

**THE REPUBLIC OF TURKEY
BAHÇEŞEHİR UNIVERSITY**

**E-CADHERIN, VIMENTIN, SNAIL, AND SLUG
EXPRESSION IN GEMCITABINE TREATED
ASPC-1 AND BXPC-3 PANCREATIC CANCER CELL LINES**

Master's Thesis

LYNE EL-BADAOUİ

ISTANBUL, 2021

**THE REPUBLIC OF TURKEY
BAHÇEŞEHİR UNIVERSITY**

**GRADUATE SCHOOL
BIOENGINEERING MASTER'S PROGRAM**

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ISTANBUL, 2021



T.C.

**BAHCESEHIR UNIVERSITY
GRADUATE SCHOOL**

date

MASTER THESIS APPROVAL FORM

Program Name:	Bioengineering program
Student's Name and Surname:	Lyne El Badaoui
Name of The Thesis:	E-cadherin, vimentin, snail, and slug expression in gemcitabine treated AsPC-1 and BxPC-3 pancreatic cancer cell lines
Thesis Defense Date:	3 February, 2022

This thesis has been approved by the Graduate School and has fulfilled the necessary conditions as a master's thesis.

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ACKNOWLEDGEMENTS

First and foremost, I want to thank Allah for his never-ending grace, mercy, and for being able to complete this project with success.

I want to express my gratitude to my advisor Dr. Zeynep Tacer Caba and my co-advisor Prof. Dr. Gülay Bulut for their patience, noble guidance, and support with full encouragement and enthusiasm. I have benefited greatly from their wealth of knowledge and meticulous editing.

I would like to thank my senior Nada Alrifai who taught and provided me with the basic management and handling skills in the lab. My sincere thanks also go to my colleagues Simay Dolaner, Elif Olkun, and Ceyda Yalcin who offered the help, assistance, and support i needed in this journey.

Most importantly, I owe my deepest gratitude to my father, my mother, and Mohamad for their unconditional, unequivocal, and loving support throughout the entire thesis process and every day.

Istanbul,2021

Lyne El-Badaoui

ABSTRACT

E-CADHERIN, VIMENTIN, SNAIL, AND SLUG EXPRESSION IN GEMCITABINE TREATED ASPC-1 AND BxPC-3 PANCREATIC CANCER CELL LINES

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MASTER OF BIOENGINEERING PROGRAM

THESIS SUPERVISOR: DR. ZEYNEB TACER CABA

01, 2022, 75 pages

Pancreatic cancer is one of the most common types of cancer with PDAC (Pancreatic ductal adenocarcinoma) a subtype of pancreatic malignancies, depicting 90 percent of all pancreatic cancer incidences. Although, Gemcitabine (trade name Gemzar) is approved for the treatment of PDAC through the activation of apoptosis in malignant cells undergoing DNA synthesis but the development of tumor chemoresistance has limited the efficacy of this cornerstone. In this study, the epithelial marker E-cadherin, and the mesenchymal markers vimentin, Snail, and Slug are the lead actors. Slug (SNAI2) and Snail (SNAI1) are key transcription factors that downregulate E-cadherin and aid in tumor growth and survival. It is crucial to determine the influence of Gemcitabine on the gene expression of E-cadherin, Vimentin, Snail, and Slug in AsPC-1 and BxPC-3 pancreatic cancer cell lines. The IC₅₀ values of Gemcitabine were first identified in both AsPC-1 and BxPC-3 cells, then they were both treated with the drug for further RNA isolation. Total RNA was extracted and Quantitative Real Time – Polymerase Chain Reaction (QRT-PCR) analysis was performed to analyze the potential effect of Gemcitabine on mRNA levels of E-cadherin, vimentin, Snail, and Slug genes. The results show that E-cadherin was significantly upregulated in AsPC-1 and BxPC-3 cell lines. Vimentin, Snail and Slug were downregulated in BxPC-3 cell line, whereas an upregulation of Vimentin and no expression of Snail and Slug were detected in AsPC-1 cell line.

Keywords: Pancreatic Cancer; E-cadherin; Vimentin; Snail; Slug; Gemcitabine

ÖZET

GEMCITABINE TEDAVİ EDİLMİŞ ASPC-1 VE BxPC-3 PANKREATİK KANSER HÜCRE HÜCRELERİNDE E-CADHERİN, VIMENTİN, SNAIL VE SLUG EKSPRESYONU

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BIYOMÜHENDİSLİK YÜKSEK LİSANS PROGRAMI

TEZ DANIŞMANI: DR. ZEYNEB TACER CABA

01, 2022, 75 sayfalar

Pankreas kanseri, tüm pankreas kanseri vakalarının yüzde 90'ını gösteren, pankreas malignitelerinin bir alt türü olan PDAC'lı en yaygın kanser türlerinden biridir. Gemcitabine (ticari adı Gemzar), DNA sentezi geçiren malign hücrelerde apoptoz aktivasyonu yoluyla PDAC tedavisi için onaylanmış olmasına rağmen, ancak tümör kemorezistansının gelişimi bu köşe taşının etkinliğini sınırlar. Bu çalışmada epitelyal belirteç E-cadherin ve mezenkimal belirteçler Vimentin, Snail ve Slug başrol oyuncularındır. Slug (SNAI2) ve Snail (SNAI1), E-cadherin'i aşağı regüle eden ve tümör büyümesine ve hayatta kalmasına yardımcı olan anahtar transkripsiyon faktörleridir. AsPC-1 ve BxPC-3 pankreas kanseri hücre dizilerinde E-cadherin, Vimentin, Snail ve Slug gen ekspresyonu üzerinde Gemcitabinin etkisini belirlemek çok önemlidir. AsPC-1 ve BxPC-3 hücrelerinde Gemcitabinin IC₅₀ değerinin belirlenmesinin ardından bu hücreler ilaçla tedavi edildi. Toplam RNA ekstrakte edildi ve Gemcitabine'nin E-cadherin, Vimentin, Snail ve Slug genlerinin mRNA seviyeleri üzerindeki potansiyel etkisini analiz etmek için Kantitatif Gerçek Zamanlı – Polimeraz Zincir Reaksiyonu (qRT-PCR) analizi yapıldı. Sonuçlar, E-cadherinin AsPC-1 ve BxPC-3 hücre hatlarında önemli ölçüde yukarı doğru düzenlendiğini göstermektedir. Vimentin, Snail ve Slug, BxPC-3'te Vimentin yukarı regülasyonu ile aşağı regüle edildi ve AsPC-1 hücre hattında Snail ve Slug ifadesi yok.

Anahtar Kelimeler: Pankreas Kanseri; E-cadherin; Vimentin; Snail; Slug; Gemcitabin

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ABBREVIATIONS

ATCC	American tissue and cell collection
BRCA2	Breast Cancer gene 2
CDA	Cytidine deaminase
CDKN2A	Cyclin Dependent Kinase Inhibitor 2A
cDNA	Complementary DNA
DMSO	Dimethyl Sulfoxide
E-cadherin	Epithelial Cadherin
ECM	Extracellular Matrix
EMT	Epithelial Mesenchymal Transition
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GDP	Guanosine diphosphate
GEFs	Guanine nucleotide exchange factors
IC50	The Half Maximal Inhibitory Concentration
IPMN	Intraductal Papillary Mucinous Neoplasm
K-RAS	Kristen rat sarcoma virus
MMPs	Matrix metalloproteinases
PanIN	Pancreatic Intra-Epithelial Neoplasia
PBS	Phosphate Buffer Saline
PDAC	Pancreatic ductal adenocarcinoma

QRT-PCR	Quantitative Real Time - Polymerase Chain Reaction
RPMI	Roswell Park Memorial Institute Medium
siRNA	RNA silencing
SMAD4	Small mothers against decapentaplegic 4
TME	Tumor Microenvironment
TP53	Tumor Protein 53
WST-1	Water Soluble Tetrazolium-1
ZEB	Zinc finger E-box-binding homeobox

1. INTRODUCTION

Cancer is a potentially fatal disease in which a single normal cell develops genetic and morphological changes, eventually transforming into a motile and invasive tumor. The major distinction between normal and malignant cells is that cancer cells have lost the growth restraints that define normal cells offering them the ability to divide for an apparently infinite number of generations, allowing a tumor formed of such cells to develop without the constraints that ordinarily limit cell proliferation (National Institutes of Health (US), 2007).

Pancreatic cancer accounts for around 3 percent of all malignancies in the United States and approximately 7 percent of all cancer fatalities. According to the American cancer society in 2021, it was estimated that 60,430 Americans will be diagnosed with pancreatic cancer in the U.S., and more than 48,220 will die from the disease (American cancer society, 2021). Pancreatic cancer is a multistep phenomenon that begins with mutations invading the oncogene K-RAS, progresses through epigenetic silencing of CDKN2A, and culminates in the inactivation of p53, the guardian of the genome. Pancreatic carcinoma is subcategorized into Exocrine cancer including Adenocarcinoma, and Endocrine cancer. Various cancer types exist in each group, each with its own set of symptoms and prognosis.

Gemcitabine is an antimetabolite medication used to treat a variety of cancers, including pancreatic cancer. This medication has a tumor remission rate of about 20 percent. It has couple of mechanisms that contribute to the inhibition of DNA replication (Manegold 2004). However, it appears that tumors can develop Gemcitabine resistance in some specific conditions. As a result, combinational therapy was developed. Its fundamental principle is to combine drugs that target different pathways, increasing the likelihood of impacting tumor cells (Mokhtari et al. 2017). In this study, Gemcitabine was assessed on E-cadherin, Vimentin, Snail, and Slug expression in AsPC-1 and BxPC-3 pancreatic cancer cell lines.

The expression of E-cadherin and Vimentin is interlinked in an irreversible way. Loss of the epithelial protein E-cadherin, together with increased Vimentin expression, is strongly associated with enhanced tumor invasion and malignancy during EMT.

SNAI1 (Snail) belongs to the SNAI family whose primary mode of action is to induce epithelial to mesenchymal transition (EMT) by inhibiting E-cadherin transcription, which is important for cell adhesion and migratory capacities (Nieto, 2002; Bolos et al., 2003). Snail downregulation via siRNA reduces tumor metastasis and immunosuppression.

SNAI2 (Slug) also corresponds to the SNAI family whose mechanism of action is similar to the zinc-finger transcription factor Snail. However, it's main aspect is E-cadherin expression alteration and tumor invasiveness (Ganesan et al., 2015).

Finally, the aim of this study is to measure the expression of E-cadherin, vimentin, Snail, and Slug under the effect of Gemcitabine in AsPC-1 and BxPC-3 pancreatic cancer cell lines. QRT-PCR results showed a significant upregulation of E-cadherin gene in both cell lines, downregulation of vimentin, Snail, and Slug in BxPC-3 cell line and an upregulation of vimentin with no expression of Snail and Slug in AsPC-1 cell line.

To emphasize the significance of this study and its findings, the "Literature Review" section provides a summary of pancreatic cancer and drug resistance. All techniques and equipment utilized are detailed and listed in sequence of application in the "Materials and Methods" section. The "Results" section quantifies and analyzes the experiment results in the form of tables and figures. Finally, the findings and results are debated in the "Discussion" and "Conclusion" sections.

2. LITERATURE REVIEW

2.1 Cancer

Our body contains trillions of highly specialized cells that grow, proliferate, and behave normally to ensure proper cell division, maintaining tissue homeostasis, selective adhesion and enabling the proper differentiation into specialized cells. Each cell carries genes that are responsible for the appropriate regulation of these molecular mechanisms. Yet, the accumulation of genetic alterations in proto-oncogenes, tumor suppressor genes and DNA repair genes can disrupt these normal processes perturbing major signaling pathways that result in masses of tissues called neoplasms (Bertram, 2000).

Neoplasms can be classified into benign tumors in which cells are encapsulated in their restricted boundaries without invading into nearby tissues and organs unlike malignant tumors that metastasize by breaking away from their primary tumor site and spread into the bloodstream and lymph nodes. Carcinomas, sarcomas, and leukemias or lymphomas present the three major cancer groups in which carcinoma presents the tumors that develop from epithelial cells, sarcomas are connective tissue's solid malignancies including bone and muscles, and leukemias and lymphomas performing the cancerous cells that arise from the blood cells and the immune cells respectively (Cooper, 2000).

2.2 Cancer's Main Hallmarks

Numerous cellular mechanisms are required for continuous cancer cell survival and maintenance; plasticity and heterogeneity in tumors, resistance to therapy, hyper vascular and immunosuppressive microenvironment, metastasis formation and progressive telomere narrowing (Orth et al., 2019).

2.2.1 Plasticity and Heterogeneity in Tumors

Since the adult pancreas lacks a stem cell population, its repair and regeneration is ensured by acinar, ductal and immune cells when injury or any stress condition strikes (Puri, Folias, & Hebrok, 2015; Orth et al., 2019). Acinar cells, exhibiting high degree of plasticity

(Ziv, Glaser, & Dor, 2013), differentiate into progenitor-like cells that express ductal markers through a phenomenon called acinar to ductal metaplasia (ADM) (Rooman & Real, 2011; Orth et al., 2019). The acinar-ductal transition is a process by which acinar cells lose their identity markers, upregulate dedifferentiation markers, and gain ductal-like phenotype (Storz, 2017). This phenomenon is activated by many factors including loss of contact-mediating signaling, mutation in the proto-oncogene KRAS, and loss of polarity (Storz, 2017). However, this morphological process helps in the regeneration of the pancreas through metaplastic cells proliferation replacing damaged or injured pancreatic cells.

During ADM, acinar cells become more sensitive to pro-oncogenic events, especially mutations in the proto-oncogene KRAS, converting them into an advanced invasive stage called PanINs (Morris, Wang, & Hebrok, 2010; Kanda et al., 2012). Additional mutations and alterations in the CDKN2A, TP53, and SMAD4 tumor suppressor genes are considered the main drivers of PDAC (Morris, Wang, & Hebrok, 2010; Kanda et al., 2012; Waddell et al., 2015).

2.2.2 Resistance to Therapy

Chemotherapy is considered one of the most effective treatments for cancer since it slows down cancer cells growth and division. More than 100 pancreatic genes, occupying a major role in apoptosis, signal transduction and cell cycle control, were discovered to be resistant to drug therapy after microarray gene expression analysis (ZHAO et al., 2007). Unlike other tumors, pancreatic cancer cells are surrounded by a thick dense stroma preventing chemotherapeutic drugs from entering the tumor cells and limiting immune cells access thus constituting a barrier against effective agents. The resistance of cancer cells to chemotherapy is mainly the result of three mechanisms: a reduced uptake of the drugs into the target cells; alterations within the cells, like altered metabolism of the drugs, increased capacity to repair DNA or reduced apoptosis; and an increased efflux of the drugs from the cells.

Gemcitabine, one of the most chemotherapeutic agents used in clinical studies and found to be highly effective for PDAC treatment, stimulates the AMPK/mTOR pathway activation by slowing down cell growth, inhibiting DNA replication and activating the autophagy cellular mechanism of degradation.

Several proteins are associated with drug resistance aiming to inhibit pancreatic cancer cells sensitivity to chemotherapeutic drugs. Integrins are transmembrane receptors, critical to ECM adhesion and transmembrane signaling. This protein family of receptors control cell differentiation, regulate tumor angiogenesis, and cooperate with EGFR to support cancer cell survival (Hynes & Zhao, 2000; Schwartz & Ginsberg, 2002). Integrin $\beta 1$, an adhesion molecule that belongs to the integrin family, triggers the activation of PI3K and CDC42 signaling pathways to diminish tumor cells sensitivity to Gemcitabine (Liu et al., 2009; Desgrosellier & Cheresch, 2010). ABC transporters, mucin proteins and some nuclear transcription factors (NF- κ B) are also highly involved in resistance to gemcitabine (Chaturvedi et al., 2007).

2.2.3 Hypervascular and Immunosuppressive Microenvironment

Extracellular matrix (ECM) deposition, cancer-associated fibroblasts (CAFs), and infiltrating immune and endothelial cells make up the desmoplastic microenvironment crucial for pancreatic cells to continue proliferating, metastasizing, and resisting therapy (Moir, Mann, & White, 2015; Kalluri, 2016; Apte et al., 2004). Pancreatic stellate cells (PSCs), usually present in a quiescent state, store vitamin A fat droplets and their activation is triggered by pancreatic injury or chronic inflammation. Following activation, PSCs lose their vitamin A-storing fat droplets and start depositing layers of extracellular matrix (ECM) stimulating the continuous proliferation and migration potentials of pancreatic cells (Moir, Mann, & White, 2015; Omary, Lugea, Lowe, & Pandol, 2007; Apte, Wilson, Lugea, & Pandol, 2013; Erkan et al., 2011). This desmoplastic hypervascularization contributes to hypoxia that supports PDAC progression through macrophage recruitment. Following

hypervascularization, CD4⁺ T cells entry into the carcinogenic microenvironment is blocked (Heinemann et al., 2014; Erkan, Kurtoglu, & Kleeff, 2015; Li et al., 2016).

2.2.4 Metastasis Formation

Metastasis is the spreading process of tumor cells from their original site to a new secondary site through a cascade that consists of three main stages: invasion, intravasation and extravasation.

Invasion begins with the loss of junctions that adhere cells together allowing tumor cells to detach from their primary tumor site and invade the surrounding stroma. Furthermore, tumor cells secrete proteins called matrix metalloproteinases (MMPs) that degrade the cell-cell and cell-matrix interactions. To metastasize, cancer cells require a source of energy: oxygen and nutrients. When the angiogenic process is activated, new blood vessels are formed from pre-existing vessels that help detaching cells to enter the circulatory system and spread into nearby sites (Brooks, 1996; Folkman & Shing, 1992; Folkman, 1996). Tumor cells keep circulating in the bloodstream, then grow and metastasize once they reach a suitable secondary tumor site in which they settle in to form new tumors; this is called the extravasation stage of metastasis (Hapach et al., 2019).

2.2.5 Progressive Telomere Narrowing

Chromosomes consist of large repeated sequences of non-coding DNA called telomeres. Present at both ends of each chromosome, they play a crucial role in genome protection from many aspects, including nucleolytic degradation and interchromosomal fusion. After each DNA replication cycle, telomeres begin to shorten and once they become aggressively short, the cell initiates a DNA damage response that activates cellular senescence (Jafri et al., 2016).

The TERT gene (hTERT), that encodes for the telomerase enzyme, is silent in normal cells and is mainly activated and overexpressed in cancer cells, enabling their replicative

immortality. The basis of oncogenesis is the infinite proliferation of tumor cells, which is accomplished by the activation of the telomerase enzyme (Trybek et al., 2020).

Cancer cells escape senescence by preserving their telomeres through activation of the DNA polymerase. The addition of few short single stranded DNA repeats to chromosomes end prevents them from shortening leading to cell senescence or cell death.

2.2.6 Epithelial-Mesenchymal Transition

2.2.6.1 EMT Phenomenon

EMT is a multi-step phenomenon where epithelial cells lose their cell-to-cell attachments, gain migratory and invasive characteristics, and downregulate E-cadherin protein to convert the epithelial state of cells into a mesenchymal state characterized by an upregulated expression of Vimentin and MMPs enzymes. E-cadherins are glycoproteins that connect cells to each other by forming adherens junctions. However, they operate as tumor suppressor proteins whose expression is blocked when mutated, dissimilar to Vimentin which is an intermediate filament that acts as an oncogene and is highly expressed in mesenchymal cells. When EMT signals strike the cells, EMT transcription factors such as SNAIL, Twist1 and ZEB family are activated; they repress the epithelial cell gene expression and activate the mesenchymal cell gene expression (Liao & Yang, 2017; Craene & Berx, 2013; Peinado et al., 2007).

EMT-TFs interact with each other to modulate the expression of target genes (Chen et al., 2018). The Twist family, a helix-loop-helix transcription factor, are upregulated in many cancers (Yang et al., 2008). Twist 1 binds to the CDH1 promoter that expresses E-cadherin thus blocking its gene expression (Yang et al., 2011). This transcription factor also downregulates let-7i to enhance the mesenchymal characteristic of tumor cells and alters the p53 and Rb signaling pathways when collaborating with the Myc protein (Yang et al., 2012; Valsesia-Wittmann et al., 2004).

The SNAIL family consisting of the C-terminal and N-terminal conserved domains bind the E-cadherin promoter and epigenetic remodeling complex respectively (Cano et al.,

2000). This transcription factor mediates cancer stemness, enabling tumor cells renewal, differentiation, and regeneration (Hwang et al., 2011).

Zeb1 is a mesenchymal cell marker protein that represses the E-cadherin protein and activates the N-cadherin and Vimentin expression driving tumor cells metastasis and malignancy (Wang et al., 2007). Zeb2 activates MMPs aiming to degrade the bonds between the epithelial cells and basal membrane. Cells will lose their polarity and their connection to the basal membrane thus transitioning into a mesenchymal cell type. This family of transcription factors is regulated by Snail 1 and Twist 1, sustaining the conversion from an epithelial to a mesenchymal cell state (Dave et al., 2011).

2.2.6.2 EMT Types

The epithelial- mesenchymal transition process occurs in three relevant types, occupying distinct functional consequences, each. Implantation, Embryonic gastrulation, and organ evolution are vital biological processes sustained by type 1 EMTs. This type of EMT forms the mesoderm and endoderm germ layers that will end up establishing the muscular system and guts respectively (Zeisberg & Neilson, 2009; Kalluri & Weinberg, 2009). Type two EMT is initiated in wound healing, tissue regeneration, and hepatic fibrosis through a repair-associated phenomenon by spawning fibroblasts and cells needed for tissue reconstruction following an injury Type three EMT arises in malignant cells favoring cancer progression and metastasis through loss of epithelial markers and activation of mesenchymal proteins gene expression (Kalluri & Weinberg, 2009).

2.2.6.3 EMT and MicroRNA

MiRNAs represent a class of small non-coding RNAs implicated in post-transcriptional gene expression regulation. Once Snail 1 is activated, Twist 1 protein is induced thereby activating the Snail 2 protein expression. Snail 2 upregulates the transcription factor Zeb expression simultaneously. All these transcription factors activate each other and control the expression of other transcription factors. Mi-29 and mi-30 are essential for the epithelial system maintenance, they inhibit the Snail 1 expression thus blocking the activation of the

Twist1-Snail 2- Zeb pathway. Mi-34 and mi-203 also suppress the Snail 1 expression as well as Snail 1 which also inhibits these micro-RNAs creating a double negative loop system where one transcription factor is inhibiting the expression of a molecule and vice versa. Mir-200f and mir-205 also create a double negative loop with the Zeb protein.

2.2.6.4 EMT and Chromatin Modifications

Histone methylation/demethylation and acetylation/deacetylation are epigenetic modifications that adjust the chromatin structure contributing to transcriptional activation or repression. The main regulators of the EMT process, Snail and Zeb, engage histone deacetylase, polycomb repressor complex and other enzymes to downregulate the gene expression of epithelial proteins by modifying the chromatin, and recruiting micro-RNAs at every level.

2.3 Pancreatic Cancer

2.3.1 The Pancreas

The pancreas is an elongated organ located in the digestive system and composed primarily of pancreatic ducts. Three main regions make up the pancreas: The head, the body, and the tail. This organ serves two vital purposes: the exocrine glands, also called the acinar cells, secrete the digestive enzymes essential for food digestion such as proteases that breakdown proteins into amino acids, while pancreatic endocrine cells organized as distinct Langerhans islets are made up of five major endocrine cell types (alpha, beta, delta, epsilon, and upsilon), each of which secretes at least five hormones into the bloodstream, includes glucagon, insulin, somatostatin, ghrelin, and pancreatic polypeptide. The synthesis of insulin and glucagon aids in the establishment of an ideal blood sugar level; however, this biological process is also regulated by the somatostatin hormone, which inhibits their secretions in order to maintain a regulated sugar level in the blood (Vergari et al., 2019). Ghrelin promotes the production of growth hormone and regulates the hypothalamic circuitry that governs food intake (Napolitano et al., 2018). Furthermore, the pancreatic polypeptide, which is also a

member of the pancreatic hormones, acts as a hepatic glucose regulator (Śliwińska-Mossoń et al., 2017).

2.3.2 Pancreatic Disorders

Several disorders disturb the normal state of the pancreas such as pancreatitis, PanIN and IPMN, and pancreatic cancer. Each disorder behaves differently showing dissimilar symptoms and requiring different treatments. Pancreatitis is the inflammation of the pancreatic organ. This is a disorder that can affect the pancreas in an acute or chronic way due to a severe build up in pancreatic enzyme secretions. PanINs correspond to the papillary lesions that occur in the pancreatic ducts. Cuboidal cells, mucin, and cytological atypia constitute the general features of these lesions. They are arranged into three stages: (i) PanIN-1 presenting low-grade flat or papillary lesions with minimal atypia; (ii) PanIN-2 corresponds to the intermediate-grade lesions having mild atypia; (iii) PanIN-3 is the most severe type of lesions with extreme cytological atypia (Distler et al., 2014). Furthermore, IPMNs account for another type of pancreatic precancerous cysts that can shift into a malignant phenotype and form tumors. Patients suffering from Diabetes and chronic pancreatitis have a high risk of developing the IPMN malignancy (Kawakubo et al., 2014). When normal cells lining the pancreatic ducts proliferate in an uncontrolled pattern, they transform into neoplasms that secrete thick mucus causing cystic dilatation of the ducts (Nissim et al., 2012).

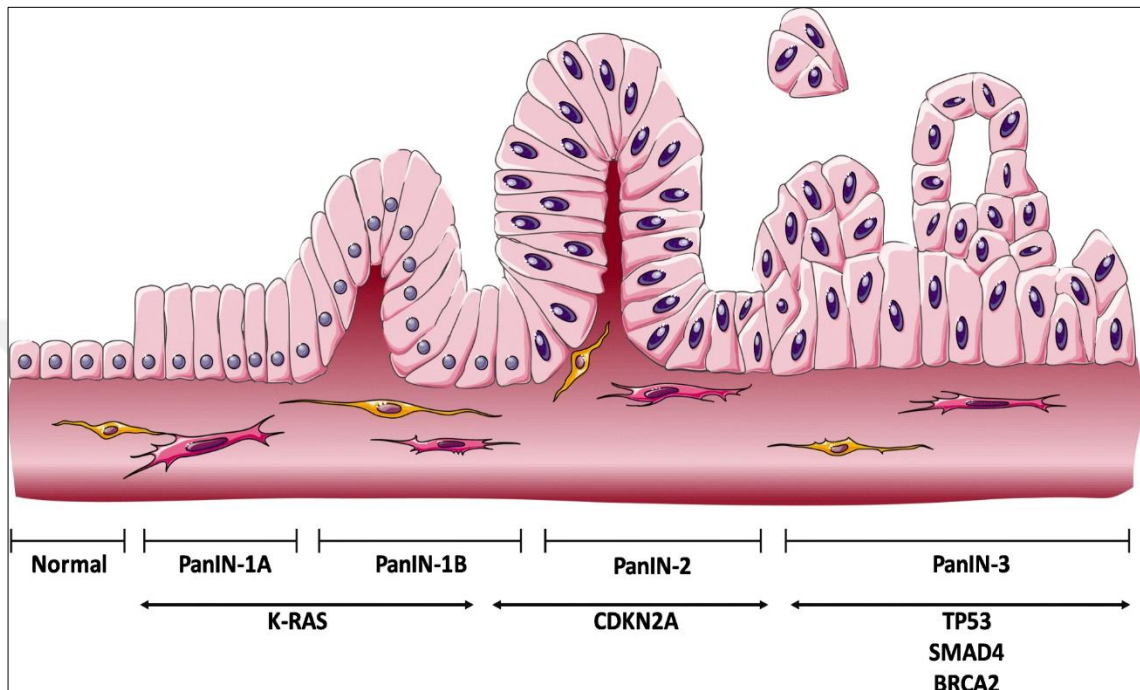
2.3.3 Pancreatic Cancer

Pancreatic cancer refers to the malignant tumors that arise when pancreatic cells divide and multiply in an uncontrolled manner. The endocrine and exocrine glands constitute the two main compartments where pancreatic cancer takes place. More than 95% of pancreatic tumors are adenocarcinomas; they represent the exocrine type of tumors that arise from cells lining the pancreatic ductal tubes (ductal carcinoma) or the cells that synthesize the pancreatic enzymes (acinar cell carcinoma). Pancreatic ductal carcinoma accounts for the majority of the malignant pancreatic cases. Most of the endocrine tumors are benign and remain confined to their primary location.

The path to pancreatic cancer involves the activation and repression of several genes dysregulating several transcriptional programs. Mutation of oncogenes and alteration of some tumor suppressor genes drive cellular malignancy maintaining the uncontrolled cell division and progression. The P53 tumor suppressor gene is a well-known protein that regulates cell division and DNA checkpoint responses. When p53 protein is altered, coping with tumor suppression becomes challenging. Thus, Apoptosis cannot be activated, keeping the invasive multiplication and proliferation of malignant cells with damaged DNA. SMAD4, a member of the SMAD family, is inactivated in most cancers. SMAD4 collaborates with SMAD2 and SMAD3 and forms a complex with FoxO that encodes for the p15 and p21 gatekeeper proteins.

Pancreatic intraepithelial neoplasia is a multistep phenomenon mastered by a series of morphological and genetic changes. Activating mutations in the oncogene KRAS and shortening of telomere initiates the first stage of pancreatic carcinogenesis transforming the low cuboidal epithelium of the pancreatic ducts into malignant lesions called PanIN-1. However, these lesions can be differentiated from the normal epithelium via the presence of a hyperplastic mucous. Epigenetic silencing of CDKN2A, a gene that encodes for the p16 and p14 tumor suppressor proteins, induces papillary infoldings of the epithelium, extends the nucleus, and develops atypia switching the PanIN-1 lesions to an advanced malignant state of lesions called PanIN-2. Additional genetic modifications inactivate the expression of the p53, SMAD4, and BRCA2 proteins contributing to a high-grade dysplasia and a significant loss of the cell polarity leading to severe cancerous malignancy. PanIN-3 lesions can evolve into an advanced level of invasion, leading to pancreatic cancer.

Figure 2.1: Pancreatic oncogenesis showing the progression from acinar cell state into PDAC state



Source: (Daoud et al., 2019)¹

2.3.4 Main Modulators of Pancreatic Cancer

2.3.4.1 KRAS

RAS is a family of genes involved in the synthesis of proteins that regulate cell growth, apoptosis and other cellular mechanisms through the activation of specific signaling pathways such as RAF/MEK/ERK, PI3K/AKT/mTOR pathways (Cicenas et al., 2017).

The K-RAS proto-oncogene, which belongs to the RAS gene family, encodes for the signal transducer K-RAS protein characterized by a GTPase activity (Cox & Der, 2010). K-RAS cycles between two states: the GDP and GTP bound states. Moreover, the activation or inactivation of this protein highly depends on the binding mechanism of K-RAS to either guanosine diphosphate (GDP) or guanosine triphosphate (GTP) (Vetter, 2001). K-RAS, which acts as a molecular master switch, is active once growth factors, hormones and

¹ Daoud, A.Z., Mulholland, E.J., Cole, G. et al. MicroRNAs in Pancreatic Cancer: biomarkers, prognostic, and therapeutic modulators. BMC Cancer 19, 1130 (2019). <https://doi.org/10.1186/s12885-019-6284-y>

cytokines interact with GEFs and enable the replacement of GDP from the nucleotide binding site for GTP. Downstream signaling pathways are not activated when K-RAS binds guanosine diphosphate (GDP). Only in the GTP-bound state, K-RAS binds and activates inflammatory mediators and other effector proteins to induce several signaling pathways (Magliano & Logsdon, 2013).

GTP and GDP are both present in the cytosol, they compete to bind RAS and since GTP has a higher binding affinity than GDP, GTP will bind RAS once GDP is released by the guanine nucleotide exchange factor (GEF); this will switch on RAS to the active state. In normal cells, GTPase activating proteins (GAP), a family of regulatory proteins, is needed to turn the RAS protein's activity off. GEF and GAP ensure normal RAS activity by maintaining a steady and balanced state. GAP hydrolyzes GTP by breaking a phosphate bond to make GDP so that RAS can be switched off again (Magliano & Logsdon, 2013).

2.3.4.2 SMAD4

Numerous studies have recorded that the tumor suppressor gene SMAD4 is downregulated in pancreatic cancer tissues. It is cited as a pancreatic cancer deletion gene 4 (DPC4), whose expression is inactivated in 50% of pancreatic cancers (Kim et al., 1996). However, this gene represents the central mediator for TGF- β family signal transduction that transmits signals from the membrane to the nucleus activating downstream signals (Schmierer & Hill, 2007). SMAD4 deficiency highly correlates with tumor expansion, and lymph node metastasis (Wang et al., 2019).

2.3.4.3 BRCA2

Deleterious mutations within the BRCA tumor suppressor proteins can be considered a risk factor for pancreatic cancer initiation (Wong et al., 2020). BRCA1 and BRCA2 belong to the BRCA family of proteins, whose main role is to repair DNA damage within cells. If impaired, blocking the proliferation of altered DNA-containing cells becomes challenging thus promoting cancer.

Table 2.1: Expression Analysis of KRAS, CDKN2A, TP53, SMAD4, and BRCA2 and their alteration status in PanINs and IPMNs

Gene	Genetic expression	Regulatory process	Alteration status in PanINs	Alteration status in IPMNs
KRAS	Upregulated	GTPase-dependent signaling	Positive	Positive
CDKN2A	Downregulated	Cell cycle regulation	Positive	Unknown
TP53	Downregulated	DNA damage response	Positive	Positive
SMAD4	Downregulated	TGF- β signal transduction	Positive	Positive
BRCA2	Downregulated	DNA repair response	Positive	Unknown

Source: Cicenás et al., 2017

2.3.5 Pancreatic Cancer Diagnosis

Despite decades of advancements in medical technology, including surgical resection and adjunctive medical therapy, the five-year life expectancy for pancreatic cancer patients remains about 7% (Zhu et al., 2018). Furthermore, due to the unusual pancreatic symptoms and the restriction in diagnostic methods, PC patients are frequently diagnosed at an advanced stage of malignancy. To enhance the prognosis of PC patients, innovative diagnostic biomarkers and revolutionary molecular therapeutic approaches are thus urgently required (Gilbert et al., 2017).

Table 2.2: Advantages and Disadvantages of the Main Diagnostic Methods

Diagnostic methods	Benefits	Restrictions
MDCT	Widely available Highly effective Cheap	Toxicity of the kidneys Exposure to radiation
MRI	Iodine-free Absence of radiation	Expensive Less available Inapplicable with some mental implants
PET/CT	Detection of metastatic malignancies Elucidate the CT findings Controls the cancer recurrence Receptive to adjuvant therapy	Expensive Less available Exposure to radiation

Source: Zhang et al., 2018

2.4 Cancer Treatments

In cancer care, several factors need to be taken into consideration: the type and phase of cancer, the potential side effects, and the patient's preferences and wellness. Tumors become extremely heterogeneous as cancer progresses, resulting in a mixed population of cells with a variety of biological characteristics and various responses to therapeutic treatments (Dagogo-Jack & Shaw, 2017). Some treatments remove the tumor without affecting and harming normal cells, others kill cancer cells impairing healthy cells. Chemotherapy, immunotherapy, radiotherapy, surgery, and targeted therapy are well-known treatments used for all cancer types.

2.4.1 Chemotherapy for Pancreatic Cancer

Chemotherapy uses a single drug or a combination of two or more drugs to eradicate cancer cells. Gemcitabine, Fluorouracil, and Nab-paclitaxel are chemotherapeutic drugs approved by the U.S. Food and Drug Administration (FDA) for pancreatic cancer. Periodic number of cycles is mandatory for this type of therapy. Gemcitabine corresponds to the antimetabolite family of drugs that gets rid of tumor cells by inhibiting a crucial enzyme involved in DNA synthesis, the ribonucleotide reductase. When this enzyme is shut down, cells find it difficult to divide properly which drives their death. This drug is given over a period of 30 mins intravenously and can be used in monotherapy or combination therapy. 5-fluorouracil is also an antimetabolite that blocks DNA synthesis and impairs the DNA repair system. However, this anti-cancer drug is injected through veins prior gemcitabine-based therapy to patients with advanced pancreatic malignancy. Nab-paclitaxel is a Taxane drug given as a combinational therapy with gemcitabine that binds to albumin receptors found on endothelial cells, activates the caveolin-1 protein and forms the wide pits of the plasma membrane called caveolae. These pits carry the albumin–paclitaxel conjugate to the interstitial fluid phase containing the tumor and stromal cells slowing down cell division and activating immune cells (Iglesias, 2009; Zhu & Chen, 2019).

2.4.2 IMMUNOTHERAPY FOR PANCREATIC CANCER

Cancer immunotherapy is widely used as a strategy that boosts the body's immune system to fight and cope with tumors. Monoclonal antibodies are synthesized proteins that mark specific tumor antigens and are highly approved in immunotherapy. However, cancer cells bypass immune cells and take advantage of small molecules located on immune cells called checkpoints to avoid being invaded by the immune system. Immune checkpoint inhibitors are drugs added to mark these checkpoints. Once checkpoints are targeted, normal cells can be differentiated from cancer cells helping the immune cells detect and combat tumors. Pembrolizumab offers a promising immunotherapeutic cure for patients developing advanced pancreatic cancer. This drug suppresses the programmed cell death protein 1 (PD-1), checkpoint protein highly expressed on T cells, to trigger the activation of the immune cells thereby dwindling tumors (Patel et al., 2019).

2.4.3 RADIOTHERAPY FOR PANCREATIC CANCER

High-energy x-rays are operated in this type of therapy to shrink and get rid of tumor cells. It is considered the most potent carcinoma treatment after surgery. Curative radiotherapy is used to heal tumors completely while neo-adjuvant radiotherapy combines radiotherapy with chemotherapy to make it more effective. Adjuvant radiotherapy is used after surgery to reduce the cancer risk restoration. Radiotherapy can be administered as small pieces of radioactive metals deposited near the tumor site (brachytherapy) or using the machine to transmit radiation beams where the cancer is located. Radioactive liquids or capsules can be also used as radioisotope therapy. These types of radiotherapy do harm normal cells unfortunately.

2.4.4 SURGERY FOR PANCREATIC CANCER

The surgical ablation of the tumor is considered the most promising cure for pancreatic cancer patients when cancer has not metastasized to nearby organs (Buanes, 2017). Unlike chemotherapy, surgery does not harm normal cells, indeed eradicates tumor cells only.

Whipple's procedure, distal pancreatectomy, and total pancreatectomy underlie surgery types for pancreatic cancer.

2.4.6 NANOMEDICINE FOR PANCREATIC CANCER

Because of their multifunctionality and improvements in cancer therapy, nanoparticles have become increasingly popular in cancer treatment and research. Combination therapy, tumor targeting, extended circulation, lower toxicity, and contemporaneous live imaging are all advantages of using nanoparticles. In nanomedicine, nanoparticles are utilized as drug carriers or as active agents. They are harnessed as therapeutic carriers for passive targeting of solid tumors via the EPR effect or active targeting using tumor specific ligands such as ligands that recognize HER2 and EGFR. Besides, these tiny particles have the potential to stimulate both the innate and adaptive immune systems, triggering an anti-cancer immune response. Furthermore, using nanoparticles to target cancer stem-like cells can help eradicate drug resistance and tumor sudden onset, as well as prevent metastasis (Patel et al., 2019).

2.4.7 TARGETED THERAPY IN PANCREATIC CANCER

This type of therapy targets proteins, genes, and specific molecules implicated in carcinogenesis using several drugs without affecting healthy cells. Erlotinib, combined with gemcitabine, downregulates the EGFR protein that aids in cancer sprouting and expansion when overexpressed in pancreatic cancer cells. Small-molecule drugs are another type of targeted therapy including angiogenesis inhibitors. They starve the tumors by blocking the sprouting of new blood vessels needed for cancer cells growth. However, targeted therapies are able to target cancer cells in distinct ways: (i) Shutting off malignant signals that activate tumorigenesis, (ii) Shattering of cancer cells, (iii) Limiting the cell survival threshold (Understanding Targeted Therapy, 2021).

2.5 RESISTANCE TO CANCER TREATMENTS

One of the major restrictions to anti-cancer treatments efficacy is the acquisition of resistance. The EMT process is controlled by specific key genes and cellular signaling processes. The E-cadherin and Zeb-1 proteins, modulators of EMT, are negatively correlated and their expression highly influences the chemoresistance phenomenon.

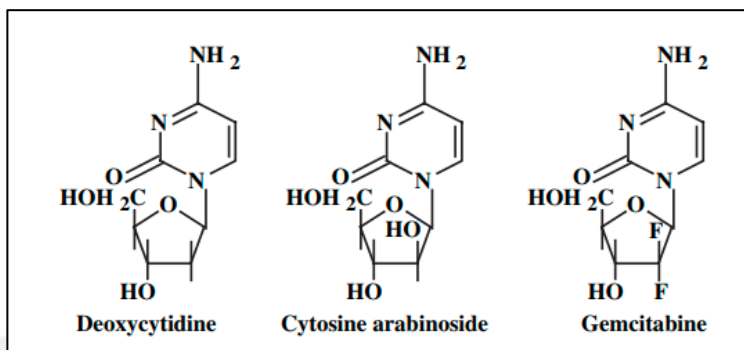
In pancreatic cancer, the dense desmoplastic stroma forms a physical barrier for drug delivery. Multiple factors such as Hedgehog, secreted protein acidic and rich in cysteine (SPARK), and cytokines help in maintaining the stromal nature and simultaneously weakening the tumor cells sensitivity to the therapeutic drugs.

2.6 GEMCITABINE

2.6.1 GEMCITABINE NATURE AND STRUCTURE

Gemcitabine is a deoxycytidine derivative exhibiting anti-proliferative characteristics whose mechanism of action is dependent on various intracellular molecular pathways. This chemotherapeutic medication with the chemical formula $C_9H_{11}F_2N_3O_4$ has always been considered as the first-line systemic treatment for pancreatic cancer with extensive antitumor efficacy (Mini et al., 2006; de Sousa Cavalcante & Monteiro, 2014). The most prominent cytidine analogue generated since cytosine arabinoside (Ara-C) is gemcitabine. Moreover, both chemotherapeutic medications are cytidine analogues that share structural and pharmacological similarities despite that Gemcitabine's metabolism and molecular mechanism is distinctive than cytosine arabinoside (Ara-C) (Mini et al., 2006).

Figure 2.2: Chemical structures of deoxycytidine, cytosine arabinoside, and gemcitabine



Source: (Mini et al., 2006)²

2.6.2 MECHANISM OF ACTION OF GEMCITABINE INSIDE THE CELL

Due to Gemcitabine's hydrophilic nature, it is transported into the cells through the nucleoside family transporters represented by two equilibrative (SLC29A1, SLC29A2) and three concentrative nucleoside transporters (SLC28A1, SLC28A2, SLC28A3). Na^+ is not required for equilibrative nucleoside transporters, however it is needed for concentrative nucleoside transporters to transmit Gemcitabine (Mini et al, 2006). Inside the cell, Deoxycytidine kinase converts gemcitabine into gemcitabine monophosphate (dFdCMP) which is then phosphorylated to generate two active metabolites: difluorodeoxycytidine diphosphate (dFdCDP) and difluorodeoxycytidine triphosphate (dFdCTP).

Both Gemcitabine molecules apply their inhibitory effects on DNA synthesis in two different ways; Gemcitabine diphosphate binds the ribonucleotide reductase's active site thereby increasing Gemcitabine's phosphorylation and causing shortage in deoxyribonucleotide pools production. In other way, Gemcitabine triphosphate incorporation into DNA is followed by a single deoxynucleotide, which prevents chain elongation and blocks DNA repairing enzymes from eliminating the Gemcitabine molecule. This molecular phenomenon is Known as the masked chain termination process constituting a main barrier against the DNA replication process (Mini et al., 2006; Amrutkar & Gladhaug, 2017).

² Mini, E., Nobili, S., Caciagli, B., Landini, I., & Mazzei, T. (2006). Cellular pharmacology of gemcitabine. *Annals of Oncology*, 17, v7–v12. <https://doi.org/10.1093/annonc/mdj941>

and the drug sensitivity. Upregulation of dCK in gemcitabine-resistant cell lines restored gemcitabine sensitivity, whereas suppression of dCK resulted in gemcitabine resistance (Saiki et al., 2012; Kroep et al., 2002).

2.6.3.3 CYTIDINE DEAMINASE

The CDA gene in *Homo Sapiens* codes for the enzyme cytidine deaminase. This gene produces an enzyme that assists in the pyrimidine salvage process. However, when the NH₂-group is removed from pyrimidine through the deamination process by converting 2',2'-difluorodeoxycytidine to 2',2'-difluorodeoxyuridine, Gemcitabine inactivation is accomplished (Maréchal et al., 2012). According to several in vitro investigations, CDA overexpression causes gemcitabine resistance whereas CDA deletion restores gemcitabine sensitivity showing that changes in CDA levels in pancreatic tumors might offer strategies for increasing the drug sensitivity (Weizman et al., 2013).

2.6.3.4 RIBONUCLEOTIDE REDUCTASE

Ribonucleotide reductase constituting of the M1 and M2 subunits plays a major role in deoxyribonucleotide formation through the conversion process of the DNA building blocks called ribonucleotides (Amrutkar & Gladhaug, 2017). The M1 subunit RRM1 has an enzyme regulatory binding site, while the M2 subunit RRM2 is implicated in the Ribonucleotide reductase activity. Unfortunately, RRM1 levels negatively correlate with survival rates in pancreatic cancer patients treated with the drug, with high expression of RRM1 being linked with low survival, implying that RRM1 plays a significant role in intrinsic resistance to gemcitabine (Nakano et al., 2007; Giovannetti et al., 2006; Nakahira et al., 2007).

Table 2.3: Gemcitabine Resistant Targets and Role in Drug Metabolism

Target	Pattern of resistance	Target's role in drug metabolism
Nucleoside transporters	Downregulated	Transport of Gemcitabine
Ribonucleotide reductase	Upregulated	Competition in DNA Synthesis
Cytidine deaminase	Upregulated	Inactivation of Gemcitabine

Deoxycytidine Kinase	Downregulated	Activation of Gemcitabine
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Source: Amrutkar et al., 2017

2.7 PRIMERS

2.7.1 E-CADHERIN

E-cadherin is a member of the cadherins family of proteins whose downregulation is one of the main initiation signals of the epithelial-mesenchymal transition (EMT) process (Acloque et al., 2009). This calcium dependent transmembrane protein is mainly engaged in cell-cell adhesion providing attachment between tissues and plays a major role in cancer suppression. However, nuclear factor- κ B signaling and several intracellular signal transduction pathways are mainly regulated by the cytoplasmic tail of E-cadherin protein (van Roy & Berx, 2008).

Loss of E-cadherin partially or completely weakens the interaction between adjacent cells, promote reduction of cell polarity and drives carcinoma progression. In pancreatic cancer, E-cadherin protein downregulation occurs due to several factors including mutations and transcriptional repression. E-cadherin loss is modulated by the activation of two oncogenic proteins: Slug and Snail and plays a key function in malignant transformation by acquiring tumorigenic activity under pathologic circumstances with mutational K-RAS activation (Kaneta et al., 2020). It was discovered that E-cadherin expression was poor or non-existent in chronic pancreatitis, which means that it is linked to a higher risk of pancreatic adenocarcinoma (PDAC) (Deng et al., 2014).

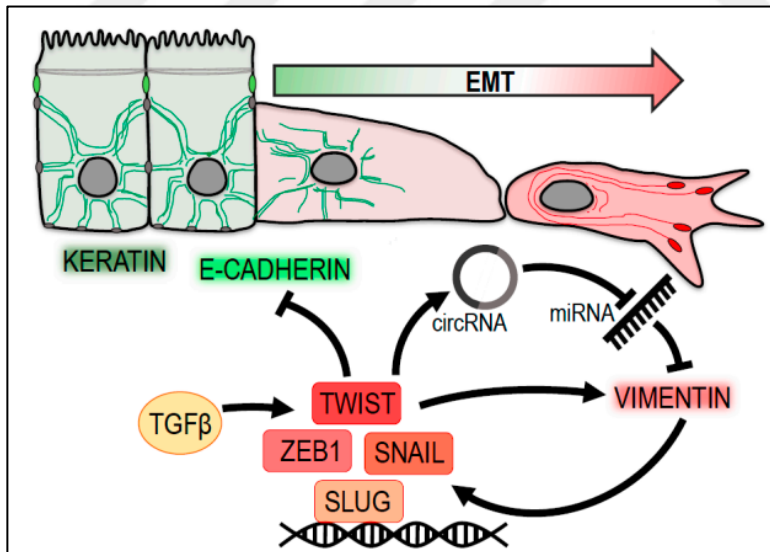
2.7.2 VIMENTIN

Vimentin is a polypeptide that belongs to the intermediate filament family of proteins mainly responsible for keeping the integrity between cells. This protein is expressed in mesenchymal cells and upregulated in many cancers enabling invasion, metastasis, and tumor growth once changes in their expression patterns strike; it is considered an active EMT marker. However, Vimentin has been found to interact with a variety of proteins including Filamen that forms cell extensions, AptA responsible for Erk1/2 mitogen-activated protein kinase activation, and

many other proteins engaged in the creation of complexes with cell signaling molecules through specific signaling pathways (Satelli & Li, 2011).

The elongated shape of mesenchymal cells is gained following Vimentin upregulation, this drives keratin IF's reorganization and rearrangement of actin and microtubules (Liu et al., 2015; Messica et al., 2017). Conversely, the downregulation of this intermediate filament inhibits invasion of cancer cells restoring their epithelial phenotype. Furthermore, the expression of Vimentin is mainly induced by four main EMT-related transcriptional through the activation of the TGF- β 1 polypeptide, overexpression of Snail and ZEB2, and Slug transcription factor phosphorylation (Cano et al., 2000; Bindels et al., 2006). Pancreatic tumorigenesis is also driven by non-coding microRNAs through miR-548an downregulation causing activation of Vimentin oncogene (Zhu et al., 2016).

Figure 2.4: Vimentin signaling pathways and cellular mechanism



Source: (Katerina et al., 2020)⁴

⁴ Strouhalova, K.; Přechová, M.; Gandalovičová, A.; Brábek, J.; Gregor, M.; Rosel, D. Vimentin Intermediate Filaments as Potential Target for Cancer Treatment. *Cancers* 2020, 12, 184. <https://doi.org/10.3390/cancers12010184>

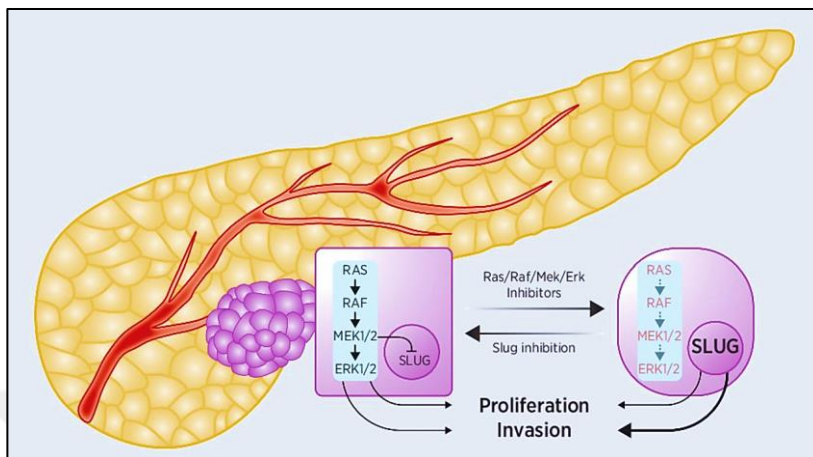
2.7.3 SNAIL

Snail is a zinc finger transcription factor that is vital for the creation of the mesoderm and neural crest during early embryogenesis (Carver et al., 2001). This protein is activated during the EMT process in which epithelial cells are transitioned into elongated shaped and migratory characterized mesenchymal cancer cells (Cano et al., 2000). When overexpressed, epithelial cells loose cell integrity and acquire invasive abilities enabling metastasis to nearby regions and lymph nodes. Snail and E-cadherin are inversely related; Snail transcription factor acting as a mesenchymal marker facilitates tumorigenesis and induces chemoresistance repressing E-cadherin expression in vivo and in vitro. However, Snail blocks E-cadherin signal transduction phenomenon by binding to E-cadherin's E-boxes located in the promoter region. However, EMT phenomenon aids in pancreatic cancer progression via Snail and Vimentin activation, and E-cadherin downregulation (Zhou et al., 2014).

2.7.4 SLUG

Slug, like Snail, is a regulatory transcription factor that belongs to the zinc finger family whose repression has been discovered to block pancreatic cancer cells invasion and malignancy. Besides its importance as a suppressor of the E-cadherin protein, this TF plays a major role in neuralization and organ formation (Zhu et al., 2016; Seki et al., 2003). Slug was targeted via suppression of the RAS–ERK1/2 route once Ras/ Raf/ Mek/ Erk inhibitors were administered to the pancreatic adenocarcinoma tissue as shown in the figure 2.5. Activation of the Slug transcription factor prior to their addition, initiated aggressiveness, and proliferation of tumor cells (Bilal et al., 2021).

Figure 2.5: Mechanism of activation and repression of Slug



Source: (Bilal et al., 2021)⁵

⁵ Bilal, F., Arenas, E. J., Pedersen, K., Martínez-Sabadell, A., Nabet, B., Guruceaga, E., Vicent, S., Tabernero, J., Macarulla, T., & Arribas, J. (2021). <https://doi.org/10.1158/0008-5472.can-20-4263>

3. MATERIALS AND METHODS

3.1 AsPC-1 and BxPC-3 Cell Line Culture

In this study, the human pancreatic cancer cell lines AsPC-1 and BxPC-3 are tested. They are collected from the American Tissue and Cell Collection (ATCC, USA) and can only be used for laboratory research purposes. These cell lines perform properties represented in the tables 3.1 and 3.2.

Table 3.1: Overview for AsPC-1 Cell Line Characteristics

Cell Line Name	AsPC-1
Organism	Humans
Tissue	Pancreas
Growth Properties	Adherent
Disease	Adenocarcinoma
Age	62 years
Gender	Female
Medium	RPMI -1640 ⁶

Table 3.2: Overview for BxPC-3 Cell Line Characteristics

Cell Line Name	BxPC-3
Organism	Humans
Tissue	Pancreas
Growth Properties	Adherent
Disease	Adenocarcinoma
Age	61 years
Gender	Female
Medium	RPMI -1640 ⁷

⁶ Catalog no: 302001 (ATCC, USA)

⁷ Catalog no: 302001 (ATCC, USA)

These two cell lines were cultured in accordance with the ATCC guidelines. They were cultured in RPMI medium supplemented with 10% Fetal Bovine Serum (FBS, Multicell, USA), with the media replaced on a regular basis.

The cells were maintained at 37°C with 5% CO₂ in a specialized cell culture incubator (NUVE, Turkey). Moreover, they were regularly examined with an inverted microscope (CKX41, Olympus). All cell culture operations were carried out in a Type II Biosafety Cabinet (NUVE, Turkey).

3.1.1 Cell's Passaging

After reaching confluency, the cells should be transferred into a fresh growth medium to allow their further propagation and proper growth. This procedure was subdivided into five main steps:

- a. After discarding the prior medium, the cells were rinsed with phosphate-buffered saline (PBS, Pan biotech, Germany) to get rid of the serum remains that may inhibit trypsin's effective role.
- b. Trypsin-EDTA (0.5 percent EDTA, Multicell) was added to the flask, enabling their further detachment.
- c. The cells were then incubated at 37°C with 5% CO₂ for 5 minutes to allow their optimal and proper detachment.
- d. Fresh medium was added to the cell suspension to shut down the trypsin's effect.
- e. The cell suspension was then subcultured into different flasks.

3.1.2 Cell's Freezing

Cryopreservation or cell freezing is a phenomenon that preserves the cells at a low temperature by using the aprotic organic solvent DMSO that prevents ice formation in cells during the freezing process.

- a. After rinsing the cells with PBS, Trypsin-EDTA was added to detach the cells.

- b. The cells were then incubated for 5 minutes at 37°C with 5% CO₂.
- c. A cell suspension was established after the addition of a complete medium.
- d. The cell suspension was then centrifuged at room temperature for 7 minutes at 1250 rpm.
- e. The supernatant was aspirated gently, and a freezing medium consisting of 90 percent FBS and 10 % DMSO was added to the remaining pellet.
- f. The whole mix, composed of the freezing medium and the cells, was transferred to a cryovial, and immediately stored at -80°C.

3.1.3 Thawing of Frozen Cells

This procedure aims to regain the viability of the preserved cells by inactivating the cryopreservation procedure, for cell culture usage.

- a. The preserved cryovial was gently placed in a warm water bath for a brief length of time (about 2 minutes) to melt the ice.
- b. The cells were then transferred to a falcon tube containing 8-10 ml of complete medium.
- c. The tube was centrifuged for 7 minutes at room temperature at 1250 rpm.
- d. The pellet was suspended with a fresh medium after further aspiration of the previous one.
- e. The new flask containing the cell suspension was further incubated at 37°C with 5% CO₂.

3.1.4 Optimization of Cell Number

Cell number optimization is a technique that calculates the number of cells required to meet the desired density in experimental studies. The number of cells obtained differs according to each cell line.

- a. Fresh medium was added to the cells enabling cell suspension after further detachment using trypsin.

- b. The average cell number was calculated by loading 10 μ l of the cell sample on a hemocytometer's slide and establishing a minimum of three counts under the microscope.
- c. Cell's concentration in the medium was performed using the equation below:
 - i. Cell concentration (cell/ml) = average of counted cells * 10.000
- d. Cell's overall number in the suspension was calculated using the equation below:
 - i. Overall number of cells = cell concentration (cell/ml) * the total volume (ml)
- e. In 6-well plates, different cell concentrations were seeded and kept at room temperature with 5% CO₂ to find out the cell's confluences.

3.2 Analyzing the Gene Expression Levels Of E-Cadherin, Vimentin, Slug, And Snail at the mRNA Level

3.2.1 Drug Treatment of AsPC-1 And BxPC-3 Cell Lines

AsPC-1 and BxPC-3 pancreatic cancer cell lines were treated with Gemcitabine drug to further analyze the expression of E-cadherin, vimentin, Slug, and Snail at the mRNA level. Gemcitabine is an anti-cancer chemotherapy acquired from Sigma drug with the catalog number G6423 and the chemical formula *C₉H₁₁F₂N₃O₄ HCl*.

For Drug treatment, this following protocol was established.

- a. Cells were seeded at a density of 650,000 cells/well for AsPC-1 and 600.000 cells/well for BxPC-3 in a 6 well plate to reach 70% confluency the following day.
- b. Cells were treated with Gemcitabine based on the IC₅₀ values after 24 hours of incubation at 37°C. Cells were treated with 0.1% DMSO as a negative control.
- c. Cells were incubated at 37°C for 48 hours.
- d. Following incubation, the growth medium was withdrawn, and the RNA isolation was carried out.

3.2.2 The RNA Isolation Principle

The extraction of the total RNA from treated cells was conducted using the GeneJET RNA Purification Kit (Thermo Scientific, USA) which is a simple yet effective technique for complete RNA purification performed through a multistep process.

- a. To avoid contamination, the working area and the materials should all be sterilized.
- b. In the hood, Lysis buffer was mixed with Mercaptoethanol.
- c. Cells were placed on ice to avoid molecules degradation where the growth medium was then removed using a serological pipette.
- d. Cells were washed with DPBS; DPBS was re-added, and scraping was established using an ice-cold scraper.
- e. After Scraping, the cells were transferred to a centrifuge tube where centrifugation was run for 5 minutes at 1250 *RCF and 4 degrees.
- f. The collection tubes were prepared and labeled during the centrifugation period.
- g. The supernatant was discarded, and the pellet was tapped very well.
- h. 600 micro liter of lysis buffer was added to the pellet. The mixture was then vortexed for 10 seconds.
- i. 360 micro liter of 96% ethanol was added. Pipetting was performed to mix the sample.
- j. 700 micro liter of the lysate was transferred to a GeneJET RNA purification column which was then inserted in a collection tube. Centrifugation for 1 minute at 1250 *RCF at 25 degrees is established. Then, the flow-through is discarded.
- k. The same steps are repeated until the lysate is all transferred.
- l. 700 micro liter of Wash Buffer 1 was added. The column was centrifuged for 1 minute at 12000 *RCF at 25 degrees. Then, the flow through was discarded.
- m. 600 micro liter of Wash Buffer 2 was added. The column was centrifuged for 1 minute at 12000 *RCF at 25 degrees. Then, the flow through was discarded.
- n. 250 micro liter was added to the column. The column was centrifuged for 2 minutes at 12000 *RCF at 25 degrees. Then, the flow through was discarded.
- o. The purification column is placed into a sterile 1.5 ml RNase-free microcentrifuge tube.

- p. In the center of the column membrane, 50 micro liter of nuclease free water was added for elution. Centrifugation is done for 1 minute at 12000 *RCF at 25 degrees.
- q. The purification column was discarded as a final step.

3.2.3 QRT-PCR Analysis

QRT-PCR principle allows for gene expression analysis because of its efficacy in detecting and precisely quantifying the target genes. In this study, this technique was used to measure the expression levels of E-cadherin, vimentin, Snail, and Slug in ASPC-1 and BxPC-3 cell lines after treatment with the drug. The qRT-PCR mixture was prepared with the iTaqTM Universal SYBR[®] Green One-Step Kit (Bio-Rad, USA). The qRT-PCR cycles were performed by the CFX96 machine (Bio-Rad, USA), and the data was analyzed by the CFX Manager software (Bio-Rad, USA). In the quantitative analysis of RT-PCR, GADPH was employed as an endogenous control.

3.2.3.1 Preparation of the QRT-PCR Reaction Mixture

Before running the machine, the qRT-PCR mix, mentioned in the table below, should be prepared.

Table 3.4: QRT-PCR Reaction Mix and Volume

Reaction mix components	Volume per 10 µl Reaction
iTaq universal SYBR [®] Green Reaction Mixture (2x)	5
iScript reverse transcriptase	0.125
Forward and reverse primers	0.4 for each primer
Template RNA, 20ng/µl	2
Nuclease-free water	2.075
Total	10 µl

3.2.3.2 QRT-PCR Cycles and Major Steps

Table 3.5: QRT-PCR Cycles and the Main Conditions Required

Steps	Conditions
cDNA Synthesis: Reverse transcription results in complementary DNA as DNA is synthesized from an RNA starting material	50°C for 10 minutes
Initial denaturation: The initial DNA template is complex and requires denaturation for further shortage	95°C for 5 minutes
40 cycles for the following steps	
Denaturation: The hydrogen bonds are disrupted allowing the separation of the double stranded DNA templates	95°C for 10 seconds
Annealing: Primers are allowed to bind the DNA template to promote extension	
Extension: The hybridized primers are extended after recruitment of the DNA polymerase enzyme	58°C for 20 seconds
Fluorescent signal measurement after every cycle	
Final Extension: The complete synthesis of all PCR products is achieved	72°C for 30 minutes
Melting Curve Analysis	

Gradual Increase of Temperature I	Between 40°C- 65°C, at 0.05°C increments
Gradual Increase of Temperature II	Between 65°C- 95°C, at 0.5°C increments

The GeneBank data and primer sequences of the genes (E-cadherin, vimentin, Snail, Slug and GAPDH) are provided, as well as the size of their amplification products.

3.2.3.3 Sequence of Primers

Primer sequences of E-cadherin, vimentin, Slug, and Snail are listed in the Table 3.6. GAPDH housekeeping gene is a key regulatory enzyme used in this experiment to normalize the data.

Table 3.6: Forward and Reverse Primers Data Sequences

<i>HOMO SAPIENS E-CADHERIN (NM_004360_L1)</i>	Forward primer 5'-TACACCATCCTCAGCCAAGA-3' Reverse Primer 5'-ACCTGACCCTTGTACGTGGT-3'
<i>HOMO SAPIENS VIMENTIN (NM_003380_L1)</i>	Forward primer 5'-AGGTGGACCAGCTAACCAAC-3' Reverse primer 5'-GGATTTCTCTTCGTGGAGT-3'
<i>HOMO SAPIENS SLUG (NM_003068_L1)</i>	Forward primer 5'-TGGTTGCTTCAAGGCCACAT-3' Reverse primer 5'-GTGCTACACAGCAGCCAGAT-3'
<i>HOMO SAPIENS SNAIL (NM_005985_L1)</i>	Forward primer 5' TGCAGGACTCTAATCCAGAGTTT 3' Reverse primer 5' CTCATCTGACAGGGAGGTCA 3'

<i>HOMO SAPIENS GAPDH (NM_002046.5)</i>	Forward primer 5' GTGAAGGTCGGAGTCAACG 3' Reverse primer 5' TGATTTTGGAGGGATCTCGC 3'
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3.2.4 Statistical Analysis

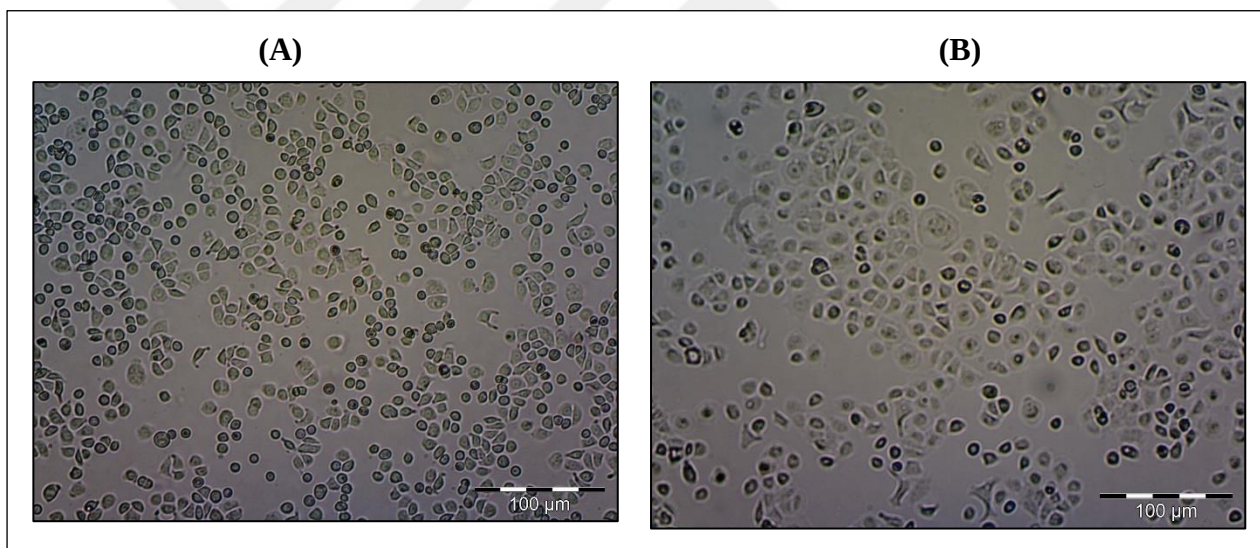
CFX Manager Software (Bio-Rad, USA) was used to analyze the results obtained. This software first determines the number of quantification cycles (Cq), then uses the Cq method to normalize the expression levels, and finally uses a paired t-test to see if the difference between the expression levels is significant. The P-value, or significance value, was adjusted to be less than 0.05.

4. RESULTS

4.1 Maintaining AsPC-1 and BxPC-3 Cells

AsPC-1 and BxPC-3 pancreatic cancer cell lines were cultured, maintained and well-nourished under the optimal conditions. The medium was changed every 48 hours and the cells were passaged when their confluency reached 70%. The figure 4.1 shows the shape and morphology of AsPC-1 and BxPC-3 cell lines under the microscope.

Figure 4.1 : (A) AsPC-1 cells, and (B) BxPC-3 cells under the microscope with magnification of 4X



4.2 Drug Treatment of AsPC-1 And BxPC-3 Cells and IC₅₀ Analysis

The IC₅₀ values of Gemcitabine on AsPC-1 and BxPC-3 cells were determined in a bachelor thesis from our group (Simay Dolaner, 2021) using the WST-1 reagent that measures the metabolic activity of cells after Gemcitabine treatment at half log concentrations (10nM, 30nM, 100nM, 300nM, 1µM, 3µM, 10µM, 30µM, 100µM). According to this previous study, the IC₅₀ value for Gemcitabine in AsPC-1 was found to be 0.2384 µM and 0.06867 µM in BxPC-3 and it was displayed by GraphPad Prism Software. In parallel with this study,

both cell lines were treated with 0.1 percent DMSO as a negative control, and with 0.2384 μ M and 0.06867 μ M Gemcitabine in AsPC-1 and BxPC-3 respectively for 48 hours.

4.3 E-Cadherin, Vimentin, Snail, And Slug Genes Expression Analysis in Gemcitabine Treated AsPC-1 And BxPC-3 Cells at the mRNA Level

4.3.1 RNA Isolation

Total RNA was isolated prior 48 hours from Gemcitabine treatment. In both cell lines, the purity values of the isolated RNA were between 2.0 and 2.1 which assure RNA purity from any contaminants including proteins and nucleic acids. The RNA concentrations are demonstrated in the Table 4.1.

Table 4.1: RNA concentration in treated and non-treated cell lines

CELL LINE	RNA CONCENTRATION
AsPC-1 (0.1 percent DMSO)	37.82 ng/ μ l
AsPC-1 (0.2384 μM GEMCITABINE)	36.34 ng/ μ l
BxPC-3 (0.1 percent DMSO)	40.16 ng/ μ l
BxPC-3 (0.06867 μM GEMCITABINE)	22.46 ng/ μ l

4.3.2 Quantitative Real Time-Polymerase Chain Reaction Analysis

Quantitative real-time polymerase chain reaction (QRT-PCR) was used to assess the expression levels of E-cadherin, vimentin, Snail, and Slug in AsPC-1 and BxPC-3 cancer cell lines prior to Gemcitabine treatment for 48 hours. GAPDH was utilized as a house-keeping gene for data normalization in this research. In the Figures 4.2 and 4.3, the melting curve and melting peak analysis of QRT-PCR data are shown respectively, with a single peak in the melting peak graph demonstrating that the primers are highly specific, and the mRNA is pure and devoid of impurities.

Figure 4.2: QRT-PCR melting curve analysis

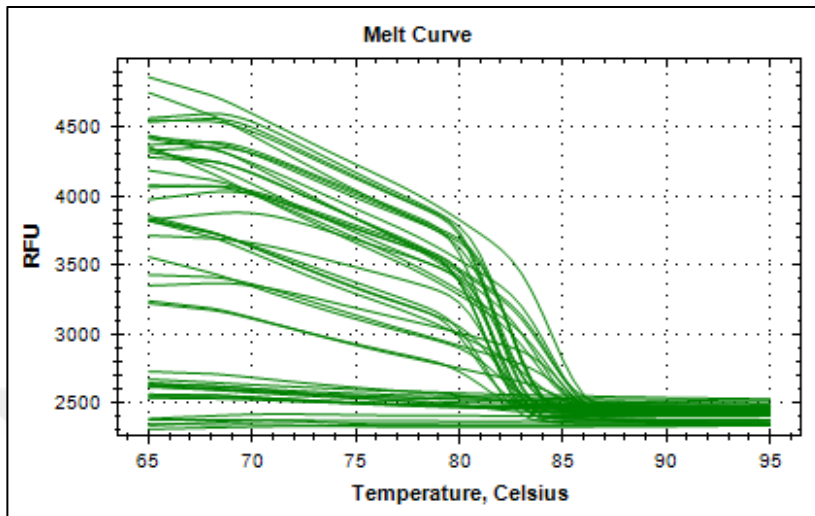
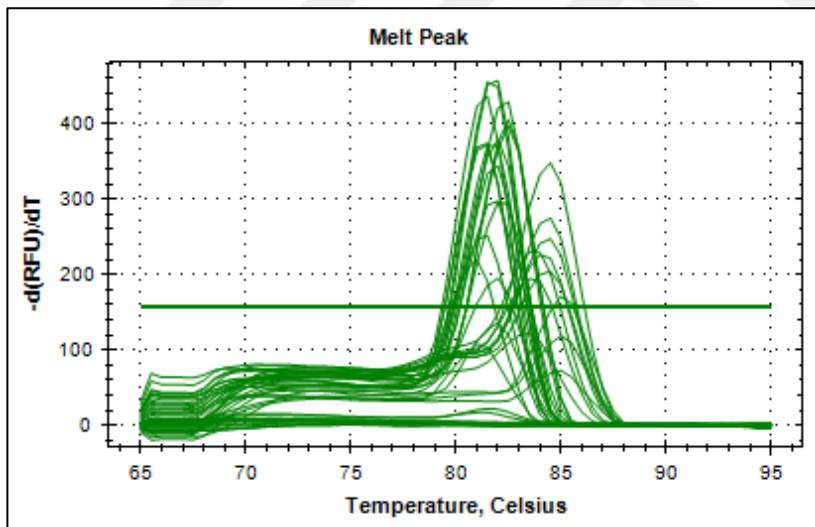


Figure 4.3: QRT-PCR melting peak analysis



Results revealed that the mRNA levels of E-cadherin were highly upregulated in BxPC-3 and AsPC-1 cell lines as shown in Figures 4.4 and 4.8 and the result was not significant. Statistical Package for the Social Sciences software (Version 20.0, SPSS Inc., Chicago, IL) was used for the statistical analyses ($p > 0.05$). Vimentin was downregulated in BxPC-3 cell line as demonstrated in Figure 4.5 not the case in AsPC-1 cells in which a higher expression of this

protein was shown after treatment with the drug compared with the non-treated control cells as demonstrated in Figure 4.9.

Figure 4.10 indicate that Snail and Slug showed no expression in Gemcitabine treated AsPC-1 cells while they showed a downregulation in BxPC-3 cancer cells as presented in Figures 4.6 and 4.7. The expression of Slug in AsPC-1 cell line only showed a significance compared to others ($p>0.05$). According to Snail, no expression was found in both treated and non-treated AsPC-1 cells.

Expression analysis of E-cadherin, vimentin, Snail, and Slug genes in BxPC-3 cells after 48 hours of Gemcitabine treatment are given in Figures 4.4, 4.5, 4.6, and 4.7.

Figure 4.4: Relative normalized expression of E-cadherin in BxPC-3 cells

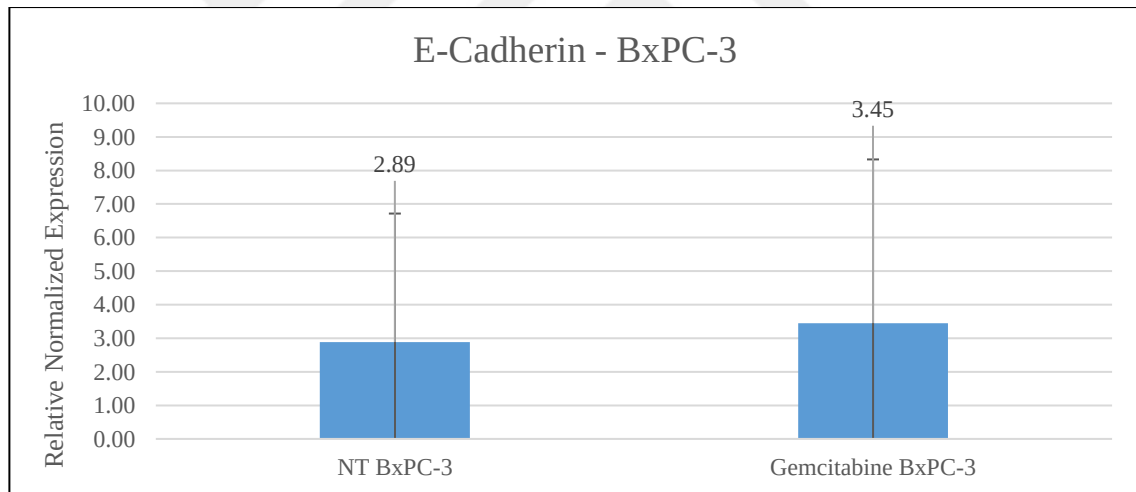


Figure 4.5: Relative normalized expression of vimentin in BxPC-3 cells

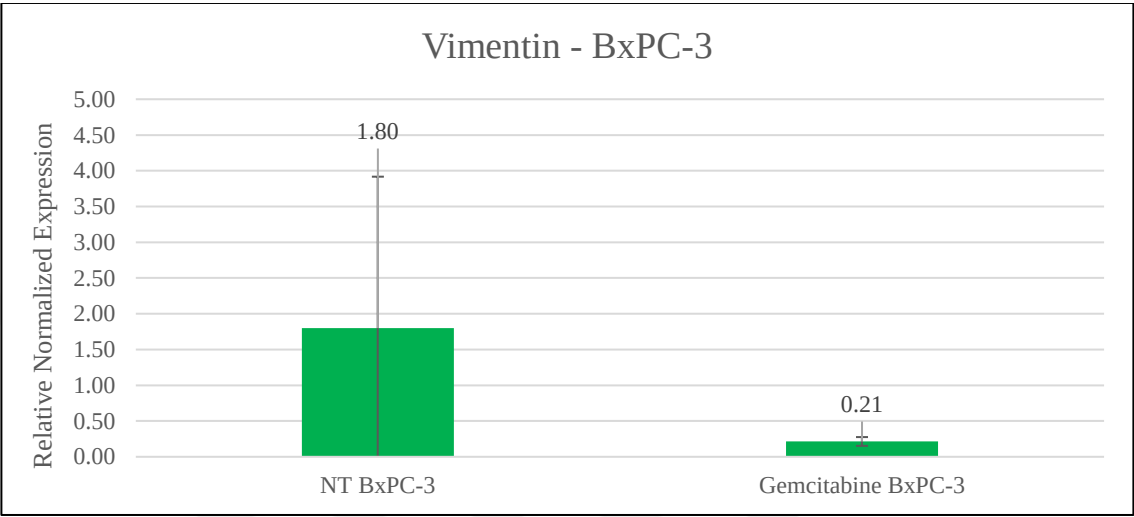


Figure 4.6: Relative normalized expression of Snail in BxPC-3 cells

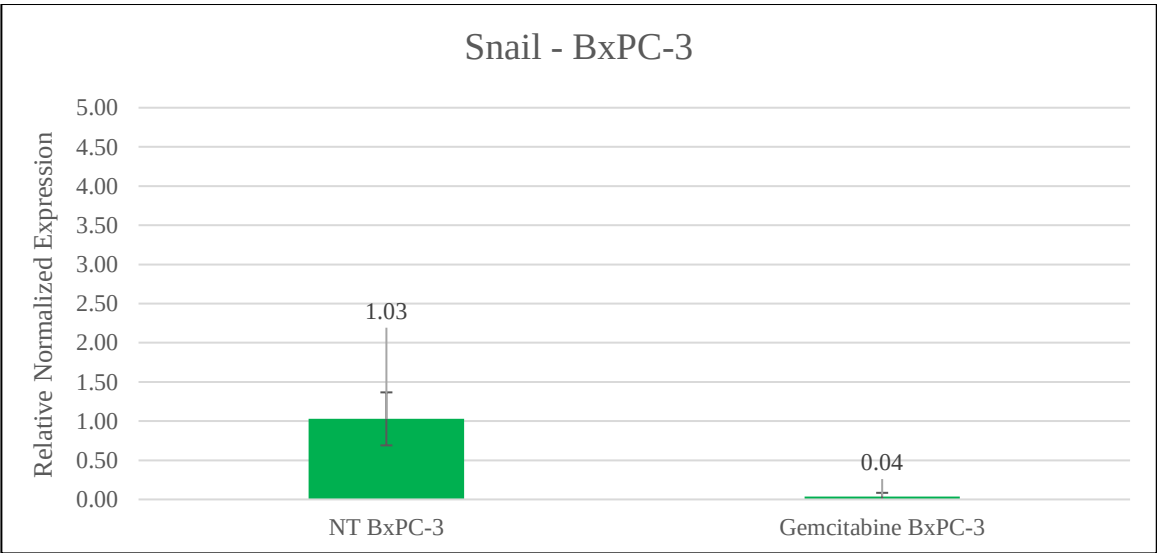
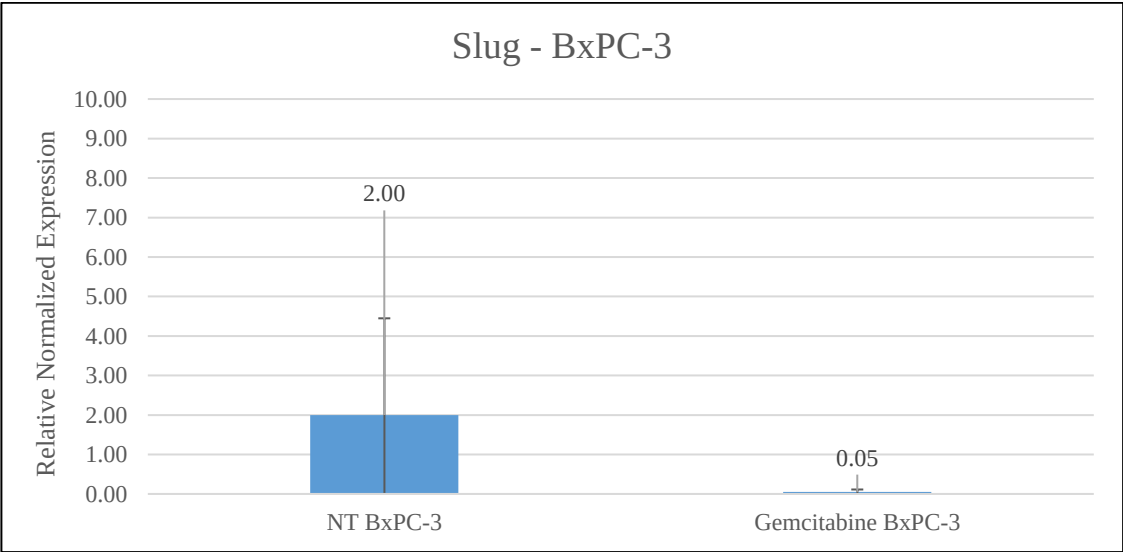


Figure 4.7: Relative normalized expression of Slug in BxPC-3 cells



Expression analysis of E-cadherin, vimentin, and Slug genes in AsPC-1 cells after 48 hours of Gemcitabine treatment are given in Figures 4.8, 4.9, and 4.10.

Figure 4.8: Relative normalized expression of E-cadherin in AsPC-1 cells

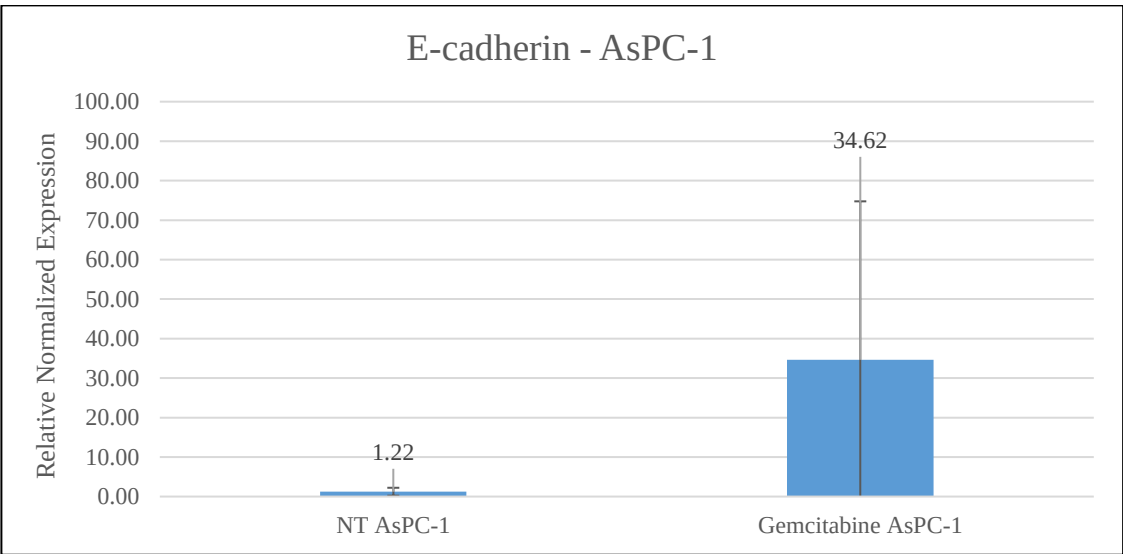


Figure 4.9: Relative normalized expression of vimentin in AsPC-1 cells

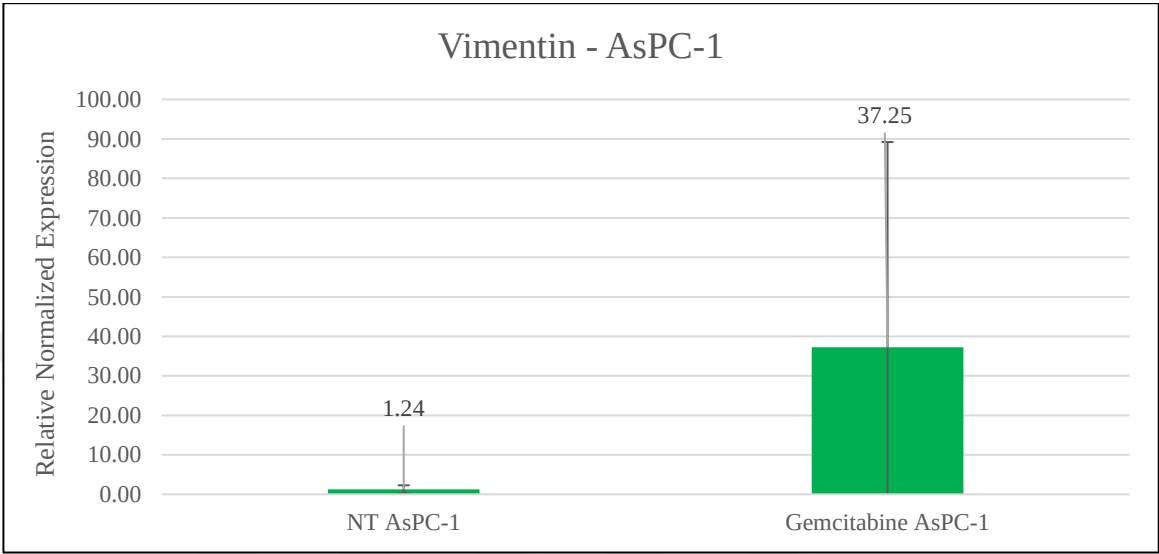
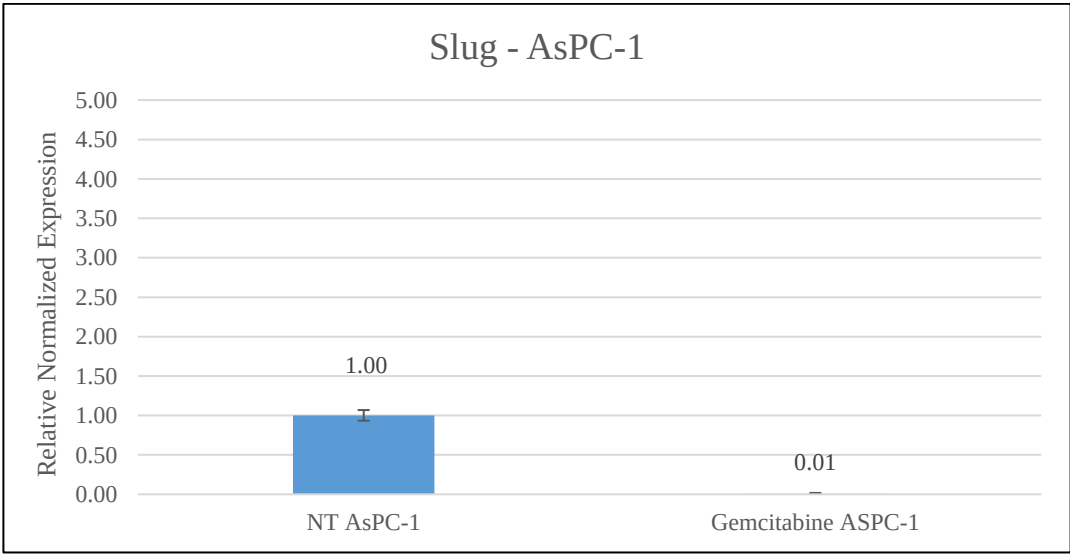


Figure 4.10: Relative normalized expression of Slug in AsPC-1 cells



1. DISCUSSION

Pancreatic cancer is considered the 7th major and leading cause of men and woman cancer mortality worldwide comprising 80-85% of cases in the USA with unresectable or metastatic malignancy (American Cancer Society, 2020; Mizrahi et al., 2020). It is predicted by the European Union that pancreatic cancer will exceed breast cancer which is considered one of the most lethal type of cancers (Global cancer statistics, 2018). Alterations in p53, SMAD4, KRAS, and BRCA2 modulating genes are the main drivers of Pancreatitis, PanIN and IPMN pancreatic disorders characterized each by distinct invasive features. Additional genetic alterations and mutations end in metastatic and malignant cancer.

Patient outcomes have improved gradually but significantly because of advances in diagnostic methods, surgical care, radiation therapies, and systemic drugs. To have a clinically significant impact, new ways for screening high-risk patients to detect pancreatic tumors at an earlier stage are badly needed.

Chemoresistance is considered a major restriction in cancer therapy. Failure to treat cancer merely helps it progress to a later stage where treatment becomes even more challenging. Chemoresistance simultaneously weakens the tumor cells sensitivity to therapeutic drugs which is why the demand for tailored therapy is mandatory.

EMT plays a fundamental aspect in pancreatic cancer malignancy and tumor growth. This phenomenon is defined by the downregulation of epithelial markers and the acquisition of a mesenchymal phenotype, which leads to basement membrane disintegration and the generation of a mesenchymal cell capable of migrating and invading other body entities. EMT is initiated by the activation of certain transcription factors, the synthesis of ECM-degrading proteins, and changes in the expression of microRNAs (Kalluri & Weinberg, 2009).

The cell–cell adhesion receptor, E-cadherin, is a tumor suppressor that slows down tumor progression. In many carcinomas, E-cadherin function is disrupted, and this cadherin

malfunction promotes cancer development and malignant transformation. Particularly, Pancreatic cancer is associated with a reduction in total E-cadherin expression or a loss in its usual placement at cell–cell interactions. However, the downregulation of this protein is highly associated with the activation of many oncogenes (Sommariva, Gagliano et al., 2020).

The intermediate filament family of proteins along with vimentin stabilizes cytoskeletal interactions and is considered a major EMT marker. However, vimentin's concentration is low in the early stages of carcinogenesis and increases gradually until tumor cells metastasize and invade the surrounding entities (Usman et al., 2021).

Snail and Slug, both members of the SNAI superfamily of proteins involved in organ formation and tissue regeneration, play a key role in epithelial cell transition to a mesenchymal cell state (Ganesan, Ramya et al., 2016).

This study aims to prove that E-cadherin, vimentin, Snail and Slug are strong potential targets for therapy due to their critical role in metastasis and invasion. For this purpose, the potential effects of Gemcitabine on E-cadherin, vimentin, Snail, and Slug gene expression levels were analyzed on AsPC-1 and BxPC-3 pancreatic adenocarcinoma cell lines. In the first step, AsPC-1 and BxPC-3 cells were seeded to reach 70 percent confluency in preparation for drug treatment the next day. After examining the healthy cells under the microscope, the AsPC-1 cell line was treated for 48 hours with be 0.2384 μ M Gemcitabine and 0.06867 μ M for BxPC-3. AsPC-1 and BxPC-3 cell lines were treated with 0.1 percent DMSO as a negative control. RNA was extracted from both cell lines, and QRT-PCR was performed to assess the effect of Gemcitabine on the mRNA expression levels of E-cadherin, vimentin, Snail, and Slug genes. GAPDH housekeeping gene was used in this experiment as a reference for data normalization.

QRT-PCR data revealed that the mRNA level of E-cadherin was upregulated in both cell lines, while the expression of vimentin was downregulated in BxPC-3 but enhanced in AsPC-

1 cell line compared with the non-treated controls. However, Snail and Slug expression in BxPC-3 cell line were highly downregulated which assures the efficient inhibitory effect of Gemcitabine.

Upregulation of the EMT markers vimentin, Snail, and Slug, as well as synergistic loss of E-cadherin, are defining characteristics of the epithelial-mesenchymal transition that drives cancer cell malignancy. The overexpression of E-cadherin in parallel with the downregulation of mesenchymal markers in BxPC-3 cancer cells after Gemcitabine treatment demonstrates Gemcitabine's potential as a promising chemotherapeutic agent.

E-cadherin and vimentin expression were increased in the AsPC-1 cell line, whereas Snail and Slug were not expressed. Even though E-cadherin expression was upregulated after drug treatment, vimentin overexpression indicates that Gemcitabine did not lower the expression of this gene, showing that an adverse effect occurred, resulting in Gemcitabine's ineffectiveness in the AsPC-1 adenocarcinoma cell line. Monotherapy may not be effective in this scenario, and a combination of drugs may be required to offset Gemcitabine's impact and downregulate vimentin. A combination of two chemotherapeutic drugs would form a good combinational therapy.

As reported in Hotz et al., 2007 study, Snail was found to have low or no expression levels in AsPC-1 cancer cells, in contrast to Slug which had a high expression level compared to Snail. In this experiment, non-treated AsPC-1 cells showed no expression level of Snail, but an elevated expression of Slug. SNAI members expression were not detectable after drug treatment. According to Slug, it was stated in Zhang et al., 2011 and Hotz et al., 2007 studies that there was no relation found between the expression of Slug and E-cadherin.

E-cadherin was increased in the BxPC-3 cell line, whereas vimentin, Snail, and Slug were downregulated, indicating that Gemcitabine was effective and might be used as a chemotherapeutic treatment to stop tumor proliferation and invasion. In a prior study published by Heinemann et al., 2006, it was found that when Gemcitabine was paired with

Cisplatin, patients with advanced pancreatic cancer had a greater median overall survival than when Gemcitabine was used alone.



2. CONCLUSION

The results drawn from this experiment show a decrease in vimentin, Snail, and Slug expression levels in BxPC-3 cell line following Gemcitabine treatment as opposed to an upregulation in vimentin expression levels in AsPC-1 cell line. Accordingly, E-cadherin and Snail prove to be promising future targets for cancer treatment while vimentin might prove to be an adverse factor. This may be related to the development of chemoresistance in AsPC-1 cell type. In future research studies, Gemcitabine and Cisplatin may form a favorable combination therapy that can enhance the inhibitory activity of Gemcitabine and diminish vimentin expression in AsPC-1 cell line.

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