Signature page

Finding Indicators to Predict How Breast Cancer Will Respond to Decitabine Treatment Based on the Drug's Mode-of-action

By
Brianne Marie Cruickshank

A Thesis Submitted to
Saint Mary's University, Halifax, Nova Scotia
in Partial Fulfillment of the Requirements for
the Degree of Honours Biology.

Halifax, Nova Scotia

April, 2017

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Abstract

The Canadian Cancer Society estimates that 13 Canadian women will die from breast cancer every day. Epigenetic modifications, like aberrant DNA methylation contribute to breast cancer progression and must be addressed to improve patient outcomes. DNA hypermethylation can inhibit the expression of tumor suppressor genes (TSGs), which contributes to the development and progression of cancer. Using a de-methylating agent such as decitabine (5-aza-2'-deoxycytidine), results in the re-expression or induction of TSGs. Although this effect has been well documented in cancer, it may not be the main contributor to decitabine sensitivity. Other aspects of decitabine treatment, such as the induction of an interferon response have also been suggested as contributors to decitabine sensitivity. Using a representative panel of breast cancer cell lines with varying sensitivities to decitabine, these possible effects of decitabine will be evaluated to reveal their value in predicting decitabine response. Using quantitative polymerase chain reaction (qPCR), expression of genes associated with TSG induction and the interferon response were analyzed to reveal the predominate class of genes that are induced upon treatment. It was found that neither class of gene was indicative of decitabine sensitivity. Alternative factors that might predict decitabine sensitivity were evaluated; these factors all have well-established roles in decitabine's mode-of-action. Decitabine must be imported, processed and incorporated into the DNA. It was found that incorporation into the DNA is also not predictive of decitabine sensitivity. Next, genes associated with import/export, processing and de-methylating effects of decitabine were evaluated for any association with decitabine sensitivity. Relatively strong correlations with the import gene *SLC28A1*, the processing gene *DCK* as well as the *DNMT1A* and *DNMT3B* de-methylating genes were found. This suggests that these four genes may be important mediators of decitabine sensitivity in breast cancer, and could be useful in predicting patient response to this new therapy.

April 21st, 2017

List of Abbreviations

AA (Antibiotic/Antimycotc)
AML (Acute Myeloid Leukemia)
ATCC (American Tissue Culture Collection)
cDNA (Complementary DNA)
Ct (Cycle Threshold)
DAC (Decitabine)
DMEM (Dulbecco's Modified Eagle Medium)
DMSO (Dimethyl Sulfoxide)
DNMT (DNA Methyl Transferase)
EDTA (Ethylene Diamine Triacetic Acid)
EOC (Epithelial Ovarian Cancer)
ERV (Endogenous Retroviruses)
ER (Estrogen Receptor)
F12 (Ham's Nutrient Mixture**)**
FBS (Fetal Bovine Serum)
GBP (Guanylate Binding Protein)
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HER2 (Human Epidermal Growth Factor Receptor-2)
IC50 (Half Maximal Inhibitory Concentration)
NCBI (National Center for Biotechnology Information)
PBS (Phosphate Buffer Solution)
PR (Progesterone Receptor)
RT-qPCR (Real-Time Quantitative Polymerase Chain Reaction)
SYBR (SYBR Green Supermix)
TSG (Tumor Suppressor Gene)
RCC (Renal Cell Cancer)

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INTRODUCTION

1.1 The Role of Epigenetics in Cancer

Cancer is a disease characterized by the uncontrolled growth and division of cells. In normal tissues, cellular mechanisms prevent unlimited cell division by carefully regulating different phases of the cell cycle. If an irreparable problem (e.g. DNA damage, nutrient deprivation, hypoxia, viral infection) is identified within the cell, normal cells will initiate regulated cell death (apoptosis) or will be identified by the immune system for destruction. Cancer cells evade these processes and continue through the cell cycle, producing too many cells, and often making errors in DNA replication and chromosome segregation. It is the accumulation of mutations and segregation errors in cells which result in the development of cancer-like characteristics such as unlimited replicative potential and the ability to evade apoptosis[1]. As cancer progresses, mutations accumulate and lead to further aberrant regulation of genes controlling cell communication, DNA repair, cellular growth, and apoptosis. These affects further contribute to the development and progression of the tumour[1].

While cancer is often associated with mutations, epigenetic modifications (which occur "on top" of the DNA) also contribute to the development of cancer[2]. One such epigenetic modifier is DNA methylation, which occurs when a methyl group is added to a cytosine residue to form 5-methylcytosine. In normal cells, DNA methylation is vital in controlling gene expression and methylation patterns can change depending on the needs of the cell[3]. For example, methylation can be used to repress the expression of repetitive DNA sequences and provides stability to the DNA by decreasing the probability of

genomic rearrangement[4]. DNA methylation also influences the expression of genes coding for important proteins. Since all the cells in the body share the same genetic material, genes must be turned "on" or "off" depending on development of different cell types, or changes in physiological state. Genes important in the development of myocardial tissue should therefore not be expressed by liver cells; this is reflected in the vastly different methylation patterns between tissue types[5]. Controlling gene expression via silencing specific genes and contributing to genome stability are the essential roles of DNA methylation; both of which are dysregulated in cancer cells[6,7].

In cancer, both hypo- and hypermethylation can play a role in driving tumor growth and progression[8]. If repetitive DNA elements and non-coding regions (which are typically methylated to aid in genomic stability) become hypomethylated, chromosomal mutations such as rearrangements, may occur and lead to the formation of cancer[6]. Cells use DNA methylation to maintain stable expression patterns of protein coding genes. In tumours, aberrant hypermethylation can silence the expression of tumor suppressor genes (TSGs), which normally down-regulate cell division. Since TSGs are important in preventing the development of cancer, repression of these genes via hypermethylation-mediated silencing contributes to cancer development [2,4]. TSGs are involved in growth-inhibiting pathways and act as "gatekeepers" to control progression through the cell cycle. They are directly implicated in growth, differentiation, and survival. When TSG expression is diminished (by DNA methylation for example), the gene products that govern the cells response to growth signals are lost and uncontrollable cell division is more easily achieved. Silencing TSGs via DNA hypermethylation has been observed in

many solid tumors[9-11]. Within the last decade, hypermethylation has been accepted as an important player in the processes of cancer initiation and progression[8-12].

Initial studies of DNA methylation in cancer were limited to testing known TSGs for hypermethylation[13]. One example is *CCNDN2*, a TSG that is often hypermethylated in some renal cell cancers (RCC)[13]. The protein product of *CCNDN2* is myopodin, an actin-bundling protein which is important in controlling and regulating the cell cycle. In kidney cancer, aberrant methylation of the promotor region of *CCNDN2* is correlated with significantly lower mRNA expression of this gene and consequently its gene product, myopodin, when compared to normal tissue[13]. In kidney tumors, myopodin levels can predict tumor progression and growth; lower levels of myopodin are associated with more aggressive RCCs[14]. In patients who received chemotherapy, hypermethylation of this gene resulted in an increased probability of recurrence as well as decreased overall survival rates[13]. *CCNDN2* in kidney cancer is therefore a prime example of a hypermethylated tumor suppressor gene which is important in driving tumor growth as well as treatment response. There are many similar examples of tumor suppressor genes silencing in other cancers by hypermethylation, which leads to more aggressive disease progression and resistance to therapy.

DNA methylation is a plastic modification and inhibitors of the DNA methylation machinery are being actively investigated. For example, DNA methyltransferases (DNMTs) are required for DNA methylation and inhibitors of DNMTs are promising cancer therapies given that global effects can be avoided and the tumor itself can be targeted. These DNMT inhibitors reduce DNA methylation levels and therefore resurrect the expression of TSGs[6]. Decitabine (5-aza-2'-deoxycytidine) is a DNMT inhibitor that is

used successfully as a therapy in myelodysplastic syndrome (MDS)[15]. MDS is a blood disorder, often considered a pre-cancerous stage of leukemia, and the effectiveness of decitabine as an MDS-therapeutic is now attributed to decitabine's ability to de-methylate genes in these pre-cancerous cells[15]. These genes have not yet been identified. Given the success of DNMT inhibitors like decitabine in MDS, the efficacy of these treatments is being explored as therapies for solid tumors. To this end, decitabine is currently being investigated in clinical trials as a cancer treatment for solid tumors found in tissues such as the kidney, lung, skin, bladder and breast[4] (Figure 1).

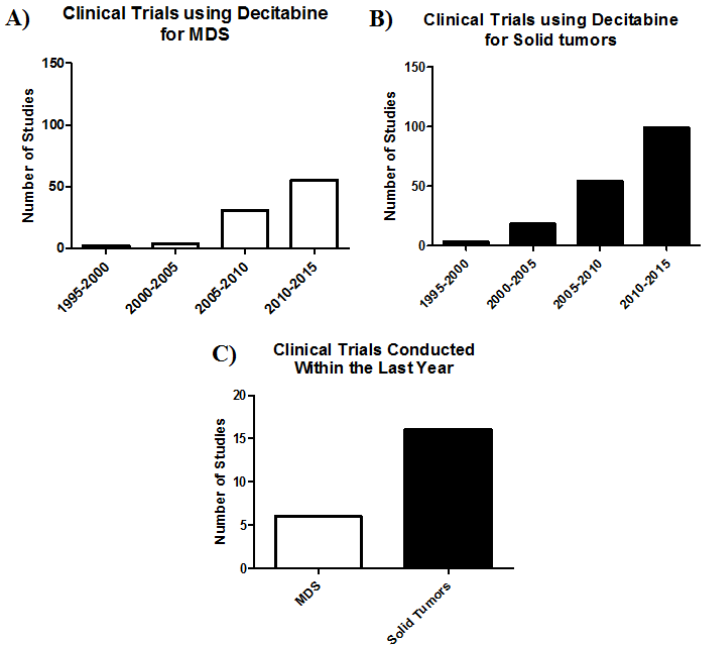


Figure 1. As of the year 2000, clinical trials using decitabine have been increasing for both myelodysplastic syndrome (MDS) and solid tumors. (A): Clinical trials for MDS using decitabine peaked between 2010-2015 (total number of studies=97) (B): Compared to MDS, more clinical trials have been based off solid tumors (totally number of studies=174) (C): Decitabine has been implemented as a successful treatment for MDS therefore less focus is placed on MDS and more is being placed on solid tumors (total number of studies=22, data compiled from North American clinical trials database, clinicaltrials.gov).

1.2 Potential Anti-Cancer Mechanisms of Decitabine

Decitabine was first developed as a cytotoxic agent to target rapidly dividing cancer cells. As a cytosine analog, decitabine induces cell death through DNA damage after it incorporates during the DNA synthesis phase of the cell cycle[16]. To act, decitabine must first be imported into the cell where it is processed into a triphosphorylated nucleotide. It must then be incorporated into the DNA upon DNA synthesis. DNMTs then bind decitabine creating a protein/DNA adduct which leads to degradation of DNMTs. This has several potential effects including de-methylation and re-expression of aberrantly silenced TSGs and the de-methylation of endogenous retroviral elements resulting in dsRNA/anti-viral responses. The induction of the DNA damage response by protein/DNA adduct formation, cytotoxicity induced by global de-methylation, and the de-methylation of silenced tumor-associated antigens increasing anti-tumor immune responses have also been reported as "downstream" effects of decitabine [17-20]. Before any of these are possible, decitabine must 1) be imported into the cell, 2) be phosphorylated into its active form and 3) be incorporated into the DNA (Figure 2).

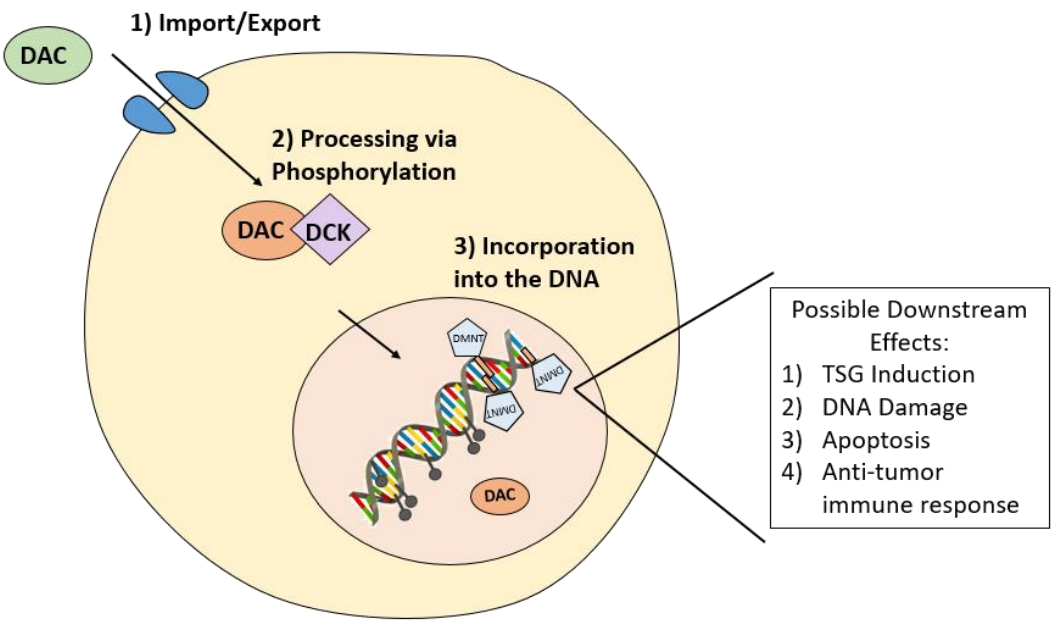


Figure 2. For decitabine (DAC) to exert an effect it first must be imported into the cell, phosphorylated, and incorporated into the DNA. This may lead to several downstream effects such as TSG induction, DNA damage, apoptosis and the anti-tumor immune response. (Bases: yellow = cytosine, blue= guanine, green =adenine, red = thymine).

Even though many TSGs have been found to be hypermethylated, we lack a complete understanding of their role and significance in relation to the development of new cancer treatments[21-23]. Current research is focused on genes with known tumor-suppressor function and evaluating whether they are methylated and if de-methylating agents can induce their expression[24-26]. Re-expression of TSGs is a desirable goal in cancer therapy and many studies that show TSG re-expression is correlated with a decrease in the growth of a tumor[27-30]. For example, Garzon et al. 2009 show that lowering levels of DNMTs led to a decrease in global methylation and a re-expression of TSG $p15^{INK4b}$ [16] resulting in the restoration of some regulatory mechanisms. Although the re-expression of TSGs is a well-known effect of de-methylating agents, the key TSGs involved in this effect and their contribution to sensitivity to de-methylating agents have yet to be identified[27].

In addition to induction of TSGs, other anti cancer mechanisms of decitabine have been reported. One of which is called the interferon response which suggests that DNMT inhibitors trigger immune responses through viral mimicry pathways[18]. Endogenous retro-viral (ERV) sequences are elements derived from retroviruses that have been inserted into the human genome, and these elements have been silenced via DNA methylation during human evolution. DNMT inhibitors like decitabine activate expression of hypermethylated ERV sequences leading to the production of viral RNA. The cell interprets these events as a viral attack, which then triggers the interferon response[18]. The interferon response results in immune recognition of cancer cells leading to the subsequent elimination of the cancer cells by programmed cell death.

1.3 Current Clinical Limitations on DNA De-methylating Therapy

Methylation is important in driving tumor growth and progression and de-methylating agents should be highly effective as cancer therapeutics[7,13,31]. Despite all evidence pointing to their potential efficacy, clinical trials have had limited success. This could be a result of a poor understanding of the mechanisms present in the cancer cells that make them resistant or sensitive to de-methylating agents like decitabine. It is unclear if mechanisms required for decitabine processing (e.g. DCK an enzyme needed for phosphorylation of decitabine) or the downstream effectors of decitabine-response (ex. tumor suppressor gene induction, DNA damage response, apoptosis and viral mimicry) are most critical in determining response of the cancer cell to decitabine[32,33]. The development of novel techniques allowing for the determination of de-methylating agents' mode-of-action in cancer cells hold promise in improving the clinical application of this class of drugs. I propose to use breast cancer cell lines as a model system to identify key determinants of response to DNA de-methylating therapy in breast cancer.

Breast cancer lends itself to the study of DNA de-methylating agents because DNA methylation is highly dysregulated in this disease[12]. One example as shown by Ottaviano et al. (1994) found that estrogen receptor (ER) expression is downregulated because of hypermethylation in cancerous breast tissues[7]. The estrogen receptor is a common target for hormone therapy in breast cancer. Without estrogen receptor expression, patients are unresponsive to hormone-based chemotherapeutics[7].

1.4 Breast Cancer

Breast cancer typically begins in the ducts of the breast which carry milk from the lobules to the nipple but can also develop in the lobules themselves. Ductal carcinomas

account for approximately 83% of all breast cancers whereas lobular carcinomas account for only 13%[34]. Some breast cancers are more aggressive than others. Triple-negative breast cancers are defined as lacking the three hormone receptors which drive the growth of most breast cancers. Specifically, triple-negative breast cancers do not overexpress HER2 receptors (plasma membrane tyrosine kinase receptors), and lack estrogen and progesterone receptors. These breast tumors are also associated with a less favourable prognosis since they cannot be treated with receptor-targeted therapy and show limited responses to generic cytotoxic chemotherapy[35,36]. Since breast tumors clearly are not identical, treatment must be tailored to each individual case.

Understanding decitabine's mode-of-action may allow us to develop more precise treatments for patients with triple-negative breast cancer. Further, using expression of certain genes as predictors of drug response may allow for more efficient use of decitabine. A literature search suggests that expression of decitabine-associated genes can predict decitabine sensitivity[37]. Specifically, the expression levels of genes implicated in import/export of the drug, processing of the drug, and de-methylation may be potential biomarkers for a favourable response to decitabine. As an example, insufficient decitabine import proteins would render patients resistant to the drug. This phenomenon is observed in acute- myeloid leukemia (AML), where levels of equilibrative transporter (ENT-1), which mediates the uptake of nucleosides, correlate with sensitivity to decitabine in mononuclear cells taken from 50 patients with AML[27].

I hypothesize that determining the factors which dictate response to decitabine will reveal potential biomarkers that will predict the effectiveness of decitabine as a cancer treatment. For this purpose, I have identified a panel of seven

breast cancer cell lines with a wide range of sensitivity to decitabine (Figure 3). These cell lines include the triple-negative breast cancer cell lines MDA-MB-231, MDA-MB-468, SUM159, SUM149 and Hs578t cells, the estrogen and progesterone receptor positive cell line MC7F cells and the HER2 overexpressing cell line SKBR3. Therefore, the cell lines used in this paper represent a diverse panel with a strong emphasis on triple-negative breast cancers which we and others prioritize as needing new therapy options. I propose the following three aims to test my hypothesis using this panel of seven cell lines:

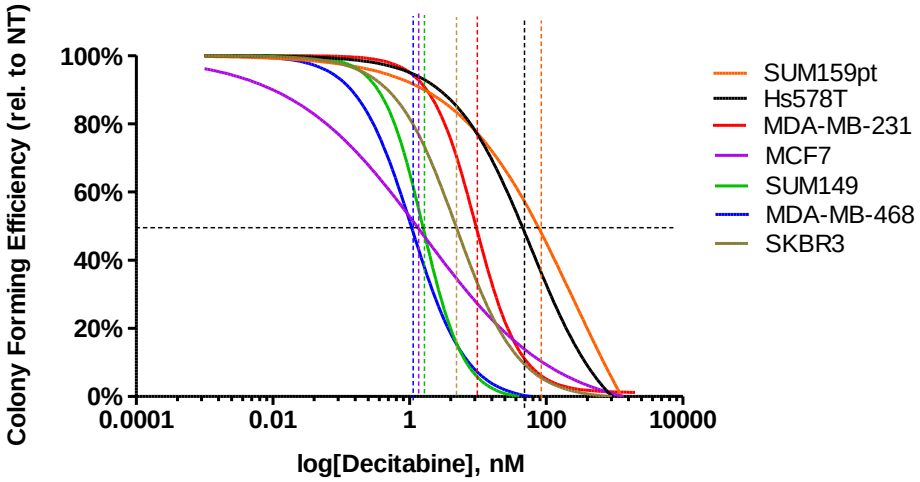


Figure 3. Breast cancer cells have varying sensitivity to decitabine. Cells were treated with increasing amounts of decitabine to determine the concentration of drug (nM) needed to kill 50% of the cells. (Thomas et al. unpublished data)

Aim 1: Identify whether the genes induced by decitabine treatment in various tumour cell lines correlate with their sensitivity to decitabine. I will compare expression levels of known decitabine response genes in the panel of seven breast cancer cell lines. The panel of 8 genes that will be assessed include, hypermethylated tumor suppressor genes and interferon response genes. Using real-time quantitative polymerase chain reaction (RT-qPCR), gene expression levels will be compared in no treatment cells and cells treated with increasing amounts of the decitabine. The analysis of all seven cell lines cells will reveal the predominate class(es) of genes induced by decitabine at different doses and if sensitive versus resistant cell lines have differing responses.

I hypothesize that induction of TSGs resurrects cell division regulation mechanisms within the cancer cells—the implication of this is that TSG induction should be observed in cell lines that are sensitive to decitabine.

It was found that genes associated with the interferon response were upregulated in ovarian and colorectal cancer cell lines when treated with decitabine[18]. It is possible that this phenomenon exists in breast cancer cell lines, where decitabine-sensitive breast cancer cell lines induce interferon response genes. Therefore, I also hypothesize that decitabine-sensitive breast cancer cell lines may have upregulated interferon response genes after decitabine treatment.

Aim 2: Investigate if replication rate determines breast cancer sensitivity to decitabine. Since decitabine incorporation is dependent upon DNA synthesis, a determining factor in sensitivity may be the cell division rate of the breast cancers. Cell lines that grow quickly should be incorporating decitabine at a higher rate than slowly

dividing cell lines. This means that the faster dividing breast cancer cell lines should be more sensitive to decitabine than the slower dividing breast cancer cell lines. Therefore, I will investigate the division of the seven cell lines, over four days, without decitabine treatment. This will reveal if cell division rate is correlated with decitabine sensitivity. I hypothesize that the faster growing cell lines will be more sensitive to decitabine than the slower growing cell lines.

Aim 3: Identify potential biomarkers to predict patient response. Expression levels of 16 genes implicated in decitabine import/export, processing and DNA de-methylation will be examined. Non-treated cells will be collected and RNA isolated. qPCR will determine expression levels of these genes in seven different cells lines with varying sensitivity to decitabine. From analyses of the genes in untreated cells, I may be able to determine if high or low expression of uptake, export, drug processing and de-methylation are important predictors of breast cancer sensitivity to decitabine. I hypothesize that cell lines with high expression of these genes will be more sensitive to decitabine treatment than cell lines with low expression of these genes.

In conclusion, my aims are to identify factors which dictate decitabine sensitivity in breast cancer and to evaluate these factors as decitabine biomarkers. This should improve the application of this drug in breast cancer treatment and promote the development of a precision medicine approach when administering de-methylating therapies. Given the ongoing decitabine clinical trials in solid tumors and breast cancer, these findings will be very timely.

MATERIALS AND METHODS

2.1 Cell Culture Conditions

MDA-MB-468, MDA-MB-231, MCF7, SKBR3, SUM159, SUM149 and Hs578t cells were purchased from ATCC Cell Lines; these breast cancer cell lines were isolated by research groups (e.g., MD Anderson Cancer Center) from patients with breast cancer and licensed to ATCC for sale. These 7 cell lines were chosen for two main reasons. First, the status of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (Her2) differs among cell lines. Five of the seven cell lines are from "triple negative" breast cancers, while the MC7F is an ER and PR positive cell line and SKBR3 is a cell line with overexpression of HER2 (Table 1). Second, the selection of cell lines was also based on their colony-forming capacity—since colony-forming ability was the metric by which decitabine sensitivity was measured (unpublished data; Thomas et al.), only cell lines with efficient colony formation were used in this study (Table 1).

Table 1. General description of seven breast cancer cell lines. Seven different types of breast cancer cell lines, their hormone receptor status (eostrogen receptor (ER), progesterone recepetor (PR) and human epidermal growth factor receptor-2 (Her2)) and clinical data (patient age and race)[38,39].

Cell Line	Hormone Receptor Status			Clinical Data
	ER	PR	Her2	
Hs 578t	-	-	-	74 years, Caucasian female
MDA-MB-231	-	-	-	51 years, Caucasian female
SUM159PT	-	-	-	Not Available
MDA-MB-468	-	-	-	51 years, Black female
SUM149PT	-	-	-	Not Available
SKBR3	-	-	+	43 years, Caucasian female
MCF7	+	+	-	69 years, Caucasian female

The MDA-MB-468, MDA-MB-231, MCF7 and SKBR3 cells were cultured using complete media which consisted of Dulbecco's Modified Eagle Media (DMEM; 2mM L-glutamine, 4.5g/L D-glucose, and 25mM HEPES buffer; Gibco) with the addition of 10% fetal bovine serum (FBS; Invitrogen) and 1X antibiotic/antimycotic (AA; Gibco). Hs578t cells were cultured using DMEM with the addition of 10% FBS, 1X AA and 0.01mg/mL bovine insulin (Invitrogen). SUM159 and SUM149 cells were cultured using Ham's F12 Nutrient Mixture (F12; Gibco), 1X HEPES buffer (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; Gibco), 1 µg/mL hydrocortisone (Invitrogen) and 5 µg/mL human insulin (Invitrogen). Cells were incubated with 5% CO_2 at 37°C and subcultured/passaged using 0.05% (MDA-MB-468, MDA-MB-231, SKBR3, MCF7) or 0.25% (Hs578t, SUM159, SUM149) EDTA-trypsin (Invitrogen) (Table 2). All cell lines were cultured as suggested by the ATCC guidelines[40].

Table 2. Cell culture conditions for the panel of seven breast cancer cell lines used in this study[39-41].

Cell Line	Base Medium	Additives	Conditions	Passaging
MDA-MB-468	DMEM	10% FBS, 1X AA	5% CO_2	0.05% EDTA-trypsin
MDA-MB-231	DMEM	10% FBS, 1X AA	5% CO_2	0.05% EDTA-trypsin
MCF7	DMEM	10% FBS, 1X AA	5% CO_2	0.05% EDTA-trypsin
SKBR3	DMEM	10% FBS, 1X AA	5% CO_2	0.05% EDTA-trypsin
Hs578t	DMEM	10% FBS, 1X AA, 0.01 mg/mL bovine insulin	5% CO_2	0.25% EDTA-trypsin
SUM159	F12	10% FBS, 1X AA, 1X HEPES buffer, 1 µg/mL hydrocortisone 5 µg/mL human insulin	5% CO^2	0.25% EDTA-trypsin
SUM149	F12	10% FBS, 1X AA, 1X HEPES buffer, 1 µg/mL hydrocortisone 5 µg/mL human insulin	5% CO^2	0.25% EDTA-trypsin

Cells were thawed from previously frozen stocks, cryopreserved in appropriate media with 5% dimethyl sulfoxide (DMSO) and then kept in liquid nitrogen (Sigma). Thawed samples were revived on 10cm cell culture-treated plates (Corning) with approximately 12mL of the appropriate media; 24hrs post-thawing, the media was refreshed. Cells were passaged once the plate reached 80% confluency; depending on growth rate of the specific cell line, passaging occurred every 3-6 days. To ensure adequate growth conditions were maintained, media was refreshed every third day if the plate was not yet ready to be passaged. To complete the experiments outlined below, cell lines were cultured for a maximum of two months (or 10-20 passages). After two months, cells were discarded and a new frozen stock was thawed.

2.2 Quantifying Growth Rate of Breast Cancer Cells

Day 1: From an 80% confluent 10cm cell culture plate, the monolayer of cells was rinsed twice with phosphate buffer saline solution (PBS, pH=7.4; Invitrogen) and dissociated using the corresponding concentration of trypsin- EDTA (Table 2). Cells were collected and a 10µL representative sample was quantified using 0.4% Trypan Blue Solution and a Bright-Line Hemacytometer (Gibco) which facilitates the counting of cells with the use of grids. The number of cells per milliliter was then calculated using Equation 1:

Equation 1. Calculation of cells/mL from a representative sample.

$$\frac{Cells}{mL} = \frac{Cells\ Counted * 10{,}000 * Dilution\ Factor}{Number\ of\ hemacytometer\ grids\ counted}$$

The appropriate number of cells were then placed on a cell-cultured treated 6-well plate (Corning) with triplicate technical replicates and 2 mL of the corresponding, fresh media was added (Table 1,3).

Table 3. Cells seeded per well for seven breast cancer cell lines. Cells are seeded at different concentrations since growth rates vary between cells lines; similar confluence was reached after three days of growth.

Cell Line	Cells Seeded per Well on Day 1
MDA-MB-468	50, 000
MDA-MB-231	50, 000
MCF7	100, 000
SKBR3	100, 000
Hs578t	200,000
SUM159	25, 000
SUM149	100, 000

Day 4: After three days of growth, cells were washed, trypsinized, and counted using the methods outlined for Day 1. Using the number of cells seeded and the number of cells counted after three days, a natural growth rate can be calculated using Equation 2:

Equation 2. Calculation of division rate.

$$\text{Division Rate} = \frac{\ln\left(\frac{Cells\ Counted\ at\ Day\ 4}{Cells\ Seeded\ at\ Day\ 0}\right)}{ln2}$$

To determine the number of cell divisions per day, division rate was divided by the number of days the cells grew (4 days).

2.3 Determining Expression of Decitabine-Associated Genes

Two possible "downstream effects" of decitabine, tumor suppressor gene (TSG) induction and the interferon response were evaluated as potential indictors of decitabine sensitivity in a panel of seven breast cancer cell lines. There has been evidence to show that 1μM decitabine (the dose recommended by most of the decitabine cancer literature) is toxic and results in the death of cells that may have induced potential sensitivity genes[36]. If this is the case, then measuring gene expression changes in the surviving cells will generate deceptive data where gene expression levels are not changing as a result of decitabine's de-methylating effect. To eliminate this possible error in interpretation of gene expression data, a sub-cytotoxic dose (unpublished data; Thomas et al.) of decitabine (0.01μM) was included in both analyses.

Each breast cancer cell line was seeded in a 6-well plate (with the cells seeded per well value given above in Table 3), allowed to adhere for 24 hours and then either treated with fresh media, 0.01 μM or 1μM decitabine (DAC; Sigma) for 72 hours, refreshing the

media daily. The media was then suctioned off and cells were detached/re-suspended in 1mL of TRIzol (Invitrogen) per sample.

RNA was extracted using the PureLink RNA MiniKit (Life Technologies) as per the manufacturer's guidelines. RNA was quantified by measuring absorbance at 260nm with a SpectraMax Microplate Reader technologies using SoftMax Pro software. Complementary DNA (cDNA) was made by reverse transcribing 0.25μg of RNA using iScript RT Supermix (BioRad) as per manufacturer's guidelines.

Using gene specific primers (Table 3), cDNA was amplified using SYBR Green Supermix (BioRad) in a CFX96 RT-qPCR thermocycler (BioRad). Primer specificity was ensured through PrimerBLAST analysis (NCBI) where primers associated exclusively with the genes of interest were designed (Supplemental Methods 1). In addition, only primers with melt curves indicating a single amplified product were used; it is therefore unlikely that this product is anything other than the gene interest. The efficiency of each primer was determined via standard curve analysis (Supplemental Methods 2).

Real-time qPCR (RT-qPCR) measures the PCR product after each round of amplification via a fluorescent dye (SYBR green) that attaches itself directly or indirectly to the accumulating DNA molecules. During each cycle of the reaction, fluorescence values are recorded. These values are directly proportional to DNA concentration. The threshold values are the points at which the fluorescence is first detected above a set threshold fluorescence and is proportional to the amount of starting cDNA of the gene of interest. Cycle threshold (Ct) values were collected and analyzed using the CFX Manager Software (BioRad). Two qPCR analyses were performed: 1) expression of decitabine-inducible genes (Table 4; relative to no treatment control for each individual cell line)

and 2) expression of decitabine processing, transport and de-methylation genes (Table 4; comparing no treatment samples between the seven cell lines).

To measure expression of decitabine-inducible genes, qPCR procedures as outlined above were used. Expression was normalized by using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and *B2M* (Beta-2-microglobulin) as reference genes that were chosen because the expression of these two genes does not change with decitabine treatment[2]. Prior to statistical analysis, the normalized expression of the decitabine-inducible genes was standardized relative to the expression of these genes in the untreated sample of each cell line.

Expression of decitabine processing, transport and de-methylation genes was also normalized to *GAPDH* and *B2M* as reference genes.

Table 4. qPCR primer sequences of genes analyzed. qPCR primer sequences for decitabine processing, transport, de-methylation genes, hypermethylated tumor suppressor genes, and interferon response genes.

Association	Gene Name	Primer Sequence	
		Forward	Reverse
Import/Export	**Solute carrier family 29 member 1** (*SLC29A1*)	ATGACAACCAGTCACCAGCC	GTTCCCAGACCCAGCATGAA
	Solute carrier family 28 member 1 (*SLC28A1*)	AAGGGTGTTTGGAAAGGAGGT	CCCAGATGATGTGCCGAAGA
	Multi-drug Resistance 1 (*MDR1*)	GAGAGATCCTCACCAAGCGG	ATCATTGGCGAGCCTGGTAG
Decitabine processing	**Deoxycytidine kinase** (*DCK*)	AGAAGCTGCCCGTCTTTCTC	GCAGCGATGTTCCCTTCGAT
	Cytidine/uridine monophosphate kinase 1 (*CMPK1*)	TCTCCTCTGCTCTCCACGTC	GCAGAAAGGTGTGTGTAGCC
	Nucleotide diphosphate kinase A (*NME1*)	ATCGTCTTTCAAGGCGAGGG	CCCCATCTGGTTTGATCGCA
	Nucleotide diphosphate kinase B (*NME2*)	GACCGACCATTCTTCCCTGG	TTGGTCTCCCCAAGCATCAC
	Cytidine aminase (*CDA*)	ATCGCCAGTGACATGCAAGA	GTACCATCCGGCTTGGTCAT
De-Methylation	**Proliferating cell nuclear antigen** (*PCNA*)	AGGTGTTGGAGGCACTCAAG	CCAAAGAGACGTGGGACGAG
	E3 ubiquitin-protein ligase (*UHRF1*)	GACAAGCAGCTCATGTCGATG	AGTACCACCTCGCTGGCATCAT
	tet methylcytosine dioxygenase 1 (*TET1*)	CCCTCCTCTCCACCTAACCA	TACCAGGCAATGTTGGCAGT
	tet methylcytosine dioxygenase 2 (*TET2*)	TTGGATACACCTGTCAAGACTCAAT	ACGCCATGTGTCTCAGTACATT
	tet methylcytosine dioxygenase 3 (*TET3*)	CCTCGGAGTTGGGACTCACT	GGACCTGCCAGGCCTTTATG
	DNA methyltransferase 1 alpha (*DNMT1A*)	CGGCCTCGTCATAACTCTCC	TGAACCGCTTCACAGAGGAC
	DNA methyltransferase 3 beta (*DNMT3B*)	TGTGGGGAAAGATCAAGGGC	ATGCCAGACATAGCCTGTCG

	TNF receptor associated factor 6 (*TRAF6*)	GCGCACTAGAACGAGCAAG	GCCACACAGCAGTCACTTTC
Tumor Suppressor Gene Induction	**Ras association domain family member 1** (*RASSF1A*)	ACAAGGGCACGTGAAGTCAT	AAAGAGTGCAAACTTGCGGG
	runt related transcription factor 3 (*RUNX3*)	CTTTGGGGACCTGGAACGG	TTCCGAGGTGCCTTGGATTG
	BRCA1, DNA repair associated (*BRCA1*)	GGAAGAAACCACCAAGGTCCA	GACACCCTGTGGGCATGTT
	cadherin 1 (*CDH1*)	GGAGAGCGGTGGTCAAAGAG	AGTCCTGGTCCTCTTCTCCG
Interferon Response Genes	**interferon regulatory factor 7** (*IRF7*)	GTGGACTGAGGGCTTGTAG	TCAACACCTGTGACTTCATGT
	2'-5'-oligoadenylate synthetase like (*OASL*)	GCAGAAATTTCCAGGACCAC	CCCATCACGGTCACCATTG
	ISG15 ubiquitin-like modifier (*ISG15*)	GCCTCAGCTCTGACACC	CGAACTCATCTTTGCCAGTACA
	retionic acid- inducible gene 1 (*RIG1*)	CCAGCATTACTAGTCAGAAGGAA	CACAGTGCAATCTTGTCATCC

2.4 Statistics

All graphs were made using GraphPad Prism 5 Software. Growth rate (as calculated above) significance was determined via one way ANOVA tests and individual relationships analyzed via a Tukey's multiple comparison test. Changes in gene expression between cell lines and treatments are analyzed via one-way ANOVA and multiple comparisons assessed via Dunnett's post-hoc test. Stars indicate the strength of the relationship ($p<0.05$*, $p<0.01$**, $p<0.001$***).

RESULTS

3.1 Tumor Suppressor Gene and Interferon Response Gene Induction

It was hypothesized that induction of TSGs resurrects cell division regulation mechanisms within the cancer cells—the implication of this is that TSG induction should be observed in cell lines that are sensitive to decitabine. Therefore, I predicted that expression of TSGs in decitabine-sensitive cell lines (ex. MDA-MB-468) may be upregulated upon decitabine treatment. Four TSGs that were identified by comparing methylation status between normal and cancerous breast tissue were selected to be studied.

None of the four tumor suppressor genes (TSGs); *RASSF1*, *RUNX3*, *BRCA1* and *CDH1* were induced upon 0.01μM or 1μM decitabine treatment in the most sensitive cell line (Figure 4, $F_{2,4}$=4.598, p=0.82). Surprisingly, 1μM decitabine treatment induced two TSGs (*RUNX3* and *CDH1*) in the most resistant cell line, SUM159 (Figure 4B, D, $F_{2,4}$=19.96, p=0.01). *RUNX3* normally encodes a transcription factor that can activate or supress transcription which is often silenced in cancer. *CHD1* encodes an important calcium-dependant- cadherin protein[42].Using a one-way ANOVA and a Dunnett's Multiple Comparisons Test it was determined that no other TSGs were upregulated in any of the other six cell lines upon 1μM or 0.01μM decitabine treatment (Figure 4).

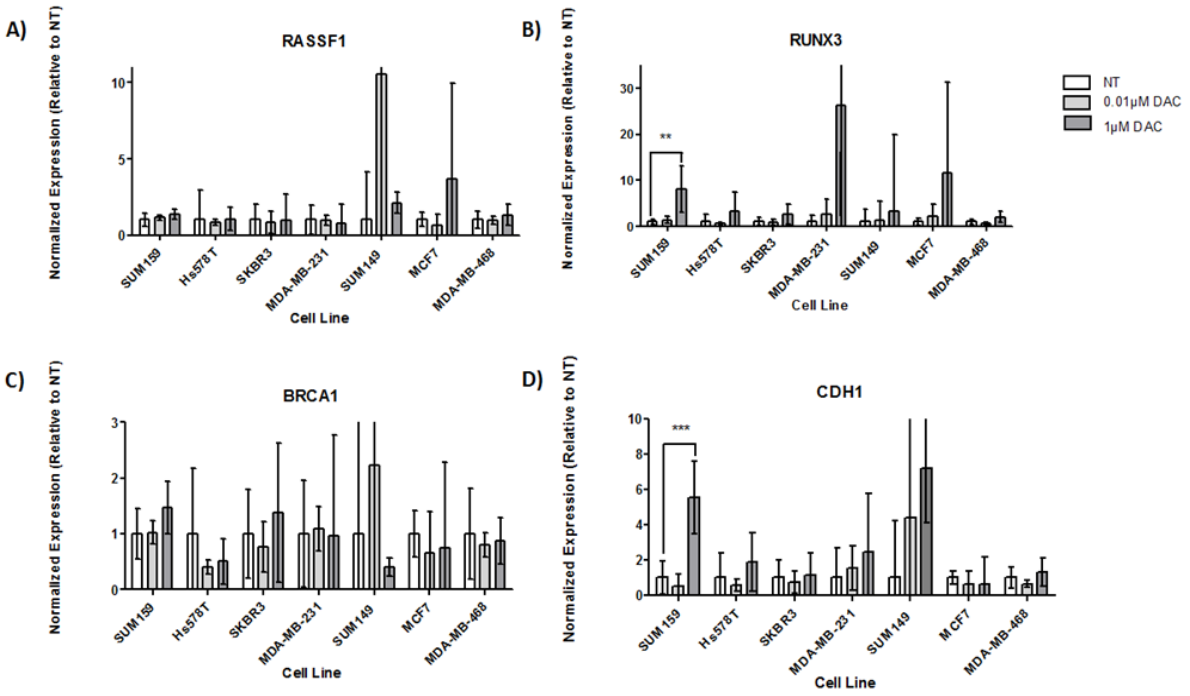


Figure 4. The induction of four known TSGs by decitabine (DAC) in seven breast cancer cell lines in order from left to right (most sensitive to most resistant). Significance determined via one-tailed ANOVA and individual relationships determined via Dunnett's Multiple Comparisons Test. Error bars represent 95% confidence intervals (CI), n=6, **p<0.001, ***p<0.0001.

Chiappinelli et al. (2015) reported that cancer cells undergo apoptosis when treated with demethylating agents via the interferon response which triggers the immune system[18]. They found that genes associated with the interferon response were upregulated in ovarian and colorectal cancer cell lines when treated with decitabine. It is possible that this phenomenon exists in breast cancer cell lines, where decitabine-sensitive breast cancer cell lines induce interferon response genes.

Each gene selected for study has been implicated differently in the interferon response. *IRF7* encodes for an interferon regulatory transcription factor which controls the transcriptional activation of virus-associated genes. *ISG15* encodes a ubiquitin-like protein that targets intracellular proteins. *RIGI* encodes a RNA helicase that can alter the structure of the RNA itself. Lastly, *OASL* is a protein coding gene that is need for proper immune signalling[42]. I predicted that these genes would increase in expression upon decitabine treatment in the most sensitive cell line. Instead, all four interferon response genes (*IRF7, ISG15, RIGI, OASL*) were induced in the most resistant cell line, SUM159 (Figure 5, $F_{2,4}=39.54$, $p<0.0001$) after 1μM decitabine treatment.

Through Dunnett's Multiple Comparisons Test it was found that two cell lines that are intermediately resistant (SKBR3) and sensitive (MCF7) induced one interferon response gene each upon addition of 1μM decitabine. An increase in *IRF7* expression was observed in the MCF7 cell line whereas an increase in the expression of *RIGI* was observed in the SKBR3 cell line upon 1μM treatment (Figure 5A/D.). No other cell lines showed increases in any interferon response genes after either 0.01μM or 1μM decitabine treatment. No significant differences were detected in any breast cancer cell lines after 0.01μM decitabine.

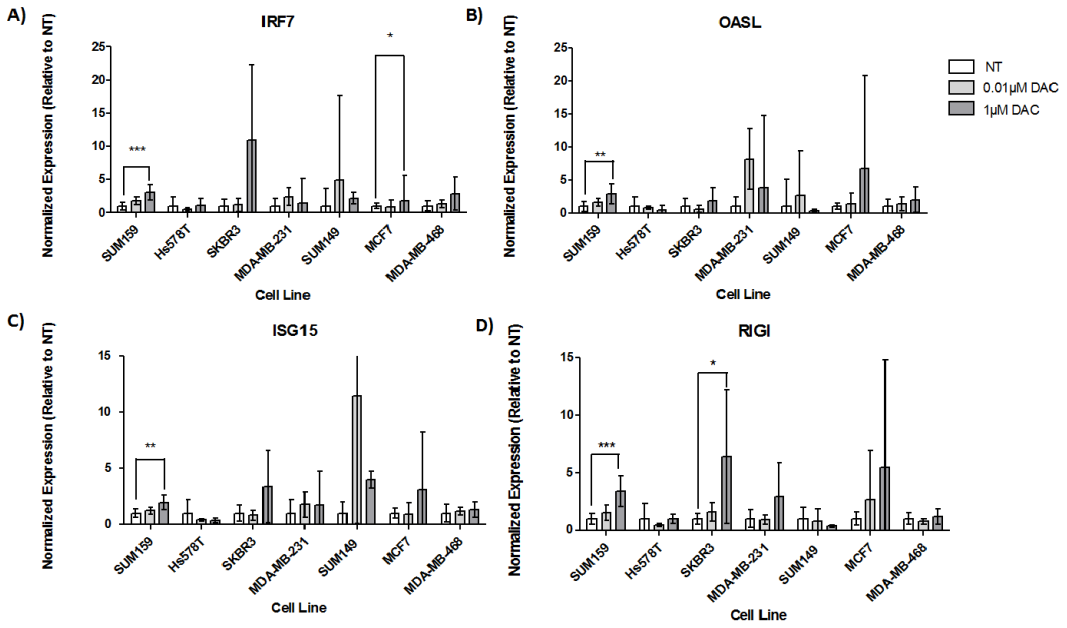


Figure 5. The induction of four known interferon response related genes by decitabine (DAC) in seven breast cancer cell lines in order from left to right (most sensitive to most resistant). Significance determined via one-tailed ANOVA and individual relationships determined via Dunnett's Multiple Comparisons Test. Error bars represent 95% confidence intervals (CI), n=6, *p<0.05 **p<0.001, ***p<0.0001.

3.2 Growth Rates of Breast Cancer Cell Lines

For decitabine to be effective, it must be incorporated into the DNA as a cytosine analog. This process is dependent on DNA synthesis which occurs before cellular division. Therefore, faster growing cell lines should be incorporating decitabine at a higher rate than slowing dividing cell lines. It was found that growth rates differed between the seven breast cancer cell lines through a one-tailed, one-way ANOVA (Figure 6, $F_{7,3}=36.41$, $p<0.0001$). The fastest growing cell line, SUM159 has a growth rate of about 0.94 replications per day whereas the slowest growing cell line has a growth rate of approximately 0.12 replications per day (Figure 6A). The fastest dividing cell lines (SUM159, MDA-MB-468) represent the most sensitive (MDA-MB-468) and the most resistant (SUM159) breast cancer cell lines in the panel studied. The growth rates of the cell lines do not correlate with the previously observed sensitivities (Figure 6B, $r= -0.25$, $p=<0.0001$).

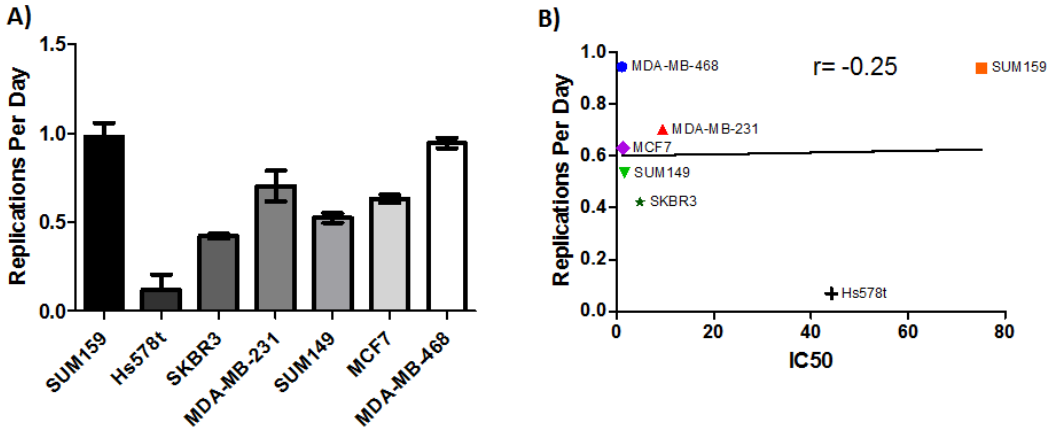


Figure 6. Growth rates of seven breast cancer cell lines and their correlations with the concentration of decitabine needed to kill 50% of the cells (IC50). A) Replication rate (replication/day) day 4 NT; Error bars represent 95% CI, n=4, $p<0.0001$. B) Correlation analysis of non-linear regression, $r^2= 0.00093$.

3.3 Induction of Genes Associated with Decitabine's "Upstream" Mediators

To begin to use decitabine effectively, it is essential to develop "biomarkers" that allow a clinician to predict if a patient would benefit from treatment. To test the predictive value of decitabine's "upstream" mediators, expression levels of genes associated with 1) import/export, 2) processing, and 3) de-methylation were analyzed without decitabine treatment.

It was found that *SLC29A1* and *MDR1* genes which are associated with the import and export of decitabine were not able to accurately predict sensitivity of the seven breast cancer cell lines used in this study (Figure 7). Spearman's correlational analysis revealed non-significant, weak correlations between the *SLC29A1* and *MDR1* genes (Figure 7B/C, r= -0.03, p= 0.9, r= -0.14, p=0.78). Interestingly, the *SLC28A1* gene has a stronger correlation but is not significant (Figure 7A, r= -0.77, p=0.18). It should be noted that the most resistant breast cancer cell line, SUM159 had such low levels of *SLC29A1* that is was not detectable.

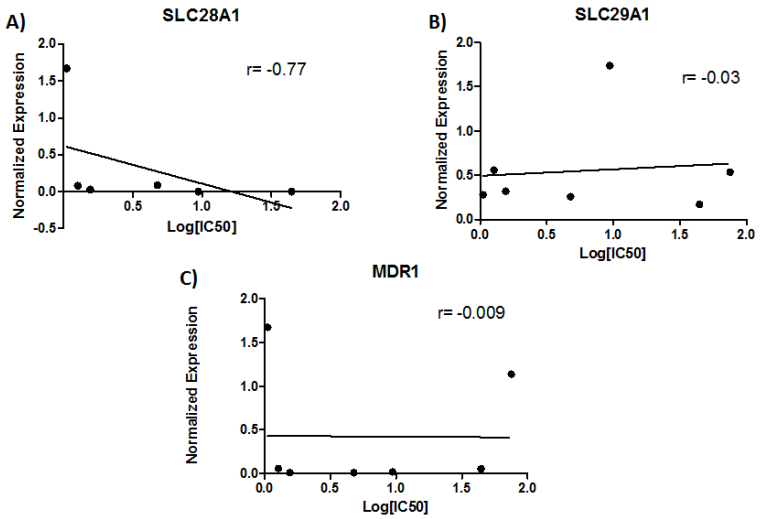


Figure 7. Expression of genes implicated in decitabine import/export correlated with breast cancer cell line sensitivity. IC50 represents the concentration of drug needed to kill 50% of cells; log transformations of these values are used as a measure of sensitivity. Spearman's correlational analysis was used to determine significance of non-normally distributed data, n=6. A) r^2= 0.23, B) r^2= 0.01, C) r^2=9.2x10^{-5}.

Genes associated with the processing of decitabine yielded similar results (Figure 8). The *DCK, CMPK1* and *NME1* genes have relatively large Spearman's correlations, but none of them are significant (Figure 8A, r= 0.71, p= 0.08; 8B, r=0.57, p= 0.2; 8D, r= 0.42, p=0.35). The *DCK* gene produces the strongest, negative correlation in relation to all the other processing-related genes (Figure 8A). The other two processing genes which are *CDA* and *NME2* produce weak correlations and are not significant (Figure 8C, r= 0.21, p= 0.66; 8E, r=-0.21, p = 0.65).

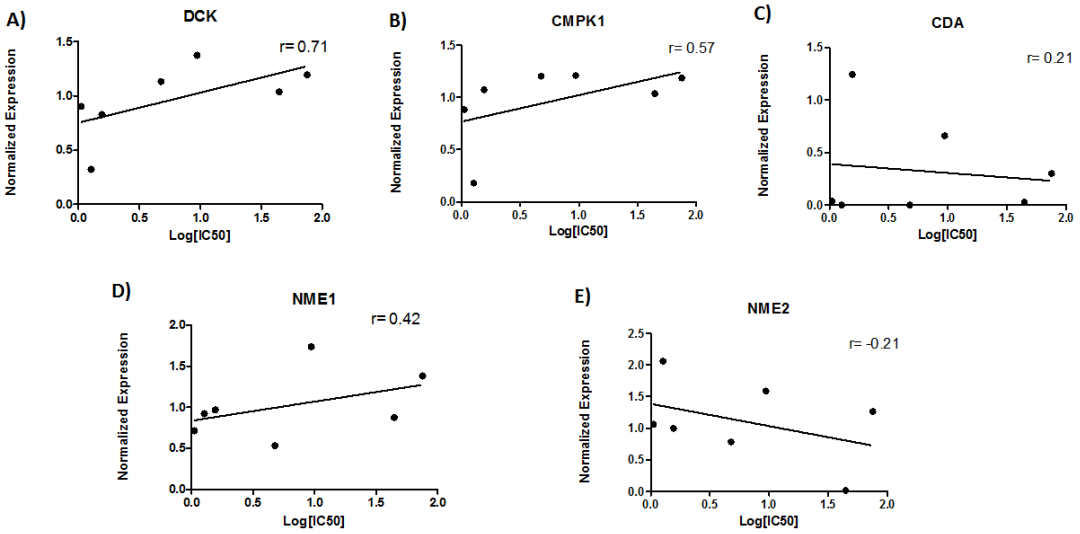


Figure 8. Expression of genes related to decitabine processing correlated with seven breast cancer cell lines. IC50 represents the concentration of drug needed to kill 50% of cells; log transformations of these values are used as a measure of sensitivity. Spearman's correlational analysis was used to determine significance of non-normally distributed data, n=6. A) r^2= 0.37, B) r^2= 0.26, C) r^2=0.01, D) r^2=0.18, E) r^2=0.16.

Eight genes associated with de-methylation were assessed and correlated with decitabine sensitivity of seven breast cancer cell lines. It was found that *TET3, DNMT1A, DNMT3B* and *TRAF6* had the strongest correlations but none of them were significant (Figure 9E, r= -0.42, p= 0.9; 9F/G, r= -0.5, p=0.26; 9H, r= -0.39, p= 0.39). The other four genes; *PCNA, UHRF1, TET1* and *TET2* all had very weak correlations that also were not significant (Figure 9A/B, r= -0.14, p= 0.78; 9D, r= -0.07, p= 0.90; 9C, r= 0, p= 0.75).

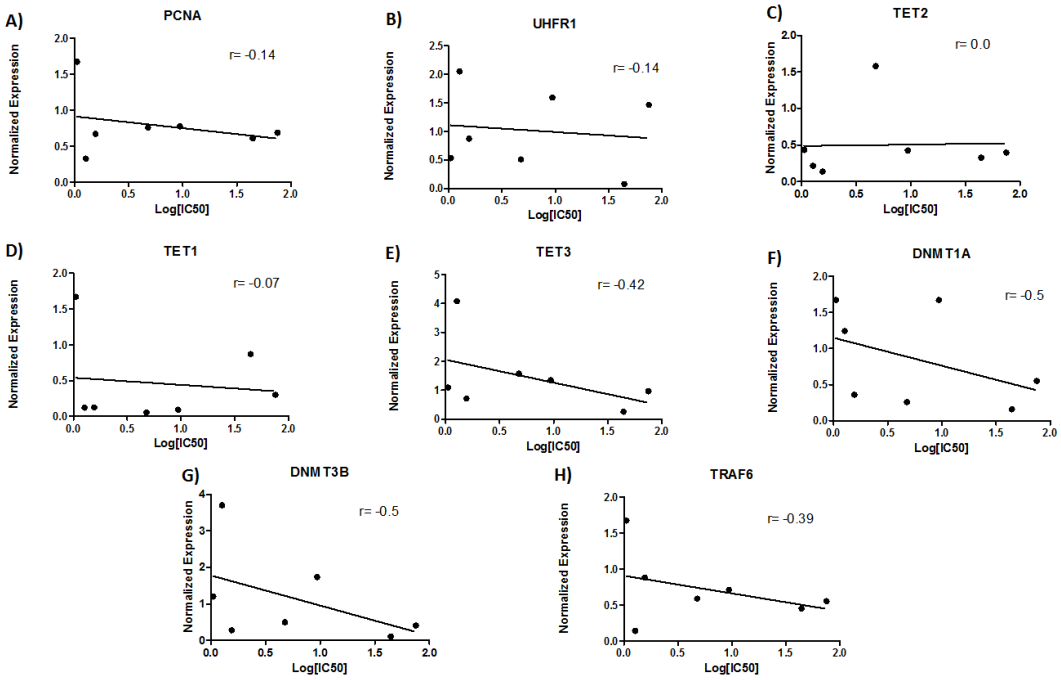


Figure 9. Expression of genes related to decitabine de-methylation correlated with seven breast cancer cell lines. IC50 represents the concentration of drug needed to kill 50% of cells; log transformations of these values are used as a measure of sensitivity. Spearman's correlational analysis was used to determine significance of non-normally distributed data, n=6. A) r^2= 0.08, B) r^2= 0.01, C) r^2=0.0008, D) r^2=0.02, E) r^2=0.23, F) r^2=0.19, G) r^2=0.23, H) r^2= 0.1467

DISCUSSION

4.1 Tumor Suppressor Genes and Decitabine Sensitivity in Breast Cancer

Loss of tumor suppressor gene expression is associated with the development of breast cancer[41]. Hypermethylation in the promotor region of tumor suppressor genes (TSGs) has been established as an important event for gene inactivation in breast cancer—and almost every tumor type[43].These types of methylation events occur genome-wide and identifying which ones are important for tumorigenesis has been a difficult task for researchers[43]. Initial studies identifying hypermethylated tumor suppressor genes compared normal tissue to cancerous tissue to reveal differentially methylated cancer-associated regions. The conclusion was that genes which are hypermethylated in cancer tissue but not in normal tissue, must be important in cancer development and progression and were putatively identified as TSGs. Four commonly accepted hypermethylated TSGs (*RASSF1, CDH1, BRCA1* and *RUNX3*) were identified using this method and were used as representative members of TSG in breast cancer in the current study.

With the development of de-methylating agents for cancer therapy, it was assumed that re-expression of these hypermethylated TSGs was a key indicator that the new de-methylating drugs were effective. It was a popular hypothesis that the responsiveness of patients resulted from the re-expression of these hypermethylated TSGs[44]. To evaluate TSG induction and its role in determining sensitivity, researchers began to look at established, functional TSGs and whether they were methylated[45]. However, the hypothesis that re-expression of these known TSGs contributed to de-methylating agent response was never tested. In fact, initial clinical trials with these drugs found that tumors that did respond, did not increase expression of these well-known

TSGs. So, while there is conjecture in the literature that induction of TSGs contributes to decitabine sensitivity, this has not yet been shown in a solid tumor model.

If TSG induction does indicate sensitivity, the most sensitive breast cancer cell lines should induce known TSGs upon decitabine treatment. Contrary to our hypothesis, the most resistant cell line, SUM159 induced two TSGs (*RUNX3*, *CDH1*), while none of the TSGs were induced in any other cell line (Figure 4). Like other studies, it was found that induction of TSGs (e.g. *p15*) was not indicative of sensitivity to decitabine[46]. There are three main reasons that explain this finding; 1) evaluating individual genes is not robust enough to explain overall TSG induction, or 2) the TSGs evaluated are not the main contributors to de-methylating therapy response or, 3) changes to the epigenomic machinery itself have a greater impact on decitabine sensitivity than TSGs.

It is possible that the induction of multiple TSGs is needed to influence sensitivity, and that examining individual genes is not robust enough to capture the interplay of overall TSG induction. The de-methylating effects of decitabine cannot be limited to specific genes of interest; its treatment can affect any hypermethylated gene in fast proliferating cells[47]. The present study analyzed the change in expression of four TSGs; the cumulative effect of multiple TSGs (which may have been changed upon decitabine treatment) was not assessed. Therefore, the induction of these four, individual TSGs was perhaps not sufficient to indicate a response to decitabine, and additional TSGs should be studied.

Using a gene expression profile composed of many TSGs, we may be able to accurately predict sensitivity to decitabine. However, this strategy will not work if the selected TSGs are not the key mediators in de-methylating agent responsiveness. The

four used in this study (*RASSF1*, *RUNX3*, *BRCA1*, *CDH1*) — which were identified by comparing DNA methylation of normal versus cancerous tissue — may not be the main hypermethylated TSGs necessary for breast cancer development. It is therefore necessary that future studies should include alternative TSGs that were not identified in this way. In addition, the use of more TSG This will aid in the process to find the most influential TSGs which dictate decitabine response.

Perhaps TSG induction is not the main contributor that influences decitabine response, but instead the changes to the epigenomic machinery itself are the key epigenomic events. With thousands of epigenetic events occurring during cancer development, it is difficult to understand the exact mechanisms by which a cancer cell develops epigenetic abnormalities, and which of these are most important[48]. Initial alterations to the epigenomic machinery create an accumulation of aberrant changes which occurs earlier on in cancer growth[48,49]. It could therefore be reversal of these early unknown epigenetic events which would more clearly indicate a response to decitabine.

4.2 Interferon Genes and Decitabine Sensitivity in Breast Cancer

Interferons are proteins that are released in response to virus entry into the cell[18]. In breast cancer, the presence of interferon inducible genes such as guanylate binding protein (*GBP*) have been associated with better prognosis for fast proliferating tumors as it seems to be a marker for an efficient T-cell response[51]. Immunotherapy, or enhancing the immune system's anti-cancer response is an increasingly popular anti-cancer strategy[52]. Pharmacological methods for improving the immune system's anti-cancer response are highly sought after, and decitabine has been suggested as such an agent.

Endogenous retroviral elements (ERVs) are viral elements in the human genome that closely resemble retrovirsues[18]. ERVs are usually hypermethylated in human tissue and de-methylation via decitabine treatment induces their expression. ERVs stimulate the transcription of immune response genes which trigger an interferon response leading to cancer cell death[18]. Although the idea of using interferons in cancer therapy has been established for some time, this specific anti-cancer mechanism was not suggested until recently[18]. Four known interferon genes (*IRF7, OASL, RIGI, ISG15*) were used to determine if interferon response indicates sensitivity. Similar results were found for the interferon response as was for TSG induction; the most resistant cell line, SUM159 showed significant induction of all four interferon genes.

The interferon response is still relatively poorly understood in relation to decitabine treatment. Although the pathway is clear, the timing and types of cells that respond remain unknown[18]. The genes *IRF7* and *OASL* are well-established in the interferon response and both genes were induced upon decitabine treatment in some epithelial ovarian cancers (EOCs)[13]. When *IRF7* is "knocked-down" in these cell lines, the observed interferon response is reduced signifigantly[13]. In the ovarian cancer cells, the interferon response was somewhat delayed and significant induction of *IRF7* and *OASL* was not observed until seven to ten days after decitabine treatment. In the current breast cancer study, expression was analyzed immediately after 72 hours of decitabine treatment. It is possible that induction of IRF7 and OASL may be occurring in other cell lines, but only after a significant delay (similar to the ovarian cancer study)[18]. Therefore, we were not able to replicate the results of this study using a breast cancer model either

because induction of these genes is time dependent or because these genes are not the main mediators of the interferon response.

It is possible that the interferon genes are observed in the response of the EOC cell lines to decitabine only because these cells have a hypermethylated *IRF7* gene. De-methylation of this gene and not the induction of ERVs could be driving the interferon-related decitabine response. The observed increase in expression of the *IRF7* gene in the SUM159 breast cancer cell line could be due to de-methylating effects rather than the expression of ERVs; and *IRF7* does not have a functional role in decitabine sensitivity.

4.3 Growth Rates

Most chemotherapies work by targeting quickly proliferating cells[51,57]. Decitabine also abides by these rules and must be incorporated into the DNA as a cytosine analog to be effective. Although many studies have shown that decitabine treatment decreases tumour growth, no one has quantified the importance of natural proliferation rates to sensitivity before this study[53-57]. It was hypothesized that faster proliferating cell lines would incorporate decitabine more quickly and therefore these cell lines would be more sensitive. It was first established that the seven breast cancer cell lines had different proliferation rates (Figure 6). Overall, no correlation was found between sensitivity and growth rate. Surprisingly, growth rate does correlate with sensitivity in all the cell lines except for the most resistant, SUM159. This indicates that importing decitabine into the cell and incorporating it into the DNA is in fact a vital step of its effectiveness. The SUM159 cell line may not follow this pattern because of an over-powering resistance mechanism.

4.3 Identifying Potential Biomarkers

To use any chemotherapy more effectively, it is advantageous to predict which patients will respond before the treatment is administered. To do this, we must understand the biology behind why they are responding. To efficiently use decitabine as a cancer therapeutic, the development of "biomarkers" of this drug are essential. In other words, indicators from patients need to be identified to tailor therapy to individual patient cases. One way to begin the implementation of this type of treatment is to look for potential biomarkers of drug response. This project evaluated representative members of three classes of genes which include 1) import/export, 2) processing of decitabine, and 3) de-methylation. We hypothesized that patients who have high expression of the genes necessary for decitabine import/export, processing and de-methylation would be more sensitive to the drug. Therefore, our most sensitive cell line should have high expression of the genes associated with this process. It was found that most of the 16 genes evaluated could not predict sensitivity in either of the seven cell lines evaluated (Figure 7-9). More specifically, most genes selected for import/export, processing and de-methylation were not correlated with the sensitivities of the seven cell lines used. Most of their individual effects were not sufficient to have predictive value. However, it was also found that the expression of four genes showed strong correlations and at least one gene from each functional category was correlated with decitabine sensitivity.

I found that the import/export gene *SCL28A1* was negatively correlated with decitabine sensitivity. The product of this gene, which is a sodium-coupled nucleoside transporter is important in transporting decitabine into the cell; therefore, high expression indicates increased decitabine uptake and increased sensitivity to this drug (Figure 7A).

This transporter efficiently transports nucleosides, so we predicted that this transporter would import decitabine (a cytosine analog) efficiently. Other members of this family, like the *SLC29A1* have lower affinity for nucleotide-like compounds explaining the lack of correlation found in this study[58]. These findings have been replicated in many other decitabine-related studies showing that high levels of these transporters contribute to increased decitabine sensitivity in AML and non-small cell lung cancer[59-61]. However, this has never been reported in breast cancer. Interestingly, the degree of sensitivity predicted by transport levels can be influenced by levels of *DCK*[61].

I also found that the *DCK* gene correlated with decitabine sensitivity. This finding has been reported in other cancers such as pancreatic cancer[62]. This is not surprising given that its protein product, the deoxycytodine kinase enzyme, is required for phosphorylation of cytidine. In addition, *DCK* has been reported as a rate determining step in molecular processing, therefore more *DCK* leads to more efficient decitabine processing and incorporation[62]. However, the direction of the correlation found in the breast cancer model differed from other studies[62]. In breast cancer, as expression of this gene increases, so does resistance of the breast cancer cells. For some reason, that has yet to be understood, this gene may contribute to decitabine resistance. Perhaps the enzyme has higher specificity for cytosine-like molecules and will preferentially process the cytosine molecule rather than its analog.

Lastly, I found that both *DNMT1A* and *DNMT3B* are negatively correlated with decitabine sensitivity. Since these genes transcribe the proteins that catalyze the addition of a methyl group onto the promotor region, and are directly inhibited by decitabine, it is logical to predict that increased expression of these genes produces less resistance.

Perhaps cell lines with high expression of DNMTs rely much more on their functioning than cell lines that have low expression of DNMTs. When decitabine degrades them, it is possible that the cell lines with high expression are effected more because of their dependency on DNMT function. Alternatively, a clinical trial which was conducted to assess decitabine's effectiveness as a treatment for AML did not find a correlation between DNMT1A levels and patient response[62]. However, this study had a sample size of only three which limits the generalizability of their result.

Identifying these four genes that are potentially important in predicting patient responsiveness contributes to our understanding of how decitabine works and which patients will benefit from its use. To further understand the effects of these four genes (*SCL28A1, DCK, DNMT1A, DNMT3B)* "knock-down clones" will be made in the breast cancer cell lines to study the direct effect of these genes on decitabine response. Treating these "knock-down" cell lines with decitabine will allow us to assess the gene's direct effect on cellular growth.

4.4 Future Directions

The goal of this study was to predict which breast cancer patients would respond to the de-methylating agent decitabine, based on its mode-of-action. It is now clear that to accurately predict response the influence of multiple genes must be considered as many of the factors currently studied, affect each other.

For example, proliferation rate, although important for decitabine incorporation, is not the main contributor of sensitivity in breast cancer possibly because a combination of effects like import and export of the drug and incorporation which, work together to decrease growth rate or initiate apoptosis[44]. The rate of decitabine uptake has been

significantly correlated with the amount of decitabine incorporated into cancer cells. This suggests that these two processes work in coordination to ensure decitabine's effectiveness within the cell[44]. In addition to this, the rate of decitabine incorporation has not been found to be genotoxic in myeloid leukemias which do in fact benefit from decitabine treatment[44]. Instead, other anti-cancer mechanisms of decitabine can occur because of the incorporation. In other words, a cascade of events such as TSG induction and the interferon response may be triggered after decitabine is incorporated into the cell[44.] Accurately predicting responsiveness to this drug, needs a holistic approach where multiple factors are taken into consideration.

The next step would be to look at the influences of the large classes of genes such as tumor suppressors and interferon genes as well as all the incorporation, processing and de-methylating genes simultaneously. Most clinical tests involve more than one gene so it is possible that finding a pattern instead of a marker may be a powerful approach.

Overall, the evolution of our search for indicators of decitabine response reflects the same process used in epigenetic research in general. Initially, the role and function of specific genes are analyzed leading to a more comprehensive understanding of how these factors interact with each other and what variables are most important. We are still at the beginning of understanding how decitabine works and which aspects influence sensitivity and responsiveness. The current study has helped to find what aspects of decitabine's mode-of-action are important in determining sensitivity in breast cancer cells. So far, there have been studies that used a single cell line treated with decitabine to understand what cellular pathways are altered by decitabine. These types of studies are important in understanding decitabine's effects but not at assigning importance to any individual

pathway. By using a variety of cell lines with different sensitivities, we could observe the differences among cell lines in relation to how decitabine works. In the future, a microarray on all the different cell lines would allow us to look for larger differences in gene expression instead of focusing on three very specific hypotheses.

So far, there is no clear answer to what factors are indicative or predictive of decitabine sensitivity. This fact simply underlines the complex nature of this drug and the need for further research to understand the biology of breast cancer epigenetics. Doing so will allow us to more efficiently and effectively treat breast cancer patients based on their individual tumor characteristics.

References

1. Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell*, *144*(5), 646-674.
2. Rodríguez-Paredes, M., & Esteller, M. (2011). Cancer epigenetics reaches mainstream oncology. *Nature Medicine*, 330-339.
3. Li, D., Da, L., Tang, H., Li, T., & Zhao, M. (2008). CpG methylation plays a vital role in determining tissue-and cell-specific expression of the human cell-death-inducing DFF45-like effector A gene through the regulation of the Sp1/Sp3 binding. *Nucleic Acids Research*, 36(1), 330-341.
4. Lehmann, B. D., Bauer, J. A., Chen, X., Sanders, M. E., Chakravarthy, A. B., Shyr, Y., & Pietenpol, J. A. (2011). Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *The Journal of Clinical Investigation*, *121*(7), 2750.
5. Zhang, B., Zhou, Y., Lin, N., Lowdon, R. F., Hong, C., Nagarajan, R. P., & Xing, X. (2013). Functional DNA methylation differences between tissues, cell types, and across individuals discovered using the M&M algorithm. *Genome research*, *23*(9), 1522-1540.
6. Hoffmann, M. J., & Schulz, W. A. (2005). Causes and consequences of DNA hypomethylation in human cancer. *Biochemistry and Cell Biology*, *83*(3), 296-321
7. Ottaviano, Y. L., Issa, J. P., Parl, F. F., Smith, H. S., Baylin, S. B., & Davidson, N. E. (1994). Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells. *Cancer Research*, *54*(10), 2552-2555
8. Lyko, F., & Brown, R. (2005). DNA methytransferase inhibitors and the development of epigenetic cancer therapies. *National Cancer Institute*, 10(20),1498-1506.
9. Kang, K. A., Zhang, R., Kim, G. Y., Bae, S. C., & Hyun, J. W. (2012). Epigenetic changes induced by oxidative stress in colorectal cancer cells: methylation of tumor suppressor RUNX3. *Tumor Biology*, *33*(2), 403-412.
10. Belinsky, S. A., Nikula, K. J., Palmisano, W. A., Michels, R., Saccomanno, G., Gabrielson, E., & Herman, J. G. (1998). Aberrant methylation of p16INK4a is an early event in lung cancer and a potential biomarker for early diagnosis. *Proceedings of the National Academy of Sciences*, *95*(20), 11891-11896.
11. Bachman, K. E., Herman, J. G., Corn, P. G., Merlo, A., Costello, J. F., Cavenee, W. K., & Graff, J. R. (1999). Methylation-associated silencing of the tissue inhibitor of metalloproteinase-3 gene suggests a suppressor role in kidney, brain, and other human cancers. *Cancer Research*, *59*(4), 798-802.
12. Widschwendter, M., Siegmund, K. D., Müller, H. M., Fiegl, H., Marth, C., Müller-Holzner, E., & Laird, P. W. (2004). Association of breast cancer DNA methylation profiles with hormone receptor status and response to tamoxifen. *Cancer Research*, *64*(11), 3807-3813.
13. Perez-Lanzac, A., Pompas-Veganzones, N., Beltran, M., Beardo, P., Vazquez, F., Cozar, J., & Sanchez-Carbayo, M. (2016). Myopodin methylation correlates to tumor progression and predicts antiangiongenic response in kidney cancer. *The Journal of Urology*, *195*(4), 1103.

14. Jing, L., Liu, L., Yu, Y. P., Dhir, R., Acquafondada, M., Landsittel, D., & Luo, J. H. (2004). Expression of myopodin induces suppression of tumor growth and metastasis. *The American Journal of Pathology*, *164*(5), 1799-1806.
15. Santos, F. P., Kantarjian, H., Garcia-Manero, G., Issa, J. P., & Ravandi, F. (2010). Decitabine in the treatment of myelodysplastic syndromes. *Expert Review of Anticancer Therapy*, *10*(1), 9-22.
16. Jabbour, E., Issa, J. P., Garcia-Manero, G., & Kantarjian, H. (2008). Evolution of decitabine development. *Cancer*, *112*(11), 2341-2351.
17. Stresemann, C., & Lyko, F. (2008). Modes of action of the DNA methyltransferase inhibitors azacytidine and decitabine. *International Journal of Cancer*, *123*(1), 8-13.
18. Chiappinelli, K. B., Strissel, P. L., Desrichard, A., Li, H., Henke, C., Akman, B., & Makarov, V. (2015). Inhibiting DNA methylation causes an interferon response in cancer via dsRNA including endogenous retroviruses. *Cell*, *162*(5), 974-986.
19. Triozzi, P. L., Aldrich, W., Achberger, S., Ponnazhagan, S., Alcazar, O., & Saunthararajah, Y. (2012). Differential effects of low-dose decitabine on immune effector and suppressor responses in melanoma-bearing mice.*Cancer Immunology, Immunotherapy*, *61*(9), 1441-1450.
20. Wood, R. D., Mitchell, M., Sgouros, J., & Lindahl, T. (2001). Human DNA repair genes. *Science*, *291*(5507), 1284-1289.
21. Mechler, B. M. (1994). When the control is lacking—the role of tumor suppressor genes in cancer development. *Bioscience*, *19*(5), 537-556.
22. Zheng, J., Xiong, D., Sun, X., Wang, J., Hao, M., Ding, T., & Shen, K. (2012). Signification of hypermethylated in cancer 1 (HIC1) as tumor suppressor gene in tumor progression. *Cancer Microenvironment*, *5*(3), 285-293.
23. Yao, K., Kuo, C., Wang, H., Lai, R., Morton, D., & Hoon, D. (2004). Prognostic significance of hypermethylated tumor suppressor genes in metastatic melanomas. *Annals of Surgical Oncology*, *11*, 85-86.
24. Catteau, A., & Morris, J. R. (2002). BRCA1 methylation: a significant role in tumor development? *Seminars in Cancer Biology*, *12*, 359-371.
25. Sarkar, S., Horn, G., Moulton, K., Oza, A., Byler, S., Kokolus, S., & Longacre, M. (2013). Cancer development, progression, and therapy: an epigenetic overview. *International Journal of Molecular Sciences*, *14*(10), 21087-21113.
26. Cameron, E., Bachman, E., Myöhänen, S., Herman, J. G., & Baylin, S. B. (1999). Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nature Genetics*, *21*(1), 103-107.
27. Garzon, R., Liu, S., Fabbri, M., Liu, Z., Heaphy, C. E., Callegari, E., & Havelange, V. (2009). MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. *Blood*, *113*(25), 6411-6418.
28. Lee, W., Reimer, L., Oh, P., Campbell, B., & Schnitzer, J. E. (1998). Tumor cell growth inhibition by caveolin re-expression in human breast cancer cells. *Oncogene*, *16*(11), 1391-1397.
29. Lee, H., Park, S., Razani, B., Russell, G., Pestell, G., & Lisanti, P. (2002). Caveolin-1 mutations (P132L and null) and the pathogenesis of breast cancer: caveolin-1 (P132L) behaves in a dominant-negative manner and caveolin-1 (−/−)

null mice show mammary epithelial cell hyperplasia. *The American Journal of Pathology*, *161*(4), 1357-1369.
30. Fang, Z., Wang, Y., Ai, N., Hou, Z., Sun, Y., Lu, H., & Yang, C. (2003). Tea polyphenol (−)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. *Cancer Research*, *63*(22), 7563-7570.
31. Greenblatt, M. S., Bennett, W. P., Hollstein, M., & Harris, C. C. (1994). Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Research*, *54*(18), 4855-4878.
32. Oki, Y., Aoki, E., & Issa, J. (2007). Decitabine—bedside to bench. *Critical Reviews in Oncology/Hematology*, *61*(2), 140-152.
33. Ewald, B., Sampath, D., & Plunkett, W. (2008). Nucleoside analogs: molecular mechanisms signaling cell death. *Oncogene*, *27*(50), 6522-6537.
34. Canadian Cancer Society's Advisory Committee on Cancer Statistics. *Canadian Cancer Statistics* 2016.
35. Foulkes, W., Smith, E., & Reis-Filho, S. (2010). Triple-negative breast cancer. *New England Journal of Medicine*, *363*(20), 1938-1948.
36. Dent, R., Trudeau, M., Pritchard, I., Hanna, M., Kahn, K., Sawka, A., & Narod, S. A. (2007). Triple-negative breast cancer: clinical features and patterns of recurrence. *Clinical Cancer Research*, *13*(15), 4429-4434.
37. Sarkar, S., Horn, G., Moulton, K., Oza, A., Byler, S., Kokolus, S., & Longacre, M. (2013). Cancer development, progression, and therapy: an epigenetic overview. *International Journal of Molecular Sciences*, *14*(10), 21087-21113.
38. Grigoriadis, A., Mackay, A., Noel, E., Wu, P. J., Natrajan, R., Frankum, J., & Tutt, A. (2012). Molecular characterization of cell line models for triple-negative breast cancers. *BMC Genomics*, *13*(1), 619.
39. Kastl, L., Brown, I., & Schofield, A. C. (2010). Effects of decitabine on the expression of selected endogenous control genes in human breast cancer cells. *Molecular and Cellular Probes*, 24(2), 87-92.
40. Freshney, I. (2005). Animal Cell Culture Basics: Tips and Techniques for Continuous Cell Lines. *A Manual of Basic Techniques*, 5th edition.
41. Lee, E., & Muller, W. (2010). Oncogenes and tumor suppressor genes. *Cold Spring Harbor Perspectives in Biology*, *2*(10).
42. Clark, K., Karsch-Mizrachi, I., Lipman, D., Ostell, J., Sayers, E. (2016). *GenBank Nucleic Acids Res*. 44(D1), 67-72.
43. Esteller, M. (2002). CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. *Oncogene*, *21*(35), 5427.
44. Öz, S., Raddatz, G., Ruis, M., Blagitko-Dorfs, N., Lübbert, M., Maercker, C., & Lyko, F. (2014). Quantitative determination of decitabine incorporation into DNA and its effect on mutation rates in human cancer cells. *Nucleic Acids Research*, 775.
45. Radpour, R., Barekati, Z., Kohler, C., Lv, Q., Bürki, N., Diesch, C., & Zhong, X. Y. (2011). Hypermethylation of tumor suppressor genes involved in critical regulatory pathways for developing a blood-based test in breast cancer. *PLoS One*, *6*(1).

46. Issa, J., Garcia-Manero, G., Giles, F., Mannari, R., Thomas, D., Faderl, S., & Kantarjian, H. (2004). Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2′-deoxycytidine (decitabine) in hematopoietic malignancies. *Blood*, *103*(5), 1635-1640.
47. Hellebrekers, D., Griffioen, A., & Van-Engeland, M. (2007). Dual targeting of epigenetic therapy in cancer. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer*, 1775(1), 76-91.
48. Sharma, S., Kelly, T.K., & Jones, P. A. (2010). Epigenetics in cancer. *Carcinogenesis*, *31*(1), 27-36.
49. Li, Y., Meeran, S. M., Patel, S. N., Chen, H., Hardy, T.N., & Tollefsbol, T. O. (2013). Epigenetic reactivation of estrogen receptor-α (ERα) by genistein enhances hormonal therapy sensitivity in ERα-negative breast cancer. *Molecular cancer*, *12*(1), 9.
50. Godoy, P., Cadenas, C., Hellwig, B., Marchan, R., Stewart, J., Reif, R., & Hengster, J. G. (2014). Interferon-inducible guanylate binding protein (GBP2) is associated with better prognosis in breast cancer and indicates an efficient T cell response. *Breast Cancer*, *21*(4), 491-499.
51. Miller, A. B., Hoogstraten, B. F. A. U., Staquet, M. F. A. U., & Winkler, A. (1981). Reporting results of cancer treatment. *cancer*, *47*(1), 207-214.
52. Rosenberg, S. A., Yang, J. C., & Restifo, N.P. (2004). Cancer immunotherapy: moving beyond current vaccines. *Nature medicine*, *10*(9), 909-915.
53. Bender, C. M., Pao, M. M., & Jones, P. A. (1998). Inhibition of DNA methylation by 5-aza-2′-deoxycytidine suppresses the growth of human tumor cell lines. *Cancer Research*, *58*(1), 95-101.
54. Chen, M. Y., Liao, W. S. L., Lu, Z., Bornmann, W. G., Hennessey, V., Washington, M. N., & Bast, R. C. (2011). Decitabine and suberoylanilide hydroxamic acid (SAHA) inhibit growth of ovarian cancer cell lines and xenografts while inducing expression of imprinted tumor suppressor genes, apoptosis, G2/M arrest, and autophagy. *Cancer*, *117*(19), 4424-4438.
55. Turcan, S., Fabius, A. W., Borodovsky, A., Pedraza, A., Brennan, C., Huse, J., & Chan, T. A. (2013). Efficient induction of differentiation and growth inhibition in IDH1 mutant glioma cells by the DNMT Inhibitor Decitabine. *Oncotarget*, *4*(10), 1729-1736.
56. Liu, Q. Y., Chen, D. W., Xie, L. P., Zhang, R. Q., & Wang, H. Z. (2011). Decitabine, independent of apoptosis, exerts its cytotoxic effects on cell growth in melanoma cells. *Environmental Toxicology and Pharmacology*, *32*(3), 423-429.
57. Fisher, D. E. (1994). Apoptosis in cancer therapy: crossing the threshold. *Cell*, *78*(4), 539-542.
58. Pastor-Anglada, M., Cano-Soldado, P., Errasti-Murugarren, E., & Casado, F. J. (2008). SLC28 genes and concentrative nucleoside transporter (CNT) proteins. *Xenobiotica*, *38*(7-8), 972-994.
59. Mini, E., Nobilli, S., Caciagli, B., Landini, I., Mazzei, T. (2006). Cellular pharmacology of gemcitabine. *Oncology*, 7-12.
60. Gati, W., Paerson, A., Larrat, M. (1997). Sensitivity of acute leukemia cells to cytarabine is a correlate of cellular ES nucleoside transporter site content measured by flow cytometry with SAENTA-fluorescein. *Blood.* 346-353.

61. Achiaw, H (2004). Determinants of sensitivity and resistance to gemcitabine : the role of human equilibrative nucleoside transporter 1 and deoxycytidine kinase in non-small cell lung cancer. *Cancer Science*, 753-757.
62. Sebastiani, V., Ricci, F., Rubio-Viqueira, B., Kulesza, P., Yeo, J., Hidalgo M. (2006). Immunohistochemical and genetic evaluation of deoxycytidine kinase in pancreatic cancer: relationship to molecular mechanisms of gemcitabine resistance and survival. *Clinical Cancer Research*, 492–497.

Supplemental

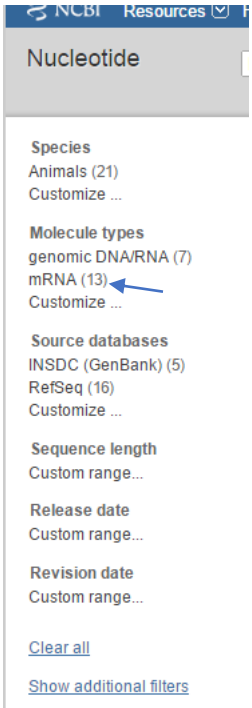
1 Primer Design Protocol

1. Go to: http://www.ncbi.nlm.nih.gov/

2. Search your gene of interest (our example is COL16A1). When making primers, you are looking for the nucleotide sequences on the database.

3. Select for human sequences only:

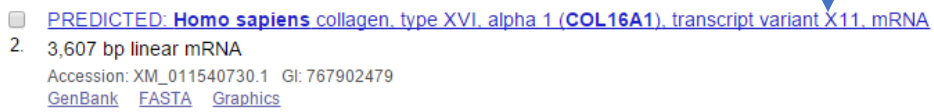
4. Select for mRNA sequences only:



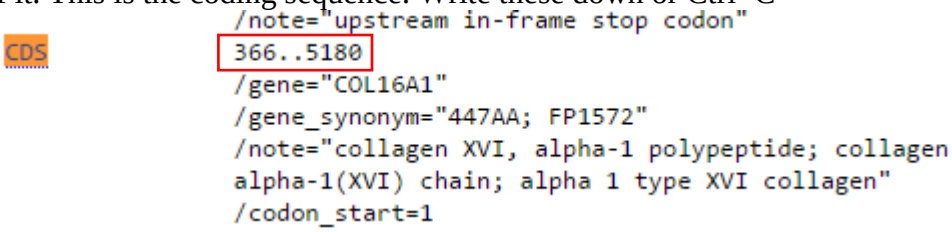
5. The resulting list will contain several things

a) Known mRNA sequences. There may be several transcript variants depending on the exon splicing etc.

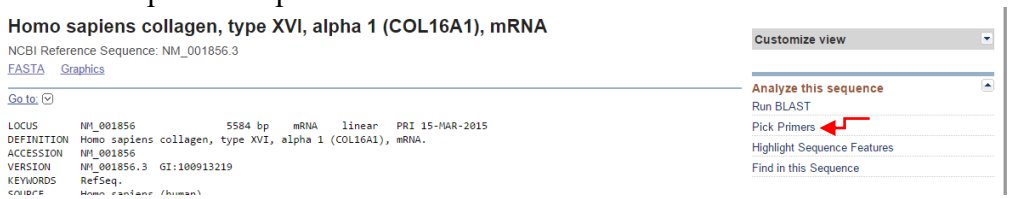
b) Predicted sequences that have not been confirmed. The "x" before transcript variant indicates that it is an unconfirmed mRNA sequence. There are 11 potential transcript variants for COL16A1 here.



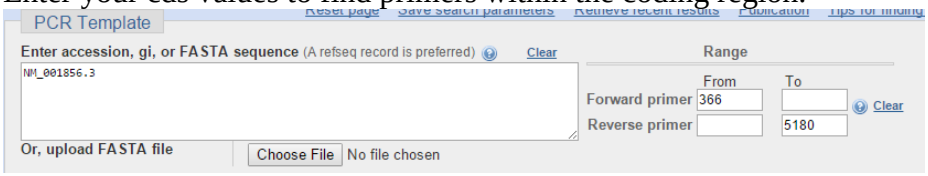
6. Click on a known mRNA. CtrL+F for "cds" and find those two number to the side of it. This is the coding sequence. Write these down or Ctrl+C



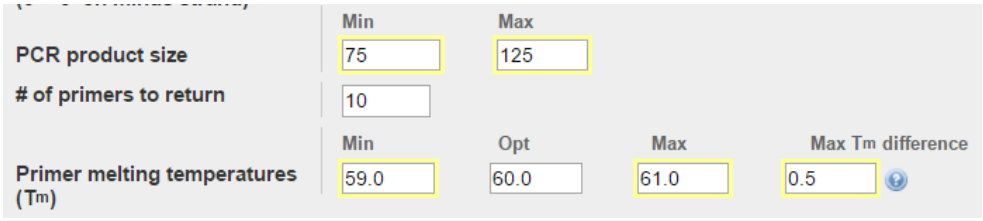
7. On the left panel of options click "Pick Primers":



8. On PrimerBlast your accession code will already be entered if you followed the previous steps. You can Ctrl+V in the FASTA code from your gene of interest. Enter your cds values to find primers within the coding region.

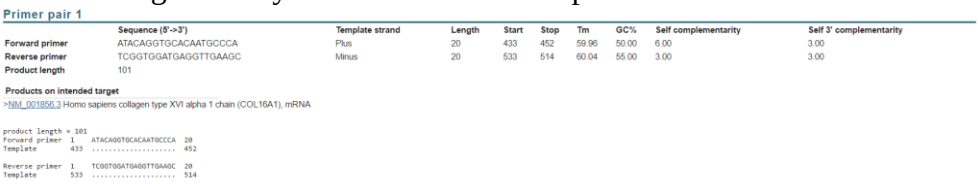


9. Set your PCR product length and melting temperature. For QPCR it should be between 75-125 bases in size. Limit the temp to 1°C around optimal temperature of 60°C with a max difference between forward and reverse primers of 0.5°C.

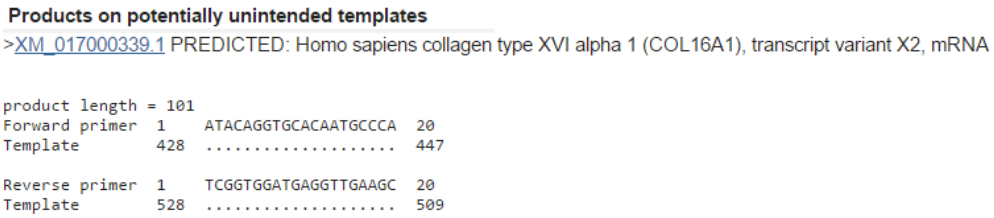


10. Select GO.

11. You will then get a list of potential primers. Check that the primer on your intended target is fully matched with the template.



12. Below your intended targets are potentially unintended targets. It is important to check that your intended targets are ONLY YOUR GENE OF INTEREST- these can be predicted or known splice variants. If there are unintended hits, it's better to move down the list of primer pairs to see if you have a more specific primer set.



13. Save the primer sequences (distinguish F and R) and order.

2 Primer Efficiency

Primer efficiencies determined via standard curve analysis:

Gene	Efficiency (%)
BRCA1	99.5
CDA	124.7
CDH1	126.7
CMPKI	92.2
CTPS	121.6
DCK	87
DNMT1	109.1
DNMT3B	108.0
IRF7	98.5
ISG15	82.7
MDR1	99.5
NME1	82.7
NME2	89.2
OASL	89.9
PCNA	100.5
RASSF1	110.1
RIGI/DDX58	106.2
RUNX3	104.9
SLC28A1	106.2
SLC29A1	100.0
TET1	124.5
TET2	116.9
TET3	90.1
TRAF6	107.2
UHRF1	97.1