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DEPARTMENT OF BIOCHEMISTRY

CYTOTOXIC EFFECTS OF ROSEMARY ESSENTIAL OIL ON PANCREATIC CANCER CELLS

M.Sc. THESIS

ABDUSSALAM YAKUBU

Nicosia

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DECLARATION

I hereby declare that all information, documents, analysis and results n this thesis have been collected and presented according to the academic rules and ethical guidelines of Graduate School of Health Sciences, Near East University. I also declare that as required by these rules and conduct, I have fully cited and referenced information and data that are not original to this study.

Abdussalam Yakubu

…../…../2021

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Abdussalam Yakubu

Cytotoxic Effects of Rosemary Essential Oil on Proliferation of Pancreatic Cancer Cells

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ABSTRACT

Objective: Rosemary essential oil is a yellowish or colorless liquid, which is characterized with a camphor smell. The aim of this research is to study the cytotoxic effects of rosemary oil on cell proliferation in pancreatic cancer cells.

Materials and Methods: Human pancreatic cancer cell line PANC-1 (ATCC: CRL-1469) was utilized. MTT assay was utilized to carry out the cytotoxic analysis. DMSO was used to prepare rosemary oil solutions and diluted in culture medium using 5 distinct concentrations (100, 200, 300, 400, 500, and 600 µg/ml).

Results: In this study, different concentration and incubation periods were compared. Cell viability was higher at 100 µg/ml for 24 hours when compared to 600 µg/ml for 48 hours. Also, cell viability at 200 µg/ml was significantly higher when compared to 600 µg/ml for 48 hours. Different concentrations were also assessed during 48 hours incubation period with large difference between 100 µg/ml and 600 µg/ml observed. It was also observed that 500 µg/ml concentration was the most effective in reducing PANC-1 cell proliferation more than any other concentration for a duration of 48 hours. An inverted microscope was used to study the morphological changes in the PANC-1 cells. The PANC-1 cells possess an epithelial morphology and cultured with monolayer adhere feature. After treatment with rosemary essential oil, the shapes of the cells were still epitheloid. However, reduction in the size of PANC-1 was observed.

Conclusions: Components of the rosemary essential oil such as limonene, β-caryophyllene, eugenol and α-Bisaolol were able to control cancer cell development via different mechanisms.

Key Words: Rosemary oil, pancreas, cancer, cell line, cytotoxicity

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Abbreviations

ATP: Adenosine Triphosphate

BMI: Body Mass Index

CA: Carnosic Acid

CT: Computer Tomography

DM: Diabetes Mellitus

DNA: Deoxyribonucleic Acid

EUS: Endoscopic Ultrasonography

FGF: Fibroblast Growth Factor

GLUT: Glucose Transporter

HIF: Hypoxia Inducible Factor

IMN: Intraductal Papillary Neoplasm

LATS: Large Tumor Suppressor

LDH: Lactate Dehydrogenase

MCT: Monocarboxylate Transporters

MIC: Macrophage Inhibitory Cytokine

MSI: Mammalian Sterile Kinase

mTOR: Mammalian Target of Rapamycin

PanIN: Pancreatic Intraepithelial Neoplasm

PDH: Pyruvate Dehydrogenase

PET: Positron Emission Tomography

PI3K: Phosphotidylinositol 3 kinase

PP: Pancreatic Polypeptide

PPP: Pentose Phosphate Pathway

RA: Rosemarinic Acid

RE: Rosemary Extract

SDH: Succinate Dehyrogenase

TKL: Transketolase Isoform

YAP: Yes Associated Protein

CHAPTER I

Introduction

Pancreatic cancer is presumed to be among the deadliest forms of cancers. This type of cancer is rated tenth in the list of the frequent forms of cancers, with a significantly elevated occurrence frequency in females compared to males. This form of cancer is considered amongst the most harmful forms of cancers with only 20% surviving for only a single year and not more than 5% for a maximum of 5 years chance of survival. As a result of its low survival rate, this type of cancer is rated fourth in the list of most prevalent cause of cancer death in many countries that are developed (*German et al., 2011*).

In the field of medicine, discovering the antidote for cancer is amongst the most chased fields of research. Statistics have revealed that from the year 2012 to 2035, the number of cases related to cancer will rise from 14 million to 24 million. Fatalities as a result of cancer is expected to rise from 8.2 million to 14.6 million per annum (*Stewart et al., 2016*).

As a result, treatments for cancer are massively being discovered, tested and applied. Different methods that are that are generally applied to treat patients suffering from cancer include: radiation, chemotherapy and surgery.

However, the techniques applied in cancer treatments are not only limited to experimenting with drugs that are synthesized. In the last couple of years, usage of different pants and products obtained naturally has been given significant attention.

Rosemary plant is a perpetual shrub found in the mediterranean area, which belongs to the botanical family Lamiaceae. It generally grows in Asia, Africa and Europe. Rosemary plant can attain a height of about 2m and the optimum weather for its growth is the hot climate, with the sun promoting its growth, however rosemary plant cannot grow in terrible winter conditions *(Porte and Godoy, 2001, Beninca et al., 2011)*.

Rosemary is among the products obtained naturally which has gotten substantial recognition as of recent as a possible anti-cancer agent that can be utilized in the for treating and possibly preventing different forms of cancers.

Aim

This research is aimed to study the cytotoxic effects of rosemary oil on cell proliferation in pancreatic cancer cells.

Objectives

The objectives of this study are:

- To extract essential oil from dried rosemary leaves using hydro distillation method
- To determine the cytotoxic effects of rosemary essential oil on pancreatic cancer cells

Hypotheses

- Rosemary oil has inhibitory properties on cell proliferation in pancreatic cancer cells.
- Rosemary oil has a reducing effect on cell growth in pancreatic cancer cells.
- Rosemary oil has potential anti-cancer effect on pancreatic cancer cells.

Although this study attempts to determine the cytotoxic effects of rosemary essential oil on pancreatic cancer cells, further studies need to be carried to fully determine the specific component of the oil extract responsible for the desired effect.

CHAPTER II

Literature Review

Pancreas

Pancreas is a crucial organ which has both exocrine and endocrine functions. Structurally, the pancreas contains two organs namely; the endocrine and exocrine glands. Components of the exocrine gland include the duct and acinar cells that produce enzymes involved digestion and sodium bicarbonate which functions as a biological buffer. The main purpose of the exocrine part of the pancreas is to produce enzymes involved in digestion which ultimately aids the digestion foods and finally absorption of the nutrients found in the food into the biological system. Alternatively, the exocrine part comprises of 5 forms of islet cells that produce peptide hormones which are utilized in the maintenance of optimum glucose levels in the biological system. Paracrine, neuorine and intracrine systematically influence the secretory functions of the pancreas. Therefore, improper alteration of the pathways controlling the pancreas detailed regulatory mechanism poses a serious health risk. (*Beger, 2008*).

The human pancreas is an organ located on the upper part of the abdomen which measures around 15cm in length and weighs around 150g. Around 1kg of pancreatic juice is produced daily which is secreted into the duodenum through the ampulla of vater where the main pancreatic duct connects with the common bile duct.

As for the anatomy of the pancreas, it is attached to different organs of the abdomen which includes the colon, duodenum and spleen duodenum. The pancreas structurally is partitioned into three segments namely; the head, body and tail. The head portion is moderately flat and situated within the duodenum's first loop. The tail portion located closely to spleen's hilum, which is the only portion of the pancreas that possess the pancreatic polypeptide cells which are capable of manufacturing the peptide hormone.

Although the pancreas's exocrine and endocrine functions are different, they have functions which are physiologically interrelated. Pancreas can be considered as four different parts structurally: the exocrine portion which generally constitutes the

duct and acinar cells. The endocrine portion of the pancreas which is where the islet cells are situated, extracellular space as well as the blood vessels. The exocrine portion of the pancreas which arguably accounts for the larger portion of the pancreas; functions to secrete enzymes involved in digestion together with sodium bicarbonate.

Alternatively, the endocrine portion of the pancreas constitutes the clusters of cells known as the islets of Langerhans which produce the pancreatic peptide hormones responsible for glucose homeostasis in the biological system. This endocrine portion of the pancreas is significantly vascularized and structurally distinct from the endocrine part of the pancreas. Fundamentally, these islets possess a shape that I spherical and account for around 2% volume of the entire mass of the pancreas. The islet comprises of five main cells namely; alpha, beta, delta, PP and to lesser degree epsilon cells which secrete glucagon, insulin, somatostatin, pancreatic polypeptide and ghrelin respectively (Kulkarni, 2004).

Signaling factors within and outside the pancreas control the entire secretory function of the islet cells. Communication amongst each other and affecting each other's secretion is possible between the islet cells. Mode by which the islet cells communicate include: cell to cell, humoral and neural communication. Moreover, the insulin acinar axis mediates communication between the exocrine and endocrine portion of the pancreas (Williams and Goldfine, 1985).

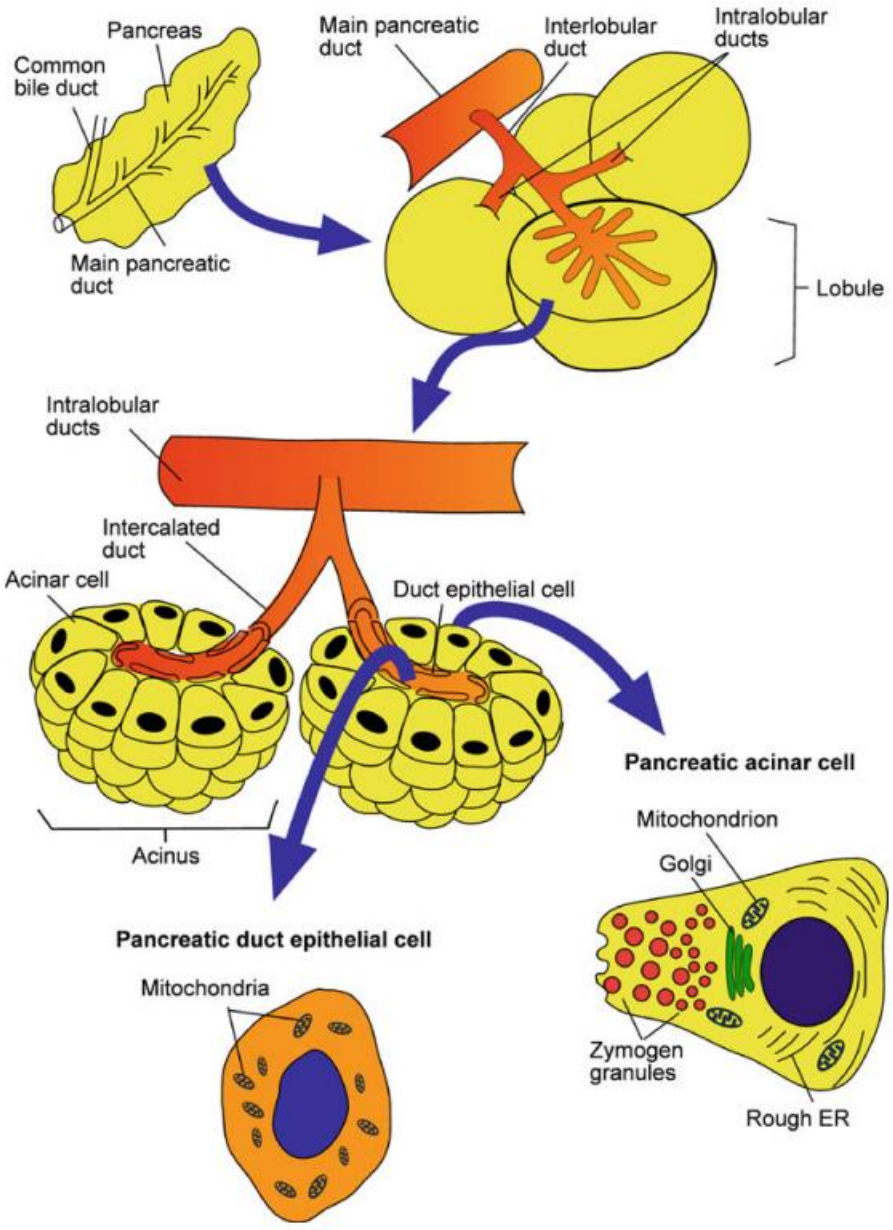

Figure 1. Structure of the Pancreas (Leung, 2006)

Pancreatic Cancer

Pancreatic cancer is presumed to be among the deadliest forms of cancers. This type of cancer is rated tenth in the latest forms of cancers, with a significantly elevated occurrence frequency in females compared to males. This form of cancer is considered amongst the most harmful forms of cancers with only 20% survival chance for only a single year and not more than 5% for a maximum of 5 years chance of survival. As a result of its low survival rate, this type of cancer is rated fourth in the list of most prevalent cause of cancer death in many countries that are developed (German et al., 2011).

As a result of its high death rate, this form of cancer is rated 4th amongst most prevalent cause of fatalities due to cancer in a significant number of countries that are developed. In a recent pancreatic cancer research where 24 different cases were carefully studied, evidence showed that there are 63 different forms of genetic alteration found in pancreatic cancer cells, comprising the 12 main signal transduction pathways. This evidence shows that the genetics of this form of cancer is heterogenous and complex (Jones et al., 2008).

Medically, the major features and indicators of cancer of the pancreas might involve abnormal weight loss, indigestion, new onset of diabetes, nausea, jaundice, back pain, and depression, which generally depends on the location of the cancer within the pancreas. Nonetheless, people suffering from this type of cancer do not generally have symptoms. During the late period of the disease jaundice and abdominal pain may be present, on the contrary other indicators that are non-specific, like abnormal loss of weight and improper digestion, might be simply misread for different illnesses. As a result, a large number of people suffering from this cancer have a tumor size of around 3cm when they are diagnosed, making it unlikely for a surgery to be successful (Ariyama et al., 1998).

Nevertheless, a few studies indicated that subjects having a tumor with a diameter of about 2cm, whether they had a surgery, possess a close survival rate of around 5 years to those whose pancreatic cancer is advanced. This is due to the spread of the cancer to areas in the body such as lymphatic vessels, lymph nodes,

nerves, blood vessels and different areas around the pancreas itself. In the case pancreatic cancer, prompt identification and diagnosis are very important to achieve the best clinical outcome (Ariyama et al., 1998).

Risk Factors

Significant risk factors for developing pancreatic cancer include the following: excessive body weight, old age, excessive smoking, excessive alcohol consumption, scaring of the pancreas, history of pancreas in the family, history of radiotherapy on the abdomen. Excessive smoking poses a high risk as studies have shown that people that smoke are twice as likely to develop this form of cancer compared to people that do not smoke. A recent research method known as the fingerprint has shown that mutations are more likely to occur in the cancer of pancreas for people that smoke compared cancer of the pancreas in people that do not smoke. Prolonged diabetes mellitus is related to elevated cancer of the pancreas. Furthermore, sudden emergence of diabetes mellitus might be the first symptom of pancreatic cancer. People with excessive body weight are at a higher risk of developing pancreatic cancer. Excessive alcohol intake also poses a higher risk of developing this form of cancer compared little consumption. Scaring on the pancreas also poses a higher risk of developing pancreatic cancer compared to a normal pancreas (*Wolfgang et al., 2013*).

Genetic Causes of Pancreatic Cancer

There are several genetic factors that can play a significant role in pancreatic cancer. A small proportion of this form of cancer can occur as a result of mutation of genes. A typical example of a mutated of gene is the Oncogene; which promotes cancer development. Abnormalities in genes involved in cell cycle apoptosis generally promotes the progression and spread of cancer. Furthermore, normal pancreatic cells can become cancerous due to instabilities in genome, telomerase becoming shortened and cell turnover (*Fang et al., 2013*).

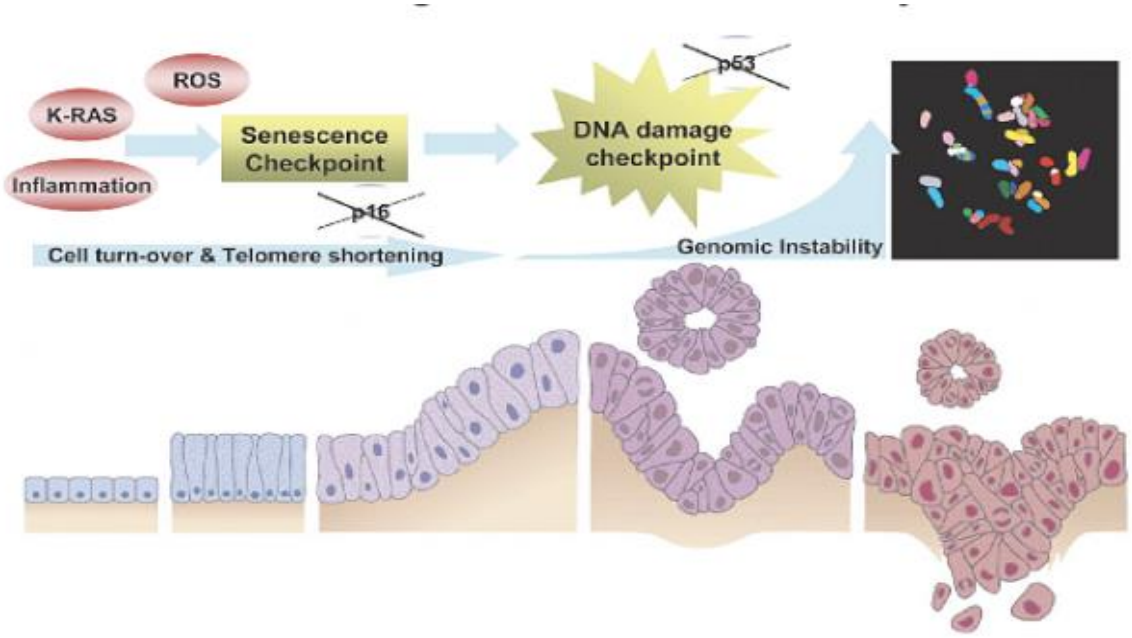

Figure 2. Genomic instabilities as a result of pancreatic cancer (*Fang et al., 2013)*

Precursor Conditions

Pancreatic cancer has precursor conditions which are as follows:

PANIN (Pancreatic Intraepithelial Neoplasm)

IPMN (Intraductal Papillary Neoplasm)

MCN (Mucinous Cystic Neoplasm)

The stem cells of pancreatic cancer are speculated to be the genesis of the above-named precursors.

PANIN: These are microscopic epithelial neoplasms which are considered non-invasive. They are found around the duct of the pancreas which are further categorized into 3 types namely: PanIN-1 2 and 3.

Mutations are generally involved with PANIN, having a high occurrence rate in adults and is normally found on the pancreas's head. This is normally attached with chronic pancreatitis and invasive carcinoma, with endoscopic ultrasonography (EUS) as an effective diagnostic tool (*Gnoni et al., 2013*).

IPMN: This is mostly small with no symptoms. It progresses at a very slow rate and is predominant in smokers. It might be found along with FAP or with familial pancreatic carcinoma. This is further categorized into 2 subgroups namely: main duct and branch duct type. This comprises of about 2% 0f the entire exocrine tumors on the pancreas and over 20% of the entire cystic tumors. This mostly forms from the major duct of the pancreas and discharges mucin.

A recent publication by the WHO in 2010 classified IPMNs by malignant transformation properties as low, intermediate, high grade dysplasia and by invasive cancer characteristics. They can be categorized by mucin antibody staining properties by making use of immunohistochemical dyes into four distinct subtypes namely: gastric, intestinal, pancreatobiliary and oncocytic with the intestinal type is the most prevalent one. It is usually located at the head of the pancreas, around the ampulla of Vater, at the inlet of the pancreatic duct (*Gnoni et al., 2013*).

MCN: This is mostly solitary and might differ in size but can reach a size of around 30cm. MCN is rare cases might contain mucin but it is generally made up of fibrotic wall. This is generally considered unusual, mostly found in women with an average of 45 years. Its diagnosis is mostly incidental and, in most cases, has no symptoms. Due to lack of symptoms, it is mostly diagnosed accidentally. Although some minor symptoms such as pain on the back, abnormal loss of weight and nausea can be observed. Tail and are usually its location. When observed on a smaller scale, 3 subtypes can be seen according to the severity of dysplasia namely: severe, moderate and mild MCN. On a larger scale, MCN is categorized into three subtypes namely: multilocular, unilocular and solitary. They normally grow at a slow rate and sometimes bring about stomach upset and might be misdiagnosed when screening abdominal mass. Pain on the back, abnormal weight loss, nausea and regurgitation are some of the symptoms. Also, the

chance of survival lies between 20 to 60% for a duration of about 5 years (Gnoni et al., 2013).

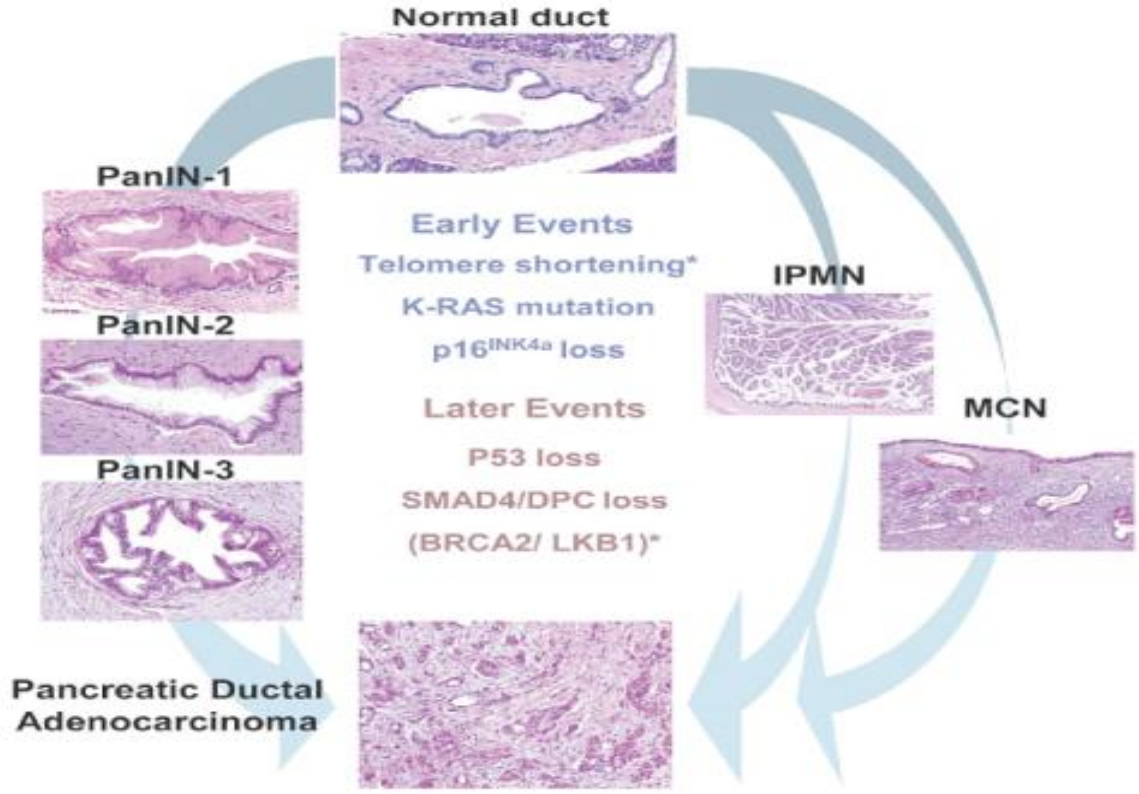

Figure 3. Regular duct of the pancreas progressing to Pancreatic Adenocarcinoma (*Gnoni* et al*., 2013*).

Diagnostic Methods in Pancreas Cancer

Carcinoembryonic antigen is a known tumor marker utilized when diagnosing cancer of the pancreas. A few new tumor markers have been discovered in the past couple of years. Considering the rate at which new discoveries are being made in the field of proteomics and genomics, it is only a matter of time before new tumor markers are discovered.

Pancreatic Cancer Detection Using Imaging Methods

ERCP: This is mostly used to detect cancer which is found on the pancreas's head. It gives room for diagnostic test for tumors. When carrying out this procedure, fluid from the pancreas can be extracted and used to run more tests such as testing growth factor binding protein.

Another imaging procedure that is advised to be carried out is MRCP which. It is important to get all the available details regarding the pancreatic and bile duct.

Abdominal CT: This is useful in diagnosing pancreatic cancer, providing useful information on its location and metastases.

Pancreatic Cancer Growth Mediated by Glutamine via a Kras-regulated Pathway

Cancer cells possess metabolic dependencies which separates them from normal cells. Some of these dependences include the utilization of glutamine to fuel anabolic processes (Vander et al, 2009).

Majority of cancer cells make use of the enzyme glutamate dehydrogenase in order to produce alpha-ketoglutarate from glutamate located in the mitochondria to fuel the TCA cycle. However, pancreatic cancer cells depend on a different pathway whereby aspartate s channeled to the cytoplasm where the enzyme aspartate transaminase finally converts aspartate to oxaloacetate. Eventually, the oxaloacetate produced is converted into malate and then pyruvate, to maintain the cellular redox state by elevating the NADPH/NADP+ ratio.

Pancreatic cancer cells mostly depend on the above series of reactions because genetic inhibition or glutamine deprivation in this pathway results to elevated levels of reactive oxygen species and reduction in the reduced form of glutathione. Also, removal of any component of the enzyme in this pathway results in the suppression of pancreatic cancer cell growth.

In addition, the reprogramming of glutamine metabolism is facilitated by oncogenic KRAS which is the signature genetic alteration found in pancreatic cancer cells, via repression of major enzymes in this pathway.

Furthermore, pancreatic cancer cells were discovered to be sensitive when glutamine is deprived, showing that glutamine s critical for pancreatic cancer cells development (Wise et al., 2010).

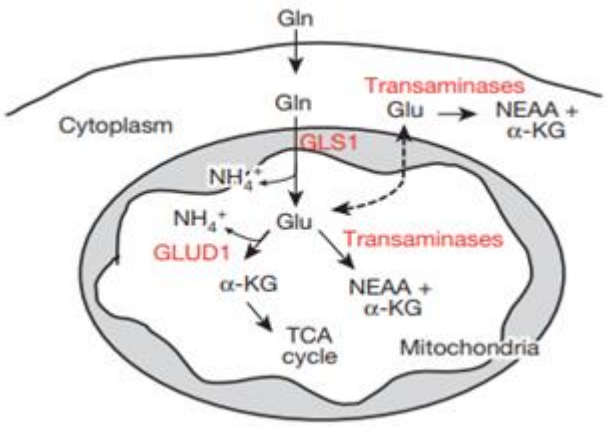

Figure 4. Pancreatic cancer cells utilize a non-canonical glutamine metabolism pathway (*Ying et al., 2012*)

Deregulation of the HIPPO Signaling Pathway Found in Cancer of the Pancreas

This signaling pathway was first found in researches carried out on Fruit fly (*Drosophila melanogaster)* (*Harvey et al., 2003*). In normal physiological conditions, this pathway controls development of tissue and normal organ development (Maugeri and De Maria, 2018).

Alteration in this signaling pathway is common in different types of cancers including cancer of the pancreas. Alteration of this pathway has been linked to prognosis in patients with cancer together with behavior of tumor cells (*Poma et al., 2018*).

This signaling pathway comprises of the mammalian sterile kinase I and 2 as well as large tumor suppressor 1 and 2. These sterile kinases (MST1 and MST2) function together with a protein called the Salvador homolog to phosphorylate and activate the Salvador homolog 1 in order to phosphorylate and activate the large tumor suppressors which are kinases.The activated large tumor suppressor in turn combine with the MOB kinase activator which serves as an adaptor and finally phosphorylate the transducers of this pathway; YES associated protein (YAP) and its transcriptional

coactivator (TAZ). These Hippo transducers via phosphorylation, are prevented from accumulating in the nucleus and blocked from interacting with transcription factors namely TEA transcriptional factor TEAD 1, 2, 3and 4. Deactivation of the regulatory complex or stimulation that activates these kinases, brings about the accumulation of these transducers in the nucleus and interactions with other transcriptional cofactors. This entire process leads to the transcription of specific genes such as the connective growth factor and cysteine rich angiogenic inducer (Maugeri and De Maria, 2018).

A recent research has shown that the YES-associated protein is overexpressed in tumor samples obtained from patients suffering from pancreatic cancer (Kapoor et al., 2014). Some of the common occurrences in pancreatic cancer include activation of KRAS mutation. This protein has also been shown to be an important partner to the KRAS that is mutated in promoting pancreatic carcinogenesis in several experimental models. The protein serves as a transcriptional regulator of the KRAS, enabling the expression of genes that bring about stromal response, cellular growth as well as development cancer (*Zhang et al., 2014*).

Evidence from a recent study has shown the YES-associated protein together with the its transcriptional coactivator (TAZ) stimulate pancreatic cancer cells to grow and spread. Evidence from a research indicated that tumor growth was drastically decreased when small interfering RNA oligonucleotides were used to target the YES-associated protein (*Diep et al., 2012*).

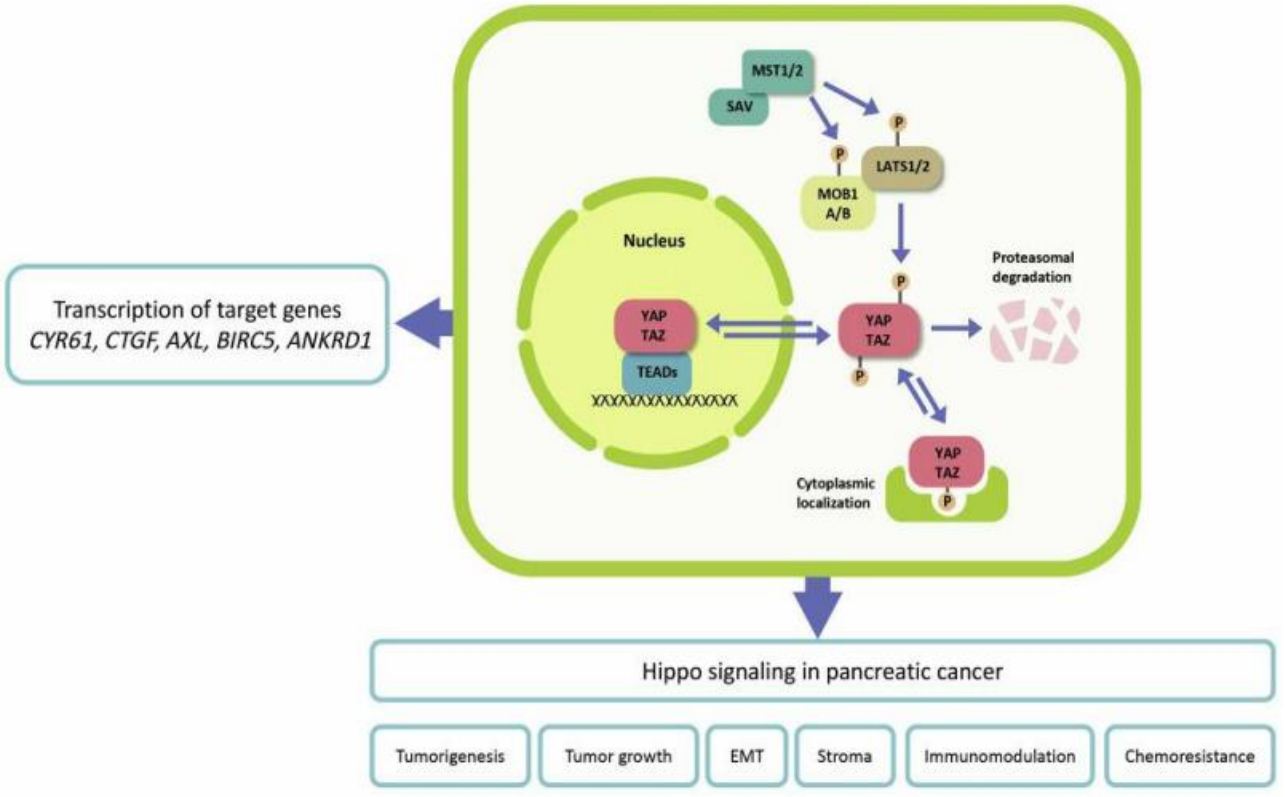

Figure 5. Hippo signaling pathway (*Ansari et al., 2016*)

The Role of FGF in Pancreatic Cancer

According to recent studies, important signaling pathways as well as genes are significantly associated with tumor growth and progression of pancreatic cancer. A few examples of these signaling pathways are the mTOR, Wnt β-catenin, Hedgehog and Phosphotidylinositol 3-kinase (*Javadinia et al., 2018*).

Other researches indicated that different signaling pathways' growth factors are involved in tumor growth and spread of pancreatic cancer, such as Fibroblast growth factor, hepatocyte growth factor, insulin-like growth factor and epidermal growth factor (*Ndlovu et al., 2018*).

Fibroblast growth factor (FGF), which is made of protein, has been shown to attach to its distinct receptors found on the membrane of the cell and to control cellular growth. Fibroblast growth factor is named as a result of its support for fibroblast proliferation and it is found in different organs. Fibroblast growth factor is also referred to as heparin conjugate growth factor due to its high affinity for heparin. At the moment, over 20 members of the FGF family have been discovered, all of which are encoded by different genes (*Turner and Grose, 2010*).

In pancreatic cancer cells, fibroblast growth factor 1 and 2 are overexpressed in pancreatic cancer cells, which can be linked to advanced stage of tumor and shorter survival (*Yamanaka et al., 1993*). In agreement with this fact, one research has showed that the expression of Fibroblast growth factor 1 and 2 expression together with their receptors were significantly elevated in pancreatic cancer cells compared with pancreatic cells that are normal (*Vickers et al., 1999*).

Furthermore, elevated levels of FGF and FGFR were linked to protein tyrosine nitration in pancreatic cancer tissues, foreseeing the possible relationship of oxidant stress with Fibroblast growth factor pathway-mediated pancreatic cancer development (*Vickers et al., 1999*).

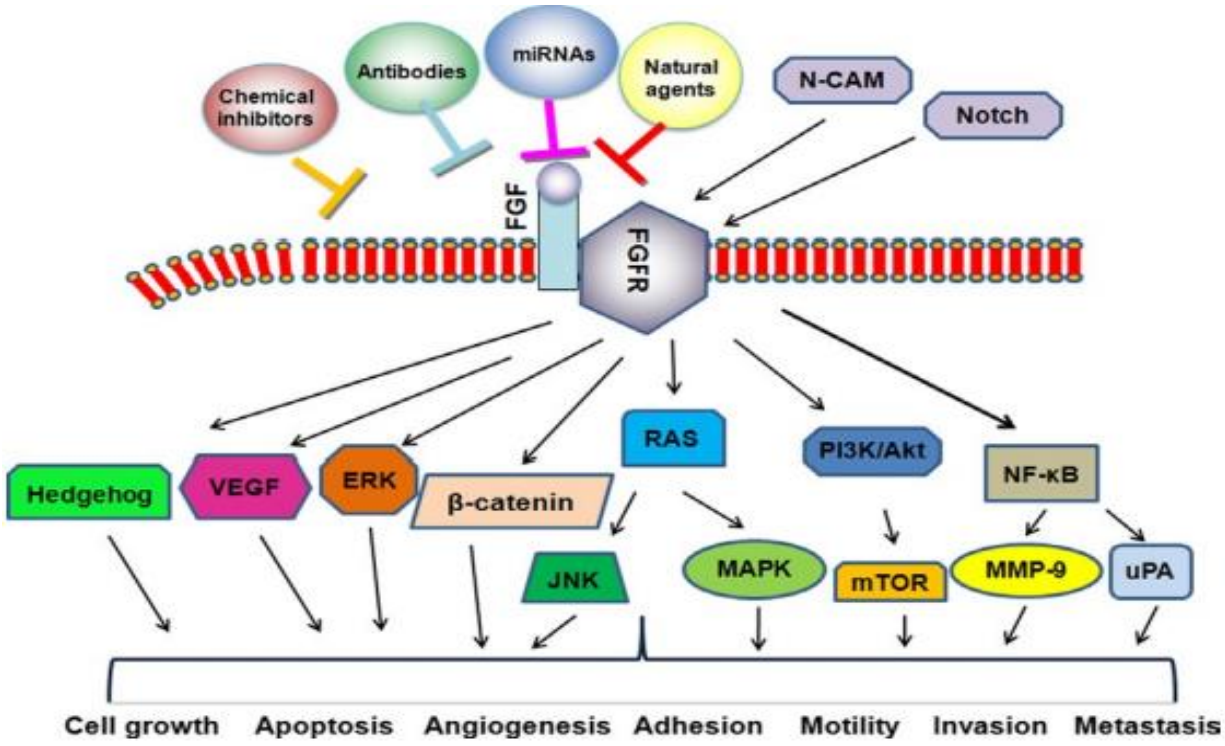

Figure 6. Role of FGF in the Development and Progression of Pancreatic Cancer (*Xang et al., 2019*).

Exploiting the Wnt/ β-catenin Pathway in the Treatment of Pancreatic Cancer

According to recent findings, this pathway, a crucial part of regular embryogenesis and organ development. It is also an important fundamental origin and an encouraging medicinal target for treating this form of cancer (*Sano et al., 2016*).

Deregulation of this pathway is found in a substantial part of people suffering from pancreatic cancer. People suffering from this form of cancer shelter a form of Mutation. This is the ring finger 43 deactivation that is present on the Wnt ligands present in pancreatic cells. This mutation brings about the alteration of ordinary pancreatic tissue to cancerous forms. (Jiang et a*l*., 2013)

According to another study, people with this form of cancer usually possess other forms of mutations on the Wnt ligands. Therefore, it is becoming clear that the onset as well as the advancement of pancreatic cancer are influenced by deregulation of this pathway (*Sano et al., 2016*).

There are existing attempts in order to discover specific, more targeted, and more fruitful methods in the treatment of cancer (Javadinia et al., 2018). The therapeutic possibilities of exploiting this pathway in the management of certain cancers has been shown (*Bahrami et al., 2017*). As of now, many therapeutic drugs have been suggested for pancreatic cancer treatment by exploiting the Wnt pathway, some of which include: OMP-54F28 and OMP-18R5. They are capable of altering this signaling pathway directly or indirectly (*Messersmith et al., 2016*).

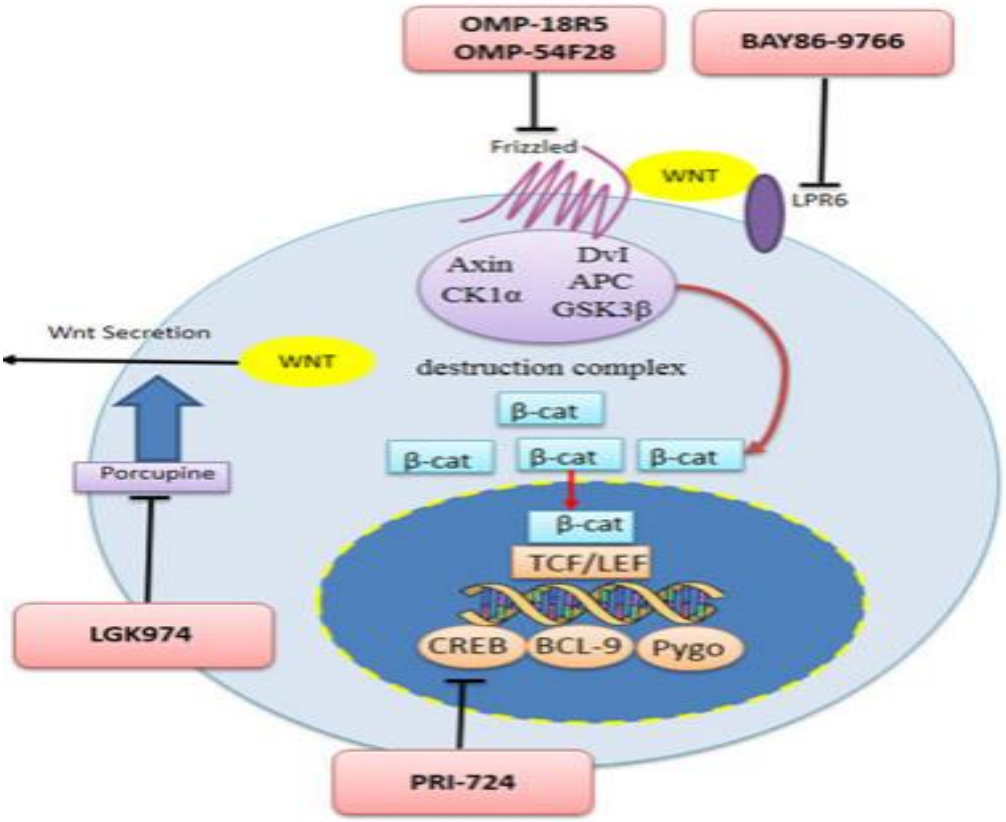

Figure 7. Anticancer agents targeting the Wntβ-catenin signaling in the treatment of pancreatic cancer (*Javadinia et al., 2018*).

Metabolic Reprogramming to the Advantage of Cancer Cells

In 1920, a Noble prize winner named Otto Warburg discovered the first tumor-specific irregular metabolism. This ‘‘Warburg phenomenon’’ entails an elevation in glucose metabolism which is subsequently kept in an environment of elevated oxygen tension and brings about increased production of lactate (Brahimi et al., 2007).

For the carbon source during anabolic reactions, cancer cells utilize high levels of glucose. Even though this phenomenon might not generally be relevant to all forms of cancers, elevated utilization of glucose is mostly common. This can be exploited for cancer diagnosis by clinics. For example, PET and CT scan can be exploited for cancer imaging techniques to detect glucose analogs. These 2 techniques are used the same time to detect the spread of most cancers, having over 90% specificity and sensitivity (Mankoff et al., 2007).

Possible explanations to why elevated glucose breakdown for forming ATP is many. Cancer cells utilize these methods to grow in an uncontrolled manner.

During glucose breakdown in the presence of oxygen, cells are able to survive in an environment where the oxygen tension is changing which could be deadly for cells that produce ATP via oxidative phosphorylation (*Pouyssegur et al., 2006*).

Also, lactic acid and bicarbonic acid are produced by cancerous cells, with glucose metabolic end product in the presence of oxygen being lactate. The above-mentioned acids dictate their surroundings, favor tumor invasion while also inhibiting anticancer immune effectors (*Fischer et al., 2007*).

Cancer cells produce lactate which is assimilated by stromal cells using various transporters ultimately reproducing pyruvate that either can be channeled to replenish the tumor cell or can be utilized for oxidative phosphorylation. The mentioned process produced an environment whereby tumor cells together with stromal cells participate in metabolic pathways that are hand in hand. This process promotes tumor cells survival as well as proliferation by forming components that are produced in the absence of oxygen. (*Koukourakis et al., 2006*).

Third, tumors are able to utilize glucose via the pentose phosphate pathway to produce NADPH which boosts the normal cell's defense against reactive oxygen species (*Gatenby and Gillies, 2004*).

Lastly, and most important of all, cancer cells utilize intermediates of glycolysis to fuel reactions that are anabolic such as glucose 6-phosphate for synthesis of glycogen as well as formation of nucleotides (*Gatenby and Gillies, 2004*).

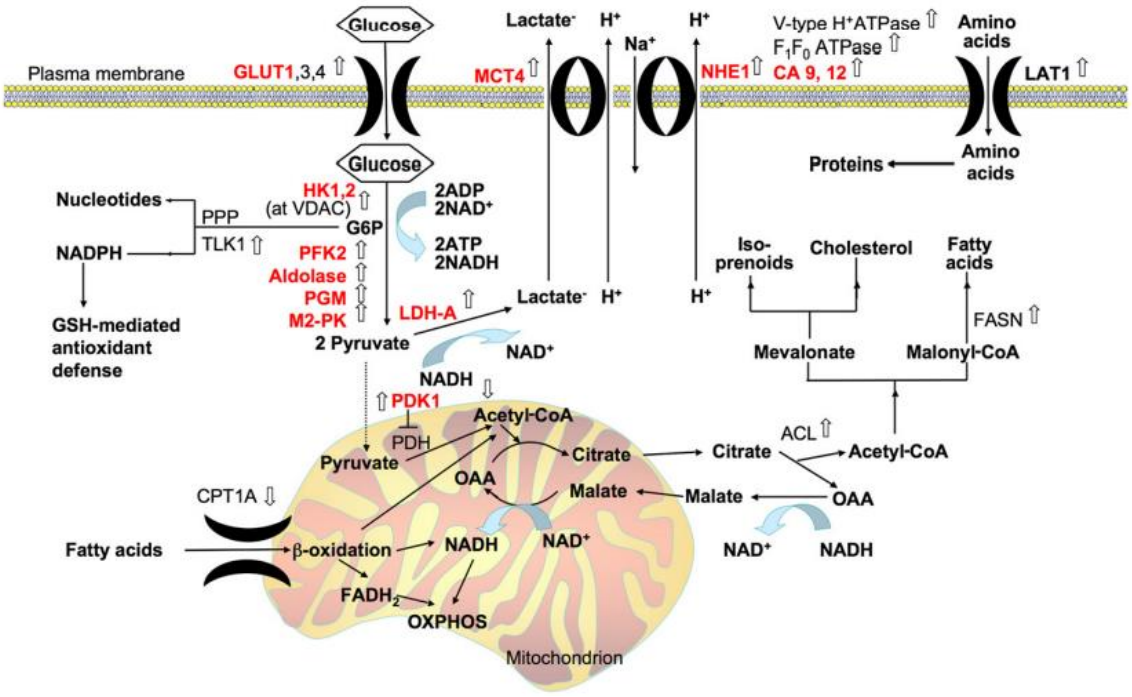

Figure 8. Metabolic reprogramming in cancer cells (*Guido et al., 2005*)

Mechanism of Metabolic Reprogramming

Reprogramming of the metabolic pathway involved in tumor cells has a complicated mechanism. Oxidative phosphorylation with flaws has been cited in order to discuss this phenomenon. Tumors found in the mitochondria are mostly small in size, lacking cristae, and ATP synthase lacks the bF1 subunit (Lopez et al, 2007). Growth of the tumor might cause the DNA present in the mitochondria to become mutated. Therefore, cancer cell growth as well as well as ROS production is brought about when the DNA in the mitochondria which is mutated becomes expressed (*Zhou et al., 2007*)

A major process that involves glucose breakdown in the presence of oxygen is found when transcriptional coactivator called hypoxia inducible factor is switched on as a result of tension due to hypoxia (*Taylor and Pouyssegur, 2007*).

This hypoxia inducible factor promotes the production of end products such as lactate and pyruvate via the glycolytic pathway. This is achieved via activating key components of this particular pathway such the glucose transporters, as well enzymes that catalyze the irreversible steps in the glycolytic pathway such as hexokinase (*Semenza, 2007*)

Furthermore, Hypoxia inducible factor decreases the production of acetyl CoA from pyruvate. This is achieved by the deactivation genes involved in encoding the enzyme pyruvate dehydrogenase kinase (*Kim, 2007*).

There is generation of electron donors such as $FADH_2$ and NADH when acetyl CoA is channeled into the TCA cycle. The above-mentioned donors act on the respiratory chain complex and in the process inhibit pyruvate dehydrogenase, hypoxia inducible factor ultimately oxidative phosphorylation (*Fukuda et al., 2007*).

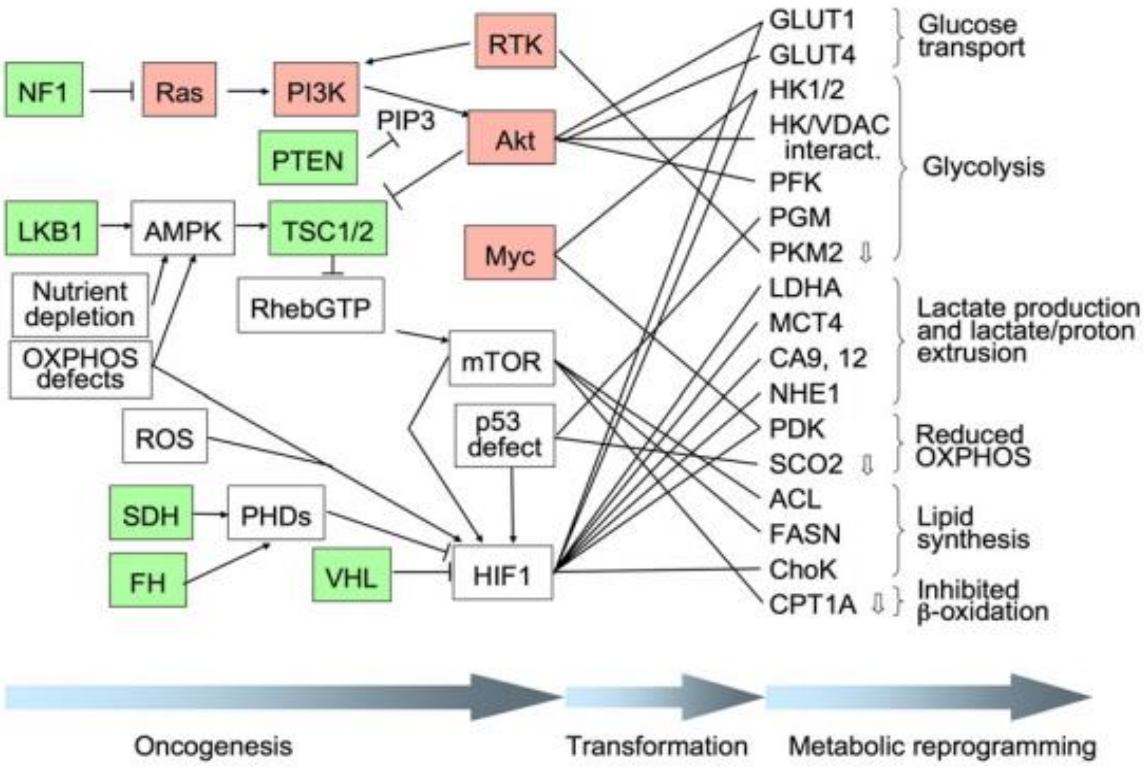

Figure 9. Mechanism of Metabolic Reprogramming (*Guido et al., 2005*)

Rosemary Plant

Rosemary (*Rosmarius officinalis)* which belongs to the family *Lamiaceae* is a fragrant plant with needle-like leaves, with different flower colors like pink, purple, white and blue; native to the Mediterranean region.

Rosemary normally forms range from upright to trailing, with the upright part reaching around 1.5m in height. The leaves are generally evergreen around 2-4cm long and 2.5mm broad mostly white below and green above containing woolly and short hairs.

Rosemary normally flowers during summer and spring weathers however it can bloom in warm climates. Rosemary can also flower in weathers different from its normal flowering season, as it known to as early as parts of December and sometimes as early as February.

According to recent studies, rosemary plant is able to exert it's anti-cancer effects as a result of the compounds that are present. Some of these compounds include carsonic and rosmarinic acids. Research has shown that these compounds possess anticancer effects as they have been shown to be effective against different forms of cancers. (*Gonzalez-Vallinas et al., 2014*).

Figure 10. Rosemary plant (http;//www.bonnieplants.com)

Rosemary Essential Oil

The oil present in this plant has a yellowish color and sometimes can be colorless. It has generally sweet smell. Research has shown that over 90% of compounds present in rosemary oil are mostly monoterpenes and similar compounds obtained from it. Other compounds present include verbenone, α-pinene with 1,8-cineole also present. Camphor is also present which is responsible for the sweet scent of the plant in general (Calo et al., 2015).

A liquid without color known as Pinene is extracted industrially by hydro distillation of the rosemary leaves. Pinene contains α and β isomeric forms due to its solubility in water and as well as it being unstable thermodynamically.

Compounds extracted from rosemary are utilized industrially in the synthesis of insecticides, flavor as well as cosmetics. Some of these compounds are also used industrially to recycle polymers. A typical of such compound is limonene which is used by industries to recycle polystyrene (*Buhl et al. 1999*).

Many researches have examined the antioxidant as well as the antimicrobial activity of the oil found in rosemary. A common feature, found in other essential oils, is its microencapsulation which can be used in active packaging, field use and for medical use. Biological pests (especially fungi) are also controlled by the use of oil extracted from rosemary leaves *(Satyal et al. 2017*).

Table 1. Compounds present in rosemary oil (*Ronoel et al., 2000*).

Compounds	Percentage	Compounds	Percentage
tricyclene	0.1	*cis*-pinocamphone	0.3
α-thujene	0.2	terpinen-4-ol	0.8
α-pinene	11.5	α-terpineol	1.2
camphene	4.3	myrtenol	0.1
thuja-2,4(10)-diene	0.2	verbenone	2.4
sabinene	0.1	citronellol	t
β-pinene	5.0	pulegone	t
myrcene	12.4	(E)-ocimenone	t
α-phellandrene	0.2	piperitone	t
α-terpinene	0.5	p-mentha-1,8-dien-3-one†	t
p-cymene	1.0	bornyl acetate	0.3
limonene	2.9	α-ylangene	0.1
1,8-cineole	22.1	methyl eugenol	t
(Z)-β-ocimene	t	β-caryophyllene	1.4
(E)-β-ocimene	t	α-humulene	0.2
γ-terpinene	1.4	γ-muurolene	0.1
terpinolene	0.8	valencene	t
linalool	1.1	α-muurolene	t
camphor	26.0	β-bisabolene	0.1
iso-isopulegol	t	γ-cadinene	t
trans-pinocamphone	0.3	δ-cadinene	0.1
pinocarvone	0.9	*trans*-calamenene	t
borneol	0.2	Total (%)	98.3

t = trace (< 0.1%); † also known as isopiperitenone

Anticancer Activity of Rosemary Essential Oil

Different studies have explained the mechanism by which Rosmarinus officinalis has exerted its anticancer effects. It has shown tremendous anti-cancer activities against many human cancer cell lines. A vast majority of compounds present in this plant's extract include: rosemarinic acid, carnosol and carnosic acid. These comounds have been proven to promote programed cell death within these cancer cells, most likely via nitric oxide production (Kontagianni et al., 2013). The strongest promoter of apoptosis seems to be Carnosic acid. Rosemary extract also effective against cancer cells (Dilas et al., 2012).

Antioxidant Activity of Rosemary Essential Oil

Rosemary oil was extensively examined in order to figure out it's antioxidant activities. The antioxidant activity can be linked to chemical constituents of the plant's essential oils. Although collaborative mechanisms among different oil components most probably brought about its antioxidant activity, phenolic diterpenes including rosemarinic acid, carnosol and carnosic acid have been shown as the most effective antioxidants found in rosemary essential oil (Yesil-Ceiktas et al., 2007).

Rosemary oil applies its antioxidant effect via multiple metabolic pathways. Rosemary essential oil together with its extract have been shown to inhibit the formation free radicals (Zegura et al., 2011). It is also able to inhibit lipid peroxidation, a harmful activity that brought about by oxidative stress (Bulbul et al., 2012).

Other than reducing the level of reactive species in the biological system, rosemary has been shown to elevate the antioxidant enzymes activities (Afonso et al., 2013). The above-mentioned actions boost the body's immune response to oxidative damage and harmful reactive oxygen species. Oxidative stress promotes the formation of several diseases. Absence of antioxidant defenses and the eventual damage caused by reactive oxygen species have been proven to cause cancer and diabetes (Dilas et al., 2012).

Antimicrobial Activity of Rosemary Essential Oil

According to recent studies, rosemary possesses both antifungal and antimicrobial activities; both of which depend on the chemical composition of the essential oil, which can differ enormously depending on the weather, location and time of harvest. The antimicrobial activity is by the components present in the oil (Jordan et al., 2013). The essential oil was also found to inhibit the growth of several bacteria such as *Staphylococcus aureus* and *Escherichia coli* (Marinas et al., 2012).

According to recent research, rosemary possesses the ability to suppress the drug resistance of certain bacteria through overcoming and decreasing the impermeability of these bacterial membranes (Oluwatuyi et al., 2004).

CHAPTER III

Methodology

Isolation of the Essential Oil

100g of air-dried rosemary leaves were hydro distilled using 1000ml of distilled water for a duration of 3 hours using a Clevenger-type apparatus. The oil extracted was stored at a temperature of 4 degree Celsius until it was required for analysis. The oil yields were calculated as w/w.

Cell Line and Cell Culture

For this study, the human pancreatic cancer cell line PANC-1 (ATCC: CRL-1469) was utilized. The cells were grown in a Dulbecco's modified eagle medium and supplemented with fetal bovine serum as the medium does not provide growth factors for cells to reach confluency. The cells were then cultured in a humidified environment at 37 degree Celsius in 5% CO_2 in order to maintain an optimum PH similar to normal biological system. When the cultured cells reached confluency, they were sub-cultured by utilizing a 0.25% trypsin-EDTA solution.

Cell Viability and Growth Assay

The cytotoxicity analysis was performed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. Rosemary essential oil solutions were prepared in Dimethyl sulfoxide and diluted in culture medium by utilizing five distinct concentrations (100, 200, 300, 400, 500, and 600 µg/ml). The human pancreatic cancer cells were obtained, suspended in the medium and seeded in 96-well cell culture plates with a density of five thousand cells in each well. Negative control row did not contain rosemary oil or any cells, however positive control row only had seeded cells and all of them were incubated for 24 h and 48 h. After incubation, 10 ml MTT solution was added

into each well for 4 h at 37 C in 5% CO_2. 50 ml of Dimethyl sulfoxide was then added to dissolve the formazan crystals. A colored solution was then generated whose absorbance was measured spectrophotometrically at 570 nm. All experiments were conducted in triplicate for the rosemary oil.

Statistical Analysis

This was performed using graph method. The entire results were shown as mean ±SD. One-way analysis of variance (ANOVA) was applied to check different groups. The values of $p<0.05$ were regarded as significant.

CHAPTER IV

Findings

Cell Viability and Cytotoxicity

The human pancreatic cancer cells were treated with 100, 200, 300, 400, 500, and 600 µg/ml concentrations of rosemary oil for 24 hours and 48 hours. Cell viability was evaluated using the MTT assay. All tested concentrations of rosemary oil decreased the PANC-1 cell growth in a dose- and time-dependent manner as shown in figure 12. We showed that rosemary oil at 500 µg/ml concentration was more effective in reducing the PANC-1 cell proliferation than the other concentrations for 48 hours incubation and was used to show the anti-cancer effect.

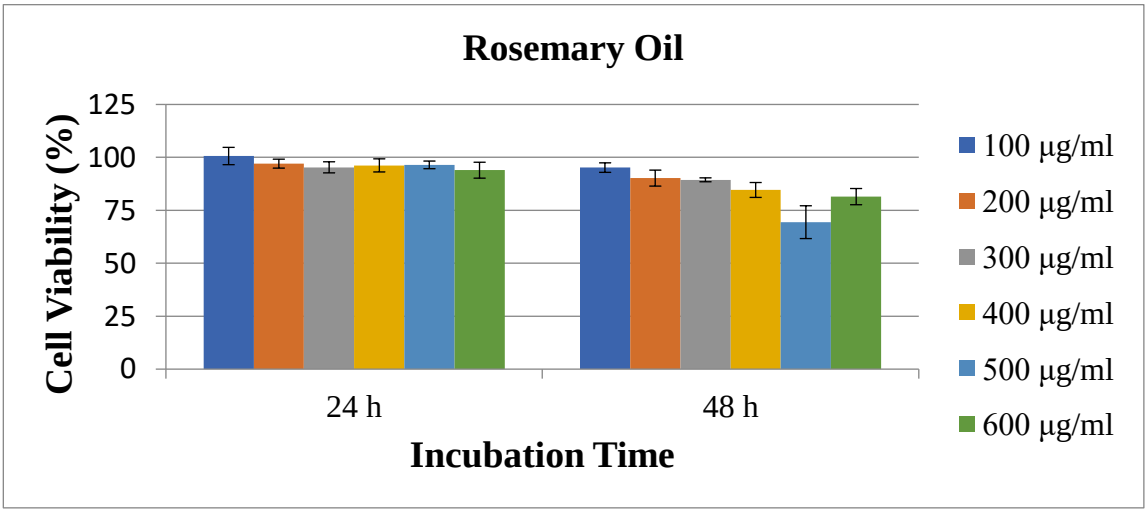

Figure 11. Dose-response columns and IC_{50} values of rosemary oil. Cell viability was evaluated by the MTT assay. PANC-1 cells were exposed to different concentrations of rosemary oil (100, 200, 300, 400, 500, and 600 µg/ml) for 24 and 48 h.

When different concentrations (100, 200, 300, 400, 500, and 600 µg/ml) and incubation times (24 and 48 h) were compared, cell viability was significantly higher at

100 μg/ml for 24 h (100.64%) than 600 μg/ml for 48 h (81.48%), (p= 0.0048). We also found that significantly higher cell viability at 200 μg/ml for 24 h (97.05%) in comparison with 600 μg/ml for 48 h (81.48%), (p= 0.0177). Furthermore, when different concentrations were assessed during the 48 h incubation period, significant difference was detected between 100 μg/ml and 600 μg/ml concentrations (p= 0.0019). Similarly, it was shown that there was a significant difference between 200 μg/ml and 600 μg/ml for 48 h (p= 0.0476). Cell viability was significantly lower at 600 μg/ml (81.48%) than 100 μg/ml (95.18%) and 200 μg/ml (90.23%) concentration levels.

Cell Morphology

The human pancreatic cancer cells possessed an epithelial morphology and cultured with monolayer adhere features. After rosemary oil treatment, the shapes of cells were still epithelioid. However, the sizes of PANC-1 cells were smaller (Figure 13).

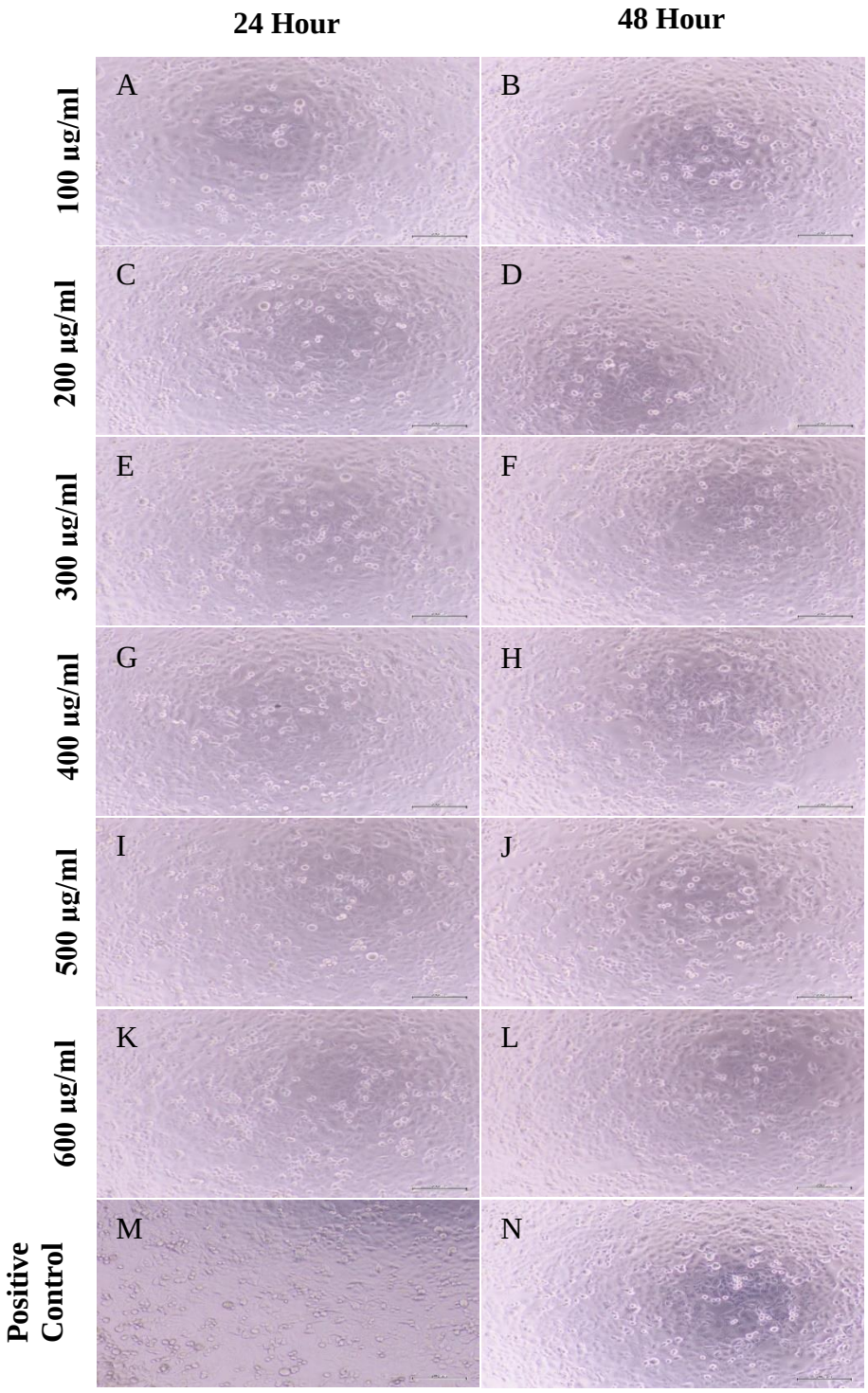

Figure 12. PANC-1 cells imaged under the inverted microscope after rosemary oil treatment. (A-N, scale bars= 200 µm)

CHAPTER V

Discussion

In this study, different concentration and incubation periods were compared. Cell viability was higher at 100 µg/ml for 24 hours when compared to 600 µg/ml for 48 hours. Also, cell viability at 200 µg/ml was significantly higher when compared to 600 µg/ml for 48 hours. Different concentrations were also assessed during 48 hours incubation period with large difference between 100 µg/ml and 600 µg/ml observed. It was also observed that 500 µg/ml concentration was the most effective in reducing PANC-1 cell proliferation more than any other concentration for a duration of 48 hours.

According to Gonzalez et al., (2014), exposing pancreatic cancer cells to rosemary extract resulted in tremendous inhibition of cell viability with increasing concentration resulting in a more significant inhibition. An astonishing 60% of cell viability inhibition was achieved using a concentration of 40µg/mL for a duration of 48 hours. Results from this study supports the suggestion by Gonzalez et al., (2014) as these results indicated that an increase in the concentration of rosemary oil resulted in significant inhibition.

Preedy, (2016) reported that rosemary essential oil generally constitutes of oxygenated and hydrocarbon terpenes; sesquiterpenes might appear in small quantities. Myrcene, limonene, geraniol and p-cymene have also been reported to be present in rosemary essential oil. Studies have indicated that limonene is effective against leukemia and other forms of cancer.

Conforti et al., (2012) reported that the anti-cancer activity of rosemary essential oil is due to the main components such as 1,8-cineole, camphor, alpha-pinene and beta-carbophyllene. When it comes to tumor development, rosemary and its components displayed antiproliferative effects in tumor cells including leukemia, liver, breast, colon, prostrate and pancreas.

Several studies have described how these components of essential oil exert their anti-cancer effects. According to Park et al., (2014), β-caryophyllene present in essential oil specifically bring about the production of reactive oxygen species in the

mitochondria of cancer cells without elevating the oxidative stress n non cancer cells. Hussain et al., (2011) reported that eugenol reduces inflammation by affecting inflammatory factors such as 1L-1β, 1L-6, TNF-alpha and PGE2, and other factors such as cyclooxygenase.

Kim et al., (2011) reported that the beneficial effects of terpenoids in essential oil are related to a change in the polarization of the cancer cells membrane and mostly in the mitochondrial membrane. Terpenoids are significantly lipophilic and possess a high affinity for cell membranes.

Seki et al., (2011) described the NF-KB pathway as a possible route for the control of cancer cell development. The NF-KB signaling pathway is involved in cellular growth control and in the metabolism of glucose. It affects protein synthesis through the Mtor enzyme and influences the uptake and usage of glucose. α-Bisaolol utilize this pathway to exert its anti-cancer effects particularly in pancreatic cancer.

The morphology of PANC-1 cells was observed using an inverted microscope. The PANC-1 cells possess an epithelial morphology and cultured with monolayer adhere feature. After treatment with rosemary essential oil, the shapes of the cells were still epitheloid. However, reduction in the size of PANC-1 was observed.

Results obtained from morphological observations under an inverted microscope indicates that the possible mechanism by which rosemary oil exerts its cytotoxic effects might be as a result of activation of apoptosis; resulting in the loss of organelles found in the cytoplasm.

CHAPTER VI

Conclusion and Recommendations

Conclusion

In conclusion, the anti-cancer effects of rosemary essential oil were studied in PANC-1 pancreatic cancer cells utilizing different concentrations and incubation periods. Components of the rosemary essential oil such as limonene, β-caryophyllene, eugenol and α-Bisaolol were able to control cancer cell development via different mechanisms.

Recommendations

In order to fully understand the effects of rosemary essential oil on pancreatic cancer cells, further studies utilizing human and animals are required to illustrate the effects of rosemary essential oil on pancreatic cancer cells.

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