HELLENIC REPUBLIC
National and Kapodistrian University of Athens
EST. 1837

"ALEXANDER FLEMING"
Biomedical Sciences Research Center

International M.Sc. in "Molecular Biomedicine - Disease Mechanisms, Molecular and Cellular therapies, and Bioinnovation" Medical School, University of Athens – BSRC "Alexander Fleming"

"Study of the effect of Sirtuins polymorphisms in pancreatic cancer"

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Athens, 2024

ACKNOWLEDGEMENTS

This dissertation thesis was conducted within the framework of the Master's program "Molecular Biomedicine - Disease Mechanisms, Molecular and Cellular therapies and Bioinnovation" of the Medical school of National and Kapodistrian University of Athens and Biomedical Sciences Research Center "Alexander Fleming". The experiments were carried out in the laboratory of Biology of the Medical school of Athens under the supervision of Professor Maria Gazouli.

First of all, I would like to thank my supervisor, Professor Maria Gazouli, for her continuous support as well as excellent guidance and communication throughout our collaboration, from the 3-month internship to the conduction of my thesis. I would like also to thank Professor Maria Roubelakis and Professor Athanassios Kotsinas for kindly accepting being members of my dissertation 3-member committee.

I would like to thank all of the members in our lab, in which I had the opportunity to collaborate smoothly and pleasantly. Matina, Orestis, Ektoras, Evangelia, Veatriki, Sophia and Vivian were excellent colleagues and I hope our friendship continues outside the laboratory.

Finally, I would like to thank my family, my friends and Lena for the continuous support during these 2 years of the Master's programme.

CONTENTS

Abstract

Pancreatic ductal adenocarcinoma represents 90 % of pancreatic cancer and is considered one of the deadliest malignancies in the 21st century. It is estimated to become the second cause of cancer-associated mortality only behind lung cancer in a few decades. Even if survival statistics have improved over the last decade through the understanding of biological basis of disease and the improvement in clinical care, the 5-year survival rate remains very poor and does not exceed 12% while less than 20% of patients survive for more than 1 year. The absence of straightforward, early detection techniques contributes to the disease's late diagnosis and subsequently high mortality since symptoms do not manifest until cancer has advanced and spread. Pancreatic neuroendocrine tumors (PNETs) on the other hand, arise from neuroendocrine cells in the pancreas, exhibiting slower growth and hormonal hypersecretion compared to pancreatic ductal adenocarcinoma (PDAC). Despite their rarity, PNETs display heterogeneity in histology and clinical behavior. Prognosis varies based on factors such as tumor size, grade, and metastasis, with 5-year survival rates ranging from 40% to 90%. Despite advancements in treatment, including surgery and targeted therapies, challenges in managing PNETs persist, warranting ongoing research efforts for improved outcomes.

The purpose of this study is to investigate the potent association of 3 single nucleotide polymorphisms of Sirtuin 1 (SIRT1), a protein deacetylase involved in various cellular processes including aging, metabolism, and tumorigenesis. The samples were genotyped for the polymorphisms: rs3758391, rs144124002 and rs369274325.

Rs3758391 and rs144124002 polymorphisms were tested both in PDAC and PNET patients, whereas rs369274325 was tested only in PDAC patients. Genotype frequency comparison showed no association of rs144124002 with PDAC and PNET. Rs369274325 polymorphism showed also no significant association with PDAC. In case of rs3758391, there was a very strong association with PDAC, both in genotype and allele level. Unfortunately, due to a small number of samples of PNET patients, no safe conclusions could be extracted.

In conclusion, the rs3758391 polymorphism could serve as a potential biomarker for pancreatic adenocarcinoma due to its strong association with the disease, as it was shown at this study.

Περίληψη

Το πορώδες παγκρεατικό αδενοκαρκίνωμα αντιπροσωπεύει το 90 % του καρκίνου του παγκρέατος και θεωρείται μία από τις πιο θανατηφόρες κακοήθειες του 21ου αιώνα. Εκτιμάται ότι σε λίγες δεκαετίες θα γίνει η δεύτερη αιτία θανάτου που σχετίζεται με τον καρκίνο, μετά τον καρκίνο του πνεύμονα. Παρόλο που τα στατιστικά στοιχεία ως προς την επιβίωση έχουν βελτιωθεί την τελευταία δεκαετία μέσω της κατανόησης της βιολογικής βάσης της νόσου και της βελτίωσης της κλινικής φροντίδας, το ποσοστό 5ετούς επιβίωσης παραμένει πολύ φτωχό και δεν ξεπερνά το 12%, ενώ λιγότερο από το 20% των ασθενών επιβιώνει για περισσότερο από 1 έτος. Η απουσία απλών, πρώιμων τεχνικών ανίχνευσης συμβάλλει στην καθυστερημένη διάγνωση της νόσου και στη συνέχεια στην υψηλή θνησιμότητα, καθώς τα συμπτώματα δεν εκδηλώνονται μέχρι ο καρκίνος να προχωρήσει και να εξαπλωθεί σε άλλες περιοχές. Από την άλλη πλευρά, οι παγκρεατικοί νευροενδοκρινείς όγκοι (PNET) προέρχονται από νευροενδοκρινικά κύτταρα του παγκρέατος, παρουσιάζοντας βραδύτερη ανάπτυξη και ορμονική υπερέκκριση σε σύγκριση με το πορώδες αδενοκαρκίνωμα του παγκρέατος (PDAC). Παρά τη σπανιότητά τους, τα PNETs εμφανίζουν ετερογένεια στην ιστολογία και την κλινική συμπεριφορά. Η πρόγνωση ποικίλλει με βάση παράγοντες όπως το μέγεθος του όγκου, ο βαθμός και οι μεταστάσεις, με τα ποσοστά 5ετούς επιβίωσης να κυμαίνονται από 40% έως 90%. Παρά τις εξελίξεις στη θεραπεία, συμπεριλαμβανομένης της χειρουργικής επέμβασης και των στοχευμένων θεραπειών, οι προκλήσεις στη διαχείριση των PNET εξακολουθούν να υφίστανται, γεγονός που επιτάσσει συνεχείς ερευνητικές προσπάθειες για τη βελτίωση των αποτελεσμάτων.

Σκοπός της παρούσας μελέτης είναι η διερεύνηση της πιθανής συσχέτισης 3 μονονουκλεοτιδικών πολυμορφισμών (αλλαγή μίας νουκλεοτιδικής βάσης) της Σιρτουίνης 1 (SIRT1), μιας πρωτεϊνικής αποακετυλάσης που εμπλέκεται σε διάφορες κυτταρικές διεργασίες, συμπεριλαμβανομένης της γήρανσης, του μεταβολισμού και της καρκινογένεσης. Πραγματοποιήθηκε γονοτύπηση των δειγμάτων για τους πολυμορφισμούς: rs3758391, rs144124002 και rs369274325.

Οι πολυμορφισμοί rs3758391 και rs144124002 εξετάστηκαν τόσο σε ασθενείς με PDAC όσο και σε ασθενείς με PNET, ενώ ο rs369274325 εξετάστηκε μόνο σε ασθενείς

με PDAC. Η σύγκριση της συχνότητας των γονοτύπων δεν έδειξε καμία συσχέτιση του rs144124002 με την εμφάνιση PDAC και PNET. Ο πολυμορφισμός rs369274325 δεν έδειξε επίσης σημαντική συσχέτιση με το PDAC. Στην περίπτωση του rs3758391, υπήρξε πολύ ισχυρή συσχέτιση με το παγκρεατικό αδενοκαρκίνωμα, τόσο σε επίπεδο γονότυπου όσο και σε επίπεδο αλληλόμορφου. Δυστυχώς, λόγω του μικρού αριθμού δειγμάτων ασθενών με PNET, δεν ήταν δυνατή η εξαγωγή ασφαλών συμπερασμάτων.

Συμπερασματικά, ο πολυμορφισμός rs3758391 θα μπορούσε να χρησιμεύσει ως δυνητικός βιοδείκτης για το παγκρεατικό αδενοκαρκίνωμα λόγω της ισχυρής συσχέτισής του με τη νόσο, όπως φάνηκε στην παρούσα μελέτη.

1.INTRODUCTION

1.1 Pancreas anatomy and physiology

The pancreas is a vital organ with dual roles in the body, functioning as both an endocrine and exocrine gland. Located in the abdominal cavity, nestled between the stomach and the spine, it plays a crucial role in both digestive and metabolic processes. The pancreas measures approximately 14-25 cm in length, weighs 100-150 g and is anatomically divided into five main parts: the head, uncinate process, neck, body, and tail. [1,2]The head of the pancreas lies in the curve of the duodenum, which is the first part of the small intestine. The neck connects the head to the body, which extends transversely across the abdomen, while the tail is the thin, tapered end of the pancreas that lies near the spleen. [1]

The endocrine function of the pancreas is performed by clusters of cells known as the islets of Langerhans. These islets contain several types of hormone-producing cells, including alpha cells that secrete glucagon, a hormone that raises blood glucose levels by stimulating the liver to convert stored glycogen into glucose. Beta cells produce insulin, which is crucial for the regulation of blood glucose levels, facilitating the uptake of glucose into cells for energy production and storage. Delta cells release somatostatin, which regulates the secretion of both glucagon and insulin, as well as other hormones.[3] Additionally, PP cells produce pancreatic polypeptide, which helps regulate pancreatic secretion activities and the hepatic metabolism of glycogen, and epsilon cells secrete ghrelin, which stimulates appetite and plays a role in regulating energy balance. [2]

The exocrine function of the pancreas is managed by acinar cells, which produce digestive enzymes, and ductal cells, which secrete bicarbonate. These enzymes and bicarbonate are delivered through a network of ducts into the duodenum. The key enzymes produced include amylase, which breaks down carbohydrates into simple sugars; lipase, which splits fats into fatty acids and glycerol; and proteases (such as trypsin and chymotrypsin), which degrade proteins into amino acids. [4]

The pancreas plays a pivotal role in digestion and metabolism. The exocrine function is triggered primarily by the food ingestion. When food enters the stomach and

duodenum, it stimulates the release of hormones like secretin and cholecystokinin (CCK). Secretin prompts the pancreas to release bicarbonate, neutralizing the stomach acid entering the small intestine, creating an optimal pH for digestive enzymes. CCK stimulates the secretion of pancreatic enzymes essential for digesting carbohydrates, proteins, and fats.

The endocrine function, particularly the regulation of blood glucose levels, is critical for maintaining energy homeostasis. After eating, rising blood glucose levels stimulate beta cells to release insulin, facilitating glucose uptake by cells and reducing blood glucose levels. Between meals, or during fasting, alpha cells release glucagon to maintain blood glucose levels by promoting glucose production in the liver. Insufficient insulin production or the body's inability to use insulin effectively leads to diabetes mellitus, a chronic condition that affects millions worldwide. Type 1 diabetes is characterized by autoimmune destruction of beta cells, while type 2 diabetes involves insulin resistance and eventual beta cell dysfunction. [1]

In summary, the pancreas is a complex organ with essential endocrine and exocrine functions. It plays a central role in digestion through enzyme production and in metabolism by regulating blood glucose levels via hormone secretion. The intricate balance of its functions is crucial for overall health, and disruptions can lead to significant metabolic disorders. Understanding the pancreas’s organization and physiological roles highlights its importance in maintaining homeostasis and supporting life.

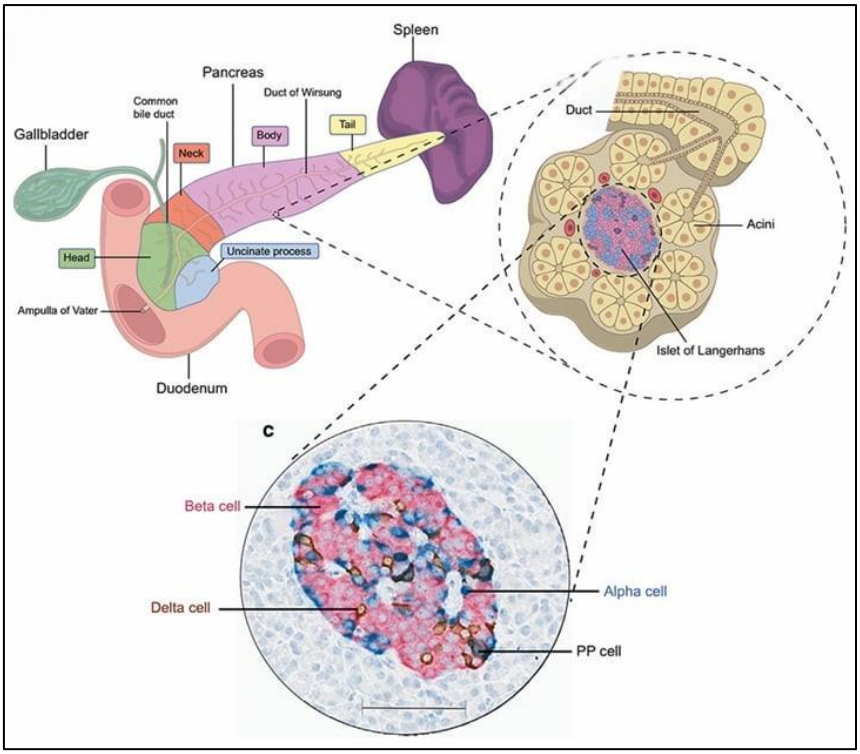

Figure 1: Organization of the human pancreas, organ parts and cell types. Atkinson, M. A., Campbell-Thompson, M., Kusmartseva, I. & Kaestner, K. H. Organisation of the human pancreas in health and in diabetes. *Diabetologia (2020)*

1.2 Pancreatic Ductal Adenocarcinoma: Characteristics, Mortality, and Epidemiology

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer, accounting for over 90% of all pancreatic malignancies. Originating from the ductal cells of the pancreas, PDAC is characterized by its aggressive nature and poor prognosis. The tumor is typically hard, scirrhous, and often involves desmoplastic stroma, which contributes to its resistance to chemotherapy.[45]

Clinically, PDAC often presents late in its course due to the retroperitoneal location of the pancreas, leading to nonspecific symptoms like abdominal pain, weight loss, jaundice (if the tumor obstructs the bile duct), and new-onset diabetes. Diagnostic tools include imaging studies like CT scans, MRI, and endoscopic ultrasound (EUS) with fine-needle aspiration for histological confirmation. Microscopically, PDAC is marked by gland-forming cells that produce mucin, with significant cellular atypia and a high degree of mitotic activity. Common genetic alterations include mutations in *KRAS* (found in over 90% of cases), *CDKN2A, TP53*, and *SMAD4*.[5]

PDAC is associated with one of the highest mortality rates among cancers. The five-year survival rate is less than 10%, primarily due to late-stage diagnosis.[6] The majority of patients have either locally advanced or metastatic disease at the time of diagnosis. Prognostic factors include the stage at diagnosis, with early-stage PDAC (confined to the pancreas) having a relatively better prognosis with potential for surgical resection, though only about 20% of cases are resectable at diagnosis. Achieving negative margins (R_0 resection) is critical for improving outcomes. Elevated CA 19-9 levels are associated with worse prognosis, though not specific to PDAC.[7]

PDAC is the 12th most common cancer worldwide but ranks as the 7th leading cause of cancer-related deaths. Its incidence is rising globally. The global incidence of PDAC is approximately 4.9 per 100,000 person-years.[8] Higher incidence rates are observed in developed countries, with North America and Europe reporting the highest rates. Incidence increases with age, typically affecting individuals over 65. Males are slightly more affected than females. Risk factors include genetic predisposition, such as familial pancreatic cancer syndromes, *BRCA1/2* mutations, and Lynch syndrome. Environmental factors like smoking, which increases the risk by two to three times, chronic pancreatitis, and diabetes mellitus are significant risk factors. Lifestyle factors, including diets high in red and processed meats, obesity, and a sedentary lifestyle, have been linked to increased risk.[4,9]

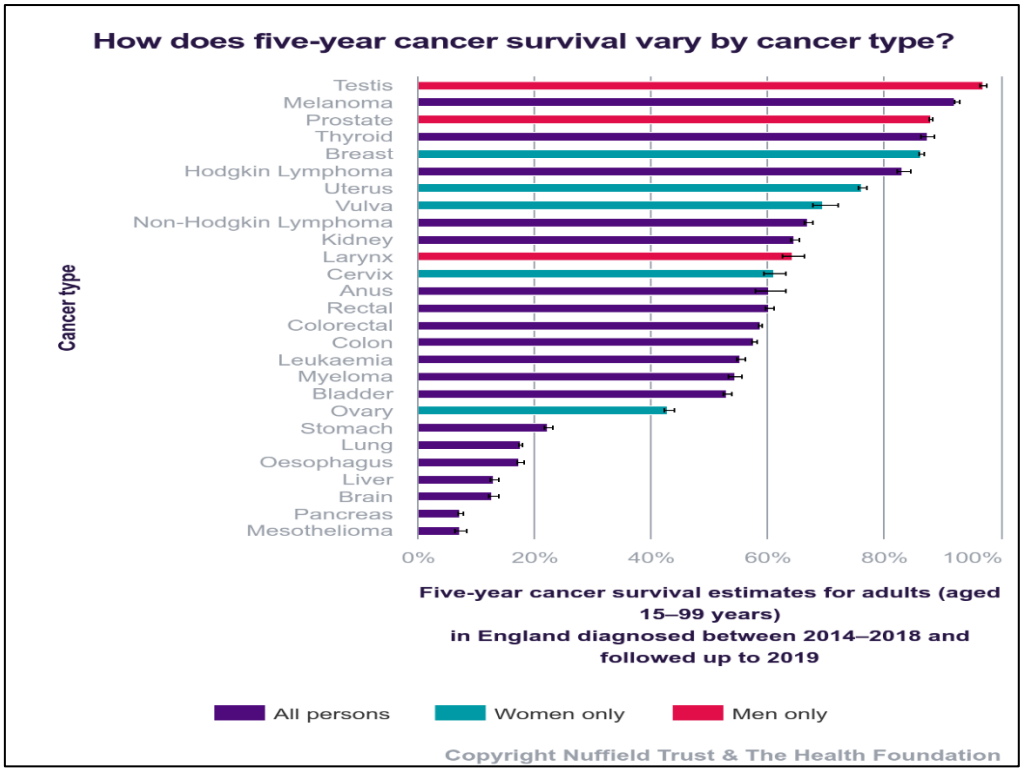

Figure 2: 5-year survival rates among cancer types in England between 2014-2018. Cancer survival in England for patients diagnosed between 2014 and 2018, and followed up to 2019

Prevention of PDAC involves lifestyle modifications such as smoking cessation, maintaining a healthy weight, and managing diabetes, which may reduce the risk. High-risk individuals, particularly those with a strong family history or genetic predispositions, may benefit from regular screening using imaging and endoscopic techniques. Management of PDAC involves surgical treatment, with the only curative treatment being surgical resection, typically a Whipple procedure for tumors in the pancreatic head. Adjuvant chemotherapy, often with agents like gemcitabine or FOLFIRINOX, is standard. Radiation therapy may be used in certain cases. Due to the high rate of advanced-stage diagnosis, many patients require palliative care to manage symptoms and improve quality of life.[5,6]

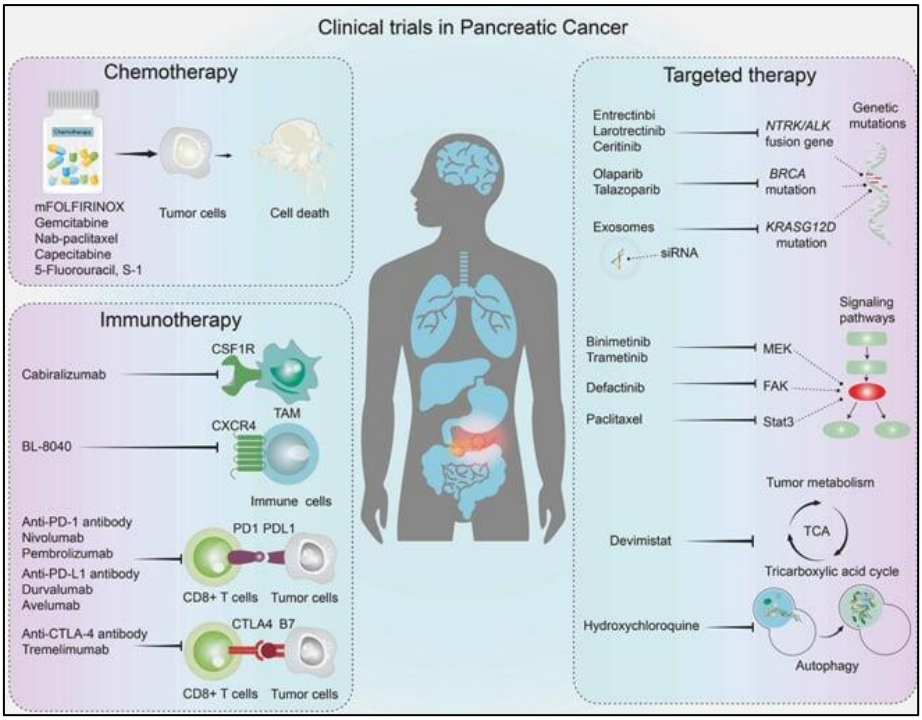

Figure 3: Clinical practices in Pancreatic Cancer. Wang, S. *et al.* The molecular biology of pancreatic adenocarcinoma: translational challenges and clinical perspectives. *Signal Transduction and Targeted Therapy* vol. 6 Preprint at https://doi.org/10.1038/s41392-021-00659-4 (2021).

In summary, PDAC is a highly aggressive cancer with poor prognosis and increasing incidence. Early detection remains challenging, emphasizing the need for continued research into better diagnostic markers and treatment options.

1.3 Pancreatic Neuroendocrine Tumors: Characteristics, Mortality, and Epidemiology

Pancreatic neuroendocrine tumors (PNETs) are a rare subset of pancreatic neoplasms, representing about 1-2% of all pancreatic cancers. These tumors arise from the endocrine (hormone-producing) cells of the pancreas and exhibit a wide range of behaviors, from indolent to highly aggressive. PNETs are often classified based on their ability to secrete hormones, leading to either functional (hormone-producing) or non-functional tumors.[10]

Clinically, PNETs may present with symptoms related to hormone overproduction in functional tumors, such as insulinomas causing hypoglycemia or gastrinomas leading to Zollinger-Ellison syndrome. Non-functional tumors often present later with

nonspecific symptoms like abdominal pain, weight loss, or jaundice due to mass effect. Diagnostic modalities include CT scans, MRI, and somatostatin receptor scintigraphy (SRS), along with biopsy for histological confirmation. Microscopically, PNETs show a variety of patterns, from well-differentiated neuroendocrine tumors to poorly differentiated neuroendocrine carcinomas. Common genetic alterations include mutations in MEN1, DAXX, ATRX, and mTOR pathway genes.[11,12]

The prognosis of PNETs varies widely. Well-differentiated tumors generally have a better prognosis compared to poorly differentiated carcinomas. The five-year survival rate can exceed 50% for localized tumors, but drops significantly for metastatic disease. Prognostic factors include tumor grade, stage at diagnosis, and functionality. Non-functional tumors are often diagnosed at a later stage, resulting in poorer outcomes. Elevated levels of chromogranin A and specific hormones can serve as prognostic markers.[13]

PNETs account for less than 2% of pancreatic cancers but their incidence appears to be rising, likely due to improved detection and increased awareness. The global incidence is about 0.48 per 100,000 person-years. PNETs are more common in patients with genetic syndromes such as Multiple Endocrine Neoplasia type 1 (MEN1), Von Hippel-Lindau syndrome, and Neurofibromatosis type 1. Unlike pancreatic ductal adenocarcinoma, there is no strong link between PNETs and smoking or other common environmental factors.[8,14]

Prevention strategies are not well defined due to the rarity and genetic predispositions associated with PNETs. Genetic counseling and screening are recommended for individuals with familial syndromes. Management of PNETs involves a combination of surgery, medical therapy, and targeted treatments. Surgical resection is the mainstay for localized tumors. For advanced or metastatic PNETs, therapies include somatostatin analogs, targeted agents like everolimus and sunitinib, and peptide receptor radionuclide therapy (PRRT). Chemotherapy is reserved for high-grade tumors. Palliative care is essential for managing symptoms and maintaining quality of life in advanced cases.[13,15]

In summary, PNETs are a diverse group of tumors with varying clinical behaviors and outcomes. While less common than pancreatic ductal adenocarcinoma, their prognosis is often better, especially for well-differentiated and localized tumors. Continued research and improved therapeutic strategies are crucial for better management and outcomes in patients with PNETs.

1.4 Genetics of Pancreatic Ductal Adenocarcinoma

Pancreatic ductal adenocarcinoma (PDAC) is characterized by well-defined somatic genetic mutations, with over 90% of cases involving KRAS gene mutations. These mutations are commonly found in early lesions such as low-grade pancreatic intraepithelial neoplasia (PanINs) and intraductal papillary mucinous neoplasms (IPMNs), indicating that *KRAS* mutations are likely the initial genetic event in exocrine pancreatic neoplasia. Besides *KRAS*, the genomic landscape of PDAC includes frequent loss-of-function mutations in *TP53, SMAD4*, and *CDKN2A*, which typically occur later in the disease progression, often in high-grade lesions or invasive cancer.[16,17]

Each of these genetic alterations uniquely contributes to PDAC's pathophysiology. For instance, loss of *CDKN2A*, often through homozygous deletions on chromosome 9p21, is associated with an immunosuppressive tumor environment and resistance to immunotherapy. *SMAD4* loss correlates with a worse prognosis and higher rates of metastasis. *TP53* mutations, the most common tumor-suppressor gene alteration in PDAC, lead to increased genomic instability, altered metabolism, and enhanced metastatic potential. Interestingly, specific "hotspot" *TP53* missense mutations may have different impacts compared to truncating mutations or deletions.[5,18]

While *KRAS* mutations are central to PDAC, not all PDACs rely on *KRAS* for survival. Some PDAC cell lines do not depend on *KRAS*, and inducible mouse models have shown that deactivating mutant *KRAS* can lead to tumor regression and even differentiation of cancerous cells back to normal acinar cells. However, tumors that have lost other tumor suppressor functions can recur independently of *KRAS* signaling, posing challenges for *KRAS*-targeted therapies.

Recent advances have brought about the development of allele-specific inhibitors targeting *KRAS* mutations, such as *KRASG12C* and *KRASG12D*, and broader "pan-RAS" inhibitors. However, the *KRASG12C* mutation is relatively rare in PDAC, found in only about 1.6% of cases, while *KRASG12D* mutations are more common. Clinical responses to *KRASG12C* inhibitors in PDAC have been limited and transient due to the emergence of resistance mechanisms, shifting focus toward combination therapies that might sustain responses more effectively.

Beyond the major driver mutations, next-generation sequencing (NGS) has revealed a variety of less common mutations in PDAC. These include genes involved in DNA maintenance, epigenetic regulation, and axon guidance pathways. Although PDAC exhibits a relatively low overall mutational burden compared to other cancers like lung cancer and melanoma, about 5-7% of PDAC patients harbor germline mutations in genes associated with homologous recombination repair, such as *BRCA1, BRCA2*, and *PALB2*. Tumors with these mutations may respond well to platinum-based chemotherapy and PARP inhibitors, especially when there is bi-allelic loss of these genes.

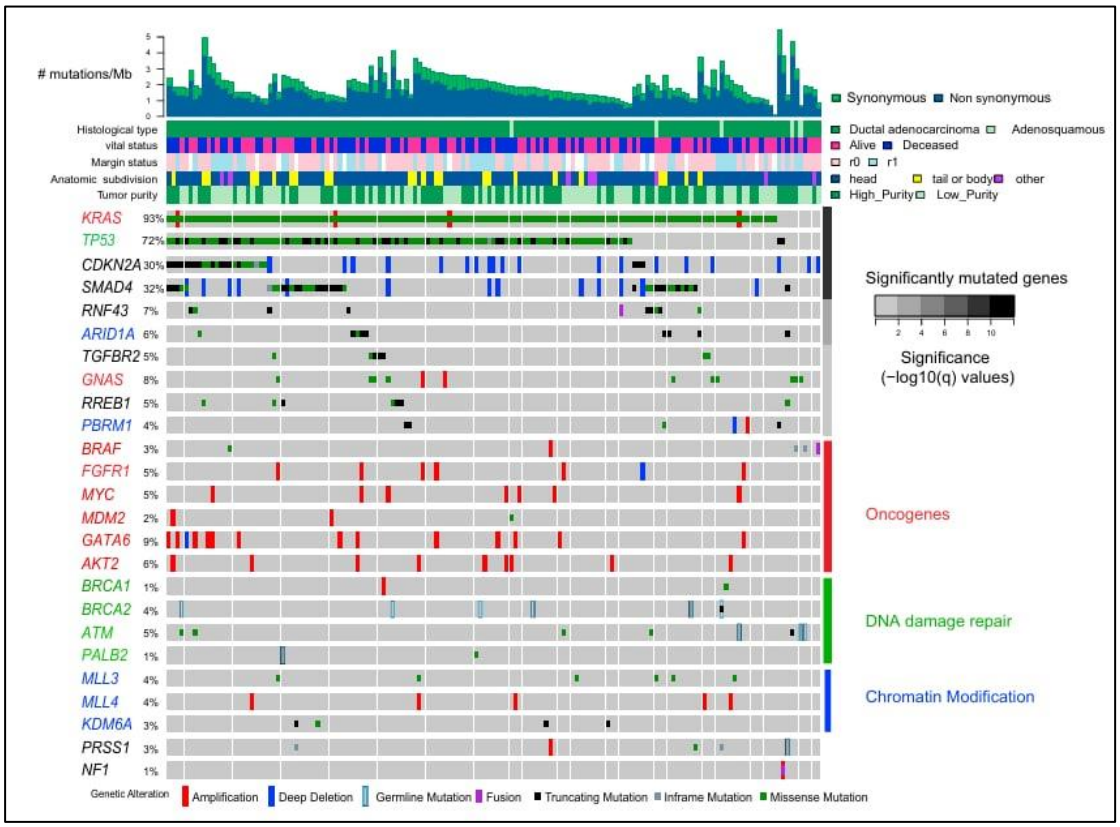

Figure 4: Landscape of genomic alterations in Pancreatic ductal Adenocarcinoma. Raphael, B. J. *et al.* Integrated Genomic Characterization of Pancreatic Ductal Adenocarcinoma. *Cancer Cell* 32, 185 (2017).

Mutations in chromatin-remodeling genes and SWI/SNF complex components are also common in PDAC. These genes may act as tumor suppressors or oncogenes depending on the context. Additionally, around 8-10% of PDAC cases are *KRAS* wild-type and often involve alternative drivers like *ALK, TRK, RET, NRG1, BRAF*, and *EGFR*, which activate the MAP kinase signaling pathway. Identifying these alternative drivers is crucial, particularly in younger patients, as targeted therapies for these mutations are available and can significantly impact treatment outcomes. [5,19,20]

In summary, the genetic landscape of PDAC is dominated by mutations in *KRAS, TP53, SMAD4*, and *CDKN2A*, each contributing to the disease's complexity and therapeutic challenges. The advent of targeted therapies and the identification of less common but actionable mutations offer new avenues for treatment, emphasizing the need for comprehensive genetic profiling in PDAC management.

1.5 Tumor microenvironment

The tumor microenvironment (TME) in pancreatic adenocarcinoma (PDAC) plays a crucial role in the progression, resistance to therapy, and overall aggressiveness of this malignancy. PDAC is notorious for its poor prognosis and high mortality rate, which can be attributed significantly to its unique and complex TME. [21]

1.5.1 Components of the Tumor Microenvironment

The TME in PDAC is characterized by a dense stromal matrix, composed of various cell types and extracellular components that interact dynamically with tumor cells. Key components include cancer-associated fibroblasts (CAFs), immune cells, endothelial cells, and the extracellular matrix (ECM).

1.5.2 Cancer-Associated Fibroblasts (CAFs)

CAFs are one of the predominant cell types in the PDAC TME. They originate from pancreatic stellate cells (PSCs) and play a significant role in promoting tumor growth and invasion. CAFs contribute to the desmoplastic reaction, a hallmark of PDAC, by

secreting large amounts of ECM components such as collagen and fibronectin. This dense fibrotic tissue not only provides structural support for tumor cells but also acts as a physical barrier to drug delivery, contributing to chemotherapy resistance.[22]

1.5.3 Immune Cells

The immune microenvironment in PDAC is typically immunosuppressive. Tumor-associated macrophages (TAMs), regulatory T cells (Tregs), and myeloid-derived suppressor cells (MDSCs) are often found in high numbers. These cells inhibit anti-tumor immune responses and promote tumor progression. For instance, TAMs and MDSCs secrete cytokines like IL-10 and TGF-β, which suppress cytotoxic T cell activity and promote a pro-tumorigenic environment. Additionally, the expression of immune checkpoint proteins such as PD-L1 on tumor and stromal cells further inhibits effective immune surveillance.[7]

1.5.4 Extracellular Matrix (ECM)

The ECM in PDAC is exceptionally dense and stiff, which is a result of the excessive deposition of ECM proteins by CAFs. This altered ECM not only facilitates tumor cell migration and invasion but also affects cellular signaling pathways that promote tumor survival and growth. Moreover, the abnormal ECM architecture impedes the effective delivery of therapeutic agents, leading to intrinsic resistance to conventional treatments.[7,21]

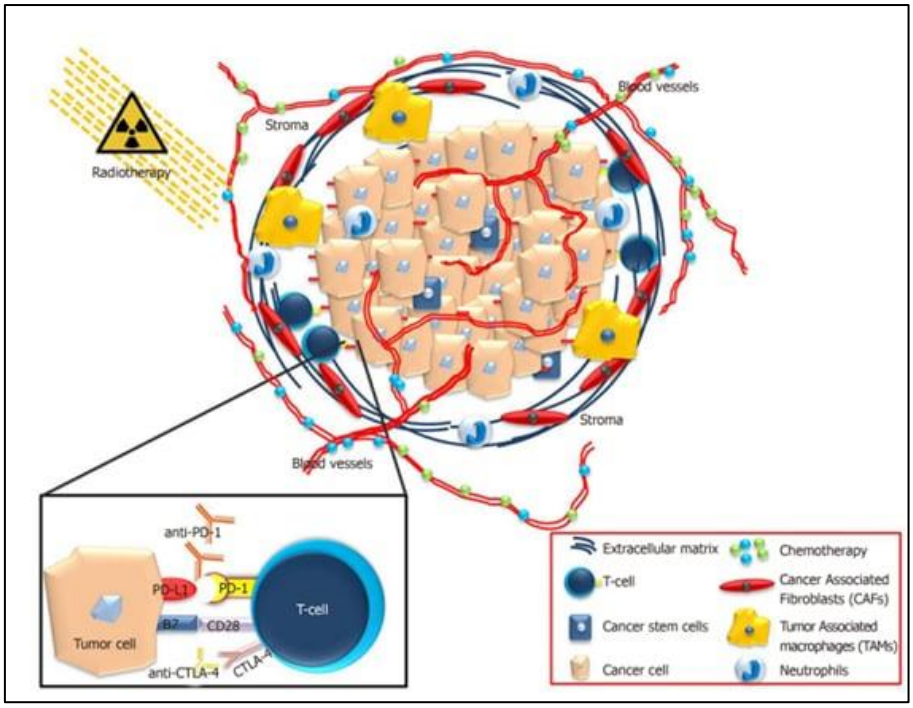

Figure 4: Tumor microenvironment components in Pancreatic Adenocarcinoma. Sarantis, P., Koustas, E., Papadimitropoulou, A., Papavassiliou, A. G. & Karamouzis, M. V. Pancreatic ductal adenocarcinoma: Treatment hurdles, tumor microenvironment and immunotherapy. *World Journal of Gastrointestinal Oncology* vol. 12 173–181 Preprint at https://doi.org/10.4251/wjgo.v12.i2.173 (2020).

1.5.5 Hypoxia

The dense stromal environment and aberrant vasculature in PDAC result in poor oxygenation, leading to hypoxia within the tumor. Hypoxia-inducible factors (HIFs) are upregulated in response to low oxygen levels and contribute to a more aggressive phenotype by promoting angiogenesis, metabolic reprogramming, and further immune evasion. [23]

1.5.6 Therapeutic Implications

Understanding the TME in PDAC has significant implications for the development of effective therapies. Targeting the stromal components, such as inhibiting CAF activity or modifying the ECM, could enhance the delivery and efficacy of chemotherapeutic agents. Immunotherapeutic strategies that reprogram the immune microenvironment to boost anti-tumor immunity are also under investigation. For example, combining

immune checkpoint inhibitors with agents that deplete immunosuppressive cells or modulate the ECM might overcome the resistance seen with monotherapies.

In conclusion, the TME in pancreatic adenocarcinoma is a complex and dynamic entity that significantly contributes to the disease's malignancy and resistance to treatment. Strategies that can effectively target and modify the TME hold promise for improving the prognosis of this devastating cancer.[5]

1.6 Epigenetics in Pancreatic Cancer

Epigenetics in pancreatic ductal adenocarcinoma (PDAC) represents a crucial area of research, highlighting how reversible modifications to DNA and chromatin structure can profoundly influence gene expression without altering the underlying DNA sequence. These epigenetic changes contribute significantly to the pathogenesis, progression, and treatment resistance of PDAC.[24]

1.6.1 DNA Methylation

DNA methylation, the addition of methyl groups to the cytosine residues of CpG islands, is a common epigenetic alteration in PDAC. Hypermethylation of tumor suppressor gene promoters leads to their silencing, facilitating unchecked cell proliferation and survival. For instance, the promoter regions of genes such as CDKN2A (p16), which regulates cell cycle progression, are often hypermethylated and inactivated in PDAC. Conversely, global hypomethylation can result in genomic instability and activation of oncogenes, contributing to the aggressive nature of this cancer.[24,25]

1.6.2 Histone Modifications

Histone proteins, which package and order DNA into nucleosomes, undergo various post-translational modifications (PTMs) such as methylation, acetylation, phosphorylation, and ubiquitination. These modifications can either promote or suppress gene transcription. In PDAC, dysregulation of histone-modifying enzymes is frequently observed. For instance, histone deacetylases (HDACs) are often overexpressed, leading to the deacetylation and repression of tumor suppressor

genes. Inhibitors targeting HDACs are being explored as potential therapeutic agents to restore normal gene expression patterns.[24]

1.6.3 Non-Coding RNAs

Non-coding RNAs, including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), are pivotal regulators of gene expression at the epigenetic level. In PDAC, the expression profiles of numerous miRNAs are altered, contributing to tumor progression and metastasis. For example, miR-21 is frequently upregulated and promotes oncogenic processes by targeting tumor suppressor genes. Similarly, specific lncRNAs are implicated in regulating chromatin states and gene expression in PDAC, adding another layer of complexity to its epigenetic landscape.[26]

1.6.4 Therapeutic Implications

The reversible nature of epigenetic modifications offers promising avenues for therapeutic intervention. Epigenetic therapies aim to restore normal gene expression patterns by targeting enzymes involved in these modifications. DNA methyltransferase inhibitors (DNMTis) and HDAC inhibitors (HDACis) are being evaluated in preclinical and clinical settings for PDAC. Combining these agents with conventional chemotherapies or immunotherapies holds potential for synergistic effects, overcoming resistance mechanisms inherent to PDAC.[24]

In conclusion, epigenetic alterations in PDAC play a fundamental role in its pathogenesis and resistance to therapy. By understanding and targeting these epigenetic changes, new therapeutic strategies can be developed, offering hope for improved outcomes in this challenging malignancy. The ongoing research in this field underscores the potential of epigenetic therapies to transform the treatment landscape for PDAC.

1.7 Overview of Sirtuins in Cellular Regulation and Disease

Sirtuins are a family of NAD+-dependent deacetylases and ADP-ribosyltransferases that play pivotal roles in cellular regulation, metabolism, and aging. These proteins, which are highly conserved across species, influence various cellular processes, including gene expression, DNA repair, and stress responses. Their functions have significant implications for diseases such as cancer, metabolic disorders, and neurodegenerative diseases.[27]

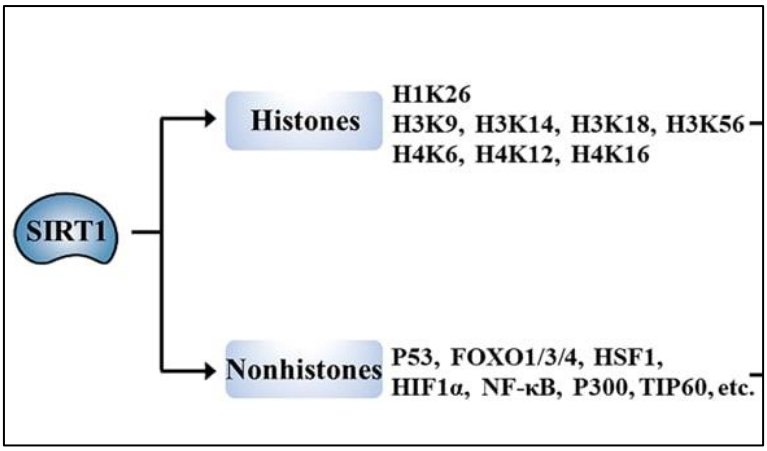

Figure 6: SIRT1 histonic and nonhistonic targets.

SIRT1, primarily a nuclear deacetylase, is essential for regulating gene expression and protein activities that control cell proliferation, differentiation, apoptosis, metabolism, and genome stability. It can move between the nucleus and cytoplasm to exert its effects. The deletion of *SIRT1* in mice results in severe developmental issues and early postnatal death, highlighting its critical role in cellular homeostasis.[28]

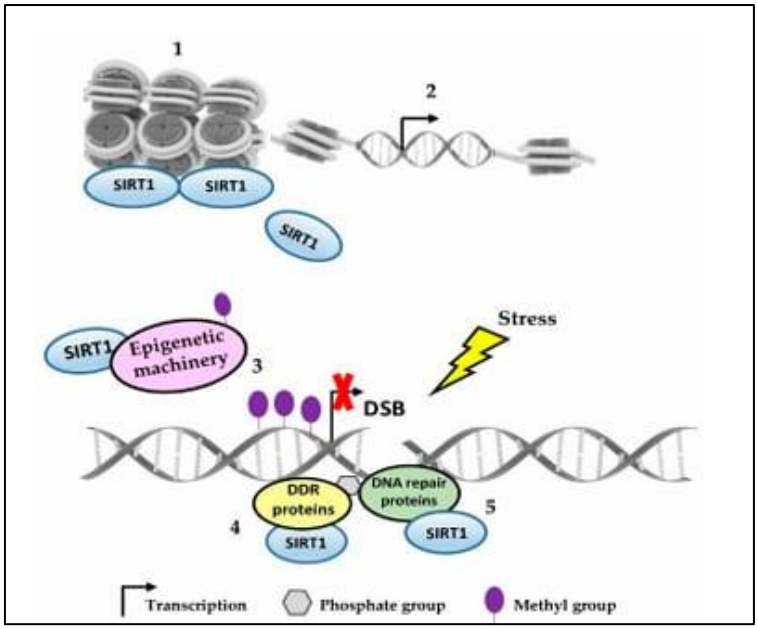

Figure 6: *SIRT1* in Double Strand Break repair. Alves-Fernandes, D. K. & Jasiulionis, M. G. The role of *SIRT1* on DNA damage response and epigenetic alterations in cancer. *Int J Mol Sci* 20, (2019).

SIRT2 is mainly found in the cytoplasm but relocates to the nucleus during mitosis. It deacetylates substrates like histone H4K16 and α-tubulin, thereby regulating cell cycle progression and stress responses. Mice lacking *SIRT2* show gender-specific tumor development, with females developing mammary tumors and males developing hepatic and intestinal tumors, indicating its tumor-suppressive functions.

SIRT3, predominantly located in mitochondria, is crucial for cellular energy metabolism and redox regulation by deacetylating key mitochondrial enzymes. Mice without *SIRT3* display metabolic syndrome and a higher susceptibility to cancer, underscoring its importance in mitochondrial function and metabolic balance.

SIRT4, also mitochondrial, acts as an ADP-ribosyltransferase, regulating metabolic processes such as insulin secretion and fatty acid oxidation. It also has anti-apoptotic functions during genotoxic stress. *SIRT4*-deficient mice develop hyperinsulinemia and lung tumors, demonstrating its role in metabolic regulation and tumor suppression.

SIRT5 is present in both mitochondria and cytoplasm, deacetylating metabolic enzymes essential for ammonia detoxification and various metabolic pathways. Mice

lacking *SIRT5* experience urea cycle defects and hyperammonemia, illustrating its role in nitrogen metabolism.

SIRT6, a nuclear protein, has both deacetylase and ADP-ribosyltransferase activities and is involved in transcription regulation, genome stability, and metabolism. SIRT6-deficient mice show premature aging, hypoglycemia, and genomic instability, emphasizing its role in metabolic regulation and aging.[29]

SIRT7, localized to the nucleolus, deacetylates histone H3K18 and regulates ribosome biogenesis and cell survival. Mice lacking *SIRT7* exhibit premature aging and enhanced cardiomyopathy, indicating its essential role in maintaining cellular survival and stress responses.

Understanding the diverse functions of sirtuins in cellular physiology and their disease implications offers valuable insights for developing targeted therapeutic strategies in cancer, metabolic disorders, and aging-related conditions.[30]

1.8 *SIRT1* in cancer

The role of *SIRT1* in cancer is complex and somewhat paradoxical, displaying both tumor-suppressive and oncogenic traits depending on its cellular context and the type of tissue involved.[31] This duality arises from *SIRT1*'s ability to target a wide range of substrates, thereby influencing various pathways related to tumor development. Key to understanding *SIRT1*'s role in cancer is its interaction with critical tumor suppressors such as *p53, FOXO*, and *HIC1*.[32]

SIRT1's relationship with *p53*, a pivotal tumor suppressor, is particularly significant. Known as the "guardian of the genome," *p53* maintains genomic integrity by inducing cell cycle arrest, apoptosis, or senescence in response to DNA damage. *SIRT1* deacetylates *p53*, leading to its functional inactivation, which reduces *p53*'s ability to activate genes involved in growth arrest and apoptosis, thereby potentially promoting tumor survival and growth. In this way, *SIRT1* can act as an oncogene by undermining *p53*'s tumor-suppressive functions, allowing damaged cells to survive.[33,34]

Nevertheless, the *SIRT1-p53* relationship is not entirely antagonistic. *SIRT1* also enhances DNA repair processes, indirectly supporting *p53*'s role in preserving genomic stability. By deacetylating and activating DNA repair proteins such as *WRN, Ku70, APE1*, *XPA*, and *XPC, SIRT1* bolsters the cell's ability to repair DNA damage, which complements *p53*'s protective role. This dual capacity of SIRT1 to both inhibit and support *p53*-related functions highlights its complex involvement in cancer.

FOXO transcription factors, especially FOXO3a, are another important target for *SIRT1*. These proteins regulate genes that mediate cell cycle arrest, apoptosis, and oxidative stress resistance. *SIRT1* deacetylates FOXO proteins, which can either enhance or inhibit their transcriptional activity, depending on the context. This modulation can have mixed effects on cancer. *SIRT1*-mediated activation of FOXO3a can promote cellular resistance to oxidative stress and support longevity, acting as a tumor suppressor. Conversely, it can also induce cell cycle arrest and apoptosis, limiting cancer cell proliferation. In some situations, *SIRT1*'s deacetylation of FOXO may inhibit its pro-apoptotic functions, aiding tumor cell survival.[34]

HIC1 (hypermethylated in cancer 1) is another tumor suppressor interacting with *SIRT1. HIC1*, often silenced in various cancers through hypermethylation, forms a regulatory loop with *SIRT1: HIC1* represses *SIRT1* expression, while *SIRT1* deacetylates and suppresses *HIC1*'s activity. This interaction suggests that SIRT1 may contribute to tumorigenesis by downregulating *HIC1*, thereby preventing it from repressing genes involved in cell proliferation and survival. The mutual regulation between *HIC1* and *SIRT1* further exemplifies the delicate balance *SIRT1* maintains in cellular processes.[35]

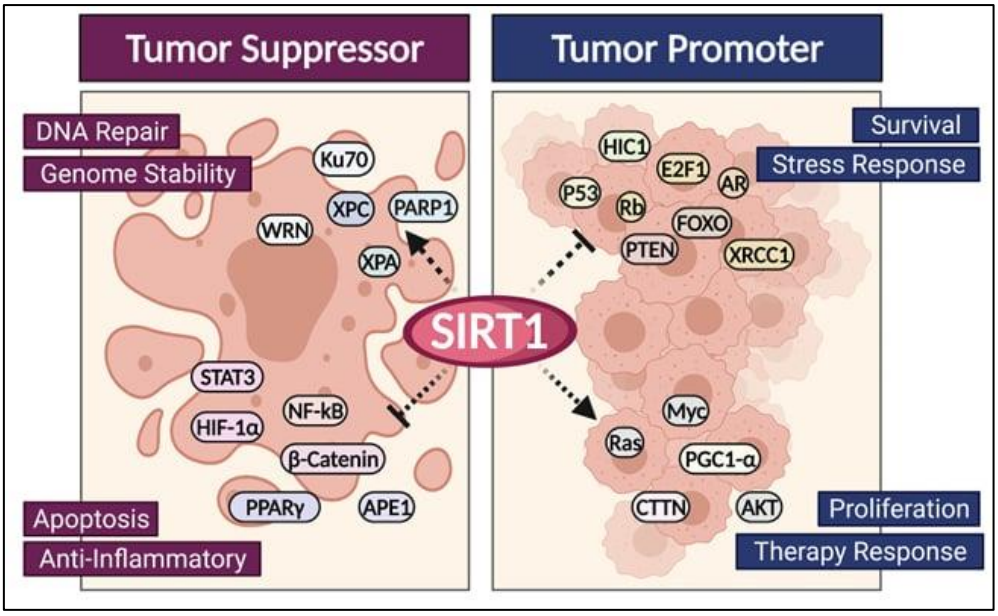

Figure 8: *SIRT1* dual roles in cancer. Garcia-Peterson, L. M. & Li, X. Trending topics of *SIRT1* in tumorigenicity. *Biochimica et Biophysica Acta - General Subjects* vol. 1865 Preprint at https://doi.org/10.1016/j.bbagen.2021.129952 (2021).

The complexity of *SIRT1*'s role in cancer is further illustrated by its regulation of various nuclear receptors and cofactors involved in tumorigenesis, such as the androgen receptor (AR), estrogen receptor-alpha (ERα), PPARγ, and PGC-1α. SIRT1 can modulate these factors either positively or negatively, depending on the cellular context and environmental conditions. For instance, *SIRT1*'s deacetylation of AR and ERα can influence hormone-dependent cancer growth, highlighting its broad regulatory impact.[34]

Moreover, *SIRT1*'s influence on oncogenes like *β-catenin, cortactin* (*CTTN)*, *c-Myc*, and *N-Myc* adds another layer of complexity. These interactions can drive tumorigenesis by promoting cell proliferation, survival, and metastasis. The dose-dependent effects of *SIRT1* further illustrate its dual nature, as different levels of *SIRT1* expression can lead to varying outcomes in tumor development.[35,36]

In summary, *SIRT1* plays a highly intricate role in cancer, acting as both a tumor suppressor and an oncogene depending on its targets and cellular context. Its interactions with p53, FOXO, and HIC1 are central to this duality, as *SIRT1*'s deacetylase activity can either inhibit or promote tumorigenic processes. Understanding the roles

of *SIRT1* in these pathways is crucial for developing targeted cancer therapies that can modulate its activity to achieve desired therapeutic outcomes.

In case of pancreatic cancer, little is known about the role of sirtuins and especially *SIRT1*. What is observed is the fact that *SIRT1* is upregulated at both mRNA and protein level in pancreatic cancer tissues. It has been shown that *SIRT1* knockdown induces apoptosis, inhibits invasion and enhances chemosensitivity in pancreatic cancer cells. These could indicate the tumor-promoting role of *SIRT1* in PDAC.[37]

1.9 Single nucleotide polymorphisms selection

The selection of the polymorphisms was based on previous studies. More specifically, studies concerning other malignancies were selected. The second criterion was the potential strong association of the polymorphisms with the disease under study. All selected polymorphisms are characterized by the Uniprot database as variants of unknown significance.

2. AIM OF THE THESIS

Even if a lot of effort has been made to unravel the genetic basis of pancreatic cancer, no specialized biomarkers have been discovered so far, that could provide evidence about susceptibility to this fatal disease. The aim of this study is to investigate the potential association of specific polymorphisms (rs3758391, rs144124002 and rs369274325) of the protein deacetylase, Sirtuin 1, with pancreatic adenocarcinoma and pancreatic neuroendocrine tumors within a Greek cohort of patients. Even though these polymorphisms have been previously investigated in other malignancies, this is the first time that they are being used in a study in pancreatic cancer.

3. MATERIALS AND METHODS

3.1 Sampling

For the purposes of this study, peripheral blood was collected from 94 patients with Pancreatic ductal adenocarcinoma and 15 patients with Pancreatic neuroendocrine tumors. Blood sample were recruited from Hippocratio General Hospital of Athens. Patients were diagnosed based on imaging and histopathological criteria.

Furthermore, peripheral blood was collected from healthy volunteers, who agreed to attend a health survey conducted by the National and Kapodistrian University of Athens. All subjects signed an informed consent regarding their participation in the study and the study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethical Committee of the participating centers.

3.2 DNA extraction

Firstly, genomic DNA from peripheral blood samples was extracted using the Nucleospin Blood Kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). A volume of 200 µl per sample was lysed by adding 25 µl of Proteinase K and 200 µl B3 buffer to the eppendorf tube. The mix was vortexed and incubated at 70 °C for 15 minutes. Then, 210 µl of ethanol (100%) was added to each sample, the tubes were vortexed and the samples were loaded into NucleoSpin® Blood Columns, which were placed in Collection Tubes. After centrifugation at 11,000 x g for 1 minute all collection tubes containing the flow-through were discarded, and the columns were placed into new collection tubes. The next steps involved two washes of the silica membrane, first by the addition of 500 µl of BW buffer to the column, centrifugation at 11,000 x g for 1 minute and discarding the collection tube, and secondly by the addition of 600 µl of B5 buffer, and again centrifugation at 11,000 x g for 1 minute. The flow-through was discarded, the column was placed back to the collection tube and centrifuged at 11,000 x g for 1 minute, so as to dry the silica membrane. Finally, the column was placed into a 1.5 mL eppendorf tube, 100 µl of elution buffer that was preheated at 70 °C was added directly onto the membrane and with 1 minute centrifugation at 11,000

x g the DNA eluted. The quality and concentration of the eluted DNA was evaluated using a spectrophotometer and checking the A260 / A280 ratio. All DNA samples were stored at -20 °C for later use.

3.3 Polymerase Chain Reaction-Restriction fragment length polymorphism (PCR-RFLP)

For the determination of the samples genotype the method of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was selected. The SNPs for which the samples were genotyped with this method were the two SNPs of the SIRT1 gene (rs3758391 and rs144124002)

Each PCR reaction was set up in a total volume of 50 µl using 5 µl DNA, 5 µl Taq Buffer A (Kapa Biosystems, USA), 1 µl dNTPs (200 µM), 0.25 µl primers (0.5µM), 0.25 Taq Polymerase (Kapa Biosystems, USA) and 38.25 µl H_20. The reaction conditions for the rs3758391 were the following: initial denaturation at 95°C for 5 minutes, then 40 cycles of denaturation at 95°C for 45 seconds, annealing at 56 ^oC for 45 seconds, extension at 72°C for 45 seconds and a final extension at 72°C for 5 minutes. In case of rs144124002 the conditions were the following: initial denaturation at 95°C for 5 minutes, then 40 cycles of denaturation at 95°C for 35 seconds, annealing at 59 ^oC for 35 seconds, extension at 72°C for 35 seconds and a final extension at 72°C for 5 minutes. The primers (Eurofins Genomics AT GmbH, Vienna, Austria) that were used for all reactions are derived from former studies and are listed in the following table.[38,39]

Table 1: Primers sequences for the rs3758391 and rs144124002 polymorphisms

SNP	Primer Sequence (5'-3')	Tm (^oC)
rs3758391	F: ACGCAGGTAATTGATGCAGT R: CGTGAGCTATCTAGCCGTTT	56
rs144124002	F: GCCTTGACTGACTTGGTTTCTT R: CATACCTATCCGTGGCCTTG	59

After the reactions were done and the desired DNA segments were amplified, the PCR products were digested with restriction enzymes at 37°C overnight. The restriction enzymes (New England Biolabs) used were chosen, so as to have their restriction site on the same site of the studied polymorphism and produce a different number of smaller segments, depending on the alleles that are present. In order to visualize the digestion products electrophoresis in 3% agarose gels stained with Gel Red (Biotium, USA) was performed. The restriction enzymes for each SNP and the products of enzyme digestion are listed in the following table

Table 2: **Base changes, restriction enzymes and digested products of rs3758391 and rs144124002**

SNP	**Base change**	**Restriction enzyme**	**Digested products (bp)**
rs3758391	T/C	NcoI	TT: 500 TC: 500 , 280 , 220 CC: 280 , 220
rs144124002	A/G	NlaIII	AA: 105 , 45 AG: 150 , 105 , 45 GG: 150

3.4 Tetra-primer amplification refractory mutation system–polymerase chain reaction (ARMS–PCR)

For the rs369274325, the method which was used for genotyping, was the Tetra-primer amplification refractory mutation system–polymerase chain since the polymorphism's locus does not contain any known enzyme's restriction site. In this type of reaction, 4 primers were totally used, 2 outer ones which produced a control product and 2 inner primers, each one of them was specific for the 2 alleles and produced 2 allele-specific products. The primers (Eurofins Genomics AT GmbH, Vienna, Austria) that were used for this reaction are derived from a former study and are listed in the following table. [40,41]

Table 3: Primers sequences for the rs369274325 polymorhism

SNP	Primer sequence (5'-3')	Tm (°C)
rs369274325	F_o : TAGGTTCCATACCCCATGAAG R_o: CATTACTCTTAGCTGCTTGGTC F_I (G allele): GAATTGTGTCATAGGTTAGGAGG R_I (A allele): ACAGCAAAGTTTGGCATATTGAT	56

This reaction is synthesized in a way that each inner allele can synthesize an allele specific product by using an outer primer. So, the forward outer primer and the reverse inner primer are used in order to produce a 152 bp amplicon specific for the A allele and the forward inner primer and the reverse outer primer are producing a 229 bp amplicon, specific for the G allele. A control product is always produced at 381 bp.

This PCR reaction was set up in a total volume of 50 µl using 5 µl DNA, 5 µl Taq Buffer A (Kapa Biosystems, USA), 1 µl dNTPs (200µM), 0.25 µl primers (0.5µM), 0.25 Taq Polymerase (Kapa Biosystems, USA) and 38.25 µl H20. The reaction run under the following conditions: initial denaturation at 95°C for 5 minutes, then 40 cycles of denaturation at 95°C for 45 seconds, annealing at 56 °C for 45 seconds, extension at 72°C for 45 seconds, and a final extension at 72°C for 5 minutes. The PCR products were visualized by electrophoresis in 3% agarose gels stained with Gel Red (Biotium, USA). The products of the reaction are listed on the following table:

Table 4: PCR products for every genotype of the rs369274325

SNP	Base change	Products (bp)
rs369274325	G/A	GG: 381 , 229 GA: 381 , 229 , 152 AA: 381 , 152

3.5 Statistical analysis

Genotype frequencies were analyzed by the χ^2 test with Yate's correction, using S-Plus (version 6.2 Insightful, Seattle, WA, USA) software. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated with GraphPad (version 300, GraphPad Software, San Diego, CA, USA). All P-values are 2-sided. P-values < 0.05 were considered significant.

4. RESULTS

The determination of each sample's genotype by the methods described in the above sections, led to the accumulation of the data needed to extract the genotypic frequencies of the SIRT1 polymorphisms. All frequencies conformed to Hardy–Weinberg equilibrium ($p>0.05$).

Table 5: rs3758391, rs144124002 and rs369274325 polymorphisms distribution in Pancreatic ductal adenocarcinoma patients and healthy individuals

SNPs	**PDAC (%) (n=94)**	**Controls (%) (n=73)**	**P [OR; (95% CI)]**
rs3758391			
TT (wt)	54 (57.4)	21 (28.8)	1.00
TC	33 (35.1)	37 (50.7)	0.0039; 0.3468 (0.1742 - 0,6249)
CC	7 (7.5)	15 (20.5)	0.0015; 0.1815 (0.0648 – 0.5080)
Alleles			
T (wt)	141 (76.6)	79 (54.1)	1.00
C	47(23.4)	67 (45.9)	0.0001; 0.3930 (0.2472 – 0.6249)
rs144124002			
AA (wt)	88 (91.5)	72 (98.0)	1.00
AG	6 (8.5)	1 (2.0)	0.2246; 4.909 (0.5724 – 41.738)
Alleles			
A	182 (95.6)	145 (99.3)	1.00
G	6 (4.4)	1 (0.7)	0.2297; 4.780 (0.5688 – 40.174)
rs369274325			
GG	74 (78.7)	62 (84.9)	1.00
GA (wt)	20 (22.3)	11 (15.1)	0.4106; 1.523 (0.6779 – 3.423)
Alleles			
G (wt)	168 (89.3)	135	1.00

A	20 (10.7)	11	0.4356; 1.461 (0.6765 – 3.156)

Table 6: rs3758391, rs144124002 and polymorphisms distribution in Pancreatic neuroendocrine tumors patients and healthy individuals

SNPs	**PNET (%) (n=15)**	**Controls (%) (n=73)**	**P [OR; (95% CI)]**
rs3758391			
TT (wt)	6 (40)	21 (28.8)	1.00
TC	6 (40)	37 (50.7)	0.5702; 0.5676 (0.1623 – 1.985)
CC	3 (40)	15 (20.5)	0.1506; 0.7000 (0.1506 – 3.254)
Alleles			
T (wt)	18 (60)	79 (54.1)	1.00
C	12 (40)	67 (45.9)	0.6971; 0.7861 (0.3532 – 1.749)
rs144124002			
AA (wt)	15 (100)	72 (98.0)	1.00
AG	0 (0)	1 (2.0)	0.6485; 1.559 (0.06057 – 40.133)
Alleles			
A	30 (100)	145 (99.3)	1.00
G	0 (0)	1 (0.7)	0.6494; 1.590 (0.06321 – 40.0003)

4.1 Genotypic and allelic frequencies of SNPs

4.1.1 Rs3758391

The first single nucleotide polymorphism which was genotyped was rs3758391 on PDAC patients. The figure from below shows the bands which were produced after the polymerase chain reaction and the treatment with the restriction enzyme (NcoI , New England Biolabs). The visualization resulted after treatment with Gel Red in a 3%

agarose gel. A bp step ladder (Nippon Genetics Europe, Düren, Germany) was also used in order the bands to be discriminated.

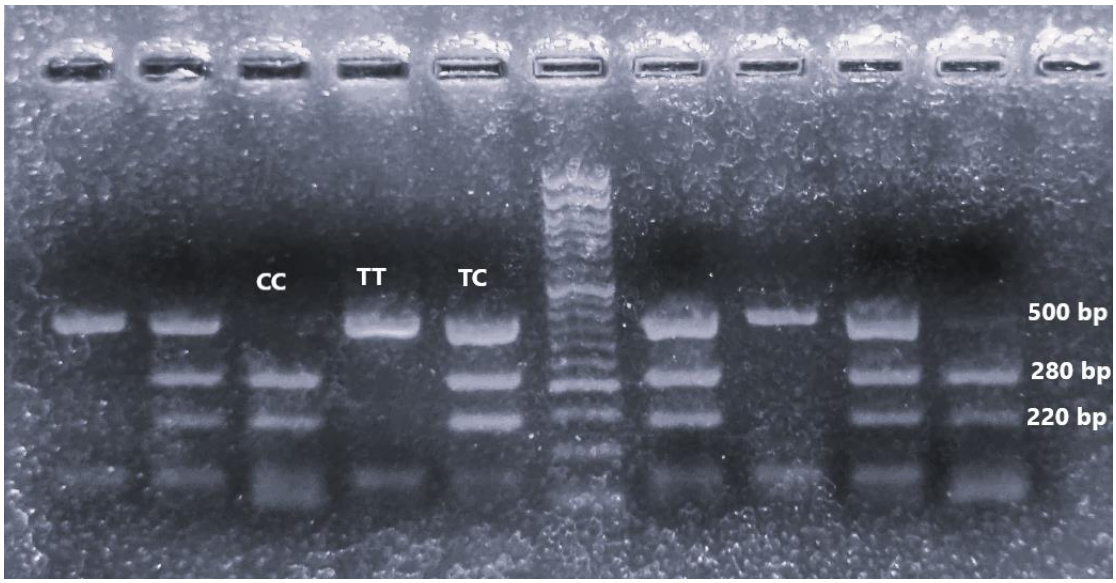

Figure 9: **Representative results of rs3758391 genotyping on a 3% agarose gel stained with Gel Red.**

According to the results of the genotyping of the rs3758391, very significant differences were found (p-value < 0.05) in both allele and genotype level between PDAC patients and healthy controls. The T allele (wild type) was found to be represented with higher frequency in PDAC patients (p=0,0001). TT genotype was also overrepresented in PDAC patients, followed by the TC genotype. Comparisons between genotypes were made in pairs and the genotype TT was used as the reference genotype.

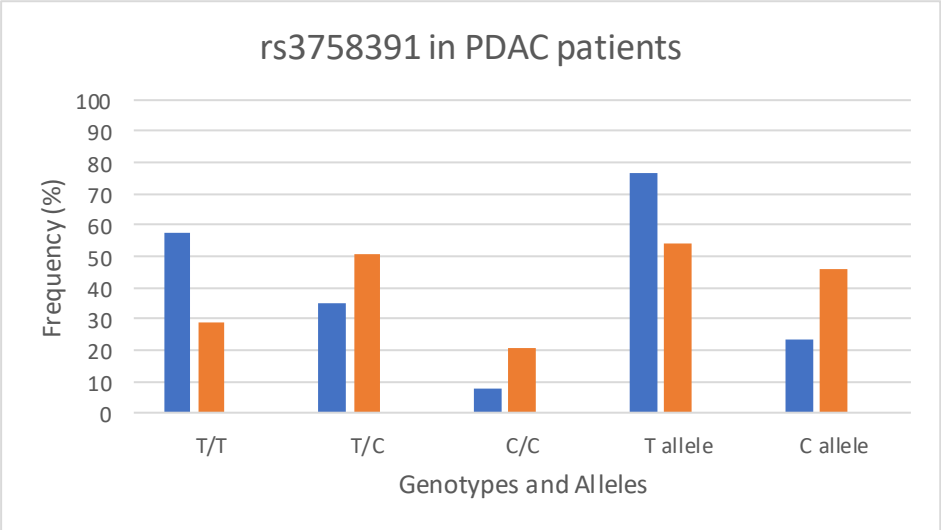

Diagram 1: Representative frequencies of the rs3758391 polymorphism between PDAC patients (blue) and healthy controls (orange) in genotypes and alleles level.

More specifically, the T/T genotype was found in 57.4 % of patients and 28.8 % of the controls. The T/C genotype was found in 35.1 % of patients and in 50.7 % of controls and lastly the C/C genotype was found in 7.5 % of PDAC patients and 20.5 % of healthy individuals. In allele level, the T allele was found in 76.6 % of PDAC patients and 54.1 % of the controls and so the C allele showed higher frequency in healthy controls 45,9 % compared to the patients (23,4 %). All of the differences described, both in genotypes and allele level, are statistically significant ($p<0.05$).

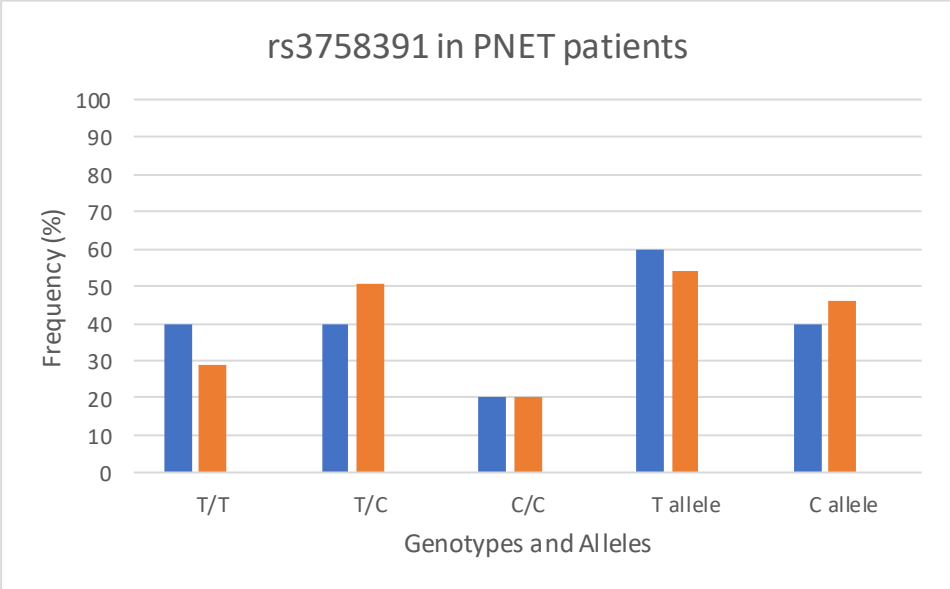

Diagram 2: Representative frequencies of the rs3758391 polymorphism between PNET patients (blue) and healthy controls (orange) in genotypes and alleles level.

In case of PNET patients, the genotyping of the rs3758391 polymorphism was conducted in the same manner. The number of patients was too small, so no statistically significant differences were found both in allele and genotypes level.

More specifically, the T/T genotype was found in 40 % of patients compared to 28.8 % of healthy controls, the T/C genotype was found also in 40 % of patients compared to 50.7 % of controls. Lastly the C/C genotype showed almost the same frequency (20 versus 20.5 %). In allele level, the T allele was represented in 60 % of patients and in 54.1 % of controls and so the C allele was found in 40 % of patients and in 45.9 % of controls.

4.1.2 Rs144124002

The second single nucleotide polymorphism which was genotyped was rs144124002. This SNP was also genotyped by the PCR-RFLP method. The figure from below shows the bands which were produced after the polymerase chain reaction and the treatment with the restriction enzyme (NlaIII, New England Biolabs). The visualization resulted after treatment with Gel Red in a 3% agarose gel. A bp step ladder (Nippon Genetics Europe, Düren, Germany) was also used in order the bands to be discriminated.

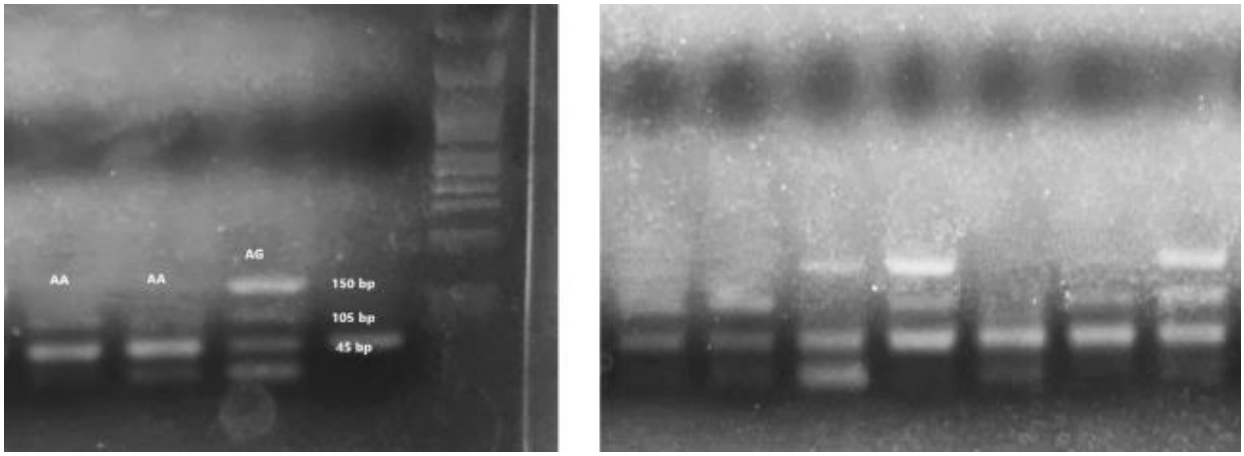

Figure 10: Representative results of rs144124002 genotyping on a 3% agarose gel stained with Gel Red.

According to the results of the genotyping of the rs144124002, no significant differences were found (p-value < 0.05) in both allele and genotype level between PDAC patients and healthy controls.

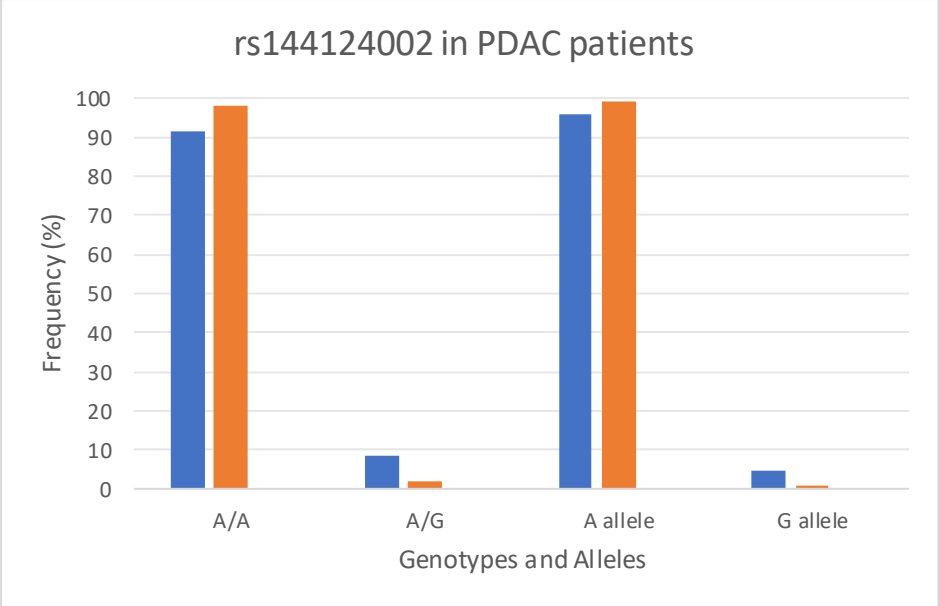

Diagram 3: Representative frequencies of the rs144124002 polymorphism between PDAC patients (blue) and healthy controls (orange) in genotypes and alleles level.

In particular, the A/A genotype and thus the A allele was overrepresented in both patients and healthy controls, leading to no statistically significant differences. A/A genotype was found in 91.5 % of patients and in 98% of controls while the A/G genotype was found in 8.5 of patients and in 2 % of healthy individuals. The A allele, in the same way, was found in 95.6 of patients and in 99.3 of controls and so the G allele showed 4,4 % frequency in patients and 0.7 frequency in controls.

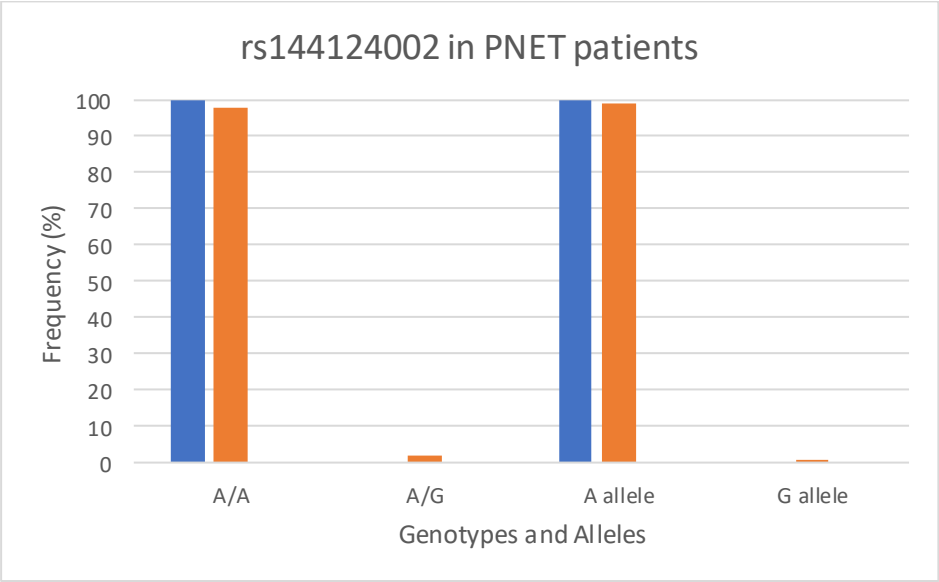

Diagram 4: Representative frequencies of the rs144124002 polymorphism between PNET patients (blue) and healthy controls (orange) in genotypes and alleles level.

In case of PNET patients, no significant differences were also found. The A/A genotype was overrepresented in both patients and controls (100% vs 98 %). The A allele in the same way, was found in 100% of patients and in 99.3 % of healthy controls and so the G allele was found only in 0.7 % of healthy controls.

4.1.3 Rs369274325

The third polymorphism which was genotyped was rs369274325. The polymorphism was genotyped by TETRA-ARMS PCR, so no restriction enzyme was used in this reaction. The figure from below shows the bands which were produced after the polymerase chain reaction. The visualization resulted after treatment with Gel Red in a 3% agarose gel. A bp step ladder (Nippon Genetics Europe, Düren, Germany) was also used in order the bands to be discriminated.

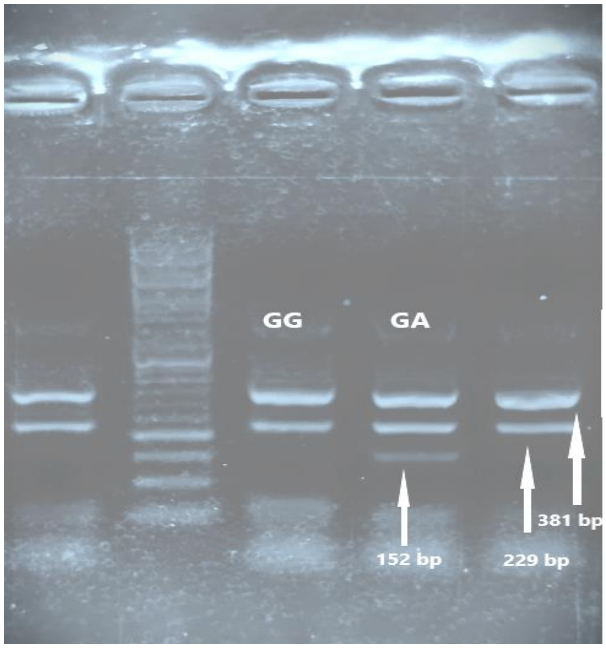

Figure 11: Representative results of rs369274325 genotyping on a 3% agarose gel stained with Gel Red.

According to the results of the genotyping of the rs369274325, no significant differences were found (p-value < 0.05) in both allele and genotype level between

PDAC patients and healthy controls. In this case no genotyping of this SNP was conducted in PNET patients.

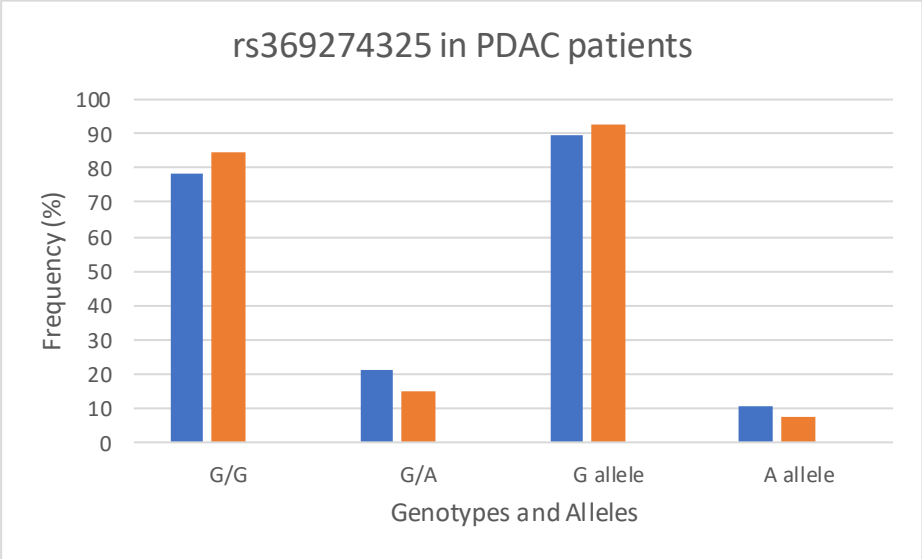

Diagram 5: Representative frequencies of the rs369274325 polymorphism between PDAC patients (blue) and healthy controls (orange) in genotypes and alleles level.

More specifically, the G/G genotype was found in 78.7 % in PDAC patients and in 84.9 % in healthy controls, whereas the G/A genotype was found in 21.3 % in patients and in 15.1 % in controls. In allele level, the G allele was found in 89.4 % of the patients and in 92.5 % of controls. Finally, the A allele was found in 10.6 % of the PDAC patients and in 7.5 % of healthy individuals. These results show no statistically significant differences between the two groups.

5. DISCUSSION

The aim of this study was to unravel any potential association among 3 different single nucleotide polymorphisms of a protein deacetylase, Sirtuin-1, the most studied among the Sirtuins family, with Pancreatic ductal adenocarcinoma and Pancreatic neuroendocrine tumors. The reason why this gene was chosen was the fact that polymorphisms of SIRT1 have been studied before in other malignancies and pancreas-associated diseases, but never before in pancreatic cancer. The polymorphisms of the SIRT1 were rs3758391, rs144124002 and rs369274325.

As previously described, SIRT1 is closely related to p53.[42] SIRT1 deacetylates p53, leading to its functional inactivation, which reduces p53's ability to activate genes involved in growth arrest and apoptosis, thereby potentially promoting tumor survival and growth. In this way, SIRT1 can act as an oncogene. In pancreatic cancer, SIRT1 is overexpressed both in mRNA and protein level.[37] The first two polymorphisms, rs3758391 and rs144124002 are both localized in or very close to the SIRT1-p53 binding area and are characterized by the Uniprot database as variants of unknown significance.

Interestingly, in case of rs3758391, there was a strong correlation of the polymorphism with Pancreatic adenocarcinoma both in genotype and allele level. When the frequencies between PDAC patients and healthy controls were compared, the T allele (wild type allele) appeared significantly more frequently than C allele in PDAC patients ($p= 0.0001$) rather than healthy controls. In genotypes level also, the TT genotype was significantly over-represented in the PDAC patients, followed by the CT genotype and lastly by the CC genotype. The TC genotype is overrepresented in healthy controls.

This specific polymorphism has been studied before in diffuse large B-cell lymphoma patients,[42] Urinary blader cancer patients[40,43] and laryngeal squamous cell carcinoma patients,[43] showing significant correlation between the T allele and the appearance of the disease. These findings in combination with the previous literature could indicate the strong association of the polymorphism with the occurrence of cancers in which the Sirt1 gene acts as a tumor promoter. Therefore, the mutation in this case is

protective against the occurrence of the disease and the polymorphism could serve as a potential biomarker.

Pancreatic neuroendocrine tumors on the other hand are very rare and represent 1% of the pancreatic cancer incidents. The findings concerning the rs3758391 polymorphism could not show a significant difference either in allele or genotype level between the PNET patients and the healthy controls due to the small number of samples which were available.

In case of rs144124002, the polymorphism was studied both in PDAC and PNET patients. This was the first time that this SNP was studied in cancer patients.[38,44] The comparison of genotypic and allelic frequencies showed no correlation since the A allele (wild type allele) was shown to be represented nearly in all PDAC and PNET patients and healthy controls in comparison with G allele.

As it was previously said, the polymorphisms were chosen mainly based in previous studies. In case of the last polymorphism, rs369274325 has been previously studied in Urinary blader cancer patients in two different cohorts, Iranians and Turkish.[40,45] Rs369274325 is located also in the SIRT1 promoter region, near the 5' end of the gene.[46] This SNP in the Iranian cohort showed a significant difference between patients and healthy individuals in genotype level concerning the A/G genotype. The comparison of frequencies in both genotypic and allele level showed slightly no difference in the Greek cohort of PDAC patients which were investigated in this study.

In conclusion, the results of this study strengthen the evidence that a specific polymorphism which has been well studied in other malignancies, is also implicated in Pancreatic ductal adenocarcinoma. Even if the other two SNPs (rs144124002 and rs369274325) showed no susceptibility to pancreatic cancer, rs3758391 and its protective role in PDAC could potentially serve as a biomarker for this devastating disease. Genotyping a larger number of samples, especially in Pancreatic neuroendocrine tumors patients could possibly provide us with even more safer conclusions.

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