plays role in the negative regulation of cell growth and proliferation. It is an important tumor suppressor genes inactivated in pancreatic cancer [9]. P53 mutations have been found to occur in 40% to 70% of pancreatic adenocarcinoma cases and its contribution to tumorigenesis is suggested to be through the inhibition of apoptosis [10]. In contrast to K-Ras oncogene, whether p53 abnormalities are an early or late event in pancreatic cancer is inconclusive [6].

Growth factors and their receptors pose meaningful functions in the progression of tumor [11]. Over expression of growth factors such as EGF, TGF alpha, TGF beta 1-3, growth factor receptors including EGF receptor, c-erbB-2, c-c-erbB-3, TGF beta receptor I-III together with genetic mutations mainly *K-ras*, p53 are of significant existence in a number of gastrointestinal cancers [12]. These abnormalities stimulate tumor growth and exaggerate the metastatic behavior of pancreatic cancer cells to such a degree that may contribute to poor prognosis following treatments of this disease [6]. The mouse model selected for the study has mutations in K-ras and p53 genes, leading to pancreatic cancer manifestation, similar to human.

1.2.1 Clinical presentation and diagnosis

Symptoms presented by pancreatic cancer are not exclusive to the disease and differ depending on the location of tumor as well as the stage of the disease. Although the etiology of pancreatic cancer remains unknown, several risk factors have been suggested such as male gender, black race, meat and fat consumption, cigarette smoking, pancreatic ductal hyperplasia and chronic pancreatitis. Since the majority of tumors develop in the head of the pancreas, obstructive cholestasis is normally manifested. Rarely, a pancreatic tumor may also cause gastrointestinal bleeding or duodenal obstruction while obstruction of the pancreatic duct by

tumor may lead to pancreatitis. Pancreatic cancer generally causes abdominal discomfort, nausea and dull, deep upper abdominal pain. For most patients, systemic manifestations of this disease include anorexia, weight loss and asthenia. Other less common manifestations include deep and superficial venous thrombosis, increased abdominal girth, panniculitis, gastric-outlet obstruction and depression. Upon physical examination, jaundice, temporal wasting, hepatomegaly and ascites may be observed. Patients may also have mild liver-function test abnormalities, hyperglycemia and anemia [2, 13, 14].

Commonly, contrast-enhanced computerized tomography (CT) is sufficient to help in the diagnosis of pancreatic cancer. Other imaging tests use to diagnose this disease include ultrasound and magnetic resonance imaging (MRI). Some other diagnostic tools that is also useful are endoscopic ultrasonography and endoscopic retrograde cholangiopancreatography (ERCP). In terms of serum biomarker, CA 19-9 is the only biomarker that has demonstrated clinical usefulness for therapeutic monitoring and early detection of recurrent event of pancreatic cancer despite its limitation of not being a solely specific biomarker for this disease [15, 16]. CA 19-9 may also be elevated in other malignancies such as lung cancer, colorectal cancer and cancer of the gall bladder as well as in non-cancerous conditions such as gall stones, pancreatitis, liver disease and cystic fibrosis. In our study, tumor development and progression in live animals was monitored by MRI.

1.2.2 Pancreatic cancer staging

According to the most recent edition of the American Joint Committee on Cancer, pancreatic cancer is staged based on tumor-node-metastasis classification. Staging of pancreatic cancer comprised of five stages namely stage 0, stage I, stage II, stage III and stage IV together with tumor grade, nodal status and distant metastases. In stage 0, abnormal cells are found in the

lining of the pancreas. These abnormal cells may become cancer and spread into nearby normal tissue. Stage 0 is also called carcinoma in situ, an early form of cancer defined by abnormal cells are still within the site of origin with the absence of spreading or invasion to nearby tissues. In stage I, cancer has formed and is found in the pancreas only. Based on the size of the tumor, stage I is divided into stage IA (tumor size of 2 centimeters or smaller) and stage IB (tumor size larger than 2 centimeters). In stage II, cancer may have spread to nearby tissue and organs, and may have spread to lymph nodes near the pancreas. Stage II is further divided into stage IIA where cancer has spread to nearby tissue and organs but has not spread to nearby lymph nodes and stage IIB where cancer has spread to nearby lymph nodes and may have spread to nearby tissue and organs. As pancreatic cancer advances into stage III, cancer has now spread to the major blood vessels near the pancreas including the superior mesenteric artery, celiac axis, common hepatic artery, and portal vein, and may have also spread to nearby lymph nodes. In the final stage of pancreatic cancer, stage IV, cancer may be of any size and has metastasize to distant organs, such as the liver, lung, and peritoneal cavity. It may have also spread to organs and tissues near the pancreas or to lymph nodes [17].

In PDAC, the growth of pancreatic cancer arises from the ductal epithelium and progresses from pre-malignant lesion to fully invasive cancer [2]. The progression of this disease can also be graded by looking at the changes that happen to lesion called pancreatic intraepithelial neoplasia (panIN). PanINs are proliferations of the smaller pancreatic ducts that can be viewed microscopically. Classification of PanIN represent step by step morphological alterations that happen in the pancreatic ductal epithelium. A normal pancreatic duct is characterized by low cuboidal, non-mucinous cells in a single layer formation. Low-grade PanINs (PanIN-1a and PanIN-1b) are characterized by the change from a cuboidal duct

epithelium to elongated cells and by the abundant accumulation of mucin. PanIN-1a lesion is made up of columnar shape cells with the presence of mucin production. PanIN-1b lesion is somewhat identical to PanIN-1a except for its architectural difference. PanIN-1b has a papillary, micropapillary or basally pseudostratified architecture. As the lesions advance from PanIN-1 to PanIN-2, some nuclear abnormalities have emerged which can be viewed under a microscope. The architectural make up of mucinous epithelial lesion of PanIN-2 can either be flat or papillary. Some of the nuclear alterations that can be seen in PanIN-2 lesion include loss of polarity, nuclear crowding, enlarged nuclei, pseudo-stratification and hyperchromatism. PanIN-3 lesion is a pre-invasive form of adenocarcinoma (carcinoma in situ). It is architecturally papillary or micropapillary, however, they may also appeared flat. This lesion may form budding into the pancreatic lumen with the observance of severe nuclear atypia and some abnormal mitosis [18]. In the overall study, the PanIN system was used to confirm the presence of cancer in the pancreas and liver tissue by histological methods.

1.2.3 Treatment and prognosis

Treatment plan for pancreatic cancer depends on the possibility of performing complete surgery to remove the cancer. Surgery is the only potential treatment to cure the disease. Unfortunately this option is only limited to patients diagnosed with early stage of pancreatic cancer, mainly stage I and some of stage II cases [19, 20]. Only 20% of cases can be surgically removed at the time of diagnosis while in the remaining 80% of cases, the tumor is unresectable or curative treatment by surgical techniques alone is less beneficial [20]. The most common operative procedures involve cephalic pancreatoduodenectomy (the Whipple procedure); when the tumor is in the head or uncinat process, distal subtotal pancreatectomy; when the tumor is located the body or tail of the pancreas and in some cases may involve total pancreatectomy.

Observations from several randomized clinical trials demonstrate that a more extensive surgical resection does not improve survival due to the increase risk of postoperative morbidity [2]. This poor prognosis is contributed by several factors including large tumor size, high tumor grade, lymph-node metastases and high level of CA 19-9 that continue to elevate persistently in postoperative setting [21, 22].

Despite being the only possible curative treatment for pancreatic cancer, prognosis following total resection of tumor alone in early-stage patients is somewhat disappointing [2]. Data from several studies have shown that adjuvant therapy through postoperative administration of chemotherapy with either leucovorin and fluorouracil or gemcitabine improves overall survival [23-25]. In addition, the combination of gemcitabine with fluorouracil given as continuous infusion and radiation therapy has also shown an increase in overall survival with a median survival of 20 to 22 months [2, 26]. Hence, the use of gemcitabine alone or gemcitabine in combination with fluorouracil-based chemoradiation postoperatively can be acknowledged as the standard of care for the management of early-stage pancreatic cancer. Interestingly, the emerging use of preoperative (neoadjuvant) gemcitabine-based chemoradiation treatment has also been demonstrated to be at least as effective as postoperative (adjuvant) treatment in patients with resectable pancreatic cancer [27].

As the disease progresses to becoming locally and systemically advanced, tumor is no longer resectable. In these cases, treatment is palliative with median overall survival ranges from 9 to 10 months. Treatment options diverge from chemotherapy alone to combination of treatment with chemoradiation therapy and chemotherapy. Data from several randomized trials has established that chemoradiation therapy is better than radiation therapy alone [28, 29]. Gemcitabine, a genotoxic drug has been the treatment of choice for pancreatic cancer particularly

for patients with nonresectable pancreatic cancer. It is the current standard therapy that is known to extend survival by a matter of weeks [30]. Several new agents have been tested in combination with gemcitabine in clinical trials but with no betterment in outcome [13, 31, 32]. The only agent, used in combination with gemcitabine that has shown a small yet significant increase in survival among patients diagnosed with advanced pancreatic cancer is erlotinib though this combination has more toxicity when compared with erlotinib alone [33]. To date, the treatment approach in acceptance for patients with advanced pancreatic cancer is either gemcitabine administration alone or gemcitabine in combination with a platinum agent, erlotinib, or a fluropyrimidine [32, 33].

Newer approach through the use of phytochemicals, the naturally occurring, plant-based substances is of the current research interest in the hope to combat pancreatic cancer. Garcinol, a bioactive agent from plant is one of the phytochemicals that has drawn researchers' attention for its anticancer properties, both as therapeutics and chemoprevention.

1.3 Garcinol

Garcinol is a yellow crystalline polyisoprenylated benzophenone derivative, extracted from *Garcinia indica* fruit rind. Also popularly known as *Kokum* or *Mangosteen*, it can be largely found in tropical regions of Asia and Africa. Characterized by its sweet and sour taste, this fruit has long been incorporated in cooking, enjoyed as snack and served as a refreshing drink. It is also traditionally used in the treatment of some illnesses such as heat strokes and infections [34].

The chemical structure of Garcinol ($C_{38}H_{50}O_6$; molecular weight 602), also called Camboginol (Figure 1) was defined using NMR and IR spectroscopy back in 1980 by Rao and coworkers [35]. It shares a structure similar with curcumin, a compound known to possess

antioxidant, antiaging and anticarcinogenic properties by means of containing both phenolic hydroxyl groups and the β -diketone moiety [35]. Some other known properties of garcinol include antiulcer, antitumorigenic, antiobesity, anti-inflammatory, antibacterial and antiviral which associated with its isomer isogarcinol [36].

1.3.1 Anticancer Properties of Garcinol

The potential of garcinol as chemopreventative and therapeutic agent for different types of cancer have been reported by a number of studies conducted recently. The mechanistic targets of the anticancer activity of Garcinol is depicted as Figure 2 [37].

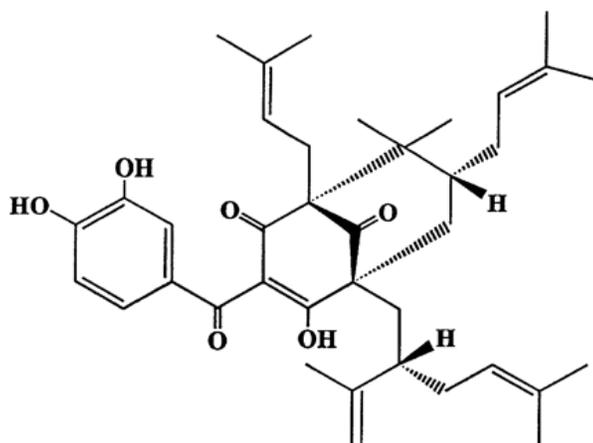


Fig. 1 Chemical structure of garcinol [38]

Its effect on oxidative and inflammatory pathways has been investigated both *in vitro* and *in vivo*. A colon cancer study on F344 rats conducted in Tanaka lab showed a significant reduction in a dose-dependent manner on the formation of colon cancer precursors known as azoxymethane-(AOM)-induced colonic aberrant crypt foci (ACF) following of 0.01% and 0.05% dietary garcinol intervention. Garcinol has also been noted to inhibit O₂⁻, iNOS, Nitric Oxide

(NO) and COX2 and interestingly found to act more potently than the green tea polyphenol, epigallocatechin gallate (EGCG) in the cell lines studied [38]. In another in vivo study, ingestion of dietary garcinol by rats exposed with 4-nitroquinoline 1-oxide (4-NQO) to induce tongue lesions has also shown to lower the expression of COX2 as compared to control diet [39]. Similar effect of inflammatory response was also observed in macrophage cell lines, by which the expression of both NF- κ B and COX2 have been down-regulated following garcinol treatment [40].

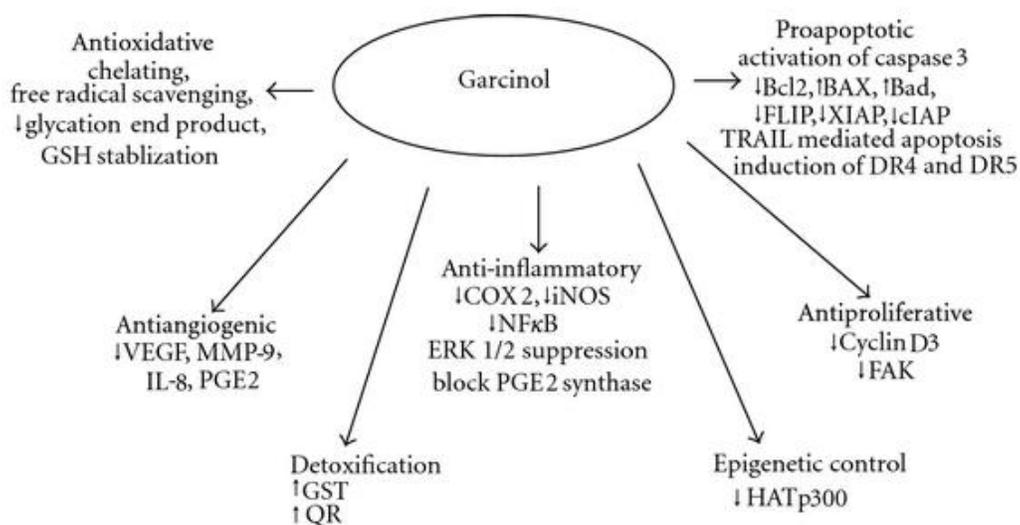


Fig. 2 Mechanistic targets of garcinol anticancer activity [37]

Apart from that, garcinol has also been documented as being potent in halting cancerous growth by targeting apoptosis, programmed cell death of cancer cells. In a human leukemia cell study by Pan and coworkers, garcinol induced apoptosis at a greater rate than curcumin through activation of caspase-3 mediated by release of cytochrome c into the cytoplasm of the cell from

mitochondria. The increase in apoptosis was also accompanied with down-regulation of an anti-apoptotic protein BCL2 together with significant up-regulation of two pro-apoptotic proteins, Bad and Bax [41]. In another cancer cell study, garcinol also exhibited a higher potency of growth inhibition against the colon cancer cells as compared to normal immortalized intestinal cell line with an increase in caspase-3 activity, indicative of apoptotic pathway activation [42].

Additionally, several *in vitro* studies on garcinol suggested its promising role as an antiangiogenic and anti-metastatic agent [43-45]. Garcinol studies in pancreatic cancer cell lines conducted in our laboratory have demonstrated down-regulation of several markers of angiogenesis and metastasis namely MMP9, IL8, PGE2 and VEGF with garcinol treatment [45]. At the present time, garcinol has not been reported to show any toxic effects and was proven by previously conducted study as safe to be given up to 0.05% in diet orally [38]. In the discovery of new anticancer agents, post-treatment gene expression is one of the tools often used to screen the potential of candidate compounds in halting cancer progression. In this study, we investigated the effect of garcinol alone and in combination with gemcitabine for potential therapeutic effect in pancreatic cancer mouse model.

1.4 Quantitative Real-Time PCR-Based Analysis of Gene Expression

A gene that encodes a protein is determined to be expressed in a cell or group of cells when its transcribed messenger RNA (mRNA) or the resulting protein product is detected. Most commonly, gene expression is being measured for comparative purposes to compare the mRNA levels of one or several genes in cells from different sources. These comparisons include studies on cancer versus normal cells, cells from an organ in genetically modified organism versus normal organism and also intervention studies to test a new drug or compound.

Gene expression can be measured by many techniques. One of the techniques is quantitative real-time polymerase chain reaction (qPCR) which enables the continuous monitoring of the amplification process as it occurs and uses fluorescent reporter dyes to merge the amplification and detection steps. The assay is based on measuring the increase in fluorescent signal, which corresponds to the amount of DNA produced during each PCR cycle.

A single PCR reaction is characterized by the PCR cycle at which fluorescence first rises above threshold background levels called threshold cycle (C_T). Hence, higher the messenger RNA (mRNA) concentration of a target gene, lower is the C_T value. The process of qPCR consists of three steps: (1) reverse transcriptase-based conversion of RNA to complimentary DNA (cDNA), (2) the amplification of cDNA by PCR (through repeated cycle of denaturation, annealing and DNA fragment synthesis), and (3) the detection and quantification of amplified products-referred as amplicons [46].

Results from a real-time PCR experiment can be calculated either using absolute or relative quantification. In absolute quantification using the Standard Curve Method, unknowns are being quantitated based on a known quantity in which the concentration of quantified serially diluted DNA sample is plotted against each dilution's C_T . The C_T value of an unknown can then be used to extrapolate a value from the standard curve. In relative quantification, the aim is to analyze changes in gene expression in a given sample relative to another reference sample such as an untreated control sample. This type of quantification is useful for comparing the level of gene expression of a particular gene of interest in response to a treatment. The most common method for relative quantitation is the $2^{-\Delta\Delta C_T}$ method which compares the C_T value of one target gene to another for example, an internal control (most commonly housekeeping gene such as β -

actin and GAPDH) in a single sample [47]. In this study, we investigated the effect of garcinol on the gene expression of genes known to be up-regulated in pancreatic cancer.

1.5 Selected Genes of Interest in Pancreatic Cancer Progression

1.5.1 Matrix metalloproteinase-9 (MMP9)

MMP9 is a type of matrix metalloproteinases (MMPs). Also called matrixins, these are zinc-dependent enzymes that are involved in the degradation of extracellular matrix components. MMPs play a central role in cell proliferation, invasion and metastasis of cancer cells. MMP9 from the family is constantly up-regulated in many types of cancer including pancreatic cancer.

1.5.2 Cyclin D1 (CCND1)

CCND1 is a member of the highly conserved cyclin family. Cyclins function as cyclin-dependent kinases subunit regulators forming a complex with CDK4 or CDK6, whose activity is required for cell cycle G1/S transition. Overexpression of CCND1 increases proliferation by accelerating the cell transit through the G1 phase.

1.5.3 B-cell lymphoma-2 (BCL2)

BCL2 is an anti-apoptosis protein found mainly at the cytoplasmic face of the mitochondrial outer membrane and other membranes such as endoplasmic reticulum membrane and the nuclear envelope. BCL2 prevents the initiation of protease cascade leading to apoptosis by directly or indirectly blocking the release of cytochrome c from the mitochondria which results in the delay of cell death [48]. Its high expression is found in various human tumors [49].

1.5.4 Notch1

Notch signaling is involved in the cellular developmental pathway including proliferation and apoptosis. Notch genes encode proteins which are activated upon interaction with its family ligands. Five ligands that have been found in mammals are Dll-1, Dll-3, Dll-4, Jagged-1 and

Jagged-1[50]. On activation, Notch is cleaved and intracellular Notch is released and shifted into the nucleus. The intracellular Notch associates transcriptional factors which play role in regulating the expression of target genes. Dysfunction of intracellular Notch is associated with tumorigenesis through prevention of cell differentiation. Notch1 is one of the four vertebrate Notch genes that have been identified: Notch1, Notch2, Notch3 and Notch4. Notch1 has been reported to cross-talk with another major cell growth and apoptotic pathway such as nuclear factor κ B (NF- κ B) and its high expression has been noted to inhibit apoptosis [51].

Hypothesis

Based on literature and *in vitro* findings in our laboratory, we hypothesized that progression of pancreatic cancer in mouse model would lead to an increase expression of MMP9, CCND1, BCL2 and also Notch1. Moreover treatment with dietary garcinol should cause a down-regulation of the above described genes in order for it to be potentially beneficial. This was shown to be true in earlier studies from our lab in pancreatic cancer cell lines. However, the *in vitro* findings need to be validated in an *in vivo* animal model of pancreatic cancer before translating the effects to a clinical level. A good animal model should be in very close resemblance to the human condition. One of the best, well-validated and clinically relevant models of PDAC to be used for *in vivo* studies is the $Kras^{LSL.G12D/+}; p53^{R172H/+}; PdxCre^{tg/+}$ or KPC transgenic mouse model.

1.6 KPC Transgenic Mouse Model of Pancreatic Cancer

The $Kras^{LSL.G12D/+}; p53^{R172H/+}; PdxCre^{tg/+}$ or KPC transgenic mouse model is generated by crossing mice with a conditional activated Kras allele ($Kras^{LSL.G12D}$) to transgenic strain that expresses Cre recombinase in pancreatic lineages ($PdxCre^{tg}$) and generating a conditionally express mutant allele of the Li-Fraumeni human ortholog,, $p53^{R172H}$. Activation of

both the $Kras^{LSL.G12D}$ and the $p53^{R172H}$ alleles occurs in tissue progenitor cells of the developing mouse pancreas through interbreeding with PdxCretg transgenic animals. KPC mice developed advanced PDAC at an early age and resembled many aspects of pancreatic cancer in human. This model developed the full range of PanIN lesion that ultimately progresses to manifest carcinoma with hundred percent penetrance. Metastases to the liver and lungs are also observed in majority of the KPC mice which is similar to what is commonly observed in humans. Furthermore, the KPC model also developed associated comorbidities to the human pancreatic cancer such as cachexia, jaundice and ascites [30, 52].

To verify our *in vitro* findings, we have conducted an *in vivo* study which aimed to investigate the effects of garcinol in pancreatic cancer mouse model. This thesis focuses on the change in gene expression observed due to dietary garcinol treatment. The following specific aims were proposed to meet the hypothesis that the effect of garcinol as a therapeutic agent in pancreatic cancer will be evident in gene expression differences of selected genes from the garcinol treated groups compared to the non-treated group.

Specific aim I: To investigate the gene expression response of the pancreatic tissue following garcinol treatment using quantitative real-time polymerase chain reaction (qPCR) technique.

Specific aim II: To examine the extent of gene expression response in the distant metastasis site following garcinol treatment of the liver tissue using quantitative real-time polymerase chain reaction (qPCR) technique.

Chapter 2

Methodology

2.1 Animals

Forty-two male, KPC (KrasLSL.^{G12D}/+; p53^{R172H}/+; PdxCretg/+) transgenic mice aged between 6-8 weeks old, obtained from Van Andel Institute (Grand Rapids, MI) were used in this study. Thirty-two of them developed pancreatic cancer by KPC mutation and ten mice with non-mutated KPC from the same litter served as control.

2.2 Housing and husbandry

All animals were housed in individual cage at Wayne State University Division of Laboratory Animal Resources (DLAR) facility under standard conditions as approved by the Wayne State University Animal Investigation Committee (AIC). All of them were kept in the same room with alternating 12 hours light alternating with 12 hours darkness under normal humidity and at room temperature. Cage bedding, diets and water were replaced weekly and their health were monitored regularly.

2.3 Experimental protocol

Upon arrival at the facility, all animals were allowed to acclimatize for one week prior to start of the experiment. Following acclimatization period, animals were randomly distributed into 4 and 2 experimental groups respectively and were subjected to different diet-treatment combination as shown in Figure 3. The KPC groups, each had similar number of animal (n=8) included the control, isocaloric diet-non treated group (KC), Garcinol diet-non treated group (KGr), isocaloric diet-gemcitabine treated group (KGm) and garcinol diet-gemcitabine treated group (KGG). The non-KPC groups received no chemotherapy drug treatment, also had equal

number of animal in each group (n=5) with one group was fed with isocaloric diet (CC) and the other was fed with garcinol diet (CGr).

2.4 Experimental diets

Three groups (KGr, KGG,CGr) were given special diet containing 0.05% Garcinol while 3 other groups (KC,KGm,CC) received standard purified diet that is isocaloric to the Garcinol diet. Garcinol for this study was purchased from Enzo Life Sciences (Plymouth Meeting, PA). Both the garcinol diet and the standard diet were formulated and produced by Dyets Inc. (Bethlehem, PA). Composition of both diets is shown in Table 1.

2.5 Experimental procedures

Experimental procedures carried out for this study as summarized in Table 2 are based on the overall project to explore the anti-cancer effects of Garcinol in pancreatic cancer mouse model. My report will describe only a portion of the main study, investigating gene expression.

All mice were provided with their respective diets for 6 weeks and had free access to water. Body weight, diet and water intake were recorded twice weekly throughout the study duration. Chemotherapy injections from 100mg/5ml Gemcitabine stock were delivered to KGm and KGG groups (5ul/g body weight) once-weekly (Week 1-5) while groups with no chemotherapy treatment were given placebo injections of saline (0.85% NaCl). Urine was collected once a week on weeks 2, 4 and 6 for urinary metabolomic profiling. To confirm the presence of pancreatic cancer and for tumor progression monitoring purpose, ultrasound was done on week 3 and 6, while MRI was done in week 1 and 5. Upon completion of the experiment at Week 6, each animal was anesthetized with the combination of 80 mg/ml Ketamine and 20mg/ml Xylazine (5ul/g body weight) and euthanized by exsanguination and major organs removal prior to blood and tissue collection. Pancreatic and liver tissues were flash-frozen in

liquid nitrogen then stored at -80°C until ready to be used for gene expression analysis (by qPCR and miRNA microarray) and histological analysis.

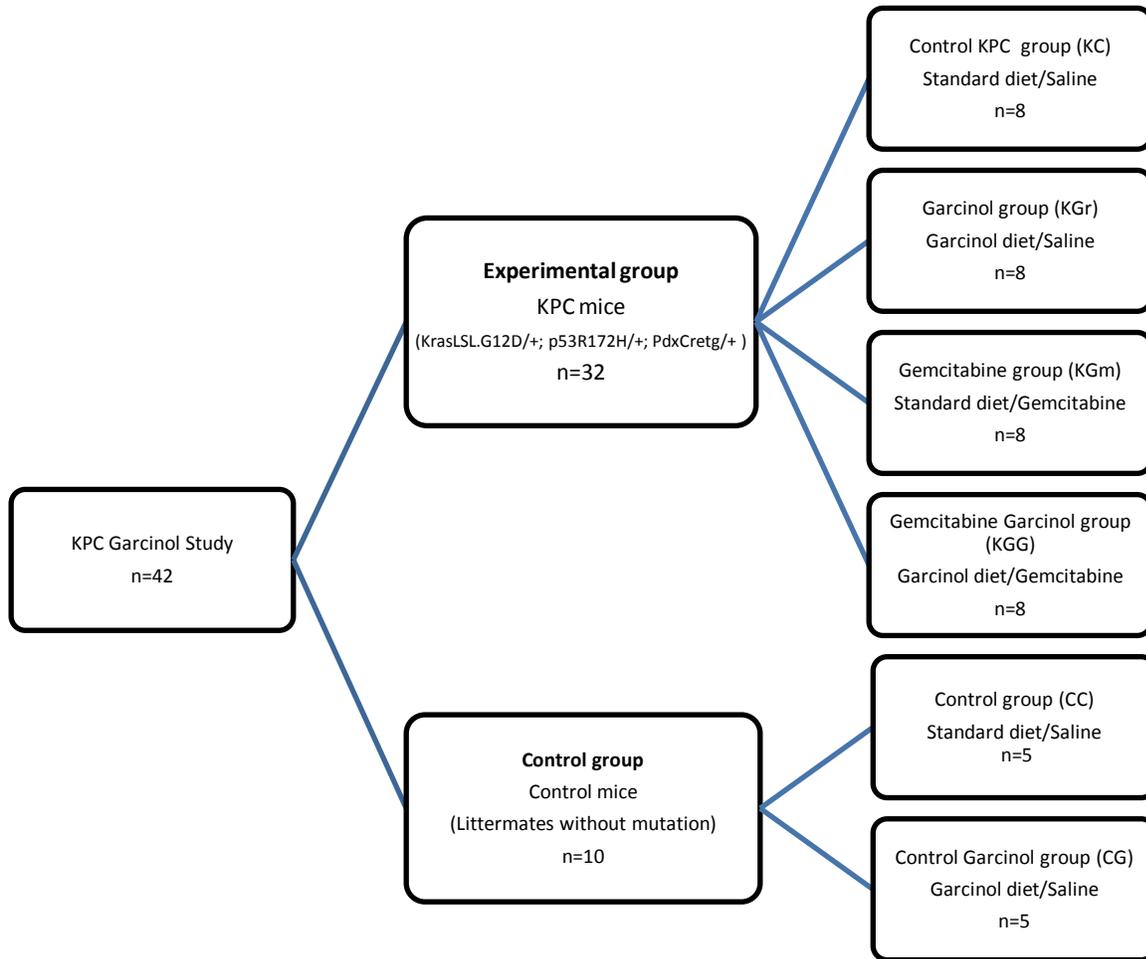


Fig. 3 Study design

Experimental groups

KC: Pancreatic cancer mouse, standard purified diet + no drug treatment.

KGr: Pancreatic cancer mouse, 0.05% garcinol diet + no drug treatment.

KGm: Pancreatic cancer mouse, standard purified diet + gemcitabine drug treatment.

KGG: Pancreatic cancer mouse, 0.05% garcinol diet + gemcitabine drug treatment.

Control groups

CC: Control mouse, standard purified diet.

CGr: Control mouse, 0.05% garcinol diet.

0.05% Garcinol added Diet				Regular Isocaloric Diet			
Ingredient	kcal/g	grams/ kg	kcal/kg	Ingredient	kcal/g	grams/ kg	kcal/kg
Casein	3.58	200	716	Casein, High Nitrogen	3.58	200	716
L-Cystein	4	3	12	L-Cystein	4	3	12
Sucrose	4	100	400	Sucrose	4	100	400
Cornstarch	3.6	396.986	1429.1496	Cornstarch	3.6	397.486	1430.9496
Dyetrose	3.8	132	501.6	Dyetrose	3.8	132	501.6
Soybean Oil	9	70	630	Soybean Oil	9	70	630
t-Butyl hydroquinone	0	0.014	0	t-Butyl hydroquinone	0	0.014	0
Cellulose	0	50	0	Cellulose	0	50	0
Mineral Mix #210025	0.88	35	30.8	Mineral Mix #210025	0.88	35	30.8
Vitamin Mix #310025	3.87	10	38.7	Vitamin Mix #310025	3.87	10	38.7
Choline Bitartrate	0	2.5	0	Choline Bitartrate	0	2.5	0
Garcinol	0	0.5	0				
			1000.00				3758.2496
							1000.00
							3760.0496

Table 1: Experimental Diets Composition

Procedures	Frequency of Measurement
Body weight, diet intake, water intake	Twice weekly (Week 1-6)
Gemcitabine/saline injection	Once weekly (Week 1-5)
MRI	Week 1, 5
Ultrasound	Week 3, 6
Urine collection	Once weekly (Week 2,4,6)
Blood and tissue collection	End of study (Week 6)

Table 2: Study timeline based on the overall study

2.6 RNA Extraction

Four pancreatic samples and five liver samples from each group were taken as representative in the gene expression analysis. Total RNA extraction was performed with a commercial kit (RNeasy Mini Kit, Qiagen Valencia, CA, USA) according to the manufacturer's instructions. First, an approximate weight of 30mg of frozen liver and pancreatic tissues were excised and placed into 700 uL QIAzol Lysis Reagent in a suitable vessel for disruption and homogenization using tissue homogenizer until sample was uniformly homogenous. Tube containing homogenate was then placed at room temperature (15-25°C) for 5 minutes. 140 uL of chloroform was then added and tube was capped securely followed by vigorous shaking for 15 seconds. The tube was then placed at room temperature for 3 minutes prior to centrifugation for 15 minutes at 12,000 rcf at 4°C. Following centrifugation, the upper aqueous phase was transferred to a new collection tube. 525 uL of 100% ethanol was added and mixed thoroughly by pipetting. Next, 700 uL of sample including any precipitate was pipetted into RNeasy Mini spin column in 2 ml collection tube and centrifuged at 10,000 rpm for 15 seconds. The flow-through was discarded and the same step was repeated for the remainder of sample. 700 uL of Buffer RWT was then added to RNeasy Mini spin column, centrifuged at 10,000 rpm for 15

seconds and flow-through was discarded. 500 uL of Buffer RPE was then pipetted onto RNeasy Mini spin column and again centrifuged at 10,000 rpm for 15 seconds. The flow-through was discarded and another 500 uL of Buffer RPE was added to RNeasy Mini column, centrifuged at 10,000 rpm for 2 minutes. RNeasy Mini spin column was then placed into a new 2 ml collection tube and centrifuged at full speed for 1 minute. The old collection tube was discarded with the flow through. Finally, the RNeasy Mini spin column was transferred to a new 1.5 ml collection tube, 40uL of RNase-free water was pipetted directly on the RNeasy Mini spin column membrane and centrifuged for 1 minute at 10,000 rpm to elute the RNA. Quantity measurement and spectrophotometric quality assessment ($A_{260/280}$ and $A_{260/230}$ ratios) of RNA were then carried out using the Nanodrop spectrophotometer.

2.7 cDNA Preparation

Reverse transcription for liver RNA was performed using High Capacity RNA to cDNA Master Mix kit (Applied Biosystems, Carlsbad, CA). 20 uL of RT buffer mix, 2 uL of RT enzyme mix, 8 uL of RNA sample (equal concentrations of 1000 ng/uL for all samples) and 10 uL of nuclease-free water were mixed into 0.2 mL PCR tube and centrifuged for few seconds. Prepared samples were then loaded into Eppendorf mastercycler realplex 4 (Eppendorf, Hauppauge, NY) for reverse transcription process with the following temperature setting; 25°C for 5 minutes, 42°C for 30 minutes, 85°C for 5 minutes and kept at 4°C until retrieve for immediate use for qRT-PCR analysis or transferred to a -20°C freezer.

Reverse transcription for pancreatic RNA was performed using miScript II RT Kit (Qiagen GmbH, Hilden, Germany). The reverse transcription master mix consisted of 4 uL 5x miScript HiFlex Buffer, 2 uL miScript Nucleics Mix, 2 uL miScript Reverse Transcriptase Mix and 4 uL RNase-free water prepared in 0.2 mL PCR tube, gently mixed and stored on ice. Next,

8 uL template RNA (equal concentrations of 1000 ng/ul for all samples) was added to each tube containing reverse transcription master mix, gently mixed and briefly centrifuged. All prepared tubes were then loaded into Eppendorf mastercycler realplex 4 (Eppendorf, Hauppauge, NY) for reverse transcription process, incubated at the following temperatures; 37°C for 60 minutes, 95°C for 5 minutes and kept at 4°C until retrieve for immediate use for qRT-PCR analysis or transferred to a -20°C freezer.

2.8 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

qPCR was performed as part of gene expression analysis. Four genes which involved in tumor progression were tested in pancreatic samples (CyclinD1, MMP9, BCL2, Notch1) and three genes were tested in liver samples (CyclinD1, MMP9, BCL2). The primer sequence of these genes are listed in Table 3. The final reaction volume of 25 uL consisted of 12.5 uL SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), 1 uL of 20 uM reverse and forward primer mixture, 9.5 uL nuclease-free water and 2 uL of cDNA (equal concentrations of 10ng/ul for all samples). qRT-PCR was carried out on the Eppendorf mastercycler realplex 4 instrument (Eppendorf, Hauppauge, NY) in Mx3000P 96-Well Plates (Agilent Technologies) with the following program; initial denaturing: 95°C for 10 minutes, 45 repeats of denaturing: 95°C (15 seconds) and elongation: 60°C (1 minute), dissociation curve: 95°C for 1 minute, 60°C for 30 seconds followed by gradual temperature increase from 60°C to 95°C in 20 minutes and finally at 95°C for 30 seconds. Each gene was analyzed in triplicate with single NTC. mRNA expression levels in the samples were calculated relative to control, isocaloric diet-no chemotherapy group (KC) using the comparative C_T method: $\Delta\Delta C_T = \Delta C_T$ sample - ΔC_T control, fold change = $2^{-\Delta\Delta C_T}$. β -actin was used to normalized the expression values (ΔC_T).

2.9 Data Analysis

A fold change cut-off of more than 2 was used in determining any significant change of gene expression relative to control.

Primer's Name	Forward Sequence	Reverse Sequence
CyclinD1	5'-CCTCCAGAGGGCTGT CGGCGCAGTAGCAGA- 3'	5'-TCTTACCTCCAGCAT CCAGGTGGCCACGAT-3'
MMP9	5'- GCTCCTGGTCTCCTG GCTT-3'	5'-GTCCCACTTGAGGC CTTTGA-3'
BCl ₂	5'-CCTGTGGATGACTGA GTACC-3'	5'-GAGACAGCCAGGAG AAATCA-3'
Notch-1	5'-CACTGTGGGCGGGT CC-3'	5'-GTTGTATTGGTTCGGC ACCAT-3'
β-Actin	5'-ACCAACTGGGACGA CATGGAGAAG-3'	5'-TACGACCAGAGGCAT ACAGGGACT-3'

Table 3: Primer sequence of the studied genes

Chapter 3

Results

The body weight, diet intake, MRI and histological data from our overall study are summarized in Table 4. In this study, we have confirmed the presence of pancreatic tumor in the experimental groups by ultrasound, MRI and histology. The 0.05% dosage of garcinol added in diet has shown to be well tolerated in this study with 100% survival rate was reported in non-pancreatic cancer group. The data of the results mentioned above will be presented elsewhere. The data on change in gene expression due to garcinol intervention, the focus of my study is presented here.

Data	Observation
Body weight	No significant difference between experimental groups
Diet intake	No significant difference between all groups
MRI	Reduction in number and tumor size in garcinol treated groups (KGr and KGG)
Histology	Significant reduction in total PanIN count in garcinol treated groups (KGr and KGG) Less number of PanIN-3 in garcinol treated groups (KGr and KGG)
miRNA microarray	miRNA related to pancreatic cancer were identified and found to be regulated in favorable manner

Table 4: Body weight, diet intake, MRI and histological data from the overall study

Specific aim I: To investigate the gene expression response of the pancreatic tissue following garcinol treatment using quantitative real-time polymerase chain reaction (qPCR) technique.

3.1 Gene Expression Analysis of Pancreatic Tissues

KPC (Kras^{LSL.G12D/+}; p53^{R172H/+}; PdxCre^{tg/+}) transgenic mouse develop tumor in the pancreas. Data from experimental group (KC, KGr, KGm and KGG) are presented. Gene expression levels are presented relative to the non-treated pancreatic cancer group (KC).

Cyclin D1 (CCND1)

As can be seen from Figure 4, the relative pancreatic mRNA expression of CCND1 for all three treatments significantly decreased compared to the non-treated cancer group with garcinol and garcinol-gemcitabine combination exhibiting more profound reduction. Garcinol significantly reduced CCND1 expression to much lower level compared to gemcitabine. Likewise, garcinol-gemcitabine combination also significantly lowered CCND1 expression than gemcitabine alone.

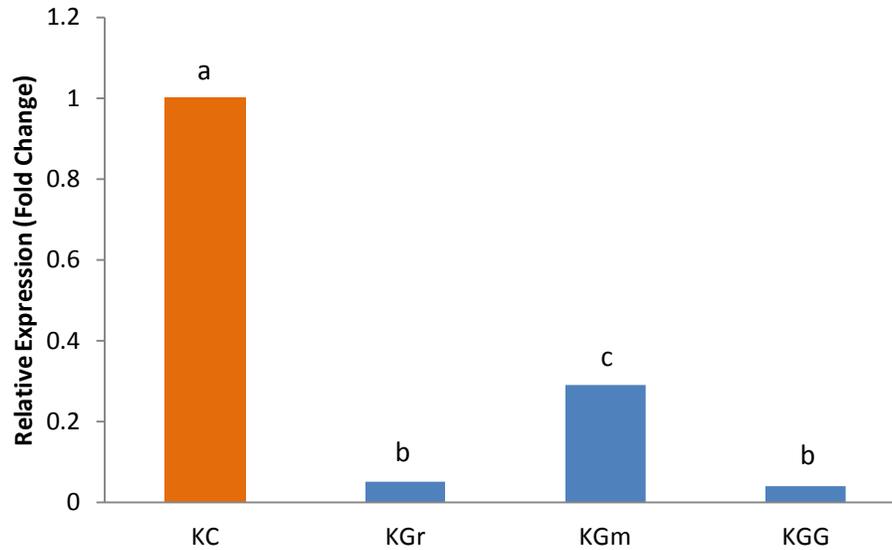


Fig. 4 Relative pancreatic mRNA expression of CCND1. The expression is presented as fold change with respect to KC. ^a is significantly different from ^b and ^c. ^b is significantly different from ^c.

Experimental groups

KC: Pancreatic cancer mouse, standard purified diet + no drug treatment.

KGr: Pancreatic cancer mouse, 0.05% garcinol diet + no drug treatment.

KGm: Pancreatic cancer mouse, standard purified diet + gemcitabine drug treatment.

KGG: Pancreatic cancer mouse, 0.05% garcinol diet + gemcitabine drug treatment.

Matrix metalloproteinase-9 (MMP9)

Figure 5 shows the relative pancreatic mRNA expression of MMP9. Treatment with garcinol, gemcitabine and garcinol-gemcitabine combination significantly decreased the mRNA level of MMP9 compared to the non-treated group. The down-regulation was most significantly pronounced with garcinol-gemcitabine combination. Garcinol significantly reduced MMP9 expression at much lower level compared to gemcitabine and also garcinol-gemcitabine combination too had significantly decreased MMP9 expression more compared to gemcitabine. Moreover, this combination decreased the expression of MMP9 more than garcinol alone at a significant level.

Notch-1

All three treatments with garcinol, gemcitabine and garcinol-gemcitabine had significantly down-regulated Notch1 compared to the non-treated cancer group (Figure 6). Garcinol lowered the Notch1 expression most significantly among the three. Garcinol significantly reduced Notch1 expression at much lower level compared to gemcitabine. The combination of garcinol-gemcitabine had significantly decreased Notch1 expression more than gemcitabine and garcinol treatment alone.

B-cell lymphoma-2 (BCL2)

The relative pancreatic mRNA expression of BCL2 is presented in Figure 7. It can be seen that garcinol slightly decreased the expression of BCL2. However this down-regulation was not significant. Gemcitabine and garcinol-gemcitabine combination increased BCL2 expression level compared to both no treatment and garcinol also at a non-significant level.

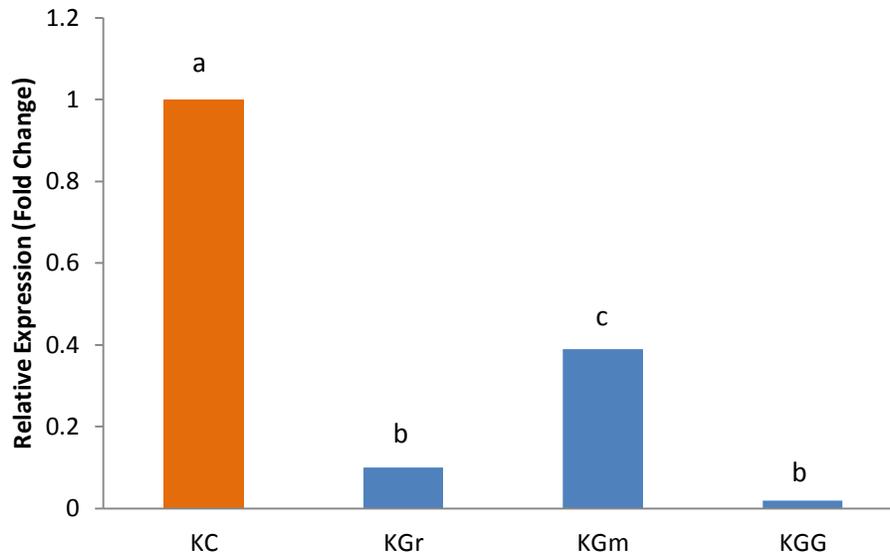


Fig. 5 Relative pancreatic mRNA expression of MMP9. The expression is presented as fold change with respect to KC. ^a is significantly different from ^b and ^c. ^b is significantly different from ^c.

Experimental groups

KC: Pancreatic cancer mouse, standard purified diet + no drug treatment.

KGr: Pancreatic cancer mouse, 0.05% garcinol diet + no drug treatment.

KGm: Pancreatic cancer mouse, standard purified diet + gemcitabine drug treatment.

KGG: Pancreatic cancer mouse, 0.05% garcinol diet + gemcitabine drug treatment.

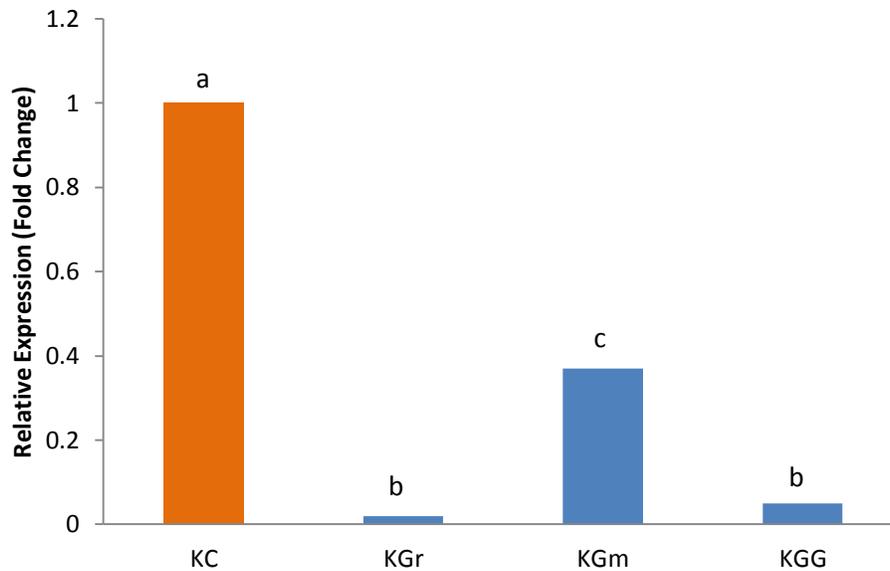


Fig. 6 Relative pancreatic mRNA expression of NOTCH1. The expression is presented as fold change with respect to KC. ^a is significantly different from ^b and ^c. ^b is significantly different from ^c.

Experimental groups

KC: Pancreatic cancer mouse, standard purified diet + no drug treatment.

KGr: Pancreatic cancer mouse, 0.05% garcinol diet + no drug treatment.

KGm: Pancreatic cancer mouse, standard purified diet + gemcitabine drug treatment.

KGG: Pancreatic cancer mouse, 0.05% garcinol diet + gemcitabine drug treatment.

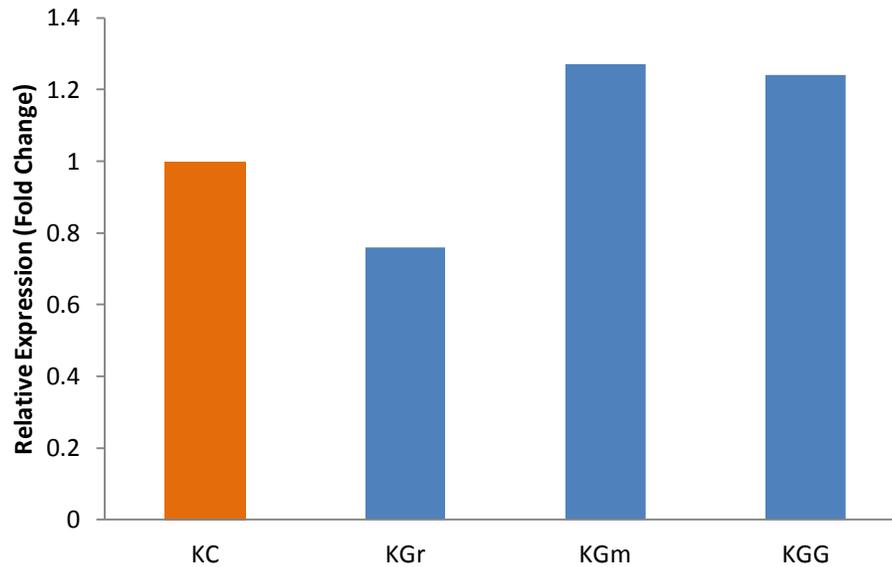


Fig. 7 Relative pancreatic mRNA expression of BCL2. The expression is presented as fold change with respect to KC. There is no significant difference in mRNA expression levels of each group in comparison with KC.

Experimental groups

KC: Pancreatic cancer mouse, standard purified diet + no drug treatment.

KGr: Pancreatic cancer mouse, 0.05% garcinol diet + no drug treatment.

KGm: Pancreatic cancer mouse, standard purified diet + gemcitabine drug treatment.

KGG: Pancreatic cancer mouse, 0.05% garcinol diet + gemcitabine drug treatment.

Specific aim II: To examine the extent of gene expression response in the distant metastasis site following garcinol treatment of the liver tissue using quantitative real-time polymerase chain reaction (qPCR) technique.

3.2 Gene Expression Analysis of Liver Tissues

Liver is one of the first organs for the primary tumor in the pancreas to be metastasized. Data from experimental group (KC, KGr, KGm, KGG) is presented. Gene expression levels are presented relative to the non-treated pancreatic cancer group (KC).

Cyclin D1 (CCND1)

Figure 8 shows the relative liver mRNA expression of CCND1. In the experimental groups, the expression of CCND1 was significantly decreased with garcinol treatment, gemcitabine treatment and garcinol-gemcitabine treatment combination with the later (garcinol-gemcitabine) showing the most down-regulation of CCND1 expression compared to no treatment. It can be seen that garcinol-gemcitabine combination decreased CCND1 expression more than garcinol and gemcitabine treatment alone but the decrease in expression was not significant, as compared to either treatment alone.

Matrix metalloproteinase-9 (MMP9)

As shown in Figure 9, the relative liver mRNA expression of MMP9 in the experimental groups was significantly decreased with garcinol treatment, and garcinol-gemcitabine treatment combination. However, down-regulation of MMP9 following Gemcitabine treatment was at a non-significant level. Garcinol and garcinol-gemcitabine combination decreased the MMP9 expression at a lower, non-significant levels compared to gemcitabine.

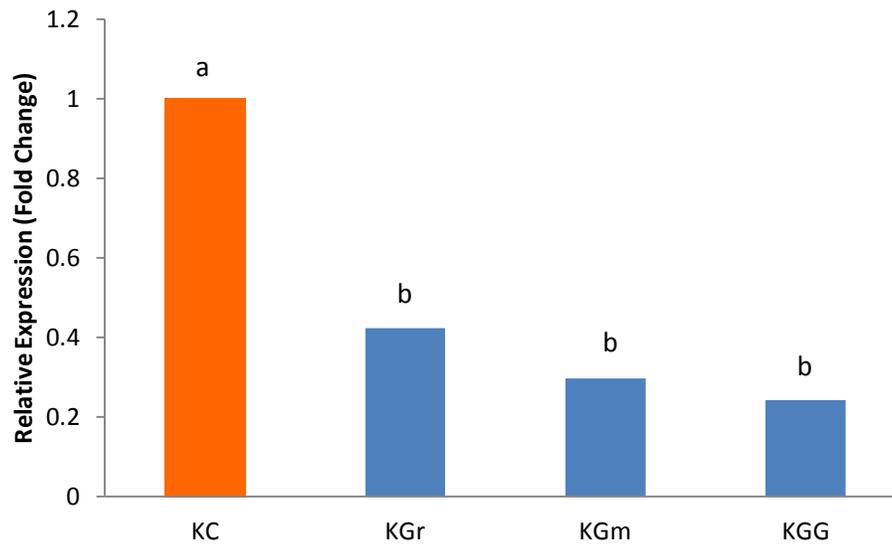


Fig. 8 Relative liver mRNA expression of CCND1. The expression is presented as fold change with respect to KC. ^a is significantly different from ^b.

Experimental groups

KC: Pancreatic cancer mouse, standard purified diet + no drug treatment.

KGr: Pancreatic cancer mouse, 0.05% garcinol diet + no drug treatment.

KGm: Pancreatic cancer mouse, standard purified diet + gemcitabine drug treatment.

KGG: Pancreatic cancer mouse, 0.05% garcinol diet + gemcitabine drug treatment.

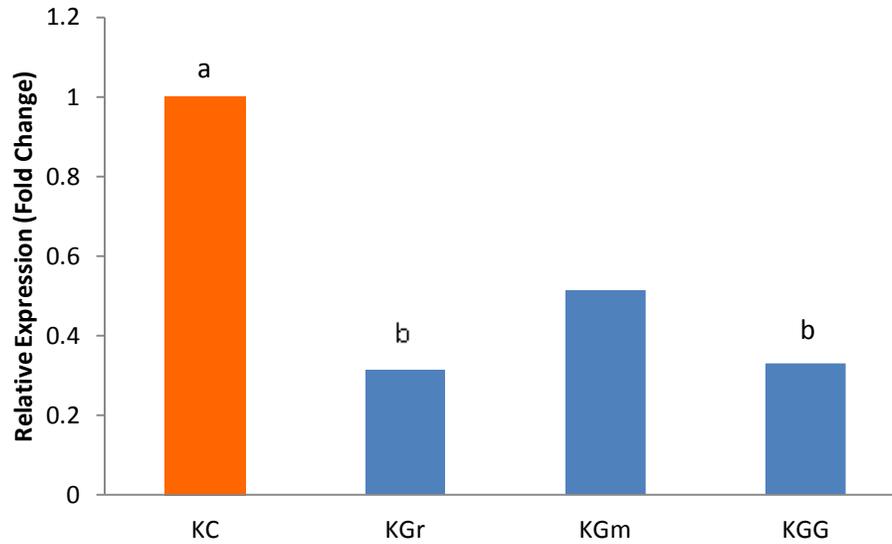


Fig. 9 Relative liver mRNA expression of MMP9. The expression is presented as fold change with respect to KC. ^a is significantly different from ^b. There is no significant difference in mRNA expression levels of KGm in comparison with KC, KGr and KGG.

Experimental groups

KC: Pancreatic cancer mouse, standard purified diet + no drug treatment.

KGr: Pancreatic cancer mouse, 0.05% garcinol diet + no drug treatment.

KGm: Pancreatic cancer mouse, standard purified diet + gemcitabine drug treatment.

KGG: Pancreatic cancer mouse, 0.05% garcinol diet + gemcitabine drug treatment.

B-cell lymphoma-2 (BCL2)

It can be seen from Figure 10 that both gemcitabine and garcinol-gemcitabine combination treatment of the experimental group significantly decreased the expression of BCL2 with the combined treatment being more pronounced. Garcinol also exhibited a reduction in BCL2 level but the decrease was not significant.

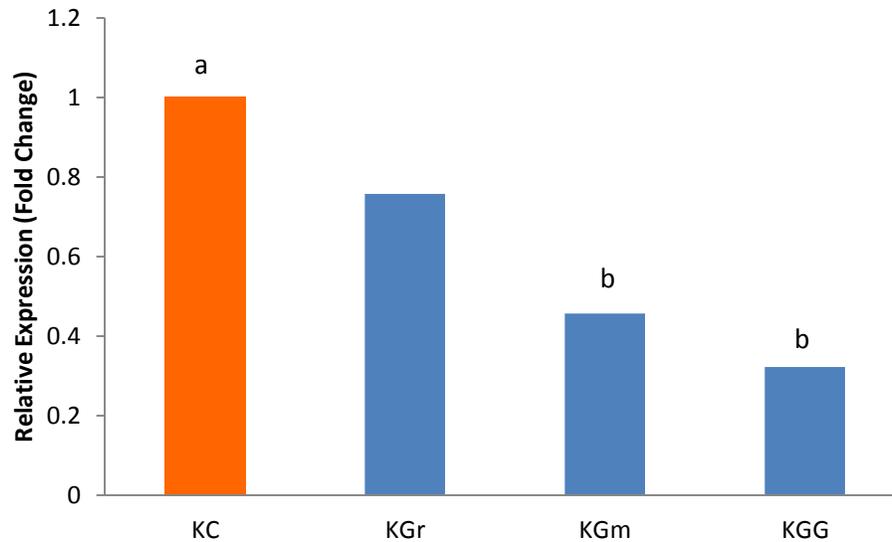


Fig. 10 Relative liver mRNA expression of BCL2. The expression is presented as fold change with respect to KC. ^a is significantly different from ^b. There is no significant difference in mRNA expression level of KGr in comparison with KC.

Experimental groups

KC: Pancreatic cancer mouse, standard purified diet + no drug treatment.

KGr: Pancreatic cancer mouse, 0.05% garcinol diet + no drug treatment.

KGm: Pancreatic cancer mouse, standard purified diet + gemcitabine drug treatment.

KGG: Pancreatic cancer mouse, 0.05% garcinol diet + gemcitabine drug treatment.

Chapter 4

Discussion

Pancreatic cancer is currently ranked as the fourth leading cause of cancer-related deaths in The United States with high mortality rate majorly contributed by late diagnosis, early metastasis of tumors and poor response to treatments, making it so important to develop new approaches in order to improve the prognosis of this lethal disease. Most chemotherapeutic treatments for pancreatic cancer are at a disadvantage of having adverse toxic reactions. Thus, development of effective preventative and/or therapeutic treatments for pancreatic cancer that are relatively nontoxic from single agents or combination with an established drug treatment is warranted. This thesis reports the potential anticancer effect of garcinol, a compound extracted from *Garcinia indica* fruit rind which may have promise for treating this lethal malignancy. Using KPC mouse-model for pancreatic cancer, this study has evaluated the mRNA expression response of selected genes in pancreatic tumorigenesis following garcinol treatment alone, gemcitabine treatment alone and also garcinol-gemcitabine combination relative to non-treated group using quantitative real-time polymerase chain reaction (qPCR) technique.

In this study, we report the potential of garcinol for its future use as a chemotherapeutic agent for pancreatic cancer. Figure 11 summarizes the *in vivo* anticancer activity of garcinol in pancreatic cancer as observed from this study. We report herein that garcinol caused a significant down-regulation of three out of four genes tested which could possibly halt the tumor progression via inhibition of proliferation, angiogenesis and metastasis and via increment in apoptosis activity. Interestingly, this study also report the similar observation with garcinol-gemcitabine treatment combination. These results show that garcinol may have potential in

pancreatic cancer chemoprevention not only limited by its use as a single treatment agent but may provide additive effect when use in combination with gemcitabine.

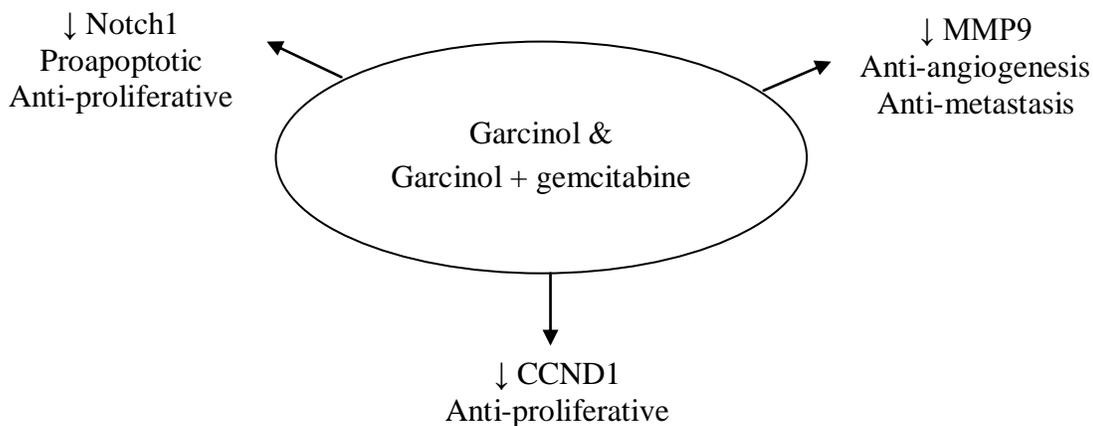


Fig. 11 *In vivo* anticancer activity of Garcinol in pancreatic cancer

The cyclin D1 (CCND1) is frequently over expressed in pancreatic ductal adenocarcinoma. Cyclins function as cyclin-dependent kinases subunit regulators forming a complex with CDK4 or CDK6, whose activity is required for cell cycle G1/S transition. Overexpression of CCND1 increases proliferation by accelerating the cell transit through the G1 phase. Moreover, there is also present evidence that CCND1 plays a role in tumor cell migration [53]. In this study, we observed that both garcinol and garcinol-gemcitabine treatment significantly reduced the expression of CCND1 thus, suggesting the anti-proliferative property of garcinol by its ability to arrest cell cycle.

Matrix metalloproteinases (MMPs) also called matrixins are zinc-dependent enzymes known to be the agent to degrade extracellular matrix components. Its role in tumor metastasis, invasion, angiogenesis and metastasis is well established. This current study demonstrates that garcinol down-regulated the level of pancreatic MMP9 relative to untreated group significantly. Moreover, it was also observed that garcinol and gemcitabine displayed synergistic effect in inhibiting MMP9 when compared to single treatment of garcinol or gemcitabine. Similar observation was obtained for liver samples. This finding accords the earlier *in vitro* observation which showed that garcinol causes inhibition of MMP9 activity [45]. These results provide further support for the hypothesis that garcinol is capable of arresting metastasis and cell invasion by interrupting MMP9 activity.

Apoptosis induction is one of the most important aspects in evaluating anticancer properties of treated animals. BCL2 is as an anti-apoptotic protein known to be overexpressed in pancreatic cancer [49] and prevents the initiation of protease caspase leading to apoptosis by directly or indirectly blocking the release of cytochrome c from the mitochondria which results in the delay of cell death [48]. It was observed from this study that garcinol slightly down-regulated the expression of BCL2 in both pancreatic and liver samples at a non-significant level. Contrary to expectations, garcinol-gemcitabine combination increased pancreatic BCL2 expression compared to both no treatment and garcinol also at a non-significant level. The opposite observation was obtained in liver samples in which combination of garcinol-gemcitabine treatment showed an additive effect resulted in a significant reduction of BCL2 expression. It is well accepted that p53 mutation leads to activation of BCL2 in pancreatic cancer. Thus, we speculate that this difference in observation is due to the animal model used in this research. The KPC mouse model selected for this study has conditional mutations in K-ras

and p53 genes. The activation of the mutant p53 in this model occurs exclusively in tissue progenitor cells of the developing mouse pancreas which leads to the activation of BCL2 in the pancreas. This high activation of BCL2 in the pancreas might provide the possible explanation on why was the significant down-regulation of BCL2 with garcinol-gemcitabine treatment observed only in liver sample but not in pancreatic sample.

The Notch signaling pathway is a fundamental signaling system used by neighboring cells to communicate with each other in order to assume their proper developmental role. Notch signaling is involved in the cellular developmental pathway including proliferation and apoptosis. Notch1 has been reported to cross-talk with another major cell growth and apoptotic pathway such as nuclear factor κ B (NF- κ B) and its high expression has been noted to inhibit apoptosis [51]. It has also been reported in the study by Wang et al., that down-regulation of Notch1 inhibited cell growth and induced apoptosis in pancreatic cancer cells [51]. However, very little was found in the literature on the question of whether garcinol is capable of down-regulating Notch1 in pancreatic cancer. Our observations highlight the therapeutic benefit of garcinol by a significant down-regulation of Notch1 level compared to the non-treated group. It was observed that the combination of garcinol-gemcitabine effectively down-regulated Notch1 more than gemcitabine and garcinol treatment alone. From these earlier findings it is likely that the potential inhibition of cell growth and apoptosis induction by garcinol treatment in pancreatic cancer is achieved through down-regulation of Notch1.

Moreover, the findings on garcinol and its synergistic effect with gemcitabine in the down-regulation of several genes as discussed above are also supported by the data from other experiments conducted in our overall study, which will be reported elsewhere. Our MRI data showed that garcinol had effectively reduced the number and size of tumor and a greater effect

was observed in the garcinol-gemcitabine treated group. From our histological observation, we found that garcinol alone and garcinol-gemcitabine combination significantly reduced the number of all PanINs (PanIN-1, PanIN-2 and PanIN-3). It was also observed that PanIN-3 lesion count in garcinol treated groups (KGr and KGG) was lowered compared to the untreated group. These histological findings are indicative of garcinol's potential in prolonging the onset of adenocarcinoma and in halting its progression.

In conclusion, despite the need for further investigation, garcinol exhibits anti-proliferative, proapoptotic, anti-angiogenesis and anti-metastatic properties which is evident by the significant down-regulation of CCND1, MMP9 and Notch1. The findings in this study add to a growing body of literature on anticancer effects of garcinol and contributes to additional evidence that suggests its chemotherapeutic effect on pancreatic cancer previously shown in *in vitro* setting is extended into animal model. Interestingly, we also showed that the promising pancreatic cancer chemotherapeutic effect of garcinol is not only limited by its use as a single treatment agent. Garcinol may also provide additive effect when use in combination with gemcitabine suggesting its potential use as a natural adjuvant in pancreatic cancer treatment regimens. Further investigation and experimentation on garcinol's anticancer effect in pancreatic cancer animal models investigating many other mechanistic targets is strongly recommended.

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ABSTRACT**EFFECT OF GARCINOL ON CHANGE IN GENE EXPRESSION IN PANCREATIC
CANCER MOUSE MODEL**

by

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Pancreatic cancer is currently ranked as the fourth leading cause of cancer-related deaths in The United States making it so important to develop new approaches in order to improve the prognosis of this lethal disease. Chemotherapy drugs as treatment for pancreatic cancer are known to have adverse toxic reactions. Thus, development of treatments for pancreatic cancer that are relatively nontoxic is warranted. This thesis reports the potential anticancer effect of garcinol, a compound extracted from *Garcinia indica* fruit rind which may have promise for treating this lethal malignancy. Using KPC mouse-model for pancreatic cancer, this study has evaluated the mRNA expression response of selected genes in pancreatic tumorigenesis following garcinol treatment using quantitative real-time polymerase chain reaction (qPCR) technique. We concluded from this study that garcinol and its combination with gemcitabine exhibit anti-proliferative, proapoptotic, anti-angiogenesis and anti-metastatic properties which is evident by the significant down-regulation of CCND1, MMP9 and Notch1 in both pancreatic and liver tissues. The findings in this study contribute to additional evidence that suggests its chemotherapeutic effect on pancreatic cancer previously shown in *in vitro* setting is extended into animal model.

AUTOBIOGRAPHICAL STATEMENT

Nurul Huda Razalli was born in Kuala Lumpur and received her early education in Malaysian public schools. In 2009, Ms Razalli earned a bachelor's degree with honors in Dietetics from Universiti Kebangsaan Malaysia and began her career in the field of dietetics as a foodservice dietitian. In 2010, Ms Razalli made a career shift into academia and was appointed as a faculty member in the Dietetic Program at Universiti Kebangsaan Malaysia. Ms Razalli is currently receiving the sponsorship from the Ministry of Higher Education of Malaysia to get her Master's degree and she is also being enrolled in the Dietetic program seeking for the Registered Dietitian (RD) credential. Upon completion of her masters, Ms Razalli will continue her studies in the Department of Nutrition and Food Science, Wayne State University and wishes to return back to Malaysia with a PhD degree in nutrition and food science.