Article title: Comparative Transcriptomic Response Of Pancreatic And Breast Cancer Cells To Anacardic Acid And Olaparib: A Quest for an Enhanced Synergistic Therapy

Authors: Abah Moses Owoicho[1], Joseph Luper Tsenum[2], Deborah Oganya Ogenyi[3], Ogu Stephen[4], Ujah Moses Okwori[5]

Affiliations: Biotechnology/Molecular Lab Department, Royal Society of Clinical and Academic Researchers International, Abuja, Nigeria, Department of Bioinformatics Moscow Institute of Physics and Technology, Dolgoprudny, Russia[1], Bioinformatics Department, Georgia Institute of Technology, Atlanta, Georgia, USA[2], Bioinformatics Department, Moscow Institute of Physics and Technology, Dolgoprudny, Russia[3], Biotechnology/Molecular Lab Department, Royal Society of Clinical and Academic Researchers International, Abuja, Nigeria[4], Integrated Science Education Department, Federal College of Education (Technical) Bichi, Kano, Nigeria[5]

Orcid ids: 0000-0001-9439-1302[1]

Contact e-mail: abakh.m@phystech.edu

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COMPARATIVE TRANSCRIPTOMIC RESPONSE OF PANCREATIC & BREAST CANCER
CELLS TO ANACARDIC ACID & OLAPARIB:
A Quest for an Enhanced Synergistic Therapy

A RESEARCH PROPOSAL

BY
ABAH, MOSES OWOICHO

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CHAPTER ONE

1.0 INTRODUCTION

Transcriptomic analysis also referred to as gene expression profiling is used to understand and predict the activity of novel therapeutic compounds in pre-clinical settings. Transcriptome analysis using bioinformatics tools gives an overview of biological processes and pathways affected by a drug; thus providing new insights about the potential cellular targets and mechanisms of action of that drug. Identification of such targets using RNA-seq would be beneficial in identifying anacardic acid (AnAc)-regulated pathways and targets in both luminal “A” breast cancer and in triple negative breast cancer (TNBC) which primarily affects premenopausal women with a predominance in women of African and Hispanic ancestry (Brewster et al; 2014).

A number of plants produce anacardic acid (AnAc) which is a mixture of 6-alkylbenzoic acid congeners, previous study shows that a specific congener, AnAc 24:1n5, acts as a concentration-dependent mixed agonist/antagonist of estrogen receptor (ER α)-induced proliferation and transcription and inhibits ER α -estrogen response element (ERE) binding by interacting with the DNA binding domain (DBD), thus acting as a nuclear receptor alternate site modulator (NRAM)² (Schultz et al; 2006). AnAc 24:1n5 also inhibited MDA-MB-231 triple negative breast cancer (TNBC) cell proliferation, although at a higher IC₅₀ and via an unknown mechanism (Schultz et al; 2006).

Pancreatic ductal adenocarcinoma comprises about 90% of all malignant pancreatic neoplasms. Of all other malignant pancreatic neoplasms (pancreatic neuroendocrine tumors, solid-pseudo papillary neoplasm, acinar cell carcinoma and pancreatoblastoma), neuroendocrine tumors are the most common, comprising approximately 5% of malignant pancreatic tumors (Kleef et al; 2016)

Olaparib was the first PARP inhibitor to be approved (December 2014 in the United States [US] and Europe) and is approved in the US for a number of indications in ovarian and breast cancer

(Oncologic Drugs Advisory Committee - ODAC, 2019)

1.1 Aim

To evaluate the comparative transcriptomic response of pancreatic & breast cancer cells to anacardic acid & olaparib: A quest for an enhanced synergistic therapy

1.2 Objectives

To evaluate the gene profile of pancreatic cancer cell lines in response to anacardic acid

To analyze the gene profile expression of pancreatic cancer cells in response to olaparib

To assess the gene profile response of pancreatic cancer cell lines in response to both anacardic acid & olaparib

To evaluate the global gene profile response of breast cancer cell lines to anacardic acid

To analyze the gene profile expression of breast cancer cells in response to olaparib

To assess the gene profile response of breast cancer cell lines to both anacardic acid & olaparib

To compare the transcriptomic response of pancreatic cancer cells and breast cancer cell lines in response to anacardic acid & olaparib respectively

1.3 Justification of Study

The poor prognosis of pancreatic cancer (~90% of patients who are diagnosed will die of the disease). This is due to the late presentation of the disease. Since there are currently no precision medicine treatment options for gBRCAm pancreatic cancer patients and also considering the fact that patients with metastatic pancreatic cancer have worse quality of life compared to the general cancer patient population (Müller-Nordhorn et al 2006); coupled with the fact that it is the most aggressive cancer with one of the worst prognoses of all malignancies (Cancer Statistics Factsheets 2019), hence the quest for a more potent synergistic therapy to combat this aggressive cancer of public health concern via this study.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 PANCREATIC CANCER

Metastatic pancreatic cancer has a very poor prognosis with a 5-year survival of only 3%. (Oncologic Drugs Advisory Committee - ODAC, 2019). First-line chemotherapy may provide initial disease and symptom control, however, those are short-lived with only a 3-month time to progression or death; furthermore chemotherapy cannot be tolerated for prolonged periods of time, requiring treatment breaks and leaving the patient untreated and at high risk for early disease progression, and early re-exposure to toxic chemotherapy (Oncologic Drugs Advisory Committee - ODAC, 2019). Pancreatic cancer is a life-threatening disease with one of the worst prognoses of all malignancies (Cancer Statistics Factsheets 2019).

Platinum-based chemotherapy is the preferred regimen for the first-line treatment of advanced pancreatic cancer, due to the evidence that overall survival is improved (Golan et al 2014) compared to that with non-platinum regimens and is advocated by national guidelines, including those for patients with known BRCA mutations pancreatic cancer (National Comprehensive Cancer Network - NCCN 2019). For gBRCAm patients with good performance status, NCCN guidelines recommend platinum-based regimens including the FOLFIRINOX regimen (a combination of 5-fluorouracil [5-FU], irinotecan, leucovorin [LV], and oxaliplatin) (Conroy et al 2011) and gemcitabine cisplatin (Oliver et al 2010).

There are two practice patterns that exist for these regimens. Physicians either intend to administer chemotherapy for 6 months and stop in patients who had not progressed (ie, the approach described the original FOLFIRINOX paper: Conroy et al 2011), or to administer chemotherapy until disease progression. However, in either case, many patients have to stop therapy early due to toxicity. Even when dosages are modified or when individual chemotherapy agents are dropped, platinum-based chemotherapy is difficult to tolerate due to cumulative

toxicities that can be significant (eg, Grade 3/4 neutropenia [45.7% of patients] and possibly irreversible (such as sensory neuropathy [9% of patients]; Conroy et al 2011, Dahan et al 2018, Kang et al 2018, Ozaka et al 2018). Furthermore, the need for a 46-hour infusion of fluorouracil every two weeks requires patients to carry ambulatory pumps with limitation of activities/travel and frequent visits to clinic. As a result, many patients who receive first-line platinum-based chemotherapy do not continue that therapy until disease progression. For those non-progressing patients who can no longer tolerate platinum-based chemotherapy, or have completed 6 months of chemotherapy, the standard practice is to stop the chemotherapy for some period of time (ie, a “chemotherapy break”), which allows the patient to recover from the challenging side effects of treatment. However, this also leaves the patient untreated and at high risk for earlier disease progression with earlier re-exposure to chemotherapy and its toxicities. This suggests a need for new treatment strategies.

2.1.1 Pancreatic Progenitors

The adult pancreas comprises three major lineages: endocrine, acinar and ductal. The endocrine compartment is in the islets of Langerhans and consists of cells that secrete insulin and whose failure to secrete this hormone leads to diabetes. Acinar cells produce digestive enzymes and, together with ductal cells, form the exocrine pancreas. The pancreas develops from part of the foregut endoderm around it. During the primary transition, a pancreas-specified and relatively homogeneous multipotent progenitor cell (MPC) population evaginates from the naive endodermal tube and proliferates to form dorsal and ventral buds. Multipotent pancreatic progenitors proliferate extensively and undergo branching morphogenesis. Later during development, scattered groups of cells undergo apicobasal polarization to initiate the formation of microlumens, which expand and coalesce to build a web-like network (or plexus) of epithelial tubes.

Recent studies indicate an essential role of the transcription factor GATA6 in pancreas organogenesis. In fact, GATA6 heterozygous inactivating mutations have been identified in patients with pancreatic agenesis, a rare birth defect characterized by complete absence of the pancreas or an extreme reduction of its size; consequently, these patients suffer from severe

exocrine pancreas insufficiency and neonatal diabetes. In line with these observations, two recent reports in various experimental systems, including human pluripotent stem cells, support a key role of GATA6 in the induction of human definitive endoderm, development of the pancreas and functionality of exocrine and endocrine pancreas (Shi et al 2017; Tiyaaboonchai et al 2017). An interaction between GATA6 and GATA4 is required for the generation of pancreatic progenitor cells (Shi et al 2017). GATA6 and GATA4 inactivation in mice determines a dramatic upregulation of Hedgehog components, not compatible with pancreatic determinations (Xuan et al 2016).

The pancreas possesses two functional cellular compartments, endocrine and exocrine. The exocrine pancreas comprises acinar, ductal and centroacinar cells. An undifferentiated pancreatic trunk epithelium, called pancreatic cords, is present during early stages of embryonic development; these pancreatic cord cells proliferate and differentiate into endocrine and exocrine lineages. Ductal cells, usually quiescent in the adult pancreas, form an intricate network of ducts representing the conduit for the flow of digestive enzymes secreted by acinar cells. Acinar cells are responsible for the secretion of digestive enzymes and represent the preponderant cell type in the pancreas; these cells have an intrinsic plasticity in that they have the capacity to undergo metaplasia to ductal or ductal-like cells: this metaplastic process is known as acinar-ductal metaplasia, it occurs during acute-chronic pancreatitis and may represent an initial step towards the pancreatic intraepithelial neoplasia formation (Sethi et al 2017). Pancreatic ductal adenocarcinoma comprises about 90% of all malignant pancreatic neoplasms.

Of all other malignant pancreatic neoplasms (pancreatic neuroendocrine tumors, solid-pseudo papillary neoplasm, acinar cell carcinoma and pancreatoblastoma), neuroendocrine tumors are the most common, comprising approximately 5% of malignant pancreatic tumors (Kleef et al 2016). Pancreatic adenocarcinoma is a highly aggressive cancer with more than 330,000 estimated deaths in the world in 2012, ranking as the seventh leading cause of cancer death, and is the most lethal of the common malignancies (Illic et al 2016). For 2017, the National Cancer Institute estimates that pancreatic cancer will represent 3.2% of all cancers and 7.2% of all cancer deaths. The incidence of pancreatic cancer has been continuously rising during the last few years, and the projected incidence and death to 2030 predicts that pancreatic cancer will be

the second cause of death by 2030 (Rahib et al 2014). The risk of developing pancreatic cancer is about three-times higher in smokers than in nonsmokers, and 5–10% of patients with pancreatic cancer have a family history of the disease. This tumor is characterized by early spread with local diffusion and metastasis to distant organs, and most patients arrive at the clinical diagnosis with surgical unresectable disease: in fact, about 80% of patients are diagnosed with locally advanced or metastatic disease. These patients have a rapid disease progression, and few of them survive more than one year. Even for patients with localized disease at diagnosis and undergoing radical curative surgery, the median survival remains low, being around 18 months (Kleef et al 2016; Rebelo et al 2017). Therefore, despite the advances in the understanding of the basic biology of pancreatic adenocarcinoma, survival rates remained basically unmodified during the last 30 years. Therefore, there is an absolute need for the development of new therapeutic strategies for pancreatic adenocarcinoma.

Infiltrating ductal carcinoma of the pancreas arises from different types of non-invasive precursor lesions. Three different types of precursor lesions have been identified: intraductal papillary mucinous neoplasms (IPMNs), mucinous cystic neoplasms (MCNs) and pancreatic intraepithelial neoplasia (PanIN). These precursor lesions meet all the criteria to be really considered as precancerous lesions, precursor to invasive cancer. MCNs are defined as mucin-producing cyst-forming epithelial neoplasms of the pancreas with a peculiar stromal tissue. IPMNs are non-invasive mucin-producing epithelial tumors, usually giving rise to the formation of long finger-like papillae. PanINs are non-invasive small epithelial tumors, located in the smaller pancreatic ducts, and characterized by cytological and architectural atypia (Hruban et al 2007).

Pancreatic intraepithelial neoplasias are classified into different stages according to the degree of histologic abnormalities in the ductal epithelium. In fact, these neoplasias range from PanIN1A and PanIN-1B to PanIN-2 and PanIN-3, with each stage showing increased cellular and nuclear atypia's. PanIN-3 is frequently observed in association with invasive pancreatic cancer and seemingly represents a precursor lesion to invasive disease. It is important to note that a typical feature of pancreatic cancer consists of the formation of a dense stroma, termed a desmoplastic reaction, composed of cellular and fibrillary elements. Pancreatic stellate cells play a key role in

the genesis of desmoplastic reaction through their activation of Transforming growth factor β 1 (TGF- β 1), Fibroblast Growth Factor (FGF), Platelet-derived growth factor (PDGF) and their differentiation into myofibroblasts that actively secrete collagen and other components of the extracellular matrix. The desmoplastic reaction plays a key role in tumor formation, progression, invasion and metastasis. A peculiarity of the pancreatic cancer stroma is given by its capacity to contribute to creating an immunosuppressive tumor microenvironment that restricts antitumor immunity. Recent studies showed that pancreatic stellate cells play also a key role as modifiers of the pancreatic tumor metabolism through the secretion of non-essential amino acids and, particularly, of alanine, which outcompetes glucose and glutamine-derived carbon to fuel the tricarboxylic acid (TCA) cycle (Sousa et al 2016). This metabolic change triggered by stellate cells decreases the tumor's dependence on glucose and serum-derived nutrients (Sousa et al 2016).

2.1.2 Genetic Abnormalities

The large majority of pancreatic cancers are represented by infiltrating ductal adenocarcinomas. These tumors at the microscopic level are composed by an infiltrating gland-forming neoplastic epithelium, associated with an intense desmoplastic reaction, this reaction being usually very intense. A fundamental step in our understanding of pancreatic adenocarcinoma is represented by the discovery of the main genetic somatic abnormalities observed in this tumor. In this context, considerable progresses have been achieved during the last few years. In 2008, the complete analysis of the pancreatic cancer exome has been reported (Jones et al 2008): the coding regions of >29,000 genes were sequenced in pancreatic adenocarcinomas, showing an average of 63 genomic alterations, in large majority point mutations. These alterations defined a core set of 12 cellular signaling pathways and processes that were altered in the majority (from 67–100%) of pancreatic adenocarcinomas: among them, the most notable were KRAS signaling, regulation of the G1/S cell cycle transition, TGF- β signaling, integrin signaling, regulation of cell invasion, homophilic cell adhesion and small guanine triphosphate (GTPase)-dependent signaling (Jones et al 2008).

2.1.3 Pancreatic Cancer Genetic Landscape

The genetic landscape of pancreatic tumor genomes is characterized by the presence of four frequently-mutated genes, represented by: KRAS (90%), CDKN2A (p16, 90%), TP53 (70%) and SMAD4 (55%). Transcription of the mutant KRAS gene determines the production of an abnormal, constitutively-activated RAS protein, determining as a consequence the uncontrolled activation of proliferation and survival pathways. On the other hand, the frequent inactivation of the CDKN2A gene results in a loss of p16 protein, a master negative regulator of the G1-to-S transition of the cell cycle, with consequent stimulation of the proliferative activity. The frequent TP53 inactivation permits the cells to bypass some important control checkpoints at the level of DNA damage and apoptotic triggering.

The frequent loss of SMAD4 gene results in the aberrant signaling by TGF- β . (Blackford et al 2009). Recent studies have confirmed that inactivation of SMAD4 is the strongest predictor of metastatic recurrence (Shin et al 2017). Furthermore, review and meta-analysis of the literature data confirmed the negative clinicopathological significance of SMAD4 loss in Pancreatic Duodenal Adenocarcinoma Cells (PDACs) (Wang et al 2017). Although SMAD4 gene deletion is associated with a poor prognosis, however, it exposes PDAC cells to a metabolic vulnerability. In fact, Dey and coworkers have analyzed PDACs that harbor the abortion of both copies of the SMAD4 gene and showed that this subset of tumors is associated with the loss of neighboring genes involved in key metabolic pathways, including mitochondrial malic enzyme 2 (ME2) (Dey et al 2017). The ME2 loss in these cells determines a compensatory increased expression of the closely related mitochondrial malic enzyme 3 (ME3) (Dey et al 2017). ME2 and ME3 are two enzymes involved in the conversion of malate to pyruvate, and PDAC cells with biallelic SMAD4 loss are vulnerable to M3 loss or inhibition (Dey et al 2017).

Recently, a new exome sequencing and copy number analysis on a large cohort (142 patients) of pancreatic adenocarcinomas has been reported (Shin et al 2017). The results of this analysis allowed defining 16 significantly mutated genes, including the four driver/founder genes KRAS, TP53, CDKN2A and SMAD4, other genes already known to be mutated in pancreatic cancer such as MLL3, TGFBR2, ARID1A and SF3B1 and unreported novel mutant genes, such as

genes involved in chromatin modification (EPC1 and ARID2) and DNA damage repair (ZIM2, MAP2K4, NALCN, SLC16A4 and MAGE/A6) (Biankin et al 2012). In addition, these authors identified frequent and various somatic genetic alterations of genes classically classified as embryonic regulators of axon guidance, particularly SLIT/ROBO signaling, thus suggesting that this pathway could play a role in pancreatic carcinogenesis (Biankin et al 2012). It is important to note that among these mutated genes, no frequent drug targets were found that could be counterbalanced with specific drugs. A notable exception was represented by the identification of rare mutations, such as the mutation of the PALB2 gene found in one individual cancer, predicting a high sensitivity to DNA damaging agents: in line with this prediction, the treatment of this patient with alkylating agents resulted in marked tumor regression and long-term survival (Vilarroel et al 2017). These four genes (KRAS, TP53, CDKN2A and SMAD4) are considered as driver genes for pancreatic adenocarcinoma.

In some patients, the mutations present in the primary tumor and in the corresponding metastases have been analyzed. Through this comparative analysis, two categories of mutations were identified: (a) the largest category of mutations corresponded to those present both in primary tumor and in their corresponding metastases (these mutations range from 48–83% of all mutations); (b) the other mutations (these mutations range from 17–51% of all mutations) are defined as progressor mutations and were present in the metastases, but not in the corresponding primary tumors (Wang et al 2017). Importantly, the driver mutations at the level of the genes encoding KRAS, TP53, SMAD4 and CDKN2A were already present in the primary tumors and remained present at the level of metastases (Yachida et al 2010). According to these observations, the driver mutations must be regarded as the founder mutations also (Yachida et al 2010).

Analysis of genetic abnormalities acquired in metastases showed that during disease progression pancreatic cancer acquires rearrangements indicative of telomere dysfunction and abnormal cell-cycle control, particularly dysregulated G1-to-S phase transition (Campbel et al 2010). The involvement of abnormalities of genes involved in chromatin remodeling was carefully assessed in a recent study of high-resolution genomic profiling of pancreatic cancer, integrated with mutational data (Shain et al 2012). This analysis allowed identifying structural alterations at the

level of multiple subunits of the switch/sucrose non-fermentable (SWI/SNF) chromatin remodelers (Shain et al 2012). In fact, Shain et al. identified somatic genetic abnormalities (genomic deletions, mutations and rearrangements) occurring at the level of genes encoding components of the SWI/SNF chromatin remodeling complex, involving the DNA binding subunits ARID1A, ARID1B and PBM1 and the enzymatic subunits SMARCA2 and SMARCA4 (Shain et al 2012). Although the frequency of mutation of each of these genes occurred at relatively modest frequency, the global incidence of all these mutations is considerable since they affected about 1/3 of all pancreatic cancers (Shain et al 2012).

2.1.4 Animal Models of Pancreatic Cancer Development

Several studies have tried to identify and define somatic genetic abnormalities that can cooperate with driver mutations to induce the development of pancreatic cancer. To identify cooperating mutations, animal models of pancreatic cancer have been developed. These animal models are based on the expression of oncogenic KRAS in the pancreas either alone or in combination with inactivating alleles of homologs of known human pancreatic cancer drivers, including TP53 and SMAD4. Some studies have explored the possible oncogenic cooperation between KRAS mutations and SMAD4 loss. SMAD4 is an intracellular common mediator of the TGF- β superfamily signaling pathways. SMAD4 loss in PDAC causes various phenotypic changes, including increased E-cadherin and CD133 expression, increased MAPK activation and increased chemoresistance (Chen et al 2014). SMAD4 loss is frequently observed in pancreatic cancer (more than 50%), but it is an event occurring at late stages of cancer development since it is rarely observed in pre-invasive pancreatic neoplasia.

The analysis of the constitutively active allele of KRAS (KRASG12D) in the ductal pancreatic epithelium elicited all the spectrum of the pre-invasive neoplastic lesions. Therefore, it seemed particularly interesting to evaluate a possible cooperation between KRAS mutation and SMAD4 loss in favoring the progression of pancreatic cancer (Kojima et al 2007). The results of these studies showed that the loss of SMAD4 in conjunction with KRASG12D stimulated the progression of pancreatic cancer development through various mechanisms, including accelerated fibrosis, enhanced acinar cell loss and accelerated development of various pre-

invasive neoplastic lesions (Kojima et al 2007).

However, it must be pointed out that the role of TGF- β signaling in PDAC development is complex. In fact, some studies have shown that conditional loss of SMAD4 or TGFBR2 in Pdx1-Cre/LSL-KRas mice develop advanced aggressive pancreatic cancer (Bardeesy et al 2006). In contrast, systematic deficiency of TGFBR1 in elastase-KRASGRD (EL-KRAS) mice induced a marked reduction in the tumoral phenotype (Adrian et al 2009). These diametrically opposed findings can be explained considering the differential effect of TGF- β signaling at the level of tumor cells and of the tumor microenvironment: in pancreas cells, TGF- β deficiency favors tumor development and is associated with tumor progression; in contrast, TGF- β deficiency at the level of microenvironment protects against tumor development by promoting fibrosis and immune invasion (Principe et al 2016).

On the other hand, other studies have shown that either p16 or p53 loss, in conjunction with constitutive KRAS, promoted pancreatic cancer progression, thus indicating that both the retinoblastoma and the p53 pathway are involved in the suppression of pancreatic cancer development (Hingorani et al 2005). All these models showed the progressive development of neoplastic lesions, corresponding to murine intraepithelial neoplasia, that in some cases become invasive or progress to adenocarcinoma. In spite that these models have considerably contributed to the progress of our understanding of pancreas cancer biology, however, they have not allowed the identification of cooperating mutations required for neoplastic disease progression. To identify these mutations, another strategy was used, consisting of performing an insertional mutagenesis screen using the inducible Sleeping Beauty (SB).

2.1.5 Epigenetic Abnormalities in Pancreatic Cancer

Besides some driver genes that are frequently mutated in pancreatic cancer, there are several oncogenes that do not carry mutations, but are deregulated in their expression and contribute to tumor maintenance/progression. A prototype of these oncogenes in pancreatic cancer is c-Myc for its overexpression observed in a part of pancreatic cancers, due, more frequently to enhanced transcriptional activity and, more rarely, to gene amplification; because of these events, the c-Myc core signaling is one of the pathways more frequently activated in pancreatic cancer

(Hruban et al 2007). To study the role of c-Myc overexpression at the level of the exocrine pancreatic lineage, Lin and coworkers have developed a mouse model implying a temporally and spatially-controlled expression of c-Myc at the level of pancreatic progenitors (Lin et al 2012). Upregulation of c-Myc expression resulted in the rapid formation of ductal precursor lesions and of adenocarcinomas, metastasizing at the level of the liver (Taki et al 2016). The downregulation of c-Myc expression in these tumors induces cell death, thus indicating their dependency on c-Myc for their survival (Lin et al 2012). However, few tumor cells survived to c-Myc downregulation: these cells remained dormant, expressed stem cell markers and gave rise to tumor recurrence upon c-Myc re-expression (Lin et al 2012).

2.1.6 Pancreatic Cancer Stem Cells

Pancreatic cancer stem cells had been initially identified in 2007 by Li and coworkers who investigated the expression of CD44, CD24 and epithelial-specific antigen (ESA) in pancreatic tumors. These authors have isolated CD44+CD24+ESA cells from xenografts in immunodeficient mice of pancreatic cancer cells and have shown that these cells are highly tumorigenic and regenerated into host immunodeficient mice the original tumor histology and heterogeneity (Li et al 2007). A second study by Hermann and coworkers provided evidence that CD133+ cells isolated from human pancreatic tumors can initiate a tumor when inoculated into immunodeficient mice (Hermann et al 2007). In this study, it was shown also that CD133+ cells are resistant to gemcitabine treatment compared to CD133- cells (Hutcheson et al 2016). Furthermore, CD133+/CXCR4+ cells were shown to be responsible for metastasis development (Hutcheson et al 2016).. Other studies have shown that ALDH+ cells isolated from pancreatic tumors have the property of cancer stem cells (Hermann et al 2007).

The presence of ALDH+ cells within pancreatic tumors was associated with the clinical and biologic properties of pancreatic cancers. Thus, an increased ALDH expression, as detected by immunohistochemistry in primary localized pancreatic cancers, was associated with reduced overall survival compared to that observed in patients with low ALDH expression (Rasheed et al 2010). Furthermore, it was observed that ALDH expression was increased in metastatic lesions compared to the primary tumors from the same patient, thus suggesting a role for these cells in

tumor metastasis (Rasheed et al 2010). Finally, it was shown that purified ALDH⁺ cancer stem cells exhibit a mesenchymal phenotype and were highly invasive compared to the bulk tumor (Rasheed et al 2010). Another study confirmed that ALDH positivity can be used as a marker to identify and to purify pancreatic cancer stem cells. In fact, it was shown that the tumor-initiating cell frequency in the ALDH⁺ cell population was significantly higher (about one in 300 cells) than in the unfractionated cells (about one in 5000 cells) (Ishizawa et al 2010). Kim and coworkers have comparatively studied from the same tumors the tumorigenic potential of ALDH⁺ and CD133⁺ cells. ALDH⁺ cells were consistently shown to be more tumorigenic than CD133⁺ cells (Kim et al 2011). Furthermore, ALDH^{high} cells are markedly more tumorigenic than ALDH^{low} cells (Kim et al 2011). Furthermore, using a triple labeling of the cells, it was shown that ALDH^{high}/CD44⁺/CD24⁻ cells are highly tumorigenic, while ALDH^{high}/CD44⁺/CD24⁺ cells are scarcely tumorigenic (Kim et al 2011). Finally, it was observed that ALDH expression was highly variable in various pancreatic cancers

2.1.7 The Difficulty of Defining Efficacious Medical Therapies for Pancreatic Cancer

Pancreatic Ductal Adenocarcinomas (PDAC) is a great health problem, with an estimated 367,000 new cases diagnosed worldwide in 2015 and an associated 359,000 deaths in the same year. Given these data, it is not surprising that PDAC is currently the fourth highest cause of cancer death in developed countries. If the treatment outcomes of PDAC are not improved, it is estimated that PDAC could become the second cause of cancer-related mortality within the next decade (Rahib et al 2014).

Many factors impede a rapid progress in PDAC treatment due to the absence of specific biomarkers for an early diagnosis, the aggressive nature of PDAC with rapid local invasion and early metastases, the limited number (15–20%) of PDAC patients amenable to curative surgical resection and the consistent resistance to most treatments, including standard chemotherapy, radiotherapy, molecular targeted therapy and immunotherapy. Furthermore, nonmalignant cells in the tumor microenvironment contribute to anticancer drug resistance. Finally, recent studies indicate that intratumor bacteria, frequently (76% of cases) observed in PDAC patients, contribute to tumor resistance to the chemotherapeutic drug gemcitabine, currently used in the

treatment of PDAC (Geller et al 2017). In spite of this impasse in the improvement of PDAC therapy, considerable progress has been made in the last few years in our understanding of PDACs. KRAS mutations are sufficient to initiate premalignant lesions, PanINs; PanINs can progress to locally invasive or metastatic cancer by way of either genomic rearrangements or stepwise acquisition of mutations in suppressor genes (CDKN2A, TP53 and SMAD4). Genome-wide studies have identified additional mutations and pathways, chromosome alterations, ubiquitin proteases and transcription factor alterations that cooperate with KRAS to drive PDAC progression. Furthermore, analysis of gene expression allowed proposing a classification of PDACs into four different groups, defined as squamous, pancreatic progenitor, immunogenic and aberrantly differentiated endocrine exocrine (ADEX); each of these subtypes is characterized by a different landscape of genetic alterations, tumor histological characteristics and different prognosis.

Unfortunately, many of the molecular abnormalities identified in PDAC are not amenable to an efficacious pharmacologic targeting. When targeting therapies are available for PDAC, these therapies have not given satisfactory results; this is due to rapid upregulation of compensatory alternative pathways removing the anti-tumor effect of targeting agents and to the tumoral fibrotic desmoplastic reaction. The studies of next generation sequencing techniques with whole-genome or exome sequencing offer the opportunity to identify, in some PDAC patients, genetic alterations suitable for therapeutic targeting. However, the practical utility of these studies has been limited in the ensemble of PDAC patients and, particularly, in those with advanced PDAC, because these methods remain costly and are still relatively rare in a clinically-certified setting.

2.2 BREAST CANCER

Loss-of-function mutations of the breast cancer susceptibility genes BRCA1 and/or BRCA2 genes are associated with an increased risk of cancers, including ovarian, breast, prostate and pancreatic cancers. BRCA genes code for proteins that are involved in homologous recombination repair (HRR) of DNA double-strand breaks (DSBs) (Murai et al; 2012). Cells with a deficiency in HRR, such as those with a germline BRCA mutations (gBRCAm) and loss of the second BRCA allele, are very sensitive to poly (adenosine diphosphate-ribose)

polymerase (PARP) inhibition. Normal tissues in people with gBRCAm still retain one wild type BRCA allele (BRCAwt). PARP inhibitors act through multiple mechanisms, including trapping of PARP on DNA at sites of single-strand breaks (SSBs) and inhibiting enzymatic activity. These processes prevent repair of SSBs, which occur commonly in cancer cells, leading to the generation of DSBs, which cannot be repaired accurately in cells with defects in HRR. Thus, PARP inhibitors cause a selective accumulation of DNA damage and cancer-cell death in cells with HRR defects

2.2.1 Basic Pathways of Breast Cancer

Breast cancer (BC) is the most frequently diagnosed cancer in women worldwide, with 1.7 million new cases diagnosed in 2012 (Sethi et al 2017). This heterogeneous disease is classified into several molecular subtypes, depending on the presence of specific cell surface receptors, including the estrogen receptor (ER), the progesterone receptor (PR), and the human epidermal growth factor 2 receptor (HER2) (Illic & Illic 2016). Luminal A BCs are ER⁺ and/or PR⁺. These are the most commonly seen BCs and have the best prognosis. Luminal B BCs are ER⁺ and/or PR⁺ and sometimes HER2⁺. These tumors have a higher proliferative index and are more aggressive. HER2⁺ BCs are ER⁻ and PR⁻. These subtypes are usually present at a younger age with a poorer tumor grade and lymph node involvement; though the prognosis has improved dramatically since the clinical implementation of Herceptin, an anti-HER2 antibody. About 20% of BCs are triple negative (TNBC) or basal-like, that is, they are ER⁻, PR⁻, and HER2⁻. These tumors are often aggressive, have poorer prognosis, and lack any targeted therapies. Research has focused extensively on the role of cell surface receptors like HER2 in the pathobiology of BC. There are numerous families of cell surface receptors.

BC is one of the most common cancers worldwide and the second leading cause of cancer-related death in women (Bankaitis et al 2015). It is a heterogeneous disease with a complex molecular etiology. A great deal of research has focused on the mechanisms underlying BC development, growth, and progression. Dysregulated RTK signaling has been identified as a critical event in breast tumorigenesis. For example, HER2 and IR are overexpressed by 20 and 80% of BCs respectively, and mutation of PI3K, a key mediator of RTK signaling, is mutated in

35% of human breast tumors (Bankaitis et al 2015; Shi et al 2017 & Adrian et al 2009). Identification of such oncogenic proteins has led to a deeper understanding of BC and allowed for the development of targeted therapies for the treatment of this disease. Nevertheless, additional research is required to characterize mechanisms of tumor initiation as well as therapeutic resistance. Indeed, crosstalk between different RTK pathways and the existence of signaling feedback mechanisms are poorly understood processes that play critical roles in BC development and resistance to therapy. In the future, fundamental studies focusing on these issues in vitro should be combined with clinical research and early phase clinical trials to further characterize the role of RTKs in BC and identify new targets for anticancer therapies.

2.2.1.1 Maintenance of Genomic Stability

Mammalian cells preserve their genomic integrity by counteracting DNA damage (Ferlay et al 2015). DNA damage can originate from exogenous and endogenous insults. Exogenous insults involve environmental stress that originates from exposure to chemicals, UV light, tobacco smoke, chemotherapy, and radiotherapy. Endogenous DNA damage can arise from impaired DNA metabolic processes, intracellular oxidative stress, and oncogene activation. Cells respond to DNA damage by activating sensors, transducers, and effectors that jointly coordinate the so-called DNA damage response (DDR). When the amount of DNA damage is manageable, DDR activates cell cycle checkpoints that arrest cell cycle progression and allow DNA repair to correct the lesion thus preventing replication of damaged DNA. When DNA damage reaches levels beyond repair, cells activate self-destruction mechanisms that include apoptosis, autophagy, senescence and necrosis. There are five different mechanisms of repair designed for specific types of DNA lesions (Siegel et al 2015):

- Base excision repair (BER) removes base damage. BER is mainly involved in the surveillance, recognition and repair of oxidative DNA.
- Mismatch repair (MMR) corrects replicative errors and mismatched base pairs caused by faulty proofreading of DNA polymerases. MMR ensures low mutation rates in replicating cells.
- Nucleotide excision repair (NER) operates on a spectrum of helix-destabilizing bulky DNA lesions (global genome NER or GG-NER) or eliminates lesions in the transcribed strand of

active genes (transcription-coupled NER or TCNER). For example, NER removes bulky DNA adducts caused by exposure to various chemicals, alkylating agents, and UV radiation.

- Single-strand break (SSB) DNA repair corrects DNA breaks on one strand of the DNA double helix arising directly on the deoxyribose moieties or indirectly as intermediates of BER. SSBs are among the most frequent DNA lesions and are major threats to genetic stability and cell survival.
- Double-strand break (DSB) DNA repair resolves lesions that appear on both DNA strands.

The DSB is the principle lesion derived from ionizing radiation and radiomimetic chemicals. It is also caused when a replicative DNA polymerase encounters a DNA single-strand break or other type of DNA lesions. DSBs are intermediates in various biological events, such as V(D)J recombination in differentiating B cells. There are two major pathways of DSB repair: homologous recombination (HR) and Non Homologous End Joining (NHEJ). During HR, the repair of the damaged strand occurs by retrieving genetic information from the undamaged complementary DNA sister strand. In contrast, NHEJ brings about the ligation of two DNA DSBs without the requirement for sequence homology between the DNA ends. Although these mechanisms are tightly regulated, DNA repair defects occur thereby promoting the acquisition of mutations. Genomic instability results from a high frequency of DNA mutations ranging from nucleotide changes to chromosomal translocation, and it is a common feature of many cancers. De novo genetic alterations can initiate tumorigenesis, augment aggressiveness, and ultimately affect the overall prognosis of cancer patients. Recent studies have shown that different tumors have specific DNA repair defect signatures that involve more than one repair pathway (Parou et al 2000).

A more comprehensive analysis of these signatures has shown that DNA repair mechanisms are not parallel distinct entities but are intimately interconnected and can influence each other. While a normal cell coordinates DNA repair to preserve its genomic integrity, cancer cells modify DNA repair hubs to maintain genomic instability without compromising survival. In fact, cancer cells are subjected to many stressful conditions including nutrient starvation, oxidative stress, hypoxia, chemotherapy, and radiation that ultimately result in genotoxic damage. Therefore, a cancer cell must rely on a certain level of functional DNA repair to cope with these additional sources of

damage. Regulation of DDR and DNA repair has a critical role in the development and treatment of breast cancer. Expression and genomic profiling studies have shown that breast cancer is a very heterogeneous disease (Yu J et al 1998). There are different subtypes of breast cancers, and each type exhibits a range of biological and clinical behaviors. Luminal subtypes A and B both express markers of the luminal epithelial layer of normal breast tissue such as keratins 8/18 and are estrogen receptor (ER) positive. The ERBB2 subtype is characterized by the amplification and overexpression of ERBB2 (HER2) and neighboring genes at 17q12-q21 locus.

The basal-like subtype expresses markers of the basal epithelial layer of normal breast tissue such as keratins 5/6. Cancer cells of this subtype do not express ER, progesterone receptor (PR), and HER2 and are therefore referred to as triple negative (TN). Other TN types include “normal-like” and the recently described “claudin-low” subtype, showing that most but not all TN are basal-like cancers. There are two main hypotheses that can explain the existence of different subtypes: (Ferlay et al 2015) each subtype arises from a distinct tumor-initiating cell and (Sergei et al 2015) all subtypes share a common cell of origin that eventually acquired different somatic DNA mutations leading to different biological phenotypes. Although a definitive answer is lacking and both phenomena can coexist, it is clear that breast cancer subtypes are the results of distinct evolutionary processes. The genomic profile of breast cancers spans from a relatively simple landscape in luminal A subtype to a highly complex scenario in basallike/TN subtype.

2.2.1.2 Genomic Instability in Luminal A/B Subtypes of Breast Cancers

Luminal A and B subtypes share similar biological characteristics. However, luminal B tumors are more genetically unstable and highly proliferative and have less favorable prognosis. Luminal A subtype tumors have few chromosome rearrangements that define a “simple” genomic landscape. The main feature of this pattern is gain of chromosome 1p and 16p with loss of 16q (Hawkins et al 1992). Luminal B subtype has a different pattern of genomic alterations called “amplifier” or “firestorm.” This pattern is characterized by focal high-level DNA amplifications, clustered on one or more chromosome arm (Lil J et al 1997). Most of luminal B DNA rearrangements affect the expression of genes involved in signaling, cell cycle regulation, and nucleic acid metabolism such as FGFR1, MYC, CCND1, MDM2, ERBB2, and ZNF217. In

these tumor cells, the amplified DNA can exist either as repeated units within chromosomes called homogeneously staining regions (HSRs) or as extrachromosomal copies called “double minutes.” Several studies have demonstrated the presence of genomic instability in luminal A/B cancers. Cyclin D1, one of the most amplified genes in luminal B cancer, contributes to tumor progression by activating a transcriptional program that promotes chromosomal instability (CIN) (Stambolic et al 1998). The analysis of a large dataset of human specimen has shown that 52% of luminal A/B tumors overexpress the gene ESPL1 that encodes for a separase, a protease that cleaves the chromosomal cohesin during mitosis (Lemon et al 2008). Indeed, overexpression of ESPL1 in the mouse mammary gland induces chromosomal instability and aneuploidy (Alessi et al 1997).

Genomic data from over 1000 luminal A tumors has identified four major subtypes defined by distinct copy number and mutation profiles. Among these types, this group has characterized an atypical luminal A subtype characterized by higher genomic instability, TP53 mutations, and increased aurora kinase signaling associated with worse clinical prognosis (Hawkins et al 1992). Moreover, a recent study has shown that in early-stage luminal breast carcinoma, genomic instability, defined as a high number of chromosomal breakpoints, is a stronger prognostic marker than proliferation (Sabassov et al 2005). Overall, these studies suggest that genomic complexity is a feature of luminal breast cancer and can be used to predict the outcome of these tumors.

2.2.1.3 Estrogen Biology

Over the last two decades, it has become evident that breast cancer should be considered as a family of diseases rather than as a unique malignancy. Pathological, molecular, and genetic analysis have revealed the existence of five to ten main subgroups (Cancer Genome Atlas Network, 2012; Curtis et al 2012 & Sorlie et al 2001). Over 70% of all patients are generally classified by the tumor dependencies on estrogenic compounds (Beatson G 1896). These dependencies are principally mediated by the nuclear receptor estrogen receptor α (ER α) (Ali & Coombes 2002 & Magnani & Lupien 2014). For all these reasons, ER α remains the key driver in the majority of breast cancers and is commonly used as a molecular biomarker for stratification

while serving as the main target for systemic adjuvant chemotherapy. A critical shift in the approaches to studying estrogen biology has occurred in the last decade. The field has gradually moved from the investigation of single genes to the study of model cell lines and finally moving to patient-derived samples. This broadening involved also the molecular toolkit used by scientists through the development of next-generation sequencing and allowed the development of unbiased, genome-wide assays (Sancak et al 2007). This transition was critical to refine our understanding and overtake long-standing dogmas. Since then, the field has become aware of the complexities of estrogen signaling and began examining the association between DNA, the scaffolding DNA structure (chromatin), epigenetic and genetic factors, and ER α . Using System Biology approaches and genome-wide annotations, we now have also linked ER α to the majority of breast cancer hallmarks thus reemphasizing the importance of this dogmatic transcription factor.

The involvement of estrogen signaling in breast cancer Biology was recognized over a century ago, when a causative link between ovariectomy and breast cancer progression was made (Beatson G 1896). Several studies have also linked estrogen and breast cancer etiology. Some of the best-characterized predisposing factors leading to breast cancer reflect endogenous estrogenic exposure (Collaborative Group on Hormonal Factors in Breast Cancer 2012). In addition, additional exogenous estrogen exposure can also favor the development of luminal breast cancer (Colditz GA 1998). Over 5×10^3 studies (source, PubMed) have evaluated the role of estrogen signaling in MCF7 cells, one of the preferred tools to investigate the dynamics of estrogen signaling at a molecular levels. The most investigated aspect of estrogen signaling is without any doubt the sustained growth promoted by activated ER α . Nonetheless, estrogen signaling is also involved in many other cancer hallmarks (Hanahan & Weinberg 2011). Some of the molecular details of how this happens will be discussed in more detail in other sections of this chapter and other chapters as well.

Estrogen signaling has been extensively associated with the evasion of cell death (Lewis-Wambi & Jordan 2009), invasion, metastasis (Nelson & Wardell 2013; Platet et al 2004), and inflammation (Franco et al 2015) phenotypes. More recently estrogens and ER α have also been associated with angiogenesis (Syed et al 2013), genome instability, mutations (Periyasamy et al

2015) and deregulation of cellular energetics (Faulds et al 2011; Nguyen et al 2015). It is important to understand that most of these biological features are modulated by activated ER α at the DNA level. In the next sections, we will discuss how breast cancer cells can access such a wide array of cellular response via a single transcription factor.

2.2.1.4 A Brief Introduction of the Chromatin Environment

ER α signaling can be broadly classified into canonic (genomic) and non-canonical (non-genomic) ER α . Non-canonical signaling involves ER α activation in the cytoplasm and the subsequent activation of complex signal transduction cascades mediated mostly by kinases. An example is provided by EGF-EGFR signaling converging on ER α phosphorylation. Even in these scenarios, ER α ultimately acts via DNA binding (Lo PK et al 2012; Wagner et al 2002). A more controversial line of investigation has addressed the potential role of ER α in the cell membrane (Alexandrov et al 2013), the data though have been challenged (Lawrence et al 2013) and the field has not matured a consensus. On the other hand, a large fraction of ER α molecules constantly shuffle between the cytoplasm and the nucleus where they alternate between a free-floating state and a DNA bound state (Makova & Hardison 2015; Perou et al 2000). More importantly, canonic ER α signaling has been associated with the majority of the breast cancer hallmarks discussed above. For all these reasons, we will focus the discussion on canonical signaling. The full-length ER α contains a ligand binding domain (LBD) and a DNA binding domain (DBD) separated by a hinge domain (Kumar et al 1987).

Once the ligands contact the LBD, conformational changes occur throughout the entire protein and allow for dimerization and DNA binding (Beekman et al 1993; Paige et al 1999). ER α then quickly contacts the DNA at genetically defined DNA sequences called estrogen-responsive elements (EREs) (Klinge et al 2001). More than 70,000 EREs are scattered throughout the human genome in addition to regions that harbor half or degenerate EREs which are also permissive to ESR1 recruitment (Bourdeau et al 2004). This poses the question of how many regions ER α binds throughout the genome, how ER α finds these ERE, what are the molecular determinants of ER α binding, and how many ER α are actually functional. The human genome consists of around 3×10^9 base pairs. Eukaryotes have evolved strategies to compact this vast

array of information in the nucleus via higher-ordered packaging (the chromatin). 147–148 bp of the DNA string wrapped around histone proteins is the minimal repeating unit of the chromatin (the nucleosome) (Luger et al 2012). ER α , similarly to 94% of all DNA binding proteins, has higher affinity for nucleosome-free DNA (Kittler et al 2013; Thurman et al 2012). Thus, chromatin accessibility represents the first barrier to ER α binding. ER α binding is the primary driver of gene expression. Activation of ER α induces the strong transcriptional response that drives breast cancer cell proliferation (Hah & Kraus 2013). ER α orchestrates transcription by binding at critical DNA regions known as regulatory elements (Carrol et al 2005). These regions can be broadly classified as promoters and enhancers based on the relative distance from the gene that is controlled.

The chromatin environment at regulatory regions is defined by several well-characterized epigenetic features (Consortium TEP, Data Analysis Coordination OC, Data Production DPL, Data Analysis LA 2012). For example, active promoters and enhancers are typically nucleosome-free and accessible to transcription factors (Thurman et al 2012). The nucleosomes surrounding regulatory regions carry special chemical modifications on the histone tails depending on their activity status (Earnst & Kellis 2010; Earnst et al 2011). These modifications, known as histone post-translational modifications (HPMTs), have been extensively used to annotate regulatory regions in the genome by several international consortiums (Earnst & Kellis 2010; Roadmap Epigenomics Consortium 2015 & Gerstein et al 2014). Promoters are characterized by histone 3 lysine 4 tri-methylation (H3K4me3), while enhancers are generally enriched for histone 3 lysine 4 mono-methylation (H3K4me1) (Earnst et al 2011). On the other end of the chromatin spectrum, inactive/repressed regulatory regions carry H3K27me3 and H3K9me3 modifications (Di croce et al 2013).

Collectively, chromatin accessibility and histone modifications are a constitutive part of the epigenome. Several integrative studies have now dissected the relationship between ER α and the epigenome. Fine mapping of ER α DNA interaction and integration with epigenetic data were central to remodel a long-standing dogma in ER α biology. For a long time, it was hypothesized that ER α controls transcription by binding to primarily the promoters of target genes. It is now well established that 97% of ER α binding occurs at distal enhancers (Lin et al 2005; Carrol et al

2005 & Ross-Innes 2012). These regions are typically enriched for active epigenetic modifications (H3K4me1, H3K27ac) and devoid of repressive marks (H3K27me3 and H3K27me9).

2.2.1.5 Relationship Between Normal Breast Development and Breast Cancer

The study of normal developmental dynamics can provide important information to understand breast cancer natural history. What is the cell of origin of breast cancer? Multiple cumulative genetic abnormalities are required to transform a normal cell. In the breast, on average one mutation per coding megabase are identified in large-scale sequencing projects (Alexandrov et al 2013; Lawrence et al 2013). As the rate of mutation accumulation at each cell division is low (Markova & Hardison 2015). Cells of cancer origin are likely to have a long proliferation history: MaSCs or progenitors surviving multiple cycles of gland regeneration are the likeliest candidates. But does this also imply that the developmental potential of the cell of origin can shape cancer phenotype? This was strongly suggested by the landmark expression microarray studies of the early 2000s (Perou et al 2000; Sorlie et al 2003), which showed that groups of commonly expressed genes that define normal lineages are also able to define clusters of cancers with common natural history (“intrinsic” subtypes). However transcriptional similarities do not necessarily extend to the cell of origin, implying for instance that a basal-like cancer had to originate from a basal-restricted cell. In fact, more recent studies on BRCA-deficient mice and humans suggest that basal-like cancers derive from luminal-committed progenitors (Lim et al 2009; Molyneux et al 2010).

The emerging consensus is that luminal progenitors are the likely cell of origin for both luminal and basal-like tumors, whereas MaSCs and/or basal progenitors are the initiators of the rarer claudin-low subtype. The origin of HER2⁺ tumors is less well understood; data in mice suggest that parity-induced stem cells might be the culprit as their ablation inhibits tumorigenesis in MMTV-neu mice (Henry et al 2004), but to what extent this model really mimics human HER2⁺ tumors is disputable since the murine expression profile is more akin to human luminal tumors (Henry et al 2004; Skibinski A, Kuperwasser 2015 & Pfefferle et al 2013). Almost completely undetermined is the cell of origin of lobular and rarer breast cancer subtypes. Transcriptome

analysis of “special” subtypes (like mucinous and micropapillary) all clustered in a separate group, revealing transcriptional homogeneity despite morphologic differences, but little about a possible common cell of origin (Weigelt et al 2008). Lobular cancers showed heterogeneous signatures resembling ductal subtypes (including luminal-like and basal-like signatures) (Weigelt et al 2008); transcriptome signatures within lobular cancers have been recently studied in more depth by the TCGA, with the definition of three new subgroups “reactive-like,” “immune-related,” and “proliferative” (Ciriello et al 2015). Again this reveals little about the cell of origin, but the only available genetically engineered mouse model showed that lobular tumors can be obtained from basal cells expressing cytokeratin (Derksen et al 2006).

2.3 OLAPARIB

Olaparib was approved for a number of indications in ovarian and breast cancer as follows:

- Treatment of patients with gBRCAm advanced ovarian cancer who have been treated with 3 or more prior chemotherapies
- Maintenance treatment of patients with recurrent ovarian cancer following platinum-based chemotherapy
- Maintenance treatment of patients with gBRCAm advanced ovarian cancer following first-line platinum-based chemotherapy
- Treatment of patients with gBRCAm human epidermal growth factor receptor 2 (HER2)-negative metastatic breast cancer (Oncologic Drugs Advisory Committee - ODAC, 2019).

2.3.1 Olaparib Pre-Clinical Study

Pre-clinical data supported evaluation of olaparib in gBRCAm pancreatic adenocarcinoma BRCAm cells which have lost both alleles lack functional BRCA1 or BRCA2 and therefore are unable to effectively undergo HRR. Normal cells in a person with a germline BRCAm retain one functional copy of BRCA and are not HRR deficient. DNA damage in the form of SSBs is common in cancer cells. PARP inhibitors trap PARP at the site of SSBs and prevent their repair, resulting in accumulation of DSBs. DSBs cannot be repaired accurately in cells which are HRR-

deficient; an alternate error prone repair pathway is used, leading to accumulation of DNA damage and selective cancer cell death. The sensitivity to PARP inhibition is dependent on BRCA mutation and is agnostic to tumor type in pre-clinical models. Pancreatic cell lines, including but not limited to a BRCA2 mutant pancreatic cell line (CAPAN-1), have been shown to be sensitive to olaparib in vitro. Further correlations between response to olaparib and cisplatin treatment have also been demonstrated in vivo, in a pancreatic patient-derived xenograft model of BRCAm (Golan et al 2018).

The correlation between response to olaparib and response to platinum-based chemotherapy is explained by the mode of action of each agent; both agents lead to DSBs in DNA, which cannot be effectively repaired in gBRCAm cells with loss of both BRCA alleles. However, chemotherapy works very differently by directly inducing DNA lesions in the form of intraand inter-strand crosslinks, irrespectively of the genetic background of the cells treated; whereas PARP inhibitors (PARPi) induce DNA damage indirectly, and the damage accumulates selectively in replicating cells with homologous recombination deficiency (HRD) (ie, BRCA deleterious mutations). As a consequence, platinum treatments led to cytotoxic effects that are only ~10-20-fold more selective for BRCAm versus BRCAwt cells; in contrast, olaparib is able to selectively kill BRCAm cells, with ~1000-fold sensitivity versus the BRCAwt or BRCA+/- heterozygote cells. This wider therapeutic window in vitro underpins the improved safety profile of olaparib compared with platinum chemotherapy (Hucl et al 2008; Tacconi et al 2019).

2.3.2 Clinical Pharmacology of Olaparib

The pharmacokinetics (PK) of olaparib tablets have been well characterized in patients with advanced solid tumors, including pancreatic cancer patients (Oncologic Drugs Advisory Committee - ODAC, 2019). Patient age, race, gender, body weight or tumor type were not found to be predictors of olaparib plasma exposure and therefore dose adjustment on the basis of individual patient demographics is not required (Oncologic Drugs Advisory Committee - ODAC, 2019).

2.3.3 Mechanism of Action of Olaparib

The anticancer activity of the PARP inhibitor olaparib works through a process called synthetic lethality (Oncologic Drugs Advisory Committee - ODAC, 2019). Olaparib inhibits and traps PARP at the sites of DNA single-strand damage, both preventing repair and during replication, leading to the generation of the more serious DNA DSBs. In HRR proficient cells, these DSBs are repaired accurately and efficiently, but in cancer cells with HRD, such as those with BRCA1 or BRCA2 mutations with loss of the second functional allele, repair is dependent on the inaccurate end-joining pathway that results in genome instability and preferential killing of cancer cells over normal cells (Oncologic Drugs Advisory Committee - ODAC, 2019).

2.3.4 Olaparib Clinical Dose Selection: 300 mg bid Tablet Formulation

The selection of the 300 mg bid tablet dose was based on the benefit risk profile established for this dose level in patients with advanced solid tumors (the majority had ovarian cancer) in Study. In this study, the 300 mg bid tablet dose was determined to be the recommended Phase III monotherapy dose, providing similar (based on non-inferiority test) efficacy to the 400 mg bid capsule dose while exhibiting a safety and tolerability profile suitable for continuous long-term dosing. In addition, the tablet formulation was found to have better oral bioavailability than the capsule formulation (Oncologic Drugs Advisory Committee - ODAC, 2019).

2.3.5 Lynparza® (Olaparib) for the Maintenance Treatment of Patients with Germline BRCA-Mutated Metastatic Adenocarcinoma of the Pancreas

A pivotal Phase III study investigated the efficacy of olaparib compared to placebo as maintenance therapy in gBRCAm metastatic pancreatic cancer patients whose disease did not progress on first-line platinum-based chemotherapy and for whom the standard practice is to stop the chemotherapy (eg, those patients who can no longer tolerate platinum-based chemotherapy or have completed the specified 6 months of chemotherapy). There was no upper limit to the duration of chemotherapy that a patient had received. The primary endpoint was PFS by BICR, with ORR, TFST, PFS2, OS and HRQoL as key secondary endpoints. – In POLO, the primary

efficacy objective was met, with the risk of disease progression or death (PFS) assessed by BICR being reduced by 47% for patients on olaparib vs placebo (HR=0.53; 95% CI 0.35 to 0.82; p=0.0038) and prolonged median PFS by 3.6 months with a median PFS of 7.4 months for olaparib vs 3.8 months for placebo (Oncologic Drugs Advisory Committee - ODAC, 2019).

In the 84% of patients with measurable disease at baseline, ORR by BICR was higher for patients taking olaparib vs placebo (18/78 [23.1%] vs 6/52 [11.5%]; odds ratio 2.30), including 2 patients (11.1%) on olaparib who had a CR compared with none on placebo. – The median DoR by BICR was much longer for patients taking olaparib (24.9 months; 95% CI 14.75, NC) vs placebo (3.7 months; 95% CI 2.10, NC) (Oncologic Drugs Advisory Committee - ODAC, 2019).

2.4 ANACARDIC ACID (AnAc)

Anacardic acid (AnAc) is a dietary agent used for the prevention and treatment of breast cancer. It inhibits the proliferation of estrogen receptor α (ER α) positive MCF-7 and MDA-MB-231 triple negative breast cancer cells (Schultz et al; 2006). To characterize potential regulators of AnAc action, MCF-7 and MDA-MB-231 cells were treated for 6 h with purified AnAc 24:1n5 congener followed by next generation transcriptomic sequencing (RNA-seq) and network analysis. It was observed that AnAc-differentially regulated miRNA transcriptomes in each cell line (Schultz et al; 2006).

In MCF-7 cells, 80 AnAc-responsive genes were identified, including lncRNA *MIR22HG*. More AnAc-responsive genes (886) were identified in MDA-MB-231 cells. Only six genes were commonly altered by AnAc in both cell lines: *SCD*, *INSIG1*, and *TGM2* were decreased and *PDK4*, *GPR176* and *ZBT20* were increased (Schultz et al; 2006). Modeling of AnAc-induced gene changes suggests that AnAc inhibits monounsaturated fatty acid biosynthesis in both cell lines and increases endoplasmic reticulum stress in MDA-MB-231 cells (Schultz et al; 2006). Since modeling of downregulated genes implicated NF κ B in MCF-7, it was therefore confirmed that AnAc inhibited TNF α -induced NF κ B reporter activity in MCF-7 cells. These data identify new targets and pathways that may account for AnAc's anti-proliferative and pro-apoptotic activity (Schultz et al; 2006).

2.5 TRANSCRIPTOMIC CONCEPT

A transcriptomic response of metformin and aspirin have been studied extensively as cancer preventative and therapeutic agents. However, the underlying molecular mechanisms for the inhibitory effects of pancreatic cancer development remain undefined (Society, A. C. Cancer Facts & Figures 2014. Atlanta: American Cancer Society (2014)). To gain further insight into their biological function in pancreatic cancer, a transcriptomic analysis using RNA sequencing to assess the differential gene expression induced by metformin (5mM) and aspirin (2mM) was carried out alone and in combination, after treatment of PANC-1 cells for 48 hours by Yue and his team (Yue et al 2014).

Compared to an untreated control, metformin down-regulated 58 genes and up-regulated 91 genes, aspirin down-regulated 12 genes only, while metformin plus aspirin down-regulated 656 genes and up-regulated 449 genes (fold-change>2, $P<10^{-5}$), of the top 10 genes (fold-change>10, P regulated by metformin plus aspirin, PCDH18, CCL2, RASL11A, FAM111B and BMP5 were down-regulated ≥ 20 -fold, while NGFR, NPTX1, C7orf57, MRPL23AS1 and UNC5B were up-regulated ≥ 10 -fold (Yue et al 2014). Ingenuity Pathway Analysis (IPA) revealed that the pathways, “cholesterol biosynthesis”, “cell cycle: G1/S checkpoint regulation”, and “axonal guidance signaling” were the most statistically significant pathways modulated by metformin plus aspirin (Yue et al 2014). Although the results need further functional validation, these data provide the first evidence for the synergistic action between metformin and aspirin in modulating the transcriptional profile of pancreatic cancer cells (Yue et al 2014).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1.0 Pancreatic Cancer Cell Culture Preparation

Human pancreatic cancer cell line PANC-1 will be purchased from the American Type Culture Collection (Manassas, VA, USA). PANC-1 cells will be maintained in Dulbecco's Modified Eagle's Media (DMEM) (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, St Louis, MO) at 37 °C and 5% CO₂. PANC-1 cells will be seeded in 6-well plates (5 × 10⁵ cells per well). 24 hours later, cells will be untreated or treated by 5mM anachardic acid, 2mM olaparib or a combination of anachardic acid (5mM) and olaparib (2mM) for 48 hours.

3.1.1 Pancreatic Cancer Cell's mRNA Library Preparation and Sequencing

A total RNA will be isolated from untreated and treated PANC-1 cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) followed by clean up with RNeasy Mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Quantity and quality of DNase treated RNA will be determined using Agilent 2100 Bioanalyzer (RNA Nano 6000) and Nanodrop, respectively. A total of 16 RNA samples will be sequenced [4 samples per condition × 4 conditions (untreated control, anachardic acid, olaparib or a combination of anachardic acid and olaparib)] using 500ng of total RNA as input. Library preparation and RNA sequencing will be carried out using the Illumina TruSeq™ RNA Sample Prep Kit v2 according to the manufacturer's protocol (Illumina, San Diego, CA, USA). Samples will be sequenced on the Illumina HiSeq 2500, 2 × 100bp paired-end reads, to a minimum depth of 30 million reads per sample.

3.1.2 Computational Analyses of Pancreatic Cancer RNA-seq Data

A total of 240 million obtained reads of high quality clean tags will be mapped and annotated via human reference genome using Bioconductor package biomaRt

(<http://www.bioconductor.org>) (Durinck et al 2009). Mapped reads with mapping quality 10 or more will be defined as uniquely mapped reads and used in the downstream analyses. Averagely, about 70.0% reads will be uniquely mapped to the annotated human genome. Ranking of genes by degree of differential expression will be performed using the Bioconductor edgeR package version 2.14 (<http://www.bioconductor.org>) and an in-house code developed in the R statistical language (<http://www.r-project.org>). Significant genes will be arbitrarily identified by using the cutoff values of $P \leq 1 \times 10^{-5}$ and fold change (FC) in mean expression of $FC \geq |2|$. (www.nature.com/scientificreports/ Scientific Reports | 5:13390 | DOI: 10.1038/srep13390 9).

3.1.3 Biological Network and Pathway Analysis

Biological networks and pathways related to anachardic acid, olaparib and the combination will be analyzed with Ingenuity Pathway Analysis (IPA) software (Qiagen, CA, USA). The lists of all genes identified in gene expression analysis will be uploaded into the IPA software. For the analysis of networks and pathways, the cutoff values will be set at $P \leq 1 \times 10^{-5}$ and $FC \geq |2|$ respectively.

3.1.4 Validation of RNA-seq Results by qRT-PCR

Quantitative RT-PCR will be used to validate the differentially expressed genes. Expression of mRNA will be determined in all 4 samples using Power SYBR® Green RNA-to-CT™ 1-Step Kit (Life Technologies, CA, USA) according to the manufacturer's protocol. All of the primers to be used in qRT-PCR will be purchased from Qiagen (Limburg, Netherland), and reference numbers used will be noted appropriately.

3.1.5 Western Blotting

The Western blotting for the selected proteins will be performed, as described by Yue (Yue et al 2015). Briefly, the treated and untreated cells will be rinsed with PBS and extracted on ice with cell lysis buffer (Cell signaling, Beverly, MA, USA). The protein concentrations will be determined with BCA Protein Assay Kit (Thermo scientific, Waltham, MA, USA). 20µg of total

proteins from each sample will be loaded and separated on a gradient 4–20% polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membrane. Membranes will be blocked with 5% fat-free milk in Tris-buffered saline-Tween 20 and incubated with the primary antibodies against RET and CCL2 from Cell Signaling Technology (Beverly, MA, USA), and antibody against ANGPTL4 from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Blots will be subsequently washed and incubated with the appropriate HRP-conjugated secondary antibodies. The immunoreactive bands will be visualized by enhanced chemiluminescence (Thermo Fisher, Rockford, IL USA) according to the manufacturer's instructions. The levels of β -actin shall be estimated to check for equal samples loading.

3.2.0 Breast Cancer Cell Culture Preparation and Treatment

AnAc 24:1n5 will be purified to greater than 95%. AnAc 24:1n5 shall be dissolved in ethanol (EtOH); thus, EtOH shall be used as a vehicle control. MCF-7 and MDA-MB-231 cells shall be purchased from the American Type Tissue Collection (ATCC, Manassas, VA) and will be used within 9 passages from ATCC. MCF-7 and MDA-MB-231 cells shall be grown prior to a 6 h treatment with established IC_{50} concentrations of AnAc 24:1n5: 13.5 μ M for MCF-7 and 35.0 μ M for MDA-MB-231 cells.

3.2.1 Breast Cancer Cell's mRNA RNA-seq Preparation

RNA shall be isolated from three separate experiments for each cell line. The Truseq Stranded mRNA kit (Illumina) will be used to prepare mRNA libraries from 1 μ g total RNA. Libraries will be confirmed on the Agilent 2100 Bioanalyzer and quantitated using the Illumina Library Quantification Kit, ABI Prism qPCR Mix from Kapa Biosystems and the ABI7900HT real-time PCR instrument. 76 cycle single read sequencing shall be performed with the 500 High-output v2 (75cycle) sequencing kit on the Illumina NextSeq 500 instrument. The sequence reads shall be mapped to the human reference genome, version GRCh37.1 (hg19) using the mapping algorithm tophat (Trapnell et al 2012) version 2.0.2. The expression levels shall be quantified at loci specified by the annotation found within the ENSEMBL release 73 gene description file (Homo_sapiens.GRCh37.73.gtf) using cufflinks version 2.2.1. Contributions to the annotation

file from both ribosomal RNA (rRNA) and mitochondrial RNA (mtRNA) shall be removed from the gtf file prior to use. Differential analyses between the specified conditions will be performed using cuffdiff version 2.2.1. The raw data shall be uploaded in the Gene Expression Omnibus (GEO) database as GSE78011.

3.2.2 Differential Gene Expression Analysis

RNA-seq reads will be assembled according to the hg19.gtf annotation file (downloaded from ENSEMBL) (Flicek et al 2014) using Cufflinks (version 2.2.1) (Trapnell et al 2012). For each comparison, both cufflinks assemblies shall be merged, and the resulting merged gtf file serves as the transcript input for differential gene expression analysis in Gene Ontology and KEGG pathways. For three of the comparisons, a p-value cutoff ≤ 0.05 shall be used to determine differential expression. For the MCF-7 vs. MDA-MB-231 comparison, differential genes will be determined using a q-value of ≤ 0.01 and a $|FC| \geq 2$. Differentially expressed genes (DEGs) for each comparison shall be used for further analysis of enriched Gene Ontology Biological Processes (GO:BP) (Haris et al 2004) and KEGG Pathways (Kanehisa et al 2000) using categoryCompare (Flight et al 2014). The Entrez gene ID for each DEG shall be obtained from the human Entrez IDs database downloaded from NCBI. Tables for the enriched GO:BP and KEGG pathways shall be generated as text files from four lists of DEGs with a unique Entrez gene ID.

3.2.3 *In silico* MetaCore Network Analysis

Pathway and network analysis of differentially expressed genes shall be performed in MetaCore version 6.27 (GeneGO, Thomson Reuters, New York, N.Y.) (Bolser et al 2012).

3.2.4 Luciferase assay

To analyze NF κ B activity, MCF-7 cells shall be transiently transfected with pGL4.32[luc2P/NF- κ B-RE/Hygro] (Promega, Madison, WI) containing five copies of a NF κ B response element and pGL4-hRluc-TK (Renilla, Promega) for 48 h and treated with 10 ng/ml TNF α and 0–40 μ M AnAc 24:1n5 for 6 h before performing dual luciferase assay (Promega). Firefly luciferase will be normalized by *Renilla* luciferase. Values shall be the average of three separate wells in one

experiment $\pm$ SEM. RNA isolation, RT-PCR and quantitative real-time PCR (qPCR) will be performed essentially as described previously in MCF-7 and MDA-MB-231 cells treated with vehicle control (EtOH) or AnAc 24:1n5 (13.5 and 35 μ M, respectively) for 6 h (Schultz et al 2017). PCR Primers will be synthesized by Integrated DNA Technologies (Coralville, IA) and sequences used will be listed in Supplementary Table (Campbell et al 2015). *GAPDH* will be used as a reference for normalization (Muluhngwi et al 2017). qPCR shall be performed in triplicate using ABI Viia 7 (LifeTechnologies). Fold change relative to vehicle-treated, control cells shall be estimated by the comparative threshold cycle (Ct) method ($2^{-\Delta\Delta CT}$) (Schmittgen & Livak 2008).

3.1.6 Statistical Analysis

The results of RT-PCR shall be normalized to expression of GAPDH using the formula $2^{\Delta CT}$. One-way ANOVA shall be used for comparing treatment with the combination of anachardic acid and olaparib to the untreated control. A P value less than 0.05 will be considered statistically significant.

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