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MicroRNA-510 as a Biomarker of Sensitivity to Platinum-Based Chemotherapy in Triple Negative Breast Cancer

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by

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Abstract

An abstract of thesis. Breast cancer is a heterogeneous disease that is clinically classified based on the expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). Associated with the poorest prognosis of all subtypes, triple negative breast cancer (TNBC) is defined as ER-/PR-/HER2-; lacking these receptors, TNBCs are insensitive to effective targeted therapies and chemotherapy remains the mainstay of treatment. Recent research has shown that platinum chemotherapy agents are particularly active in TNBC, including a clinical trial that demonstrated single agent cisplatin alone can induce response in a subset of TNBC patients. However, identification of biomarkers is still needed to distinguish sensitive patients from resistant ones, whom may benefit from other forms of chemotherapy. Recent studies have revealed that cisplatin activates the c-Abl/TAp73 apoptotic pathway, specifically in TNBCs with p53 mutations (~60% of all TNBCs). Our lab has identified miR-510 to be overexpressed in human breast tissue samples, suggesting a role in breast cancer. Our lab has validated Prdx1 to be a direct target of miR-510. Prdx1 has been shown to protect cells from cisplatin and also inhibit the tyrosine kinase c-Abl. Therefore we wanted to determine the efficacy of miR-510 as a biomarker of cisplatin sensitivity and elucidate the mechanism of this sensitivity.

Drug cytotoxicity assays indicate that miR-510 positively correlates with cisplatin sensitivity in TNBC cell lines. Furthermore, inhibition of miR-510 causes sensitive TNBC cell lines to become more resistant, while overexpression of miR-510 restores sensitivity

in resistant TNBC cell lines *in vitro*. MicroRNAs are ideal biomarkers in cancer as they regulate many processes in cancer development and progression and are also stable in biological fluids. Analysis of samples from our in vivo study reveals a positive correlation of miR-510 levels in matched tumor and serum samples, suggesting that serum expression of miR-510 is indicative of its expression in tumor samples. Analysis of breast cancer patient serum samples demonstrates that varying levels of miR-510 can be detected in human samples as well. We then went on to explore the mechanism of miR-510 mediated cisplatin sensitivity. IHC analysis of in vivo tumors suggests that miR-510 mediated sensitivity may not be through decreased proliferation or increased cleaved-caspase 3 dependent apoptosis. Prdx1 IHC analysis, along with matched tumor miR-510 expression suggests that negative regulation of Prdx1 may play a role in miR-510 mediated cisplatin sensitivity. The Prdx1 rescue experiments found that the mechanism of miR-510 mediated sensitivity is dependent upon the negative regulation of Prdx1. Our mechanistic studies reveal that cisplatin treatment leads to upregulation and increased phosphorylation of TAp73 in miR-510 overexpressing cells. In cells overexpressing miR-510, we also show that cisplatin leads to dissociation of TAp73 from its repressor ΔNp63 and induction of downstream pro-apoptotic genes, PUMA and NOXA. Cumulatively, our data demonstrates that miR-510 is a potential, non-invasive biomarker of cisplatin sensitivity in TNBC. We also show this mechanism of sensitivity is attributed to, at least in part, the negative regulation of Prdx1 and the subsequent activation of TAp73 apoptotic pathway.

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List of Abbreviations

AGO - Argonaute

ATM - Ataxia Telangiectasia Mutated

ATR - Ataxia Telangiectasia and Rad3-related Protein

BRCA1/BRCA2 - Breast cancer 1, early onset/ Breast cancer 2, early onset

BSA- Bovine Serum Albumin

CTR1 – Copper Transporter 1

DCIS - Ductal Carcinoma In Situ

ddPCR – Digital Drop PCR

ER – Estrogen Receptor

FISH – Fluorescence In Situ Hybridization

GOF - Gain of Function

HER2 – Human Epidermal Growth Receptor 2

IHC – Immunohistochemistry

LCIS – Lobular Carcinoma In Situ

LOF - Loss of Function

miRNA - MicroRNAs

MMR – Mismatch Repair

MMS – Mismatch Repair System

MRI – Magnetic Resonance Imaging

NER – Nucleotide Excision Repair

PBS- Phosphate Buffer Solution

pCR – Pathological Complete Response

PDEF – Prostate-derived Ets Factor

PR – Progesterone Receptor

PRDX1 – Peroxiredoxin 1

PSF – Progression Free Survival

qPCR – Quantitative Real Time PCR

RIP-Chip - RNA binding protein immunoprecipitation microarray

RISC – RNA-Induced Silencing Complex

RNS – Reactive Nitrogen Species

ROS - Reactive Oxygen Species

SERMs - Selective Estrogen Receptor Modulators

SILAC - Stable Isotope Labeling with Amino Acids in Cell Culture

SRB - sulforhodamine B

TNBC – Triple Negative Breast Cancer

TUNEL – Terminal deoxynucleotidyl transferase-mediated dUTP nick endlabeling

UTR – Untranslated Region

Introduction and Background

Breast Cancer

Breast cancer is a heterogeneous disease that originates from tissues of the breast. Consisting of glandular, adipose and connective tissue, the breast functions to produce, store and release milk during lactation [1]. The most common types of malignancies are carcinomas that arise from the epithelial cells of milk producing lobules or milk transporting ducts (Figure 1). Soft tissue sarcomas may also develop in the breast, although they are rare [2]. Several risk factors are well known to contribute to the development of breast cancer, including personal and hereditary factors that are not modifiable and also lifestyle factors. Non-modifiable factors include age and family history of breast cancer. Advance age (65+ years) is one of the highest risk factor for breast cancer as risk increases across all ages until the age of 80 [3]. Family history of breast cancer is important as well. Women with a first degree relative with breast cancer have a higher risk of developing the disease; this risk increases significantly with each additional relative with breast cancer [3]. A small portion of breast cancer (5%-10%) is due to inherited mutations, most notably the breast cancer susceptibility genes BRCA1 and BRCA2. Other non-modifiable factors could also increase the risk, including: benign breast disease, increased breast density, high endogenous hormone levels, early menarche and late age pregnancy [3]. Lifestyle-related factors could also add to the risk of breast cancer. Use of post-menopause hormone replacement therapy is well established to increase the risk of developing and dying from the disease [3]. Studies

have suggested that obese women and women that do not exercise regularly are at a higher risk of post-menopausal breast cancer than lean and active women [3, 4]. Women who are frequently exposed to alcohol, tobacco, radiation and various chemical pollutants are at a higher risk to have breast cancer [3]. With countless risk factors, it is not surprising that breast cancer is one of the most prevalent diseases of women in the United States and worldwide. It is estimated in the United States, there will be 231,840 new cases of invasive breast cancer in 2015, along with 60,290 cases of in situ breast cancer. This disease is also projected to claim the lives of 40,290 women in the same year [5].

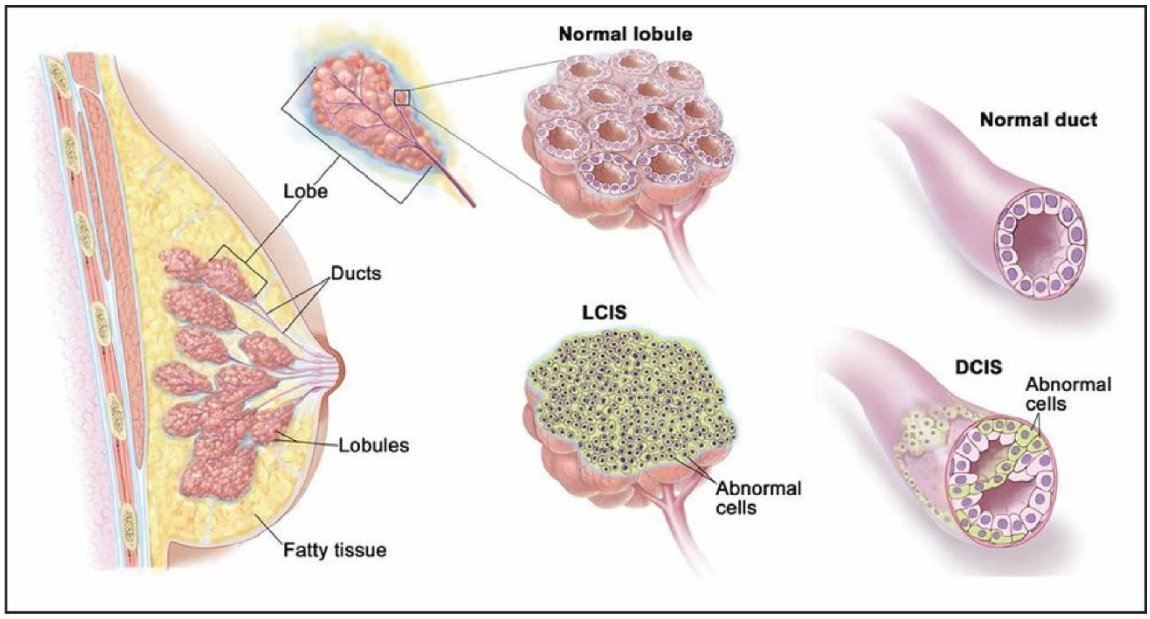

Figure 1. Breast anatomy showing the glandular structure, a complex system of lobules and ducts. Most breast cancers originate from epithelial cells of these lobules and ducts. Illustrations of lobular carcinoma in situ and ductal carcinoma in situ are also shown. Figure adapted from "Breast Cancer Treatment" 2015, National Cancer Institute PDQ®. Copyright 2011 by Terese Winslow LLC. Adapted with permission. [2]

Diagnosis: Breast cancer is usually suspected after detection of a mass during clinical breast examinations or mammograms, and occasionally through breast MRI. Biopsies are then taken to further distinguish between benign and malignant lesions [3]. Due to the extremely heterogeneous nature of breast cancer, it can be classified in a variety of ways. A common classification is by histopathology, first distinguishing in situ/invasive tumors and then classifying them into distinct subtypes based on their architectural features and growth patterns [2, 6]. In situ tumors are a noninvasive form of breast cancers that have not yet infiltrated beyond the original cell layer, most commonly ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS) (Figure 1). While most of these tumors are not life-threatening, studies suggest that approximately one-third of these cases will become invasive and may require intervention [7]. The majority of breast cancer cases are invasive, characterized by tumor cells that have penetrated into the surrounding breast tissue. Following diagnosis of invasive breast cancer, tumors are further classified by grade and stage to determine prognosis and guide treatment choice. TNM classification is commonly used to stage the tumor based on tumor size (T), spread to local lymph nodes (N) and whether the tumor has metastasized (M). Tumors are then assigned a stage of 0, I, II, III or IV, with increasingly poorer prognosis [2]. Grade is determined based on differentiation of the breast tumor (presence of ductal structure and nuclear pleomorphism), along with its proliferation rate (mitotic count). A grade of 1, 2 or 3 is assigned, where grade 1 is well differentiated with low proliferation rate and grade 3 is poorly differentiated with high proliferation. A higher grade

correlates with a poorer prognosis [8]. A pathologist will also determine the receptor status of the tumor, by immunohistochemistry (IHC) or fluorescence in situ hybridization (FISH), for the presence of estrogen receptor (ER), progesterone receptor (PR) and the amplification of human epidermal growth receptor 2 (HER2). This determines the treatment of the cancer, as there are highly effective, targeted therapies for tumors that express these receptors [2]. Various studies have shown that breast cancer can be defined by its molecular profile and classified into intrinsic subtypes: luminal A, luminal B, HER2 enriched, basal-like, claudin-low and normal-like [6, 9-11]. This new form of classification could allow for better differentiation of subtypes of breast cancer once thought to be similar, providing more specific information regarding prognosis and predicted response to treatments. However, this classification is currently limited to research as the cost of large scale microarray analysis and genome sequencing to classify breast cancers is not suitable for routine clinical analysis [6, 11, 12]. Current methodologies to assign molecular subtype of breast cancer are also not standardized as various methods of classification have been developed, some of which are still being improved upon [11, 13]. Current methods also requires the gene expression data of individual test tumors to be compared against a large, heterogeneous reference tumor set [13]. Therefore, classification of tumors into a molecular subtype is highly dependent on the reference set used in the classification method. These issues must be addressed before molecular classification of breast cancer can be effectively employed for clinical use.

Treatment Options: After diagnosis, breast cancer can be treated with an assortment of interventions. Current treatments include surgery, radiation, chemotherapy, hormonal therapy, and HER2-targeted therapies; either individually or in combination [3]. Surgeries are performed to remove the primary tumor with either breast-conserving lumpectomy or mastectomy. Radiation therapy is typically administered to lumpectomy patients and patients with a high risk of recurrence. Systemic treatments, hormonal therapies, targeted therapies and chemotherapies, can be administered prior to surgery (neoadjuvant) or after surgery (adjuvant). Tumors that are ER and PR positive are treated with tamoxifen, a Selective Estrogen Receptor Modulator (SERMs) that blocks estrogen signaling through the ER. For post-menopausal women, aromatase inhibitors, anastrozole and letrozole, are used to inhibit estrogen production through the aromatization of androgens. Patients with HER2 amplifications are treated with targeted therapies (trastuzumab, lapatinib and pertuzumab) that inhibit the HER2 signaling pathway. Chemotherapy is another systemic treatment with drugs that stop the growth of cancer cells and could be given in addition to targeted therapies. Most commonly used chemotherapies are anthracyclines, taxanes, antimetabolites, and alkylating agents.

Survival rates are promising for early stage breast cancer. The five-year survival for localized and regional diseases is extremely high, 99% and 84% respectively. But once it has progressed into metastatic breast cancer, the survival rate drops drastically to 24% [3]. Breast cancer is very treatable if detected at an early stage, when treatments are the most effective and a cure is more likely [14]. Therefore, there is an emphasis on early

detection and selecting the most effective intervention to prevent progression into late-stage disease.

Triple Negative Breast Cancer

When classifying breast tumors based on receptor status, triple negative breast cancer (TNBC) encompasses a group of tumors that lack ER, PR and overexpression/amplification of HER2 [15]. Accounting for approximately 10-20% of all invasive breast cancer cases, TNBC is known to have the poorest prognosis of all the receptor-based subtypes [16]. This is evident as the 5 and 10 year survival percentages are the lowest of all the subtypes, independent of the stage at diagnosis (Figure 2) [17]. This poor prognosis can be attributed to higher rates of distant recurrence, shorter median time from relapse till death, and higher incidences of early visceral and central nervous system metastasis compared to other subtypes [18-20]. TNBCs are more commonly observed in younger women, as the average age of diagnosis is 5-10 years younger compared to non-TNBC [21]. There are multiple reports of higher rates of TNBC in women of African ancestry, with studies showing two-fold to three-fold higher frequency of TNBC in African American women compared to Caucasian American women [21, 22]. Studies have also suggests TNBC incidences are higher among women of Hispanic ancestry and women of lower socioeconomic status [23, 24]. A lower percentage of TNBC are discovered by mammograms as it is underutilized by younger

women or women of low socioeconomic status [23] and TNBCs are more likely mammographically occult due to lower tumor calcification [21].

Treatment of TNBC: TNBC are more difficult to treat as they are generally more biologically aggressive [15] and the lack of hormonal receptors and HER2 renders them unresponsive to effective targeted therapies that are clinically available [25]. The current standard of care for TNBC is combination cytotoxic chemotherapy, since TNBC patients treated with chemotherapy showed higher response rate than non-TNBC patients in neoadjuvant, adjuvant and metastatic settings [26, 27]. A paradox of TNBC is that even with the increased sensitivity to chemotherapy, its survival rates remain lower than other subtypes [16, 25]. While TNBC patients respond well to anthracycline and taxane based regimens, there is still a high risk of relapse for patients with residual disease [15, 28]. Since TNBC shows decrease duration of response from each successive line of chemotherapy [27], it is crucial to find the most effective first-line treatment to prevent drug resistance and disease progression. Therefore, current TNBC research focuses on novel biomarkers of response to treatment along with the development of effective, targeted therapies for TNBC. Potential TNBC targets under investigation include: preventing angiogenesis (e.g. anti-VEGF antibody), inhibiting growth factor pathway (e.g. EGFR inhibitors and mTOR inhibitors), and exploiting aberrant DNA repair mechanisms (PARP inhibitors and platinum agents) [25].

Subtype	Stage	5 year OS (%)	10 year OS (%)
*DCIS	0	99	98
Luminal (non-HER2+)	I	98	95
	II	91	81
	III	72	54
	IV	33	17
**HER2+	I	98	95
	II	92	86
	III	85	75
	IV	40	15
TNBC	I	93	90
	II	76	70
	III	45	37
	IV	15	11

*Preinvasive Stage
**Estimated Overall Survival (OS) using HER2 targeted therapies

Figure 2. Prognosis of breast cancer subtypes, by stage, shown as percentage of overall survival for 5 and 10 years. Figure adapted from "SnapShot: Breast Cancer" by K. Polyak and O.M. Filho, 2012, Cancer Cell, 22, pg. 562. Copyright 2012 by Elsevier Inc. Adapted with permission. [17]

TNBC is often used as a surrogate term for basal-like breast cancer and BRCA1 associated breast cancer due to similarities between the groups. While TNBC and basal-like breast cancer are often used synonymously due to the large overlap between the two groups, studies found around 30% discordance between the two groups [29]. TNBC is distinguished solely by clinical assessment of receptors (ER, PR, and HER2) and not all TNBCs exhibit the molecular profile of basal-like breast cancer. Basal-like breast cancer is based on its molecular profile (e.g. CK 5/6, c-kit, HER1, and others) and not all basal-like breast cancers display the triple negative phenotype [15]. TNBC are also often associated with BRCA1 associated breast cancers since BRCA1 mutation carriers that develop breast cancer are often triple negative for ER, PR, and HER2 [30]. However, there are discordances between these two groups as well since majority of TNBCs are sporadic and does not harbor BRCA1 mutation. The large overlap between TNBC and basal-like/BRCA1 associated breast cancers suggest TNBC may be sensitive to similar therapeutics. BRCA1 mutation carriers and many sporadic basal-like breast cancers have altered (e.g. promoter methylation and decrease transcript levels) or loss of BRCA1 activity [31], which is involved in homologous recombination to repair double strand breaks in DNA. The aberrant DNA repair mechanisms of BRCA1 associated/basal-like breast cancer renders them vulnerable to platinum agents (generates double strand breaks) leading to tumor cell apoptosis [31]. Various *in vitro* and in vivo studies have demonstrated that BRCA1 associated and basal-like breast cancer were significantly more sensitive to cisplatin compared to other popular chemotherapies [32, 33]. A recent

randomized, phase III trial (NCT00532727) found that TNBC patients with mutant BRCA1/2 achieved an objective response rate of 68%, treated with a platinum agent (carboplatin), compared to 33% when treated with a taxane (docetaxel); whereas, these differences in response rate were not observed in non-mutant BRCA1/2 patients. This trial has also found that progression free survival (PSF) was longer in mutant BRCA1/2 breast cancer patients, treated with platinum agents compared to taxane agents. Promising trials, like NCT00532727, have sparked a renewed interest in treating TNBC with platinum agents due to the similarities of between TNBC and basal-like/BRCA1 associated breast cancer.

Platinum Agents: Platinum agents are a group of "alkylating-like" chemotherapy agents that are composed of platinum complexes. The first platinum agent, cisplatin or cis-diamminedichloroplatinum(II), was introduced in the clinics in 1970's and has made significant impact in treating cancer, particularly germ cell cancers (testicular and ovarian) [34]. Cisplatin is still widely used in the clinics to treat various solid tumors including: head and neck, bladder, cervical and small cell lung cancer [35]. The severe side effects of cisplatin (nephrotoxicity and neurotoxicity) initiated platinum drug development, leading to carboplatin and subsequently, oxaliplatin to combat mechanisms of drug resistance [34]. These are the three main platinum agents that are widely used in treating cancer (Figure 3). Oxaliplatin has a distinct structure compared to cisplatin and carboplatin, leading to activation of different pathways upon DNA damage; this is further supported by the fact that cisplatin-resistance cells response to

oxaliplatin [34, 36]. Due to its differential mechanism of action, oxaliplatin is only approved for the treatment of colorectal cancer. Carboplatin is used to treat similar types of cancers as cisplatin, as their mechanism of action are similar. Carboplatin offer a similar level of clinical activity as cisplatin, while eliminating the severe side effects [35, 36]. However, studies has shown that carboplatin is less potent than cisplatin, demonstrated by significantly higher dosage to match the effectiveness of cisplatin and slower rate of DNA adduct formation [35, 36]. Upon uptake into the cell by copper transporter-1 (CTR1), cisplatin become activated [34-36]. While cisplatin could interact with various cellular molecules, deoxyribonucleic acids (DNA) remains the primary target. Cisplatin then covalently bonds to the N^7 position of the imidazole ring of purine bases (mostly guanine) to generate, predominately 1,2 intrastrand and 1,3 intrastrand cisplatin-DNA adducts (Figure 3) [34-36]. A complex system of molecular pathways has been shown to be activated upon recognition of cisplatin-DNA adducts (Figure 4). The various pathways are involved in promoting G2 cell cycle arrest (ATM/ATR), attempt to repair DNA damage (either via nucleotide excision repair/NER or mismatch repair system/MMS), and activation of survival/apoptotic pathways depending on extent of DNA damage [34-36]. The tumor suppressor p53 plays an important role downstream of cisplatin-induced DNA damage, promoting cell cycle arrest, NER machinery, and apoptosis [34-36]. However, p53 is often mutated in many types of cancers, including TNBC [17, 36, 37]. Various studies have shown that wild-type p53 correlates with cisplatin sensitivity [37]. However, cisplatin can still induce apoptosis in the presence of

p53 mutation by activation of c-Abl through the MMR system [35]. A mechanism of cisplatin-induced cell death specifically in TNBC has also been uncovered (Figure 5) [38]. It has been shown that tumor suppressor, p53 family members, ΔNp63 and TAp73, are coexpressed in a subset of TNBC that has a mutational inactivation of p53 (significantly higher in TNBC compared to non-TNBC subtypes, 54% vs 26% [17]). In TNBC tumors, ΔNp63 acts a survival factor by binding to TAp73 and inhibiting it from promoting apoptosis. Upon cisplatin induced DNA damage, c-Abl (non-receptor tyrosine kinase) becomes activated and phosphorylates TAp73, allowing dissociation of its repressor ΔNp63; thus allowing TAp73 to transcribe pro-apoptotic genes leading to tumor cell death (Figure 5). As this molecular pathway is only responsive to cisplatin treatment and no other chemotherapeutic agents (taxanes and anthracyclines), coexpression of ΔNp63/TAp73 is being investigated as a potential biomarker of cisplatin sensitivity in TNBC clinical trials.

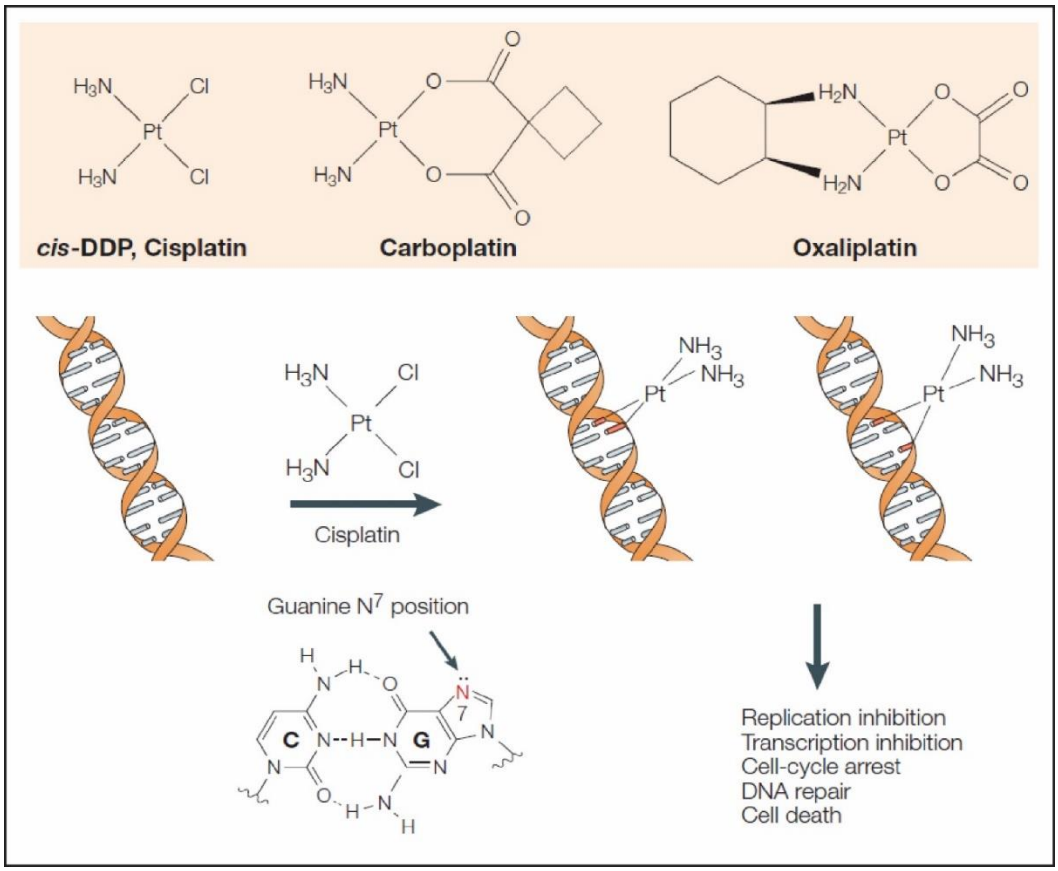

Figure 3. Chemical structures of platinum-based chemotherapy drugs: cisplatin, carboplatin, and oxaliplatin. Also included, cisplatin mechanism of action. Figure adapted from "Cellular Processing of Platinum Anticancer Drugs" by D. Wang and S.J. Lippard, 2005, Nature Reviews Drug Discovery, 4, pg. 308-311. Copyright 2005 by Nature Publishing Group. Adapted with permission. [36]

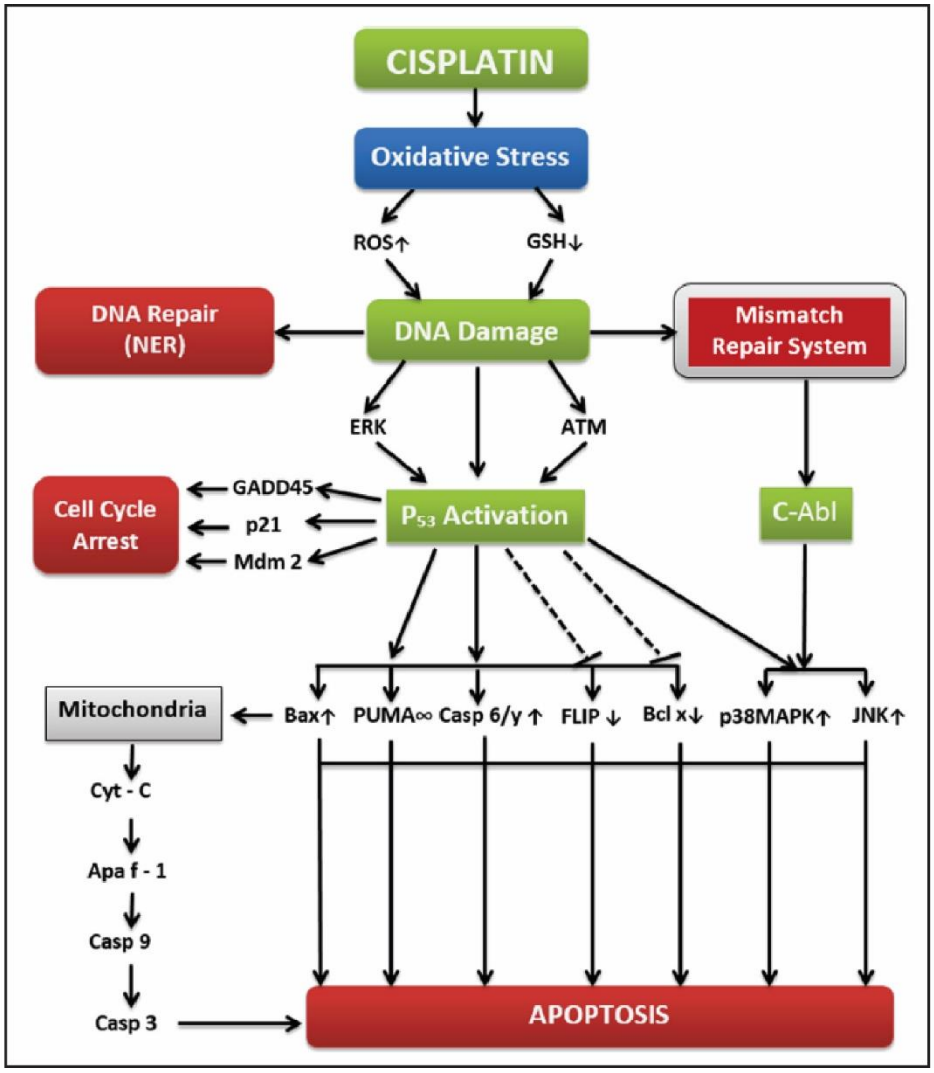

Figure 4. Downstream molecular mechanism of cisplatin induced DNA damage. Figure adapted from "Cisplatin in cancer therapy: Molecular mechanisms of action" by S. Dasari and P.B. Tchounwou, 2014, European Journal of Pharmacology, 740, pg. 370. Copyright 2014 by Elsevier B.V. Adapted with permission. [35]

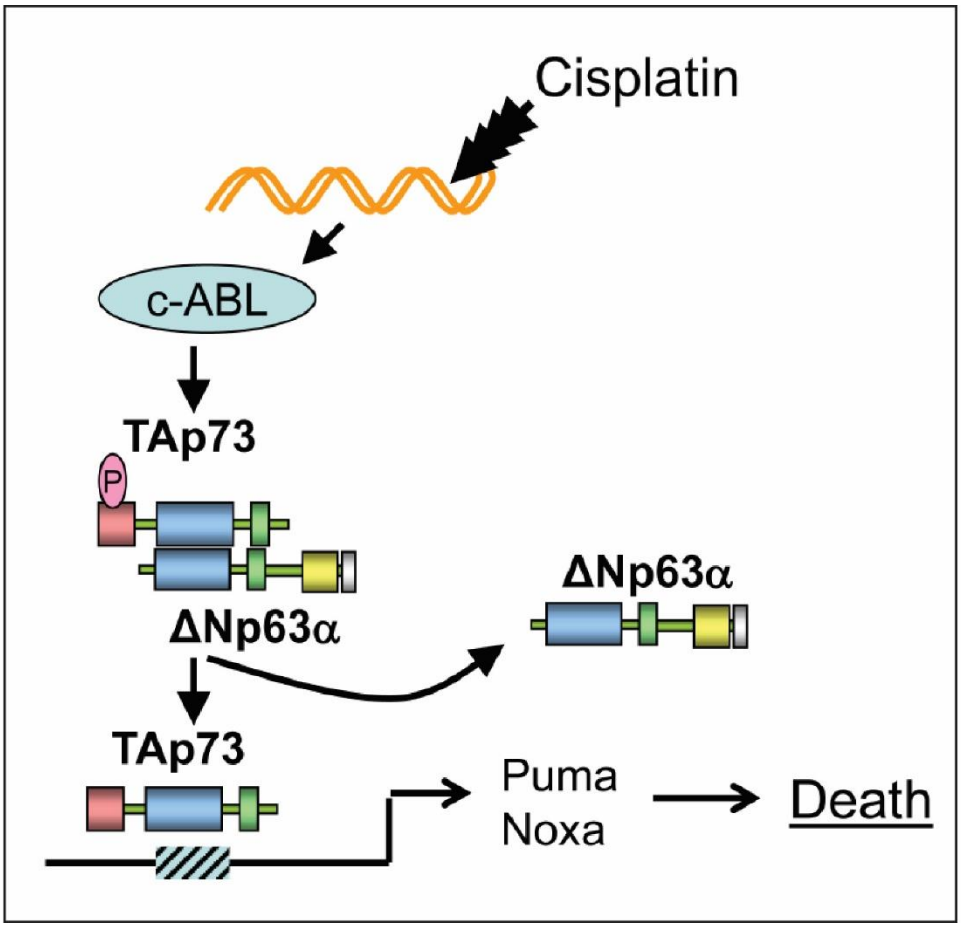

Figure 5: Mechanism of cisplatin induced cell death in TNBC. Figure adapted from "Triple Negative Breast Cancer: Role of Specific Chemotherapy Agents" by S.J. Isakoff, 2010, Cancer J, 16, pg. 59. Copyright 2010 by Lippincott Williams & Wilkins, Inc. Adapted with permission. [27]

Promising pre-clinical data led to clinical trials to investigate the potential of platinum agents in TNBC. Various clinical investigations of platinum agents in TNBC offered mixed results; although many studies investigated platinum doublet or triplet regimens (in combination with other chemotherapies) and often use carboplatin, which may be less potent than cisplatin [35, 39]. A retrospective study showed a higher complete response rate with neoadjuvant, cisplatin based chemotherapy in TNBC patients (88%) vs non-TNBC patients (51%) [40]. Similarly, metastatic TNBC patients also showed higher response rates to cisplatin based chemotherapy compared to other breast cancer subtypes [40, 41]. Many previous trials do not provide a clear assessment of the efficacy of platinum drugs in treating TNBC due to: limited number of trials since cisplatin is not a standard of care in treating TNBC, analysis of retrospective samples that lack HER2 data (prior to the discovery of HER2), investigation of platinum agents in polychemotherapy regimens, and administration of platinum as a non-first line therapy. A phase II clinical trial (NCT00148694) addressed these problems and evaluated the efficacy of neoadjuvant, single-agent cisplatin as first line chemotherapy to newly diagnosed TNBC patients. The results show a pathologic complete response (pCR) in 22% patients while 36% showed complete or near-complete responses [42]. Due to the low sample numbers (28 patients) and the relatively low pCR rate compared to other polychemotherapy regimens used to treat TNBC, the data do not justify using neoadjuvant, single agent cisplatin to treat all TNBC cases [42]. However, it indicates that cisplatin is particularly active in a subset of TNBC cases and suggests the need of

biomarkers to identify responsive patients [27]. This trial evaluated various potential markers including altered BRCA1 activities (Figure 6). While the two patients with BRCA1 mutations responded extremely well and reached pCR, the majority of the patients who showed complete or near-complete response did not harbor the mutation. Low BRCA1 mRNA levels and BRCA1 promoter methylation are also inconsistent predictors [42]. The aforementioned biomarker, ΔNp63/TAp73, was also assessed as these proteins are involved in a molecular pathway by which cisplatin induces apoptosis specifically in TNBC [38]. Of the patients that were positive for ΔNp63/TAp73, 33% (3/9) achieved pCR; whereas only one of thirteen, that were negative for the biomarker, reached pCR [42]. This biomarker was further evaluated in a phase II clinical trial (NCT00483223) in metastatic TNBC and did not proved to be a successful biomarker of cisplatin sensitivity in this setting [27, 43]. Data from these trials suggests that there is still a need for a more specific biomarker to accurately predict cisplatin sensitivity.

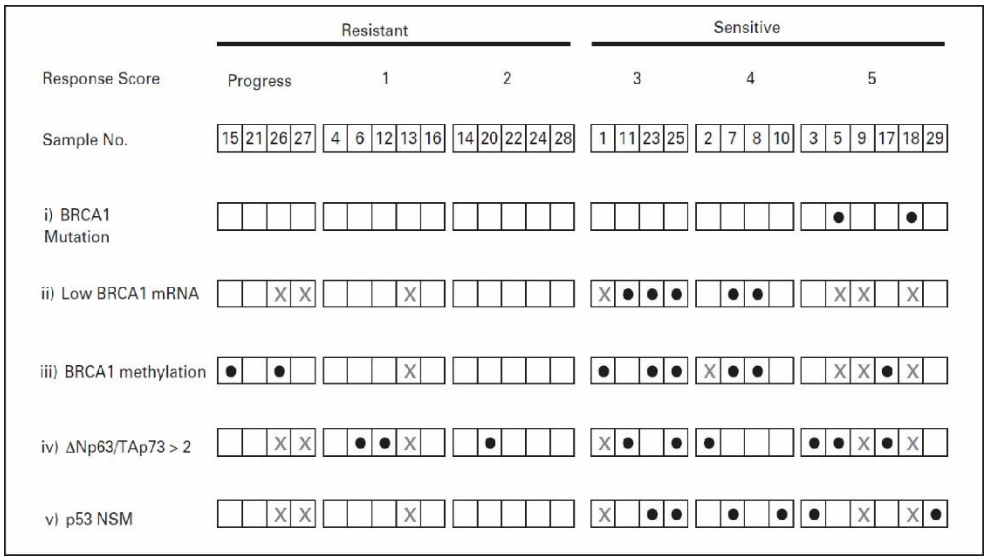

Figure 6. Potential biomarkers of cisplatin sensitivity. Assessment of potential biomarkers of cisplatin sensitivity in TNBC in the phase II clinical trial NCT00148694. Black dots indicate presence of marker, while samples with no data are indicated by gray X. Figure adapted from "Efficacy of Neoadjuvant Cisplatin in Triple-Negative Breast Cancer" by D.P Silver, 2010, Journal of Clinical Oncology, 28, pg. 1148. Copyright 2010 by American Society of Clinical Oncology. Adapted with permission.

MicroRNAs

Initially discovered in *C. elegans*, microRNAs (miRNAs) have been one of the most extensively studied molecules in their role of post-transcriptional gene regulation [44]. miRNAs are a class of endogenous, short non-coding RNAs (~18-24 nucleotides) that serve as negative regulators of gene expression by targeting specific mRNAs [45]. The majority of miRNAs are intergenic and are transcribed as primary miRNA (pri-miRNA) transcripts by RNA polymerase II. In other cases, miRNAs are intragenic, existing within introns of protein coding or non-protein coding genes and must be excised by the splicing machinery [45]. While in the nucleus, pri-miRNAs are processed by the enzyme Drosha to form precursor miRNA (pre-miRNA) hairpin structures (Figure 7). These pre-miRNAs are then actively transported to the cytosol and further processed by the Dicer enzyme, resulting in a double strand miRNA duplex [45]. As the duplex is separated, the complementary strand is degraded and the mature miRNA strand loaded into a multi-protein complex known as the RNA-induced silencing complex (RISC) by associating with the Argonaute (AGO) protein. miRNAs then bind target transcripts through base-pair complementarity of the seed sequence (6-8 nt) at the 3′ UTR (untranslated region) of the mRNA. This miRNA-RISC complex can then negatively regulate target transcripts through translational repression or transcript degradation through endonucleolytic cleavage (requires perfect complementarity of seed sequence) [45-47]. Due to the small region of complementarity needed for miRNA regulation, a single mRNA may be targeted by multiple miRNAs and a single miRNA

may target multiple transcripts, adding to their complexity. To date, over 1800 human miRNAs sequences have been identified (mirbase.org) and are predicted to regulate a third of the human genome [48].

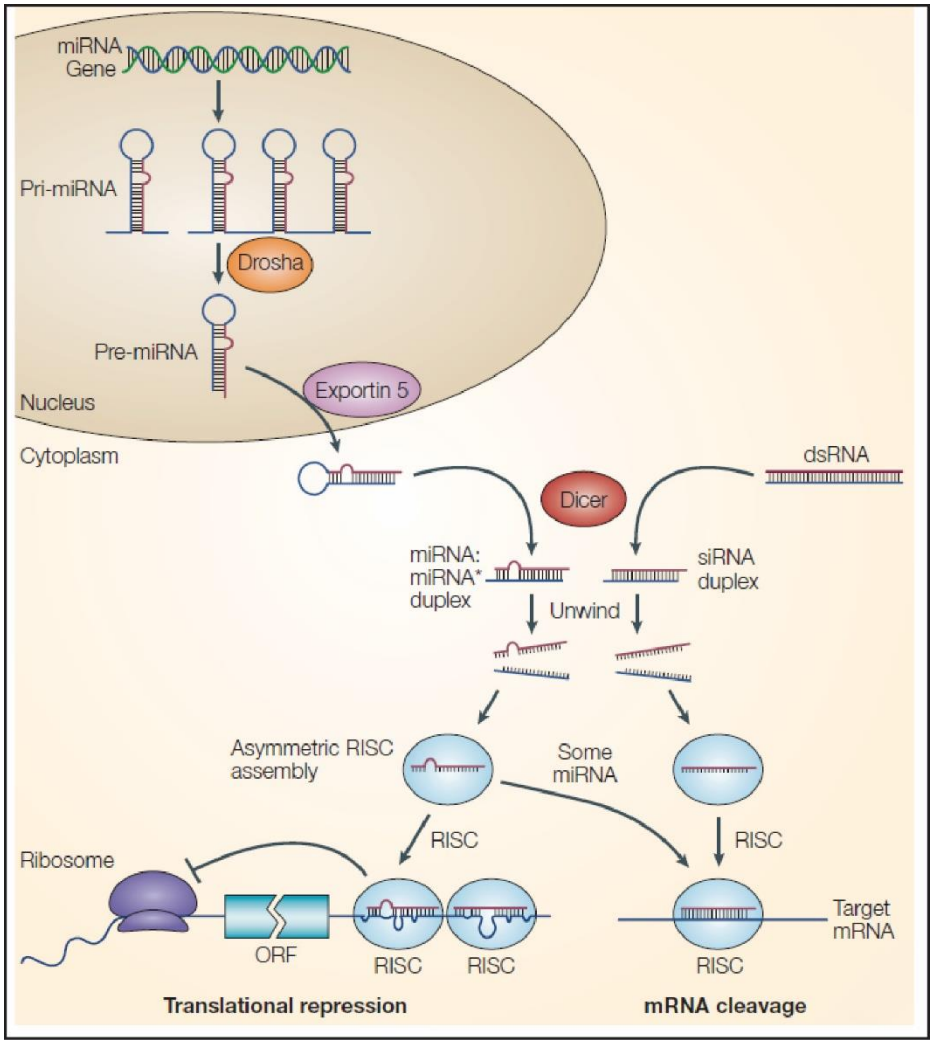

Figure 7. Biogenesis and post-transcriptional regulation of miRNAs. Figure adapted from "MicroRNAs: Small RNAs with a Big Role in Gene Regulation" by L. He and G.j. Hannon, 2004, Nature Review Genetics, 5, pg. 524. Copyright 2004 by Nature Publishing Group. Adapted with permission. [49]

The role of miRNAs have been characterized in various biological processes, including development, differentiation, apoptosis, and proliferation [49]. Accumulating evidence from studies suggest deregulation of miRNA is involved in tumor initiation and progression; one study found 72.8% of breast cancers showed miRNA gene number abnormality [50]. Considered by many to be a new class of oncogenes and tumor suppressors, miRNA promote tumorigenesis through negative regulation of genes involved in functions such as cell cycle regulation and apoptosis; and also suppress cell growth by targeting genes involved in proliferation and survival [51]. This has led to extensive research on exploring miRNAs as possible therapeutic targets in breast cancer. An example is the clinical trial NCT01829971, a phase I clinical trial to test the efficacy of MRX34, a miR-34 mimic, to treat liver cancer. Due to its involvement in a variety of breast cancer processes, miRNAs have also garnered attention as potential biomarkers of diagnosis, prognosis, and even treatment choice. Due to their small size, miRNAs have been found to be extremely stable and reproducibly detected in various biological fluids including serum and plasma [52]; carried in primarily in exosomes that could also be involved in paracrine and endocrine cell-cell communications [53].

miR-510: The miRNA of interests in this study is miR-510 (Figure 8), a miR-506 family member that is located in the intergenic region of chromosome Xq27.3; a region that has been shown to be amplified in breast cancer [54]. Tissue specificity analysis of miR-510 reveals significant overexpression of miR-510 in the testes [55]; interestingly, cisplatin has been used to successfully treat testicular cancer [34]. As a poorly conserved miRNA,

there has been limited research on miR-510. Studies have shown that miR-510 regulates serotonin receptors and may play a role in irritable bowel syndrome [56, 57]. Studies have also suggests miR-510 may play a role in various cancers, including lung cancer, gastric cancer, ovarian cancer, and lymphoblastic and myeloid leukemias [58-62]. The role of miR-510 in breast cancer was first established by Findlay et al, showing that miR-510 repressed the tumor suppressor protein PDEF in breast cancer [63]. The miR-510 mediated loss of PDEF protein expression in non-invasive breast cancer cells led to the progression into a more invasive phenotype [63]. Our lab then went on further to investigate the role of miR-510 in breast cancer [63, 64]. We have shown that miR-510 overexpression in human breast tissue samples compared to matched non-tumor samples, suggesting a role in breast cancer (Figure 9) [63]. We have also found miR-510 to be an oncogenic miRNA, promoting proliferation, migration, invasion and colony formation in breast cancer [64]. Our lab has also identified peroxiredoxin 1 (Prdx1) as a novel target of miR-510. Luciferase assays were conducted in MDA-MB-231 cells stably infected with miR_510 or scrambled controls and transiently transfected with luciferase reporter vector (empty vector; EV), vector with PRDX1 3'UTR, or vector with PRDX1 3'UTR mutated at the miR-510 seed site (PRDX mut) (Figure 10). The assay showed that an increased repression of the PRDX1 3'UTR when miR-510 was overexpressed compared to scrambled controls. However, there is no significant decrease in luciferase activity with the EV control and the PRDX mut constructs, in the presence of miR-510, suggest that miR-510 directly binds to the predicted site within the 3'UTR of Prdx1; this

luciferase assay and the downregulation of Prdx1 protein expression provides evidence that Prdx1 is a direct target of miR-510 [64]. More studies are required to further uncover the role of miR-510 in the development and progression of breast cancer.

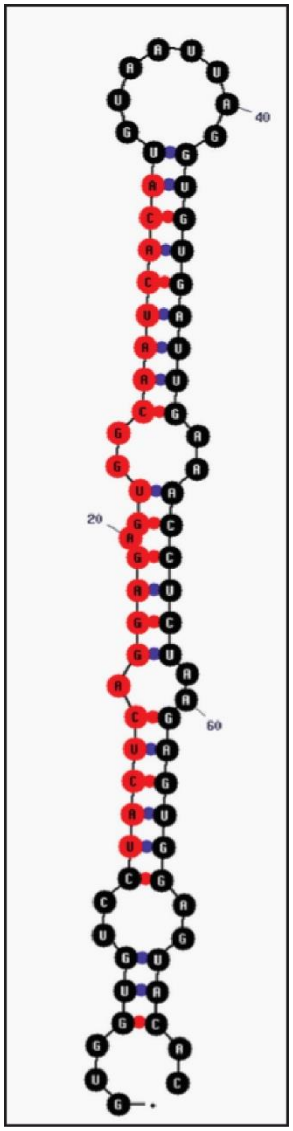

Figure 8. Predicted secondary structure of pre-miR-510 using plt22gif program (mfold program) developed by D. Stewart and M. Zuker. Sequence of mature miR-510 highlighted in red. Figure adapted from "miRNAMap 2.0: genomic maps of microRNAs in metazoan genomes" by S.D. Hsu, 2008, Nucleic Acids Research, 36. Copyright 2007 by Washington University. Adapted with permission. [55]

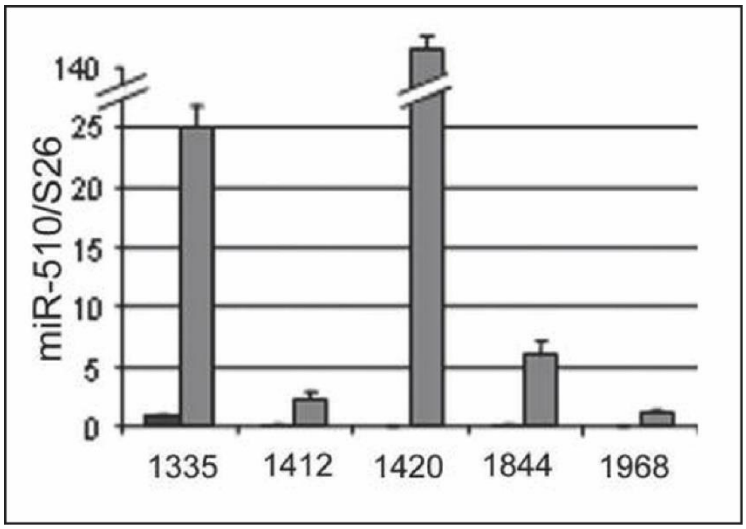

Figure 9. Quantitative real-time PCR assessing normalized miR-510 expression in human breast tumors (grey bars) compared to matched non-tumor samples (black bars). Fold differences of miR-510 in matched tumor vs. normal tissue: #1335-33.7, #1412-15.7, #1420-30884.0, #1844-45.0, #1968-32.6. Figure adapted from "MicroRNA-mediated inhibition of prostate-derived Ets factor messenger RNA translation affects prostate-derived Ets factor regulatory networks in human breast cancer." By V. Findlay, 2008, Cancer Research, 68, pg. 8505. Copyright 2008 by American Association for Cancer Research. Adapted with permission. [63]

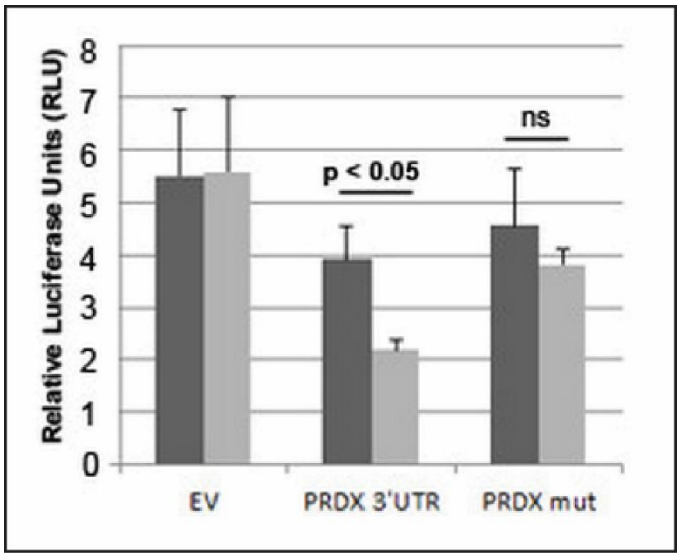

Figure 10. Luciferase assay in MDA-MB-231 cells stably transfected with scrambled control (dark grey) or miR-510 (light grey) transiently transfected with pGL3 promoter luciferase reporter vector alone (EV), the PRDX1 3'UTR reporter construct or the PRDX1 3'UTR reporter construct mutated at miR-510 seed sequence. Figure adapted from "MicroRNA-510 promoted cell and tumor growth by targeting peroxiredoxin1 in breast cancer" by Q.J. Guo, 2013, Breast Cancer Research, 15:R70, pg. 9. Copyright 2013 by Guo et al., licensee BioMed Central Ltd. Adapted with permission. [64]

Peroxiredoxin 1: Prdx1 is a thiol-specific antioxidant enzyme which protect cells from oxidative damage by reducing cellular ROS (H_2O_2 and O_2^{-}), RNS (peroxynitrite), and thiyl radicals [65]. Aside from its enzymatic activities, Prdx1 has been shown to be interact with various proteins involved in regulation of cell signaling, proliferation and apoptosis [65, 66]. Prdx1 has also been shown to act as a chaperone protein by binding to transcription factors (NF-κB and c-Myc) and modulate gene expression in the nucleus [65]. Studies have demonstrated that Prdx1 protects cells from ionizing, ultraviolet radiation [67, 68], and cisplatin induced cell death [69]. Interestingly, Prdx1 has been shown in many studies to be a physiological inhibitor of c-Abl by binding to its SH3 domain, preventing it from being phosphorylated and hindering its kinase activity [69-71]. Therefore protective role Prdx1 plays against cisplatin may be due to its suppression of c-Abl phosphorylation, inhibiting the TAp73 pro-apoptotic pathway.

Hypothesis: Elevated miR-510 expression is a predictive indicator of response to cisplatin in triple negative breast cancer (TNBC). miR-510 mediated, cisplatin-induced cell death is through activation of the TAp73 apoptotic pathway.

Specific Aim 1

Rationale

Treating TNBC remains a clinical challenge as it is not responsive to some of the most effective breast cancer therapeutics. Due to similarities between TNBC and BRCA1 associated breast cancer/basal like breast cancer, it was hypothesized that the aberrant DNA repair mechanism of TNBC could be exploited by DNA damaging therapeutics such as platinum chemotherapy agents. As this investigation progressed into clinical trials, it was determine that only a subset of TNBC patients responded to single agent cisplatin. Assessment of various biomarkers of cisplatin sensitivity proved to be unsuccessful, suggesting a need for better biomarkers to distinguish responsive patients to improve treatment outcome in TNBC. Prdx1 has been show to play a protective role against cisplatin induced cell death and our lab has validated Prdx1 as a direct target of miR-510. This suggests that miR-510 may be a potential biomarker of cisplatin sensitivity in TNBC. MicroRNAs has gained attention as potential biomarkers since dysregulation of miRNAs are known to be involved in many cancer processes. Along with their stability in many biological fluids, miRNAs are ideal non-invasive biomarkers of prognosis, diagnosis, and treatment response in cancer. The goal of this aim is to explore the efficacy of miR-510 as a biomarker of cisplatin sensitivity marker with the following tasks.

Specific Aim 1: Determine if elevated miR-510 expression correlates with cisplatin sensitivity in TNBC

Task 1: Assess cisplatin cytotoxicity in various TNBC cell lines *in vitro* to determine:

1. If a correlation exists between cisplatin sensitivity and miR-510 expression
2. If gain of miR-510 expression sensitize resistant cell lines to cisplatin
3. If loss of miR-510 expression makes sensitive cell lines more resistant to cisplatin

Task 2: Measure miR-510 levels in mouse and human serum samples to define:

1. If miR-510 could be detected in serum samples
2. If a correlation exists between miR-510 expression in tumor and serum

Methods

Cell culture: MDA-MB-231 and BT549 cell lines were a gift from Dr. Dennis Watson at the Medical University of South Carolina. SUM149 and SUM159 cell lines were a gift from Dr. Steve Ethier at the Medical University of South Carolina. MDA-MB-231 and BT549 cell lines were cultured in a 37°C, 5% CO_2 incubator and SUM149 and SUM 159 cell lines were cultured in a 37°C, 10% CO_2 incubator, all in their respective media. MDA-MB-231 cells were incubated in DMEM/High Glucose media (HyClone, South Logan, UT), with 10% fetal bovine serum (FBS) (HyClone, South Logan, UT) and 1% Penicillin/Streptomycin mixture (Fisher Scientific, Hampton, NH). BT549 cells were incubated in RPMI 1640 media (HyClone, South Logan, UT) with 10% FBS (HyClone, South Logan, UT) and 1% Penicillin/Streptomycin mixture (Fisher Scientific, Hampton, NH). SUM149 and SUM159 cell lines were incubated in Ham's F-12 media (HyClone, South Logan, UT), with 5% FBS (HyClone, South Logan, UT) and the following additives (Fisher Scientific, Hampton, NH): Insulin (5μg/ml), hydrocortisone (1μg/ml), amphotericin B (2.5μg/ml) and gentamicin (25μg/ml). Media on the cells were changed every 2-3 days and cells are passaged by using 0.05% trypsin (HyClone, South Logan, UT) when plates were ≤90% confluent.

Cisplatin cytotoxicity screen: TNBC cell lines (MDA-MB-231, BT549, SUM149 and SUM159) were plated at a density of $2.5x10^3$ cells/100μL into a 96 well plate. After 24 hours in culture conditions, the cells were treated with a range of cisplatin (MUSC Pharmacy, Charleston, SC) concentrations (0, 1, 3, 10, 30, 100, 300 and 1000μM) in

triplicates. Cells were left in culture for an additional 72 hours. After 72 hours, each well was fixed with 50µL of cold 5% trichloroacetic acid (TCA) for an hour at 4°C and then washed four times with distilled water. The wells were further stained by adding 75µl of sulforhodamine B (SRB) reagent (0.4% SRB in 1% acetic acid), incubated at 25°C for an hour and washed four times with 1% acetic acid. Next, the SRB was solubilized with 100 µl of 10mM unbuffered Tris Base solution, while shaking for 5 minutes on a shaker platform. The plate was then read in a 96-well plate reader (µQuant™, Bio-Tek, Winooski, VT) with the working wavelength 540nm. The optical density of each well was normalized to the untreated wells of each respective cell line, expressed as percent viability and plotted against cisplatin concentration. The sensitivity of cell lines were assessed by IC_{50} value, the concentration of cisplatin required to reach 50% viability, calculated by GraphPad. Each cell sample was collected and processed for RNA analysis (see below). A Pearson's Correlation test was used to determine the correlation of miR-510 expression and cisplatin sensitivity (IC_{50} cisplatin concentration) in TNBC cell lines.

Generation of stable cells: MDA-MB-231 cells were plated at a density of $5.0x10^4$ cells/well in a 24 well plate with 500µl of cell specific media with 5% heat inactivated FBS. After 24 hours in culture, a virus suspension of 2µl of Scr or miR-510 virus particles (Genecopoeia, Rockville, MD) diluted in complete media with Polybrene (8mg/ml) (Santa Cruz, Dallas, TX) was prepared for each well. The cells were infected by removing the old culture media and replacing with virus suspension and left overnight. The cells were then trypsinized and re-seeded in 6 well plates and incubated for 48

hours in regular media. Stably transduced cells were selected by replacing old media with new media containing hygromycin (Fisher, Hampton, NH) at 500µl/ml for SCR cells and puromycin (Fisher, Hampton, NH) at 0.15µg/ml for miR-510 cells. New selections media was added every 3 days until drug resistant colonies become visible by fluorescence microscopy.

In vitro mir-510 GOF and LOF: For the gain-of-function (GOF) studies, MDA-MB-231 cells stably infected with Scr or miR-510 lentivirus were plated at a density of $2.0x10^5$ cells/well in a 6 well plate. After 24 hours in culture, the cells were treated with increasing concentrations of cisplatin (0, 3 and 10µM) for another 24 hours. Cell viability was assessed by Trypan blue exclusion assay using the CelloMeter counter (Nexcelom, Lawrence, MA). Cell viability was measured as a percentage of viable cells compared to untreated wells of respective cell line. Each treatment was measured in triplicate. Cell samples were collected and processed for both protein and RNA (see below).

For the loss-of-function (LOF) studies, BT549 cells were plated at a density of $2.0x10^5$ cells/well in a 6 well plate. After 24 hours in culture, cells were transfected with either 1µg of pEZx Scr vector (control) or 1µg of pEZx anti-miR-510 (miR-510 inhibitor) vector using the XTREME Gene regent plasmid protocol (Roche, Nutley, NJ). After 48 hours, the cells were treated with increasing concentrations of cisplatin (0, 3 and 10µM) for 24 hours. Cell viability was assessed by Trypan blue exclusion assay as described above. Cell viability was measured as a percentage of viable cells compared to untreated wells. Each treatment was measured in triplicate.

RNA extraction and quantitative Real-Time PCR analysis: RNA was extracted using RNeasy Plus Mini kit (Qiagen, Valencia, CA). For miRNA analysis, 100ng of total RNA was reverse transcribed into complementary DNA (cDNA) with miR-510 specific primers using TaqMan Reverse Transcription kit (Applied Biosystems, Grand Island, NY) as per manufacturer's instructions. Quantitative real time PCR (qPCR) was then performed using 1µl of reverse transcribed cDNA, 0.5µl of TaqMan miR-510 probe (Applied Biosystems, Grand Island, NY), 5 µl qPCR master mix (BioRad, Hercules, CA) and 3.5µl of nuclease-free water as per the manufacturer's instruction on the LightCycler 480 machine (Roche, Nutley, NJ). For mRNA analysis, 1µg of total RNA was reverse transcribed into cDNA in a 20µl reaction using the iScript Kit (BioRad, Hercules, CA). qPCR was then performed to assess PRDX1 and GAPDH (for normalization) transcript expression, using 2.5µl of 1:20 dilution of reverse transcribed cDNA, 0.1µl of 20µM forward gene primer, 0.1µl of 20µM reverse gene primer, 0.1µl of UPL probe (Roche, Nutley, NJ), 5µl of qPCR master mix (BioRad, Hercules, CA) and 2.2µl of nuclease-free water, using the universal probe library (UPL) system (Roche, Nutley, NJ) as per the manufacturer's instructions on the LightCycler 480 machine (Roche, Nutley, NJ). Primer sequences and UPL probe for each gene are shown in Table 1. All samples were run in triplicates. The relative expression of each gene was quantified on the basis of Ct values in comparison to an internal standard curve for each gene using the software provided by the manufacturer (Roche, Nutley, NJ). PRDX1 expression was normalized to GAPDH.

Table 1. Primers and probes used for qPCR.

Gene	5′ primer sequence	3′ primer sequence	UPL Probe
GAPDH	agccacatcgctcagacac	gcccaatacgaccaaatcc	60
TP73	ggagggacttcaacgaagg	tcatccacatactgcgagaga	19
PUMA	ctgcctcaccttcatcagg	gcagagcacaggattcacag	79
NOXA	ggagatgcctgggaagaag	cctgagttgagtagcacactcg	67

Protein extraction and Western Blot analysis: Protein was extracted by lysis of cell pellets with RIPA buffer (50mM Tris, 150mM NaCl, 0.5% sodium deoxycholate, 1% Triton X 100 and protease inhibitor cocktail) and protein concentrations are determined by performing a BCA assay as per the manufacturers' instructions (Pierce, Fisher Scientific, Hampton, NH). 25ug protein of each sample was then separated on an SDS-PAGE gel and transferred to a PVDF membrane (Fisher Scientific, Hampton, NH) using the BioRad western blotting system (BioRad, Hercules, CA). Membranes were blocked with 10% milk in 1X Tris buffer saline-Tween (TBST) for 1 hour and then probed overnight, at 4°C with shaking, with Prdx1 (1:1000 dilution; Abcam, Cambridge, MA) and GAPDH (1:1000 dilution; Santa Cruz, Dallas, TX) antibodies. The membranes were then washed with 1X TBST and incubated with secondary antibodies (anti-rabbit, 1:2000; GE Healthcare, Piscataway, NJ) at 25°C for 45 minutes with shaking. Membranes were washed again with 1X TBST and before enhance chemilluminescence (ECL) solution was added for 5 minutes (Pierce, Fisher Scientific, Hampton, NH). Signals were detected using x-ray films (Phenix, Candler, NC) and SRX 101a film processor (Konica Minolta, Ramsey, NJ).

miR-510 expression of murine tumors: Frozen tumors (stored at -80°C) from the in vivo study previously conducted in our lab (Figure 15) were placed in a mortar with liquid nitrogen and crushed into a powder-like consistency. RNA was then extracted from the samples using TRIzol® reagent (Invitrogen, Carlsbad, CA) as per the manufacturers' instruction. RNA samples were cleaned with DNA-free Kit (Ambion, Grand Island, NY)

to remove genomic DNA. RNA concentration and quality were determined using a Nanodrop spectrophotometer. miR-510 was then reverse transcribed and levels assessed by qPCR as described above. Samples are normalized to GAPDH using the iScript kit and qPCR as described above.

Murine serum miR-510 expression: Serum samples from each experimental mouse in the in vivo study (Figure 15) were assessed for miR-510 expression. 50uL of each serum sample was used for RNA extraction using TRIzol® LS reagent (Invitrogen, Carlsbad, CA) as per the manufacturers' instructions. For miRNA analysis, 5μL of extracted RNA was reverse transcribed using miR-510 specific primers using TaqMan Reverse Transcription kit (Applied Biosystems, Grand Island, NY) as previously described. The miR-510 levels was then assessed by qPCR as previously described.

Human serum miR-510 expression: Serum samples from breast cancer patients were obtained from MUSC's Tissue Biorepository. These serum samples were processed and assessed for miR-510 expression as described above.

Correlation of murine tumor and serum miR-510 expression: A Pearson's Correlation test was performed between the relative expression of miR-510 in mouse serum and tumor samples of the in vivo experiment (Figure 15) to determine the relationship between serum and tumor expression.

Results

Previous work in our lab has tested platinum sensitivity in the non-transformed breast cell line MCF10A that were stably infected with either a scramble (SCR) or miR-510 (510) lentivirus. Treatment with cisplatin revealed that MCF10A overexpressing miR-510 had a significant increase (~3.5 –fold) in sensitivity (IC_{50} values: SCR=10.2μM vs 510=2.9μM) compared to the scramble control cells (Figure 11). Treatment with carboplatin yielded similar results (IC_{50} values: SCR=24.3μM vs 510=10.1μM). While this result is promising, it will have to be explored in a TNBC setting.

Cisplatin sensitivity correlates with miR-510 levels in TNBC cell lines. To determine the sensitivity of various TNBC cell lines to cisplatin, a cytotoxicity assay was performed with MDA-MB-231, BT549, SUM149 and SUM159 cells. Assessment of viability with a SRB assay reveals a broad range of sensitivity in TNBC cell lines. SUM149 cells showed the highest sensitivity with an IC_{50} of 0.76μM, followed by SUM159 (2.12 μM), BT549 (7.24 μM) and MDA-MB-231 (11.6 μM) (Figure 12-A). Next, miR-510 expression in these cell lines were assessed to determine if a correlation between miR-510 expression and cisplatin sensitivity exists. Analysis of miR-510 qPCR shows that SUM149 cell line expresses the highest level of miR-510, followed by SUM159, BT549 and MDA-MB-231 (Figure 12-B). Plotting miR-510 expression of these cell lines against matched cisplatin IC_{50} values reveals a Pearson's Correlation coefficient of -0.96 (Figure 12-C). This demonstrates a positive correlation between miR-510 expression and cisplatin sensitivity in TNBC cell lines.

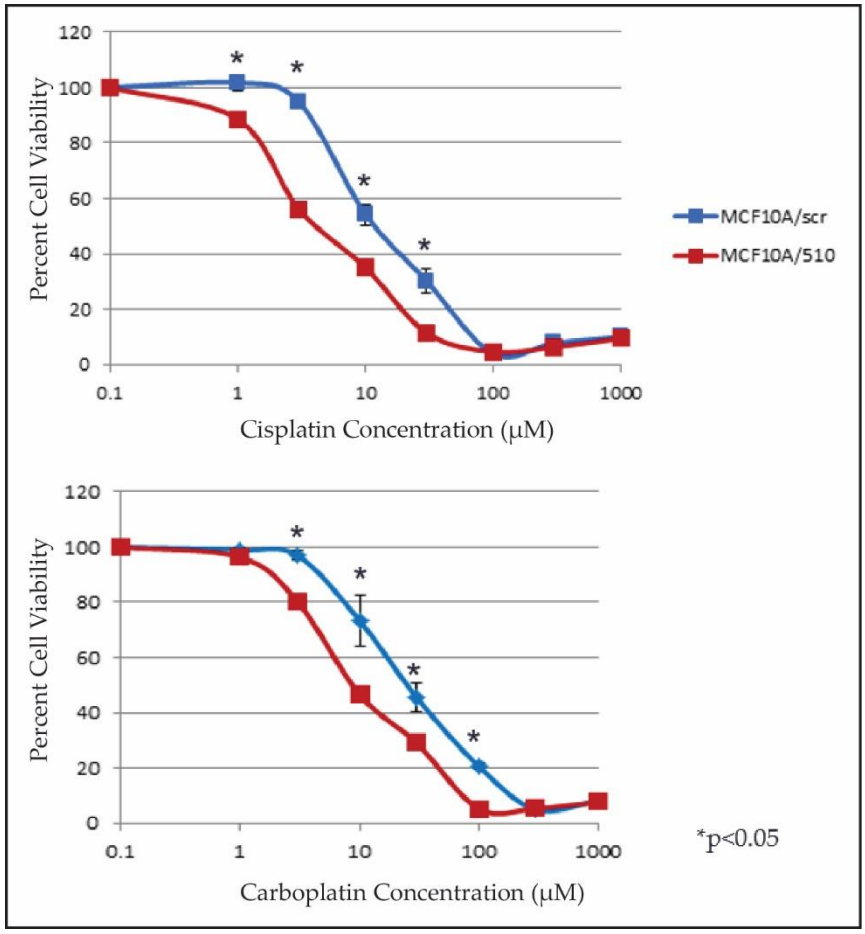

Figure 11. Cytotoxicity curve of the non-transformed MCF10A SCR & miR-510 stable cells treated with increasing concentrations of cisplatin and carboplatin for 72 hrs. IC50 values of cisplatin treatment are 10.2μM and 2.9μM for SCR and 510 cells respectively. IC50 values of carboplatin treatment are 24.3μM and 10.1μM for SCR and 510 cells respectively.

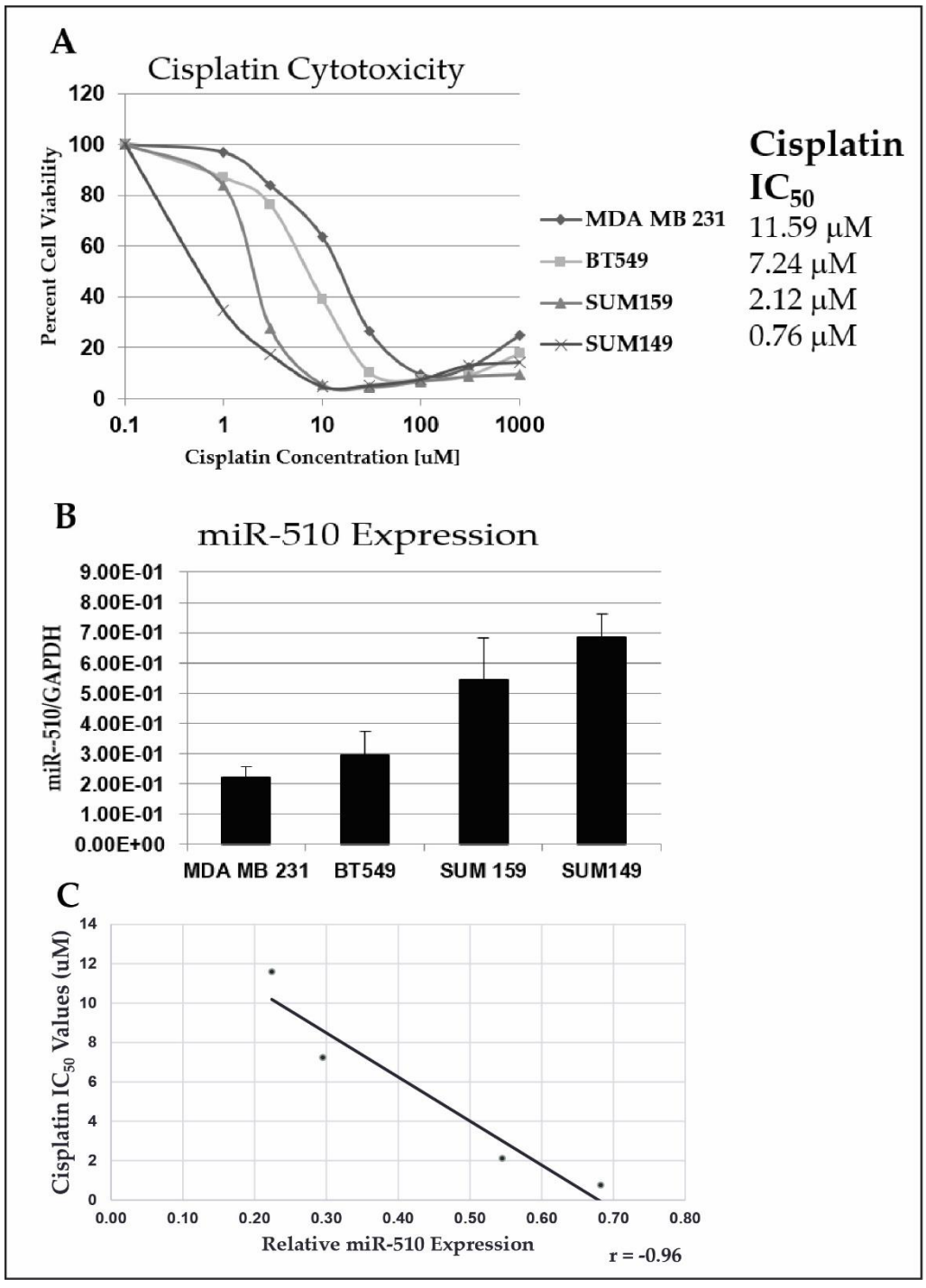

Figure 12. Cisplatin cytotoxicity curves of triple negative breast cancer cell lines treated with increasing concentrations of cisplatin for 72 hrs with IC_{50} values (A). Normalized, relative expression of miR-510 in various triple negative breast cancer cell lines though qPCR (B). Cisplatin sensitivity (IC_{50} values) plotted against matched miR-510 expression of each TNBC cell line, revealing a Pearson's Correlation Coefficient of r = -0.96 (C).

Overexpression of miR-510 sensitizes resistant MDA-MB-231 cells to cisplatin. Next, we wanted to determine if miR-510 expression could directly modulate sensitivity to cisplatin. In our GOF study, MDA-MB-231 SCR & miR-510 stables cells were treated with cisplatin to determine if overexpression of miR-510 is sufficient to sensitize a cisplatin-resistant cell line. Assessment of Trypan blue exclusion assay resulted in a statistically significant reduction in cell viability in the miR-510 overexpressing cells when compared to the SCR cells (Figure 13-A) when treated with cisplatin. Assessment of miR-510 by qPCR in these stable cells validates overexpression of miR-510 in the 510 cells (Figure 13-B). Western blot analysis of these stable cells also validates negative regulation of Prdx1 protein expression by miR-510 (Figure 13-C).

Inhibition of miR-510 causes sensitive BT549 cells to become more resistant to cisplatin. To further explore the role of miR-510 in modulating cisplatin sensitivity, we next performed a LOF study. BT549 cells were transfected with a SCR control vector or an anti-miR-510 vector and treated with cisplatin. Assessment of the Trypan blue exclusion assay reveals a statistically significant increase in cell viability in the anti-miR-510 cells, where miR-510 is inhibited, compared to SCR controls (Figure 14-A).

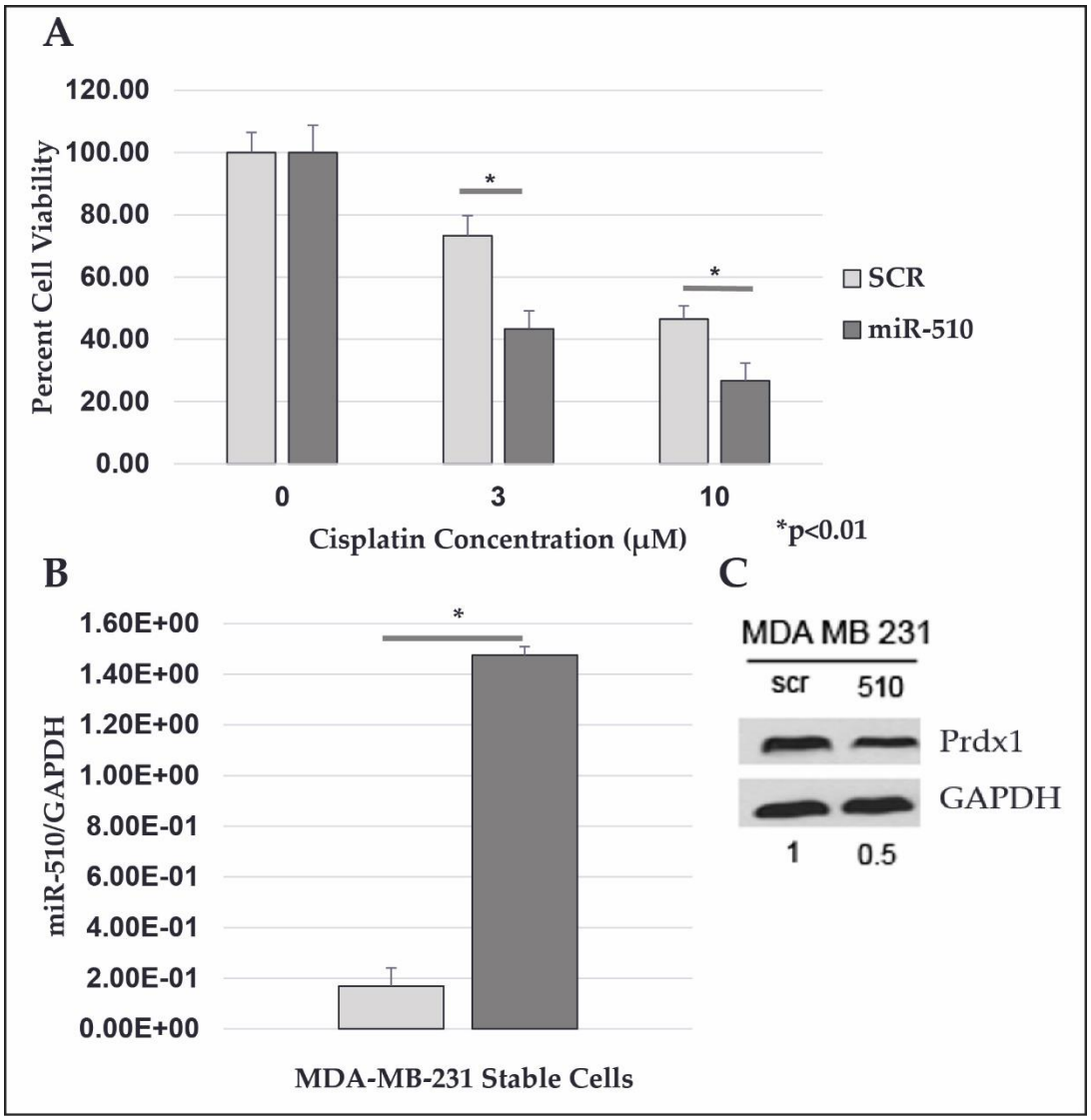

Figure 13. miR-510 GOF study in MDA-MB-231 cells. Viability of MDA-MB-231 SCR & 510 stably infected cells treated with increasing concentration of cisplatin for 24 hrs (A). Expression of miR-510 in MDA-MB-231 SCR & 510 stable cells by qPCR (B). Expression of Prdx1 protein expression in MDA-MB-231 SCR & 510 stable cells by Western Blot analysis (C).

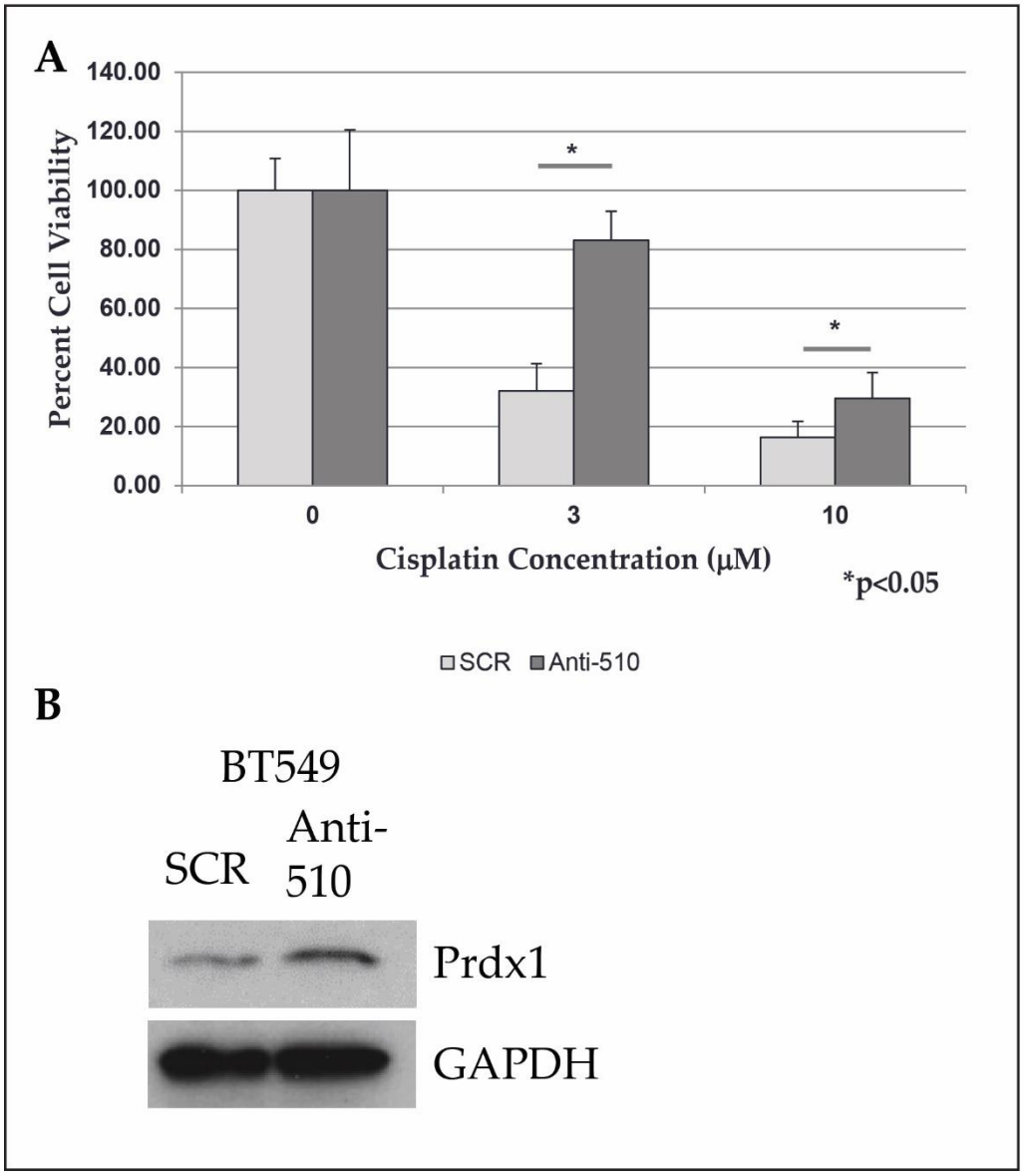

Figure 14. miR-510 LOF study in BT549 cells. Viability of BT549 cells transiently transfected with SCR or anti-510 vectors and treated with increasing cisplatin concentration for 24 hrs (A). Expression of Prdx1 protein expression in BT549 transiently transfected with SCR or anti-510 vectors by Western Blot analysis (B).

Previous work in our lab has also tested miR-510 mediated cisplatin sensitivity in an in vivo model (Figure 15). Stables of the invasive, transformed breast MDA-MB-231 (TNBC) cell lines were generated by infecting with either a scramble control (SCR) or miR-510 lentivirus. These cells were then orthotopically injected into the inguinal mammary fatpad of nude, female mice (group of n=24 per cell line). At week 1 (when tumor sizes were consistent for all groups) 12 mice from the SCR group were given intraperitoneal injection of 100μL saline while the experimental group received 100μL of 1mg/ml cisplatin (human equivalent dose of cisplatin, 15mg/m^2). The same treatment was administered to the miR-510 groups. The mice were treated twice weekly for a total of 4 weeks. Tumor volume was monitored throughout the study. Comparing the SCR and miR-510 groups treated with saline, the 510 tumors grew significantly faster and were larger at the end of the study when compared to the SCR controls; this is consistent with our previous work, supporting miR-510 as an oncogene that promotes tumor growth [63, 64]. The miR-510 mice treated with cisplatin resulted in a statistically significant reduction in tumor volume compared to the saline treated miR-510 mice; whereas no such difference was observed in the SCR groups (Figure 16). Assessment of the average tumor weights per group showed the same trend as the tumor volume (Figure 16).

MicroRNAs are the ideal biomarkers due to their stability and can be non-invasively detected in various biological fluids including serum [52]. Serum and match

tumor samples from the in vivo study were collected to assess the potential of miR-510 as a non-invasive biomarker.

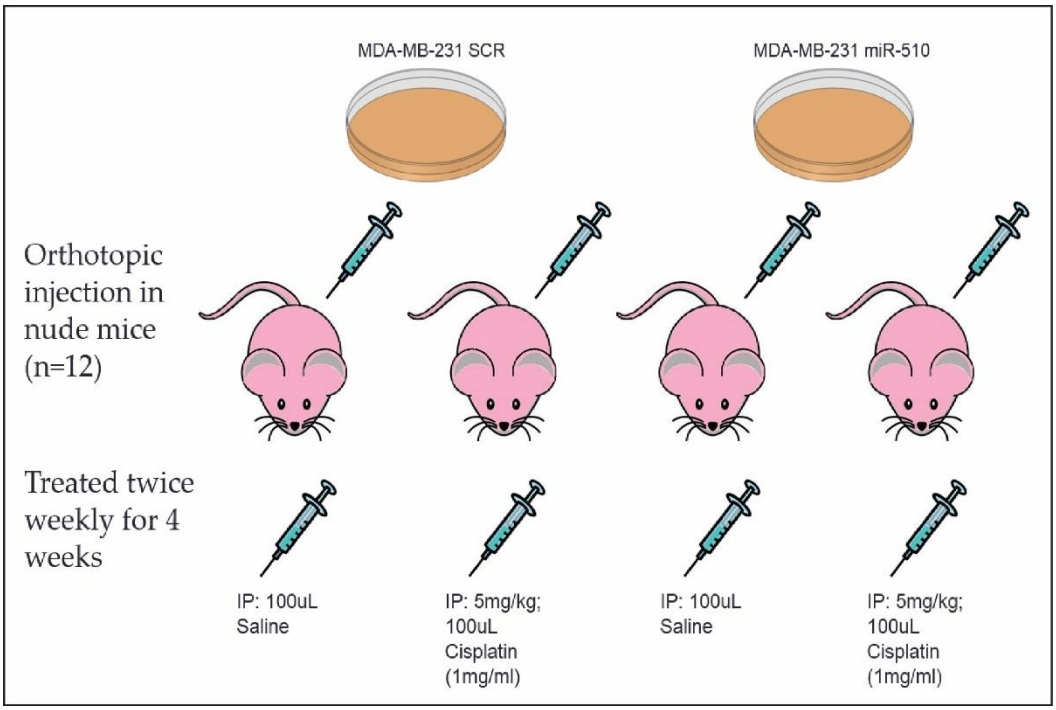

Figure 15. Schematic of in vivo study. This study was used to test miR-510 mediated cisplatin sensitivity of TNBC in an in vivo setting. MDA-MB-231 SCR & 510 stables cells were orthotopically injected into the inguinal mammary fatpad of nude, female mice (n=24 per cell line). Once the tumors reached a similar size in 1 week, mice (n=12) from SCR/510 groups were either given an intraperitoneal injection of 100µl saline or 100µl of cisplatin (1mg/ml). Treatments were repeated twice, weekly for 4 weeks.

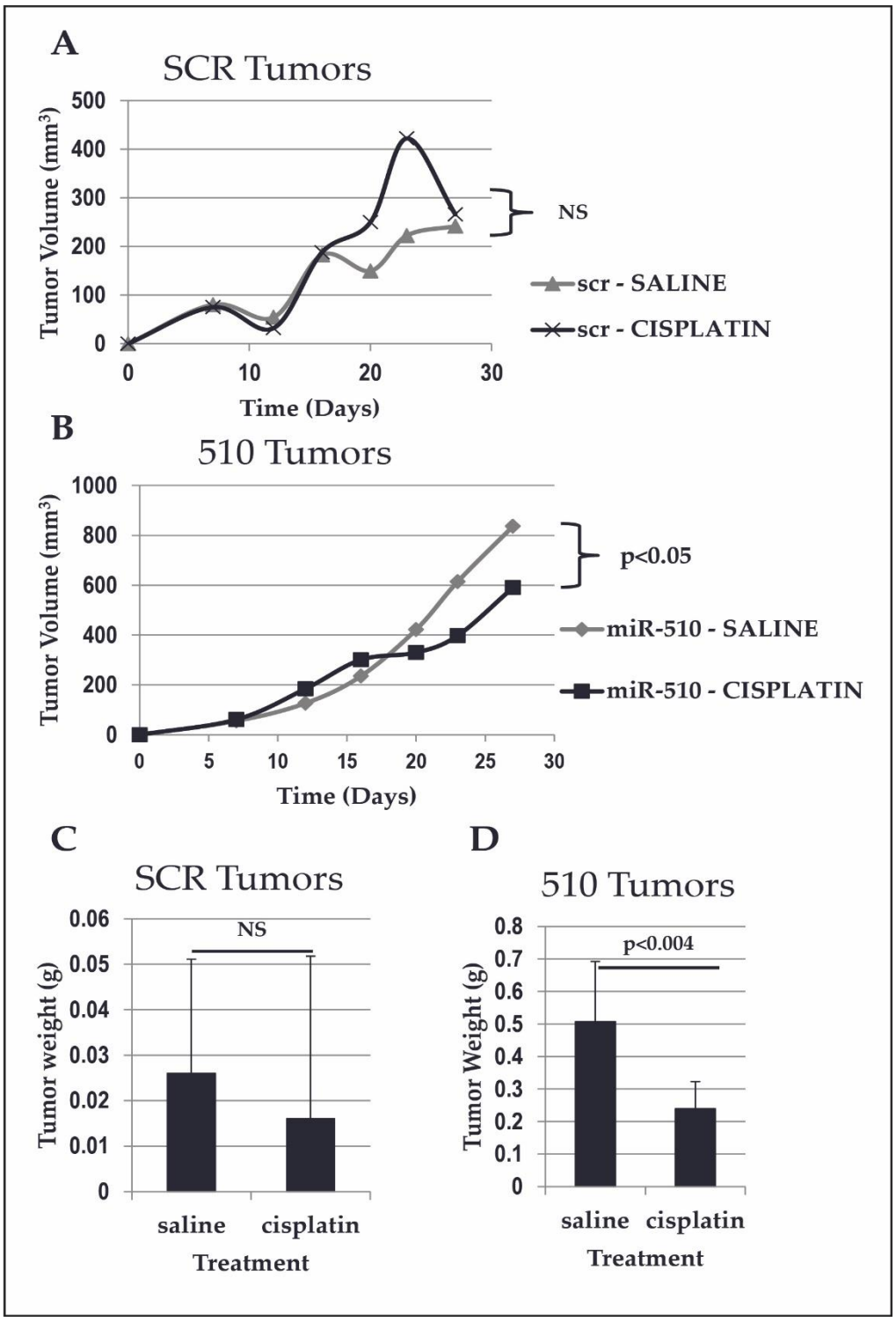

Figure 16. In vivo study results. Tumor growth curves (A & B) and final tumor weights (C & D) of SCR & 510 mice from in vivo study.

Higher miR-510 levels were detected in serum of miR-510 overexpressing tumors. To explore the potential of miR-510 as a non-invasive biomarker, we assessed miR-510 in the serum samples from the aforementioned in vivo experiment. qPCR analysis indicates a statistically significant increase in serum miR-510 in 510 tumor animals compared to SCR tumor animals (Figure 17-B). There is a broad range of serum levels of miR-510 in the 510 tumor animal samples, however, tumor expression of miR-510 in the 510 tumors displayed a broad range of expression as well (Figure 17-A).

miR-510 expression in serum correlates with tumor expression. To further explore miR-510 as a non-invasive biomarker, we wanted to determine if serum expression of miR-510 is reflective of miR-510 expression in the tumor. As expected the miR-510 tumors expressed significantly higher miR-510 than the SCR tumors (Figure 17-A). Since the serum miR-510 were higher in miR-510 tumor animals, it suggested that tumors overexpressing miR-510 will exhibit higher levels of it in the serum as well. To test this, tumor miR-510 expression were plotted against matched serum expression of miR-510. A Pearson's Correlations test yielded a Pearson's Correlation coefficient of 0.75, indicating a positive correlation between serum and tumor expression of miR-510 (Figure 17-C).

miR-510 levels detected in human breast cancer serum samples. Next, we wanted to determine if serum miR-510 could also be detected in human samples as well. Therefore we obtained breast cancer patient serum samples from MUSC Tissue Biorepository to assess miR-510 expression. qPCR analysis shows that various levels of miR-510 can be

detected in human samples as well (Figure 17-D). Serum samples of benign patients show a low basal expression of serum miR-510, whereas breast cancer patient serum samples (Grade I-III) show a higher averaged expression of serum miR-510. There does not seem to be a trend in serum miR-510 expression and tumor grade. These serum samples are from patients with various receptor subtypes of breast cancer (ER, PR, HER2), only two of which are triple negative breast cancer.

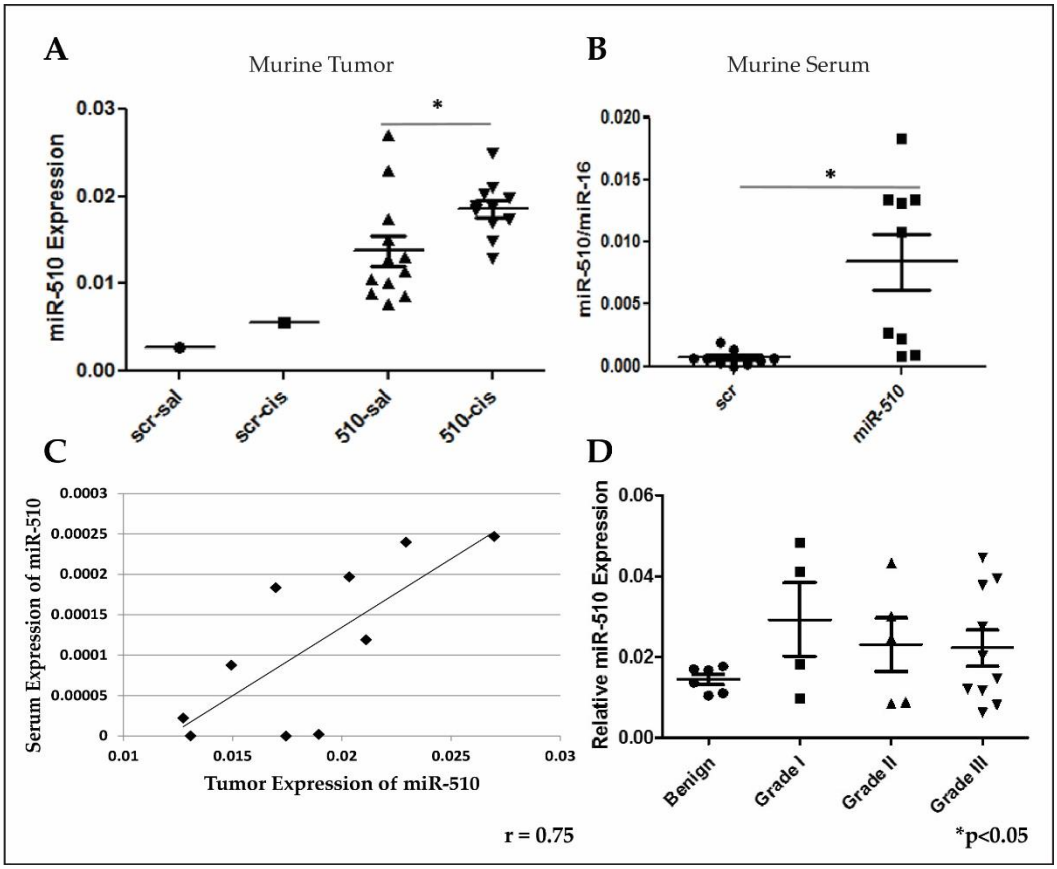

Figure 17. Serum analysis of miR-510 expression. Tumor expression of miR-510 from in vivo experiment, assessed by qPCR (A). Samples are grouped based on tumor type (SCR vs 510) and treatment (saline vs cisplatin). Serum expression of miR-510 from in vivo experiment, assessed by qPCR (B). Samples are grouped based on tumor type (SCR vs 510). Serum miR-510 expression vs tumor miR-510 expression of matched murine samples with a Pearson's Correlation coefficient of r = 0.75 (C). Serum expression of miR-510 of human breast cancer patients, assessed by qPCR (D). Samples grouped by tumor grade.

Discussion

The cisplatin cytotoxicity of TNBC cell lines reveals a broad range of cisplatin sensitivity, representative of TNBC's in the clinic. Of the four triple negative cell lines, SUM 149 cells were the most sensitive, followed by SUM159, BT549 and MDA-MB-231. This relative sensitivity to cisplatin between these cell lines were also observed in other publications [72, 73]. It should be noted that SUM149 cells harbor BRCA1 mutation and would possibly exhibit heightened sensitivity to cisplatin, regardless of miR-510 expression. However, it has been shown that the proposed cisplatin-induced p73 apoptosis pathway is active in both BRCA1-associated and sporadic TNBC (without BRCA1 mutations) [38]. It is well known that there is heterogeneity within the triple negative breast cancer subtype. Various studies have investigated elucidating subtypes within TNBCs. TCGA analysis has identified the basal-like molecular subtype of breast cancer, which significantly overlaps with TNBC [9]. Other studies have gone further, Neve et al. have further distinguish Basal A and Basal B subtypes [74]. Lehmann et al. have utilized gene expression profiling to identify six different subtypes of TNBC [73]. In this analysis, basal-like 1 subtype (SUM149) were the most sensitive to platinum chemotherapy agents. However, mesenchymal stem-like subtype (SUM159, BT549 and MDA-MB-231) still exhibit a broad range of sensitivity to cisplatin. This suggests that miRNAs may serve to further identify sensitivity in TNBC or miRNA profiling should be incorporated into the gene expression analysis to further breakdown TNBC into even more specific subtypes. Observing a tight positive correlation between miR-510

expression and cisplatin sensitivity in four TNBC cell lines provided encouraging evidence that miR-510 may serve as a potential biomarker of cisplatin sensitivity in TNBC. A limitation of this experiment is that SRB protocol stains for cellular protein content and may not a direct representation of cell viability. Assessment of cell viability with Trypan blue exclusion protocol will provide a more accurate representation of cisplatin sensitivity. Since two different assays were used to assess cisplatin sensitivity in the TNBC cell line screen and GOF/LOF experiments, the IC_{50} values for the same cell lines are different.

Correlation does not imply causation and does not provide enough evidence to show that miR-510 directly affects cisplatin sensitivity. MDA-MB-231 cells were utilized for GOF studies as they express lower levels of miR-510 and are known to be intrinsically more resistant to cisplatin. Our GOF study demonstrates that miR-510 levels are directly modulating cisplatin sensitivity in TNBC cell lines, showing that elevated miR-510 expression could sensitize resistant TNBC cells to cisplatin. While overexpression of miR-510 may have therapeutic potential, it is not advised since we have demonstrated the oncogenic potential of miR-510 in breast cancer [64]. The risk of promoting tumor growth and progression outweighs the benefits of sensitivity to cisplatin. The LOF study complements the GOF study, by showing that the loss of miR-510 is sufficient to make TNBC cells more resistant to cisplatin. A limitation of the LOF experiment is that BT549 cells may be an inappropriate choice for LOF studies as its miR-510 expression is only slightly higher than MDA-MB-231 cells. LOF studies with additional cell lines, such as

SUM159 and SUM149, would provide stronger evidence that miR-510 is directly modulating cisplatin sensitivity. The dead cells of the Trypan blue exclusion assay in the GOF and LOF studies were not quantified. Although cell viability was used to assess cisplatin sensitivity, quantification of these dead cell may provide an understanding of cell death in these studies.

GOF in MDA-MB-231 cells were validated by qPCR and showing a reduction of Prdx1 protein. The overexpression of miR-510 in the GOF study is physiologically relevant compared to overexpression of miR-510 in human breast tumor samples (Figure 9). Gain of miR-510 expression leads to a decrease in Prdx1 protein in MDA-MB-231 as expected. Since the anti-miR-510 vector does not necessarily degrade miR-510, assessment of miR-510 expression in these cells will not provide evidence of miR-510 inhibition in the LOF studies in BT549. However, assessment of Prdx1 protein expression in the LOF studies shows a restoration of Prdx1 expression, suggesting successful inhibition of miR-510 regulation. While this data is consistent with the hypothesis that attenuated Prdx1 levels, through miR-510 regulation, is sensitizing tumor cells to cisplatin, these results do not provide direct evidence and will have to be validated in the following Prdx1 rescue experiments in Specific Aim 2.

MicroRNAs are known to have many targets due to the small complementarity between the miRNA and mRNA that is required for regulation. While bioinformatic programs provide many genes that miR-510 is predicted to bind to, these targets will have to be experimentally validated. Due to the limited studies on miR-510, there are very few

validated targets for miR-510. While it is proposed here that the negative regulation of Prdx1, leading to TAp73 induced apoptosis, is the mechanism of miR-510 mediated cisplatin sensitivity, it may also be through targeting various proteins associated with platinum-resistance. Downregulation of various membrane transporters (e.g. MDR1, ATP7A, ATP7B) could lead to decreased cisplatin efflux, leading to increased sensitivity to cisplatin [34, 36, 37]. This could be tested by measuring the accumalation of cisplatin within the cell. After cisplatin binds to and damages DNA, cells could resist cell death by upregulating various repair pathways: nucleotide-excision repair, base-excision repair, mismatch repair and double-strand repair [34, 37]. This could be tested by assessing DNA damage within the cell upon cisplatin treatment. Inhibition of various signal transduction apoptotic pathways, downstream of DNA damage, could also render cells resistant to cisplatin [34, 36, 37]. This could be tested by various rescue experiments. Genes that are involved in any of these processes could also be targets of miR-510 regulation, providing an alternative hypothesis of the mechanism of miR-510 mediated sensitivity to platinum agents.

The data presented here suggests that miR-510 expression is directly modulating cisplatin sensitivity in TNBC and may serve as a potential biomarker of response in patients. MicroRNAs have emerged as candidates for novel biomarkers in cancer due to their involvement in various cancer processes particularly as noninvasive biomarkers, as they are stable in various bodily fluids and are more convenient to access than tissues from biopsies and surgeries [75]. Present in serum, associated with protein complexes or

in tumor-derived exosomes, miRNAs can be easily detected by qPCR [75-77]. Various studies have demonstrated the efficacy of using miRNA profiles in serum as biomarkers of diagnosis and prognosis in breast cancer [75] and some of these test are now available for clinical use (e.g. Prometheus Laboratories Inc and Rosetta Genomics) [76]. As expected, in mice miR-510 tumors expressed higher levels of miR-510 than SCR tumors and this expression was maintained throughout the course of the in vivo study. Interestingly, treatment of 510 tumors with cisplatin leads to upregulation of miR-510 (Figure 17-A); this trend is also evident in the SCR tumors as well. This suggests that activation of downstream pathways of cisplatin treatment may promote miR-510 expression.

Higher levels of serum miR-510 in 510 tumor animals compared to SCR samples demonstrates the potential of miR-510 as a non-invasive biomarker. However there is still high variability within the miR-510 serum group. This could be attributed to lack of standardization of serum collection, processing, normalization and interpretation; problems that hampering the application of miRNA biomarker in the clinics [76]. MicroRNAs has been shown to be secreted from cells, potentially as methods of paracrine and endocrine signaling [53], however, serum expression of miR-510 may be independent of the tumor expression of miR-510. The Pearson's Correlation Coefficient of 0.75 indicates a positive correlation between tumor expression and serum expression of miR-510. This suggests that higher levels of miR-510 in the serum is indicative of higher expression in the tumor, therefore more sensitive to cisplatin. Standardization of

the sample collection, processing and normalization, as well as more samples, is expected to improve the Pearson's Correlation coefficient. Serum detection of miR-510 is relevant to human samples as well, since we have demonstrated that varying levels of miR-510 could be detected in the serum of breast cancer patients. However, the human serum samples are from various subtypes of breast cancer. Of the twenty samples, only two were TNBC, which is similar to the distribution of triple negative subtype in all breast cancers. It should also be noted that in control serum samples, from benign tumors, all exhibited low expression of miR-510. It was expected that not all but a subset of breast cancer patients would express miR-510 as our previous studies have shown it to have an oncogenic role in breast cancer. Future studies will address whether these patients with higher expression of miR-510 are positive responders to platinum agents in the clinic. There was high variability of miR-510 expression within the human samples. It is expected that standardization of the sample collection, processing and normalization may improve specificity and reproducibility. More samples are required to determine the expression threshold to distinguish high and low miR-510 expressing patients. Limitations of these studies are the limited sample size of both in vivo murine samples and human serum samples. Matched murine serum and tumor samples from the in vivo experiment were limited, particularly in SCR tumor group. Increase in sample size would provide a stronger evidence of correlation between tumor and serum expression of miR-510. Human breast cancer serum samples were also limited, particularly samples of TNBC. More samples would allow for us to assess if miR-510

expression is differentially expressed specifically in TNBC. The issues that hamper clinical application of miRNA markers in the clinic are also limitation of this experiment. Lack of standardize protocols for sample collection and preparation for miRNA analysis may lead to inaccurate quantification. Potential factors (i.e. fasting vs non-fasting) that may affect serum expression of miRNAs has yet to be explored. There is also low correlation between various platforms that are used to measure miRNA expression. Quantitation with new platforms like Digital Drop PCR (ddPCR) that provides absolute quantitation of miRNAs may provide more accurate assessment of miRNA expression than relative quantitation through qPCR. Normalization of miRNAs, particularly extracellular miRNAs, is also not standardized. These issues need to addressed to allow for accurate and reproducible quantitation of circulating miRNA.

Specific Aim 2

Rationale

In specific aim 1, we have demonstrated the potential of miR-510 as a non-invasive biomarker of cisplatin sensitivity in TNBC. However, the mechanism of how miR-510 mediates sensitivity is yet to be elucidated. Based on our hypothesis, miR-510 negative regulation of Prdx1 allows for activation of TAp73 apoptotic pathway upon cisplatin-induced DNA damage (Figure 18). MicroRNAs are known to target many different genes due to the small complimentary seed site. As previously mentioned (specific aim 1), there are many potential mechanism of how miR-510 mediates cisplatin sensitivity. It is also possible that miR-510 mediated sensitivity is through multiple pathways. Elucidating the mechanism of this sensitivity will allow for more effective utilization of miR-510 as a biomarker. Understanding the mechanism could uncover additional markers to increase the specificity of miR-510. Understanding the pathways involved could help guide treatment choice by finding other drugs that may complement or inhibit the effectiveness of cisplatin. The goal of this aim is to experimentally uncover the mechanism of miR-510 mediated cisplatin sensitivity. We will determine if miR-510 mediated sensitivity is dependent on negative regulation of Prdx1 and if the TAp73 apoptotic pathway is activated upon miR-510 overexpression.

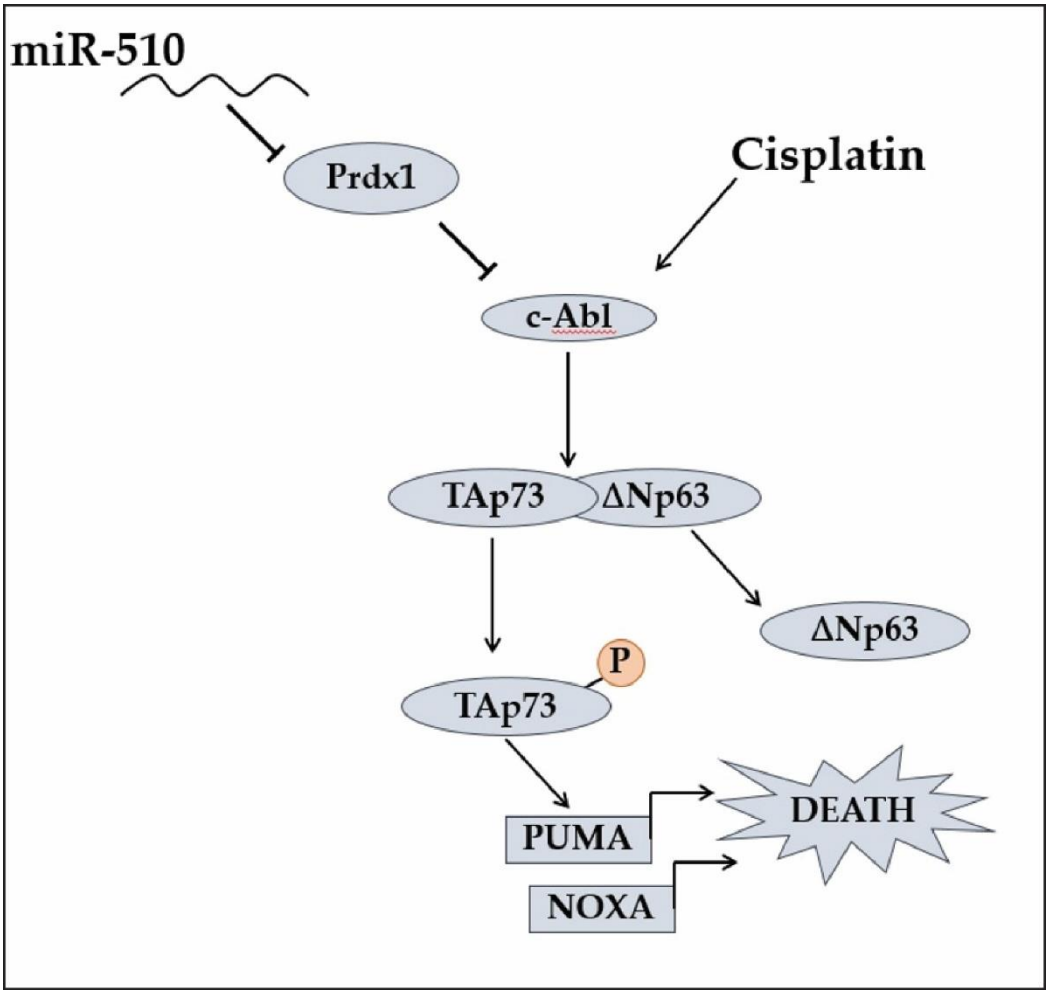

Figure 18. Model of miR-510 mediated cisplatin sensitivity in TNBC. miR-510 mediated negative regulation of Prdx1 prevents inhibition of tyrosine kinase c-Abl. Upon cisplatin treatment, c-Abl is activated and phosphorylates its downstream target, tumor suppressor TAp73. Once phosphorylated, TAp73 dissociates from its repressor ΔNp63. This allows TAp73 to transcribe pro-apoptotic genes, PUMA and NOXA, leads to cell death.

Specific Aim 2: Determine if miR-510 mediated cisplatin sensitivity is through negative regulation of Prdx1 and the subsequent activation of the TAp73 apoptotic pathway

Task 1: Characterize tumor samples from in vivo study to define:

1. If miR-510 mediated cisplatin sensitivity is through increased apoptosis or decreased proliferation in tumors
2. If miR-510 negatively regulates Prdx1 in an in vivo setting

Task 2: Perform *in vitro* mechanistic studies through RNA and protein analysis to define:

1. If miR-510 mediated cisplatin sensitivity is dependent on loss of Prdx1
2. If miR-510 mediated cisplatin sensitivity is through TAp73 pathway activation

Methods

Tissue sectioning: MDA-MB-231 SCR and 510 tumors, treated with saline or cisplatin, from the aforementioned study (Figure 15), were stored in 70% ethanol at 4°C. The tumors were dehydrated by passing through 95% ethanol twice and then 100% ethanol twice, for 1 hour each. Tumor samples were then placed into 100% toluene twice for 4 hours each. Next the sample was placed in 50:50 mixture of toluene/paraffin overnight at 60°C. The 50:50 mixture of toluene/paraffin was removed and 100% paraffin is added to the samples, twice for 2 hours each, at 60°C. The samples were embedded in cassettes with the EG1160 paraffin embedding station (Leica, Buffalo Grove, IL). The tumors are then sectioned (5 microns thickness) with Jung RM2055 microtome (Leica, Buffalo Grove, IL) and mounted onto Superfrost Plus glass slides (Fisher, Hampton, NH) for immunohistochemistry (IHC).

Immunohistochemistry: For IHC, slides were incubated in a 60°C oven for 1 hour and then placed in xylene (Fisher, Hampton, NH) for 10 minutes and then rehydrated with serial incubations in decreasing ethanol concentrations (100%, 95%, 70% and 50%) for 10 minutes each. Next, the slides were subjected to heat-induced antigen retrieval by heating slides in a vegetable steamer with 1% citrate buffer solution (Vector Labs, Burlingame, CA) for 30 minutes. Slides were further washed with 1X PBS twice for 3 minutes each and then incubated with 0.3% hydrogen peroxide (Fisher, Hampton, NH) to sequester endogenous peroxidase activity for 30 minutes. The slides were then washed with 1x PBS-Tween (0.05%) for 5 minutes before blocking with 2.5% horse

serum (Vector Labs, Burlingame, CA) at 25°C in an incubation chamber. Next, the slides were incubated with the following primary antibodies, diluted in 2.5% horse serum, overnight at 4°C: Ki-67 1:200 dilution (Santa Cruz, Dallas, TX), cleaved caspase 3 1:500 dilution (Cell Signaling, Danvers, MA) and Prdx1 1:1000 dilution (Abcam, Cambridge, MA). After overnight incubation in primary antibody, the slides were washed 3 times for 5 minutes each with 1X PBS and then incubated with peroxidase conjugated, anti-rabbit secondary antibody (Vector Labs, Burlingame, CA) for 30 minutes. The slides were washed again, 3 times for 5 minutes each with 1X PBS, stained with Diaminobenzidine (DAB) (Sigma, St. Louis, MO) for 30 seconds and then counterstained with hematoxylin (Fisher, Hampton, NH) for 2 minutes. Slides were then dehydrated with serial incubation in increasing concentrations of ethanol (50%, 70%, 95% and 100%) and xylene for 5 minutes each. Finally, slides were mounted with Permount ® (Fisher, Hampton, NH) and a glass coverslip (Fisher, Hampton, NH). Images of the slides were taken with a Nikon Eclipse 90i microscope (Nikon, Melville, NY) at 40x magnification; images of 5 random, non-overlapping fields of view were taken for each tumor. For proliferation quantitation, Ki-67 positive cells were counted and plotted as a percentage of the total number of cells in each field using ImageJ software (NIH, Bethesda, MD). The counts from 5 fields of view were then averaged and presented together with tumors of the same experimental group. The cleaved caspase 3 slides were quantified in a similar fashion, averaging total number of positively stained cells in 5 fields of view using ImageJ software (NIH, Bethesda, MD) and then averaged between all tumors of

the same experimental group. For Prdx1 quantitation, images were scored based on intensity of staining (0 for no staining, 1 for low staining, 2 for moderate staining, 3 for high staining, and 4 for very high staining). These scores were averaged and were plotted against miR-510 expression (from specific aim 1) from matched tumor. These quantitations were performed by two people and then averaged to minimize observer bias.

Prdx1 rescue experiment: MDA-MB-231 SCR & miR-510 stable cells were plated at a density of $1.5x10^5$ cells/well in two 6 well plates. After 24 hours, cells were transiently transfected either with the Prdx1 ORF vector (without 3'UTR) or an empty vector control using the XTREME Gene HP reagent protocol as previously described above (specific aim 1) (Roche, Nutley, NJ). After 24 hours, cells were treated with cisplatin (0, 3 and 10μM). After 24 hours, cell viability was assessed using Trypan blue exclusion assay as previously described. Cell pellets were collected to assess Prdx1 protein expression by western blot as described above (specific aim 1) using Prdx1 antibody (1:1000 dilution; Abcam, Cambridge, MA).

Assessment of TP73, PUMA, and NOXA mRNA: MDA-MB-231 SCR & 510 stable cells were plated at a density of $2.0x10^5$ cells/well in a 6 well plate and cultured as previously described. After 24 hours, the cells for TP73 mRNA assessment were treated with 0μM and 10μM cisplatin for 0, 6, 12, and 24 hours. Cells for PUMA and NOXA mRNA assessment were treated with 0μM and 3μM cisplatin for 0, 6, 12, and 24 hours. Cells were then collected for RNA analysis.

RNA extraction and quantitative Real-Time PCR analysis: Cells were collected and processed for RNA extraction as previously described (specific aim 1). RNA was then reverse transcribed into cDNA and TAp73, PUMA, NOXA and GAPDH transcript expression was quantified using qPCR as previously described. Primer sequences and UPL probe number for each gene are listed in Table 1. All samples were run in triplicates. The relative expression of each gene was quantified on the basis of Ct values in comparison to an internal standard curve for each gene using the software provided by the manufacturer (Roche, Nutley, NJ). All mRNA expression was normalized to GAPDH.

Immunoprecipitation and Western Blot analysis: MDA-MB-231 SCR & miR-510 stable cells were plated at a density of $1.0x10^6$ cells/10cm plate. Once the cells reached ≤ 90% confluency, cells were treated with 0 and 10μM cisplatin for 6 hours. After 6 hours, cell media was removed from the plates and washed with cold 1X PBS. Next, 500μl of RIPA buffer (specific aim 1), with Na_3VO_4 and NaF phosphatase inhibitors, were added to each plate and cell lysates were collected by scraping. Protein extraction was performed as previous described (specific aim 1). For the phospho-p73 experiment, cell lysates were subjected to immunoprecipitation with p73 antibody. Magnetic Novex™ Dynabeads™ Protein G beads (100μl) are incubated with 2 μg of p73 (s-20) antibody (Santa Cruz, Dallas, TX) for 1 hour. The magnetic bead/p73 conjugate is washed and resuspended in 100μl of 2% BSA/PBS solution. For immunoprecipitation, 500μg of protein lysate was incubated with 50μl of the magnetic bead/p73 antibody conjugate overnight at 4°C with

agitation. After incubation, beads were washed 3 times by resuspending in cell lysis buffer, isolate complex with a magnet and aspirating the supernatant. Beads were resuspended in 25µl of 2x Laemmli Buffer (Bio-RAD, Hercules, CA) and boiled for 10 minutes. Magnetic beads were captured with a magnet and the supernatant was used for Western Blot analysis as previously described (specific aim 1) using phospho-p73 antibody 1:1000 (Cell Signaling, Danvers, MA). An input blot of 50µg of total lysate was also performed using p73 (s-20) antibody (1:200; Santa Cruz, Dallas, TX) and GAPDH (1:1000; Santa Cruz, Dallas, TX), with anti-goat (1:1000; Santa Cruz, Dallas, TX) and anti-rabbit (1:2000; GE Healthcare, Piscataway, NJ) secondary antibodies, respectively. For the ΔNp63/p73 dissociation experiment, cell lysates were subjected to immunoprecipitation with p63 antibody. Magnetic Novex™ Dynabeads™ Protein G beads (100µl) are incubated with 2 µg of p63 antibody (Cell Signaling, Danvers, MA) for 1 hour. The magnetic bead/p63 antibody conjugate is washed and resuspended in 100µl of 2% BSA/PBS solution. For immunoprecipitation, 500µg of protein lysate was incubated with 50µl of the magnetic bead/p63 antibody conjugate overnight at 4°C with agitation. After incubation, beads were washed with 3 times by resuspending in cell lysis buffer, isolate complex with a magnet and aspirating the supernatant. Beads were resuspended in 25µl of 2x Laemmli Buffer (Bio-RAD, Hercules, CA) and boiled for 10 minutes. Magnetic beads were captured with a magnet and the supernatant was used for Western Blot analysis as previously described (specific aim 1) using p73 (s-20) antibody (1:200; Santa Cruz, Dallas, TX). An input blot of 50µg of total lysate was also

performed using p63 antibody (1:1000; Cell Signaling, Danvers, MA) and GAPDH (1:1000; Santa Cruz, Dallas, TX), both with anti-rabbit (1:2000; GE Healthcare, Piscataway, NJ) secondary antibodies.

Results

miR-510 mediated cisplatin sensitivity is not through decreased proliferation in vivo. Immunohistochemistry analysis of tumors from the in vivo study was performed to provide insights on the mechanism of miR-510 mediated tumor size reduction upon cisplatin treatment. To investigate whether this reduction in tumor volume is due to decrease in proliferation, Ki-67 IHC quantitation was performed. Ki-67 IHC of the tumor sections indicate similar percentage of proliferating cells between saline and cisplatin treated animals in both SCR and 510 tumors. There is a statistically significant difference of proliferation rates between the Scr and miR-510 saline treated groups (Figure 19); this is consistent with our previous publication, showing that miR-510 increases tumor cell proliferation. Similar proliferation trends between the saline and cisplatin treated groups, suggests that the role of miR-510 mediated cisplatin sensitivity may not be through inhibition of tumor cell proliferation.

miR-510 mediated cisplatin sensitivity is not through increased cleaved-caspase 3 dependent apoptosis in vivo. Next, we wanted to investigate whether miR-510 mediated reduction in tumor volume is attributed to increased apoptosis. Cleaved caspase 3 IHC of the tumor sections showed a non-significant difference in apoptosis between saline and cisplatin treatment in the SCR group. Quantification of saline/cisplatin treated 510 tumors also did not reach statistical significance (Figure 20). These data suggest that the mechanism of miR-510 mediated cisplatin sensitivity is not through an increase in cleaved caspase 3 mediated apoptosis.

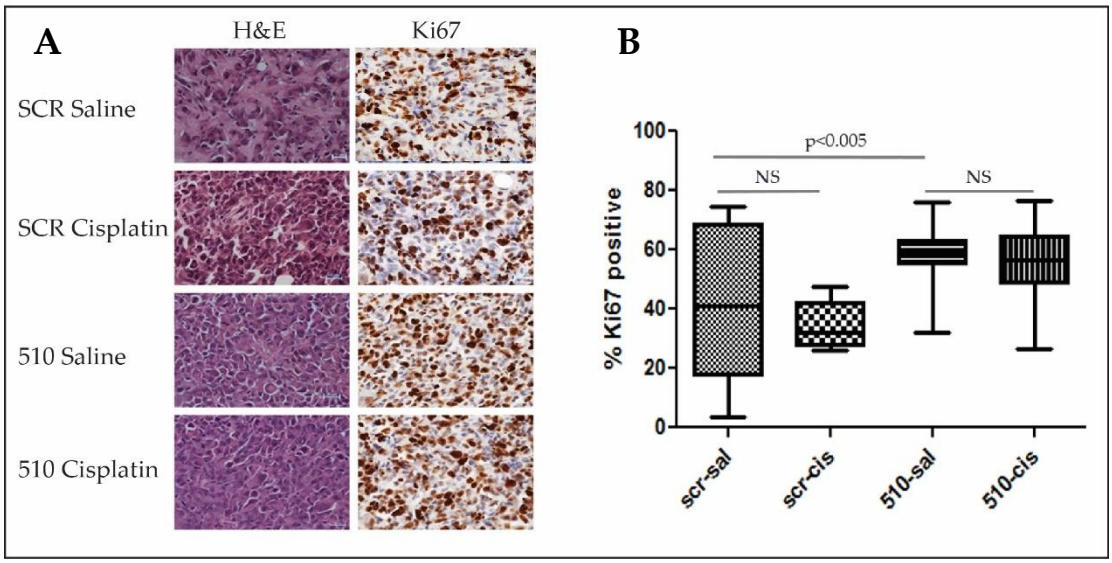

Figure 19. Proliferation profile of in vivo study murine tumors. Representative images of hematoxylin and eosin staining and Ki67 IHC staining of murine tumors (A). Quantitation of proliferation in tumors with samples grouped together based on tumor (SCR vs 510) and treatment (saline vs cisplatin) type (B).

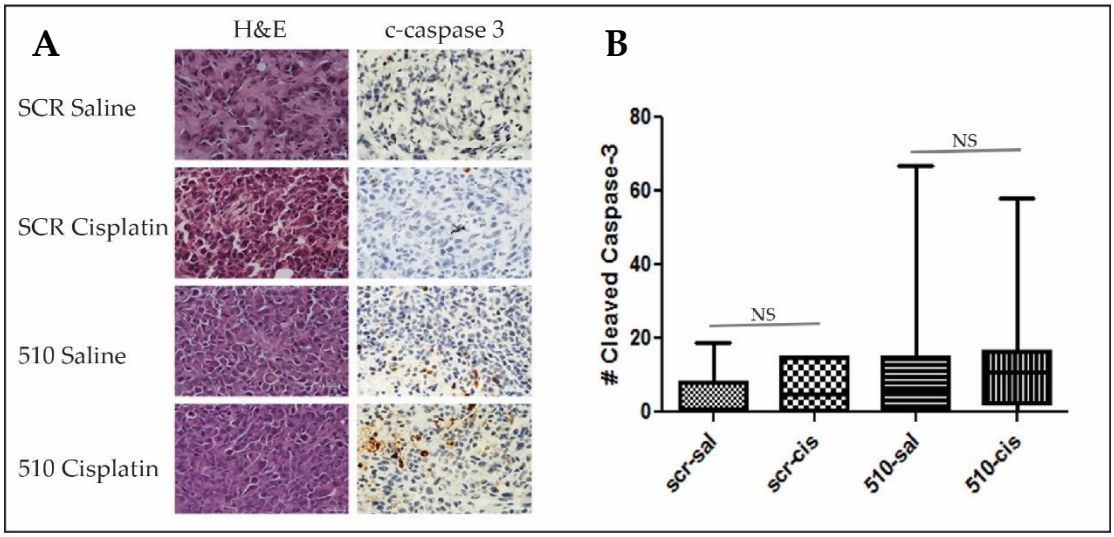

Figure 20. Cleaved-caspase 3 apoptosis profile of in vivo study murine tumors. Representative images of hematoxylin and eosin staining and cleaved-caspase 3 IHC staining of murine tumors (A). Quantitation of cleaved-caspase 3 dependent apoptosis in tumors with samples grouped together based on tumor (SCR vs 510) and treatment (saline vs cisplatin) type (B).

miR-510 negatively regulates Prdx1 expression in vivo. Assessing Prdx1 protein levels in tumor samples from the in vivo study provided evidence that miR-510 is negatively regulating Prdx1 in an in vivo setting. Quantitation of tumor Prdx1 protein expression with representative tumors from each experimental group is shown (Figure 21). Tumors were grouped together based on Prdx1 IHC score and plotted against matched tumor expression of miR-510 (specific aim 1). There is no statistical significant difference of miR-510 expression between Prdx1 IHC 1 and 2 score tumors. Tumors with Prdx1 IHC score of 3 and 4 shows a significant decrease in miR-510 expression compared to Prdx1 IHC score 2 tumors. A negative correlation between miR-510 expression and Prdx1 expression in the tumors indicates miR-510 is negatively regulating Prdx1 expression in vivo, consistent with our previous *in vitro* experiments. This also suggests that downregualtion of Prdx1 in miR-510 tumors may be a potential mechanism of miR-510 mediated reduction in tumor volume upon cisplatin treatment.

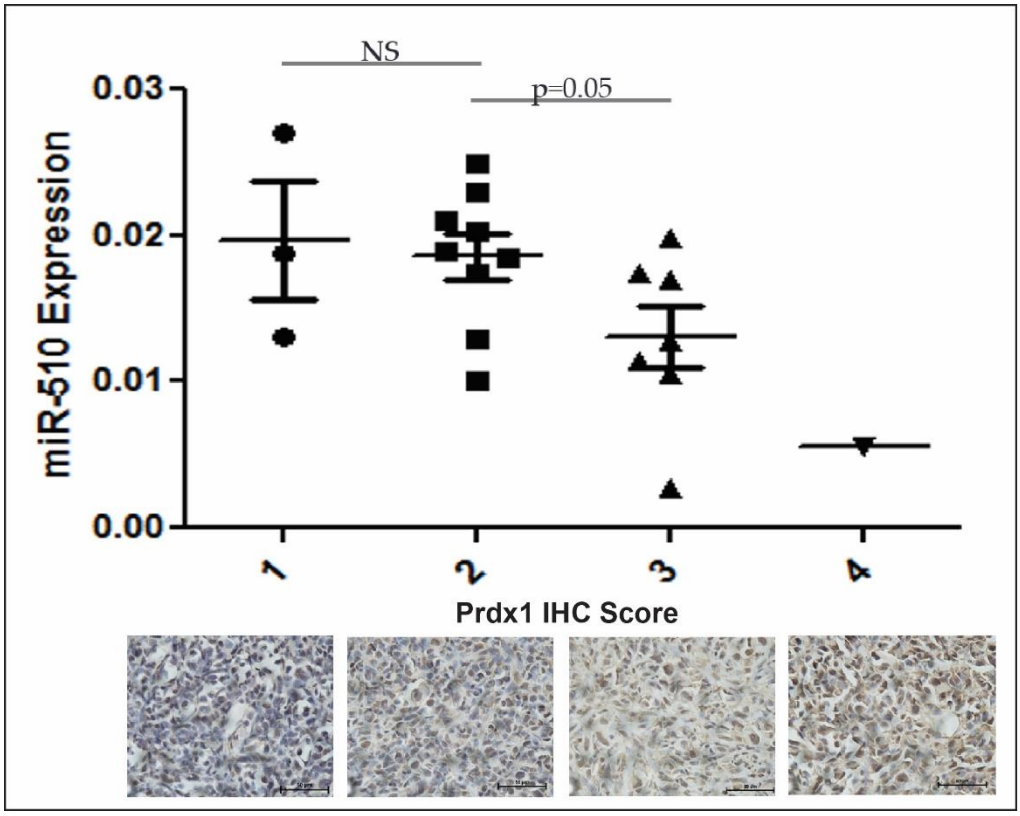

Figure 21. Prdx1 expression of in vivo study murine tumors. Quantitation of Prdx1 IHC in murine tumors from the in vivo experiment with samples grouped together based on Prdx1 IHC score. Samples are plotted against miR-510 expression of matched tumor samples. Representative images of Prdx1 IHC Scores are shown.

miR-510 mediated cisplatin sensitivity is dependent on negative regulation of Prdx1.

In order to determine whether the mechanism of miR-510 mediated sensitivity is through our proposed pathway (Figure 18), we first wanted to determine whether miR-510 mediated sensitivity is directly through Prdx1 downregulation by performing a Prdx1 rescue experiment (Figure 22-A). MDA-MB-231 SCR & 510 stable cells were transfected with an empty vector (ev) or Prdx1 rescue vector (Prdx1'). The Prdx1' plasmid encodes Prdx1 transcript without its 3' UTR, the regulatory region of which miRNAs bind, and allows for the translation of Prdx1 even in the presence of miR-510. To determine whether Prdx1 rescue was successful, a Western blot was performed (Figure 22-B). The Western blot shows a significant reduction in Prdx1 expression in 510-ev cells compared to SCR-ev cells as expected. Both SCR-Prdx1' and 510-Prdx1' cells show upregulation of Prdx1 protein expression compared to their ev cells. The 510-Prdx1' cells expression of Prdx1 is close to levels in SCR-ev cells, showing that the Prdx1' vector has successfully rescued Prdx1 expression in the 510 cells. Next, these cells were treated with increasing concentration of cisplatin and viability was assessed (Figure 22-C) to determine whether miR-510 mediated sensitivity is dependent upon Prdx1 expression. There is no difference in cisplatin sensitivity between SCR ev and Prdx1' cells. Consistent with Figure 13, there is a significant decrease in viability of the 510-ev cells compared to SCR-ev cells, indicating a higher cisplatin sensitivity with miR-510 overexpression. Treating the 510-Prdx1' cells with cisplatin revealed a similar cisplatin sensitivity as the SCR cells. This shows that rescue of Prdx1 protein expression

in miR-510 overexpressing cells ablates miR-510 mediated cisplatin sensitivity, making them as sensitive as the SCR cells. This suggests that the mechanism of miR-510 mediated cisplatin sensitivity is, at least in part, dependent upon its negative regulation of Prdx1.

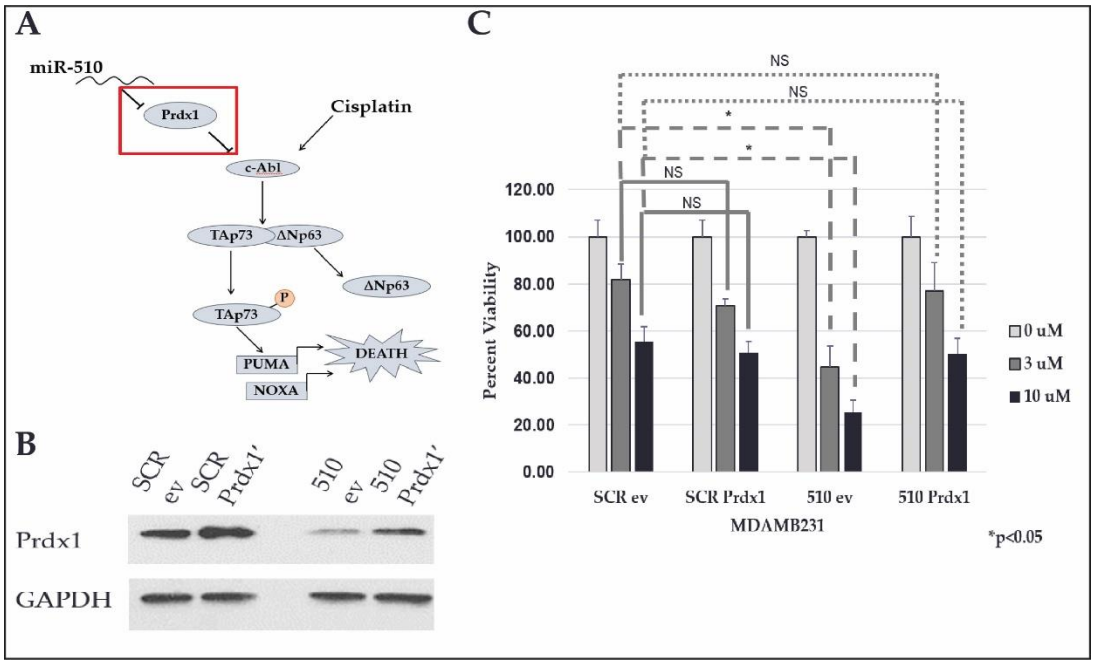

Figure 22. Prdx1 rescue experiment. Prdx1 rescue experiment will determine if miR-510 mediated cisplatin sensitivity is through its negative regulation of Prdx1, highlighted in pathway model (A). Western blot analysis of Prdx1 expression in MDA-MB-231 SCR/510 cells transfected with an empty vector (ev) control or Prdx1' rescue vector (B). Viability of in MDA-MB-231 SCR/510 cells transfected with an empty vector (ev) control or Prdx1' rescue vector, treated with increasing concentrations of cisplatin for 24 hrs (C).

Overexpression of miR-510 leads to upregulation and increased phosphorylation of TAp73 upon cisplatin treatment. Next, we wanted to determine if miR-510 overexpression leads to the phosphorylation of TAp73 upon cisplatin treatment (Figure 23-A) . MDA-MB-231 SCR & 510 stable cells, treated with 0 and 10μM cisplatin for 6 hours, were subjected to immunoprecipitation with p-73 antibody conjugated magnetic beads. Western Blot analysis with phospho-p73 antibody (Figure 23-B) reveals induction of phospho-p73 only in the 510 cells treated with cisplatin. This suggests that under these conditions the TAp73 pathway is activated only in miR-510 cells treated with cisplatin. We also wanted to assess the expression of total TAp73 by analyzing the input lysate by Western blotting (Figure 23-B). There is significant upregulation of total TAp73 protein in 510 cells treated with cisplatin, whereas this upregulation was not seen in the SCR cells. To further investigate the mechanism of TAp73 protein upregulation, qPCR analysis was performed to quantify TP73 mRNA expression at various time points after cisplatin treatment (Figure 23-C). This analysis shows similar levels of TP73 transcripts in the SCR and 510 cells at the 6 hrs time point, when the protein expression of TAp73 was assessed. Treatment for 24 hrs leads to statistically significant increase in TP73 mRNA in both SCR and 510 cells. However, a significantly higher induction of TP73 mRNA is observed in 510 cells compared to SCR cells (2.5 fold increase vs 1.5 fold increase, respectively).

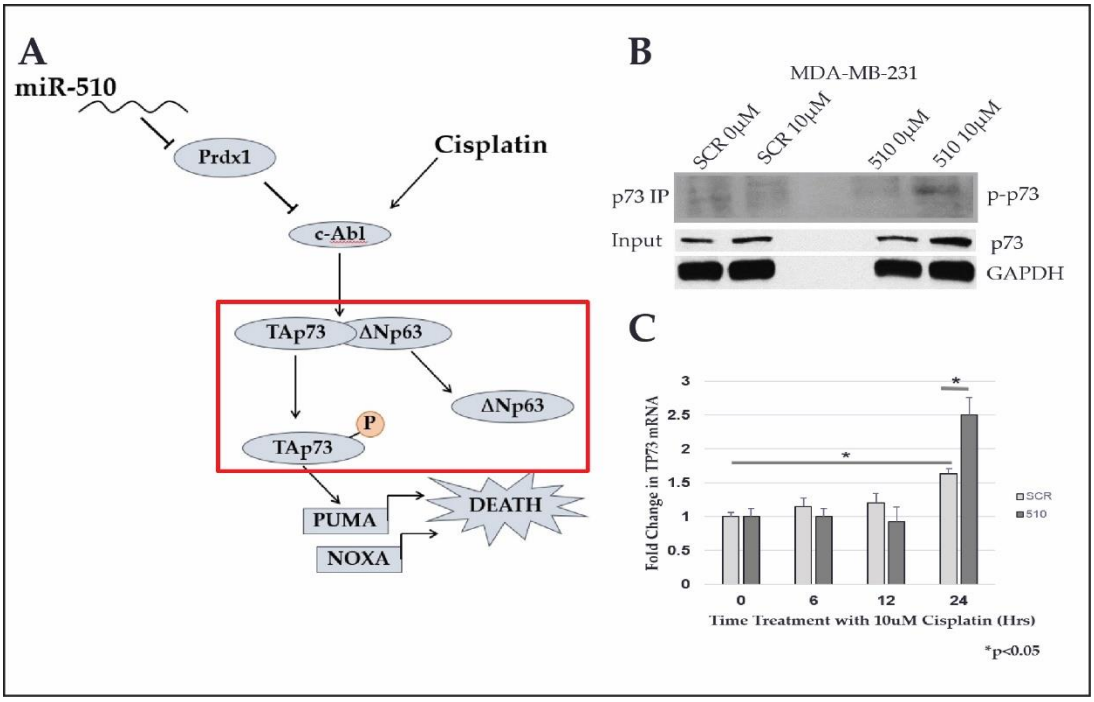

Figure 23. TAp73 phosphorylation. This experiment will provide evidence that miR-510 overexpression leads to activation of the TAp73 pro-apoptotic pathway upon cisplatin treatment through promoting TAp73 phosphorylation, highlighted in the pathway model (A). Western blot analysis of p-p73 from p73 IP lysates, along with total p73 and GAPDH protein expression in MDA-MB-231 SCR/510 cells treated with 10 μM cisplatin for 6 hrs (B). Expression of TP73 mRNA, assessed by qPCR, of MDA-MB-231 SCR/510 cells treated with 10 μM cisplatin for 0, 6, 12, and 24 hrs (C).

Overexpression of miR-510 leads to dissociation of TAp73 and ΔNp63 upon cisplatin treatment. In the proposed mechanistic pathway, phosphorylation of TAp73 leads to the dissociation of its repressor ΔNp63 (Figure 24-A). To investigate this dissociation of ΔNp63 / TAp73, cell lysates from the TAp73 phosphorylation study were subjected to immunoprecipitation with p63 antibody and then analyzed by Western blot with a p73 antibody (Figure 24-B). There was less TAp73 protein associated with ΔNp63 immunoprecipitation in the 510 cells treated with cisplatin compared to untreated cells. There was no reduction of TAp73 association with ΔNp63 in SCR cells treated with cisplatin. Western blot analysis of whole cell lysate reveals similar expression of total p63 in all the samples. This data provides additional evidence that overexpression of miR-510 leads to upregulation of the proposed mechanistic pathway upon cisplatin treatment.

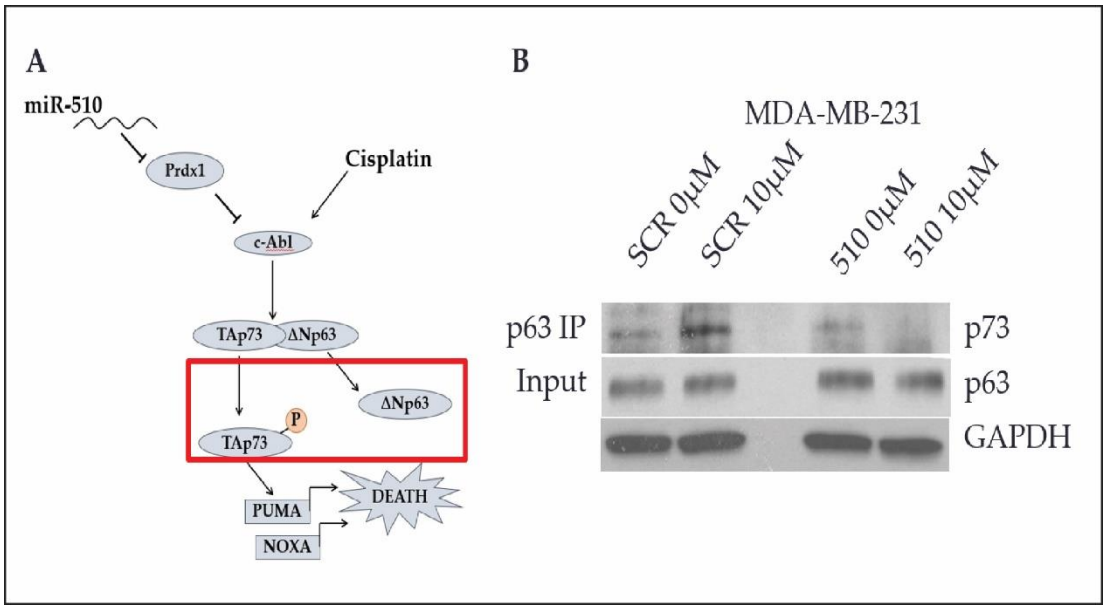

Figure 24. Dissociation of ΔNp63/TAp73. This experiment will provide evidence that miR-510 overexpression leads to activation of the TAp73 pro-apoptotic pathway upon cisplatin treatment through promoting dissociation of ΔNp63/TAp73, highlighted in the pathway model (A). Western blot analysis of p73 from p63 IP lysates, along with p63 and GAPDH protein expression in MDA-MB-231 SCR/510 cells treated with cisplatin for 6 hrs (B).

miR-510 overexpression leads to induction of p73 target genes in response to cisplatin treatment. To further uncover evidence of TAp73 activation, we assessed PUMA and NOXA mRNA expression, downstream target genes of TAp73 (Figure 25-A). PUMA and NOXA transcript levels were assessed by qPCR in MDA- MB-231 SCR & 510 cells treated with 3μM cisplatin for 0, 6, 12, and 24 hrs. A statistically significant increase in PUMA mRNA (Figure 25-B) was observed in miR-510 overexpressing cells after 6, 12, and 24 hrs of cisplatin treatment. A significant increase of NOXA mRNA (Figure 25-C) was observed in miR-510 overexpressing cells after 24 hrs of cisplatin treatment. This provides evidence that TAp73 transcriptional activity and the p73-dependent apoptotic pathway is upregulated in the presence of miR-510 upon treatment with cisplatin.

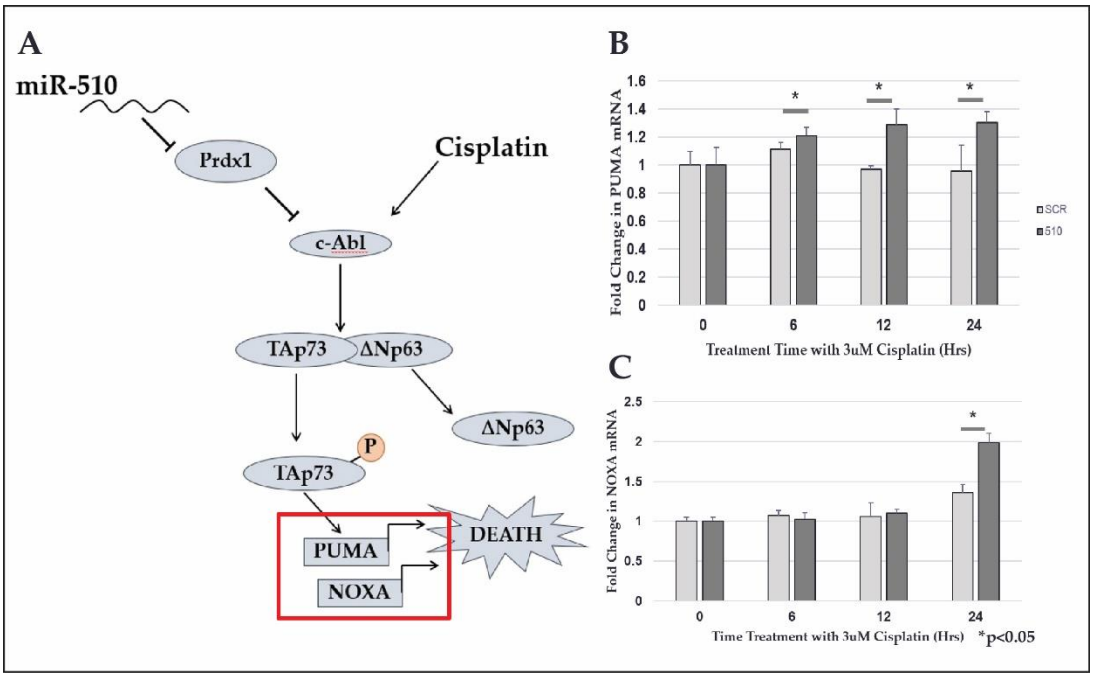

Figure 25. Induction of pro-apoptotic TAp73 genes. This experiment will provide evidence that miR-510 overexpression leads to activation of the TAp73 pro-apoptotic pathway upon cisplatin treatment through induction of pro-apoptotic, TAp73 target genes, highlighted in the pathway model (A). Expression of PUMA (B) and NOXA (C) mRNA, assessed by qPCR, of MDA-MB-231 SCR/510 cells treated with 3 μM cisplatin for 0, 6, 12, and 24 hrs.

Discussion

The data from the previous aim has demonstrated that miR-510 expression could directly modulate cisplatin sensitivity. Next, the mechanism of miR-510 mediated cisplatin sensitivity was explored to determine if it is through the proposed p73 apoptotic pathway. This will allow for more specific identification of sensitive patients by assessing additional markers (i.e. p53 mutations, c-Abl mutations, ratio of ΔNp63/TAp73) as well as identify agents that may reduce efficacy of this pathway (i.e. kinases inhibitors). To gain insight on the mechanism of miR-510 mediated cisplatin sensitivity, tumors from the in vivo study were analyzed. Reduction of tumor size could be attributed to decreased proliferation of the tumor, increased cell death (apoptosis or necrosis) or a combination of both. While the main mechanism of cisplatin is inducing apoptosis, cisplatin could also induce cell cycle arrest (S phase and G2 phase) [36]. Ki-67 is used as a marker for the assessment of proliferation, a non-histone nuclear protein that is closely linked with cell cycle and proliferation, emerging as a prognostic marker in various cancers, including breast cancer [78]. A significant increase in proliferation in the 510 overexpressing tumors compared to SCR tumors is consistent with our previous publication, that miR-510 promotes proliferation in breast cancer [64]. The similar proliferation profiles between saline and cisplatin treated 510 tumors suggests that cell cycle arrest and inhibition of proliferation may not be the main mechanism of miR-510/cisplatin induced reduction of tumor volume in the in vivo study.

The tumors were then quantified for apoptosis, to provide evidence to determine if miR-510/cisplatin mediated reduction of tumor volume is attributed to increased apoptosis. Cisplatin has been shown to induce both necrosis and apoptosis as main modes of cell death [36]. The mode of cell death is dependent upon the concentration of cisplatin. One study showed that treatment of mouse proximal tubular cells with a high concentration of cisplatin (800μM) leads to necrotic cell death, whereas, treatment with low concentration of cisplatin (8μM) leads to apoptotic cell death [79]. Since the concentrations of cisplatin used in the *in vitro* studies are similar to the low concentration used in the aforementioned study, it is believed that apoptosis may be the main mechanism of cisplatin induced cell death in miR-510 overexpressing cells. Cisplatin insult has been shown to promote activation of the caspase family of proteases that ultimately commits the cell to undergo irreversible apoptotic cell death [36, 80]. Activation of initiator caspases (caspase 8 and caspase 9) leads to cleavage and activation of effector caspases (caspase 3 and caspase 7). Activated effector caspases then begin proteolytic cleavage of various cellular proteins leading to apoptosis of the cell [36, 81]. The lack of statistically significant difference of cleaved- caspase 3 in treated and untreated 510 tumors suggests that miR-510 mediated sensitivity may not be through cleaved-caspase 3 pathway of apoptosis. Since cleavage of caspase 3 is a transient process, our experimental design and low sample numbers may limit the ability to detect the differences in cleaved caspase 3 by IHC. Another possible explanation is that caspase-dependent apoptosis is not the main mechanism of miR-510 mediated cell death

in these tumors upon cisplatin treatment. Various studies have demonstrated caspase-independent pathways of apoptosis upon cisplatin treatment [82, 83]. Lack of statistically significant differences in cleaved-caspase 3 between saline/cisplatin treated tumors and the relatively low expression of cleaved-caspase 3 in all tumor groups suggests that cisplatin induced apoptosis in these tumors are not caspase dependent. Another possible explanation is that cisplatin is known to interact with various molecules in the cell besides DNA, including molecules that are involved in maintaining cellular metabolism and energy supply. This could lead to situations, such as ATP depletion that lead to cell death prior to completion of apoptotic pathway signaling. This leads to cisplatin-induced cell death that lacks characteristic morphological and biochemical properties of "classic" apoptosis [37, 80]. A better understanding of apoptosis within these tumors could be assessed by employing additional IHC markers of apoptosis: cleaved cytokeratin-18, cleaved lamin A, cleaved poly (ADP ribose) polymerase (PARP) and translocation of apoptosis-inducing factor (AIF) [81]. An alternative method of apoptosis detection in tissue samples, like the terminal deoxynucleotidyl transferase-mediated dUTP nick endlabeling (TUNEL) assay [84], could also be employed. Limitation of these IHC analysis is the small sample size of murine tumors from the in vivo study. A larger sample size would provide a better representation of proliferation and apoptosis within these tumors.

In our previous publication, Prdx1 was experimentally validated as a direct target of miR-510 [64]. However, this direct negative regulation has not been verified in an in

vivo setting. We demonstrated here that miR-510 negatively regulates Prdx1 in an *in vivo* setting and could be a possible mechanism of cisplatin induced reduction in volume of miR-510 tumors. However, the negative correlation between miR-510 and Prdx1 does not provide direct evidence that downregulation of Prdx1 is directly leading to cisplatin sensitivity.

Quantitation of in vivo tumors indicate miR-510 mediated sensitivity is most likely through downregulation of Prdx1 and subsequently inducing apoptosis upon cisplatin treatment. In the proposed pathway, cisplatin-induced DNA damage leads to c-Abl activation and phosphorylates downstream target, TAp73. Upon phosphorylation of TAp73, it dissociates from its repressor ΔNp63. This allows TAp73 to become transcriptionally active, transcribing genes to promote cell cycle arrest and apoptosis. We hypothesize that expression of miR-510 allows for upregulation of this pro-apoptotic pathway upon cisplatin treatment in TNBC. As miRNAs are known to target many genes simultaneously, miR-510 mediated sensitivity may be through negative regulation of other genes besides Prdx1 or even a combination of genes. As previously mentioned in specific aim 1, miR-510 mediated sensitivity maybe through negative regulation of genes involved in cisplatin resistance. Negative regulation of membrane transporters involved in efflux of cisplatin, DNA damage recognition and repair proteins, proteins of pro-survival pathways and inhibitor proteins of pro-apoptotic pathways could all potentially lead to cisplatin sensitivity. A bioinformatics screen could be used to identify candidate target genes of miR-510 that are involved in these processes. To determine

whether miR-510 targets these proteins, it would require experimental validation through luciferase assay or RNA binding protein immunoprecipitation microarray (RIP-Chip). Restoration of cisplatin resistant in miR-510 overexpressing cells by rescuing Prdx1 expression provides evidence that miR-510 mediated cisplatin sensitivity is dependent upon downregulation of Prdx1. However, cisplatin is known to activate many downstream pathways and miR-510 could potentially target many proteins of these pathways. It is unlikely that negative regulation of Prdx1 is the only mechanism of miR-510 mediated sensitivity, but it may be the main mechanism. This will have to be explored through identifying novel targets of miR-510 and rescue experiments.

After demonstrating that Prdx1 plays a role in miR-510 mediated cisplatin sensitivity, we further explore the mechanism of cell death by examining pathways downstream of Prdx1. It is proposed that downregulation of Prdx1 prevents its inhibition of c-Abl, allowing for increased phosphorylation of c-Abl targets, TAp73. Besides c-Abl, Prdx1 has been shown to play a role in other pathways that may contribute to increased resistance to cisplatin. The main function of Prdx1, as an antioxidant enzyme, is to protect cells from oxidative damage by reducing cellular ROS, RNS, and thiyl radicals [65]. Through its antioxidant activities, Prdx1 has been shown to protect cells from ionizing and ultraviolet radiation induced cell death [67, 68]. Like many other chemotherapy drugs, cisplatin is pro-oxidant and known to lead to ROS accumulation and subsequent cell death [85, 86]. However, studies of cisplatin resistance through reduction of cisplatin-induced ROS formation is mainly attributed to glutathione,

another family of thiol-peroxidases [85, 86]. This implies that the role of Prdx1 in cisplatin sensitivity is not through reduction of its peroxidase activity. The catalytic activity of Prdx1 is dependent upon two cysteine residues, which could be mutated to serine to inhibit its peroxidase activity [87]. This will help determine whether the role of Prdx1 is independent of its peroxidase activity. Previous studies have demonstrated that, aside from its enzymatic activities, Prdx1 has been shown to be interact with various proteins involved in regulation of cell signaling, proliferation and apoptosis [65, 66]. Prdx1 has been shown to inhibit proteins such as ASK1, JNK, p38, ERK, $p66^{Shc}$ and c-Abl [65, 69], many of which are also involved in downstream signaling of cisplatin-induced DNA damage [36, 37]. Prdx1 has also been shown to act as a chaperone protein by binding to transcription factors (NF-κB and c-Myc) and modulate gene expression in the nucleus [65]. While all these interactions of Prdx1 could contribute to its role in cisplatin sensitivity, we focus on the interaction between Prdx1 and c-Abl. In the proposed pathway, cisplatin-induced DNA damage activates c-Abl to transmit DNA damage signals from the nucleus to the cytoplasm. Downregulation of Prdx1 prevents inhibition of c-Abl kinase activity allowing it to phosphorylate its downstream effector, TAp73. Studies have shown phosphorylation of tyrosine residue on TAp73 is required for it dissociation from ΔNp63 and its activation [38]. Immunoprecipitation with p73 antibody and subsequent Western blotting with phospho-p73 antibody reveals that there is an increase in p73 phosphorylation only in miR-510 overexpression cells treated with cisplatin. This suggests that there is an increase c-Abl kinase activity with miR-510

overexpression upon cisplatin treatment. The phospho-p73 antibody used lacks specificity, as it has been shown to cross-react with phospho-p63 due to high homology between the two proteins. Therefore, immunoprecipitation with a p73 antibody was employed to enrich p73 and help improve specific detection of phospho-p73 within our samples. Various proteins have been shown to phosphorylate p73 besides c-Abl, including PKCδ, Chk1 and JNK [88, 89]. However, these are all serine/threonine kinases that only phosphorylate serine/threonine residues on p73. The phospho-p73 antibody used recognizes phospho-tyrosine residue and since c-Abl is the only tyrosine kinase known to phosphorylate p73, this suggests that c-Abl kinase activity is enhanced during miR-510 overexpression upon cisplatin treatment. Based on our model and previous publications, phosphorylation of the tyrosine residue on TAp73 allows it to dissociate from its repressor ΔNp63 [27, 38]. This dissociation is crucial for TAp73 activation and transcription of downstream targets PUMA and NOXA and subsequent sensitivity to cisplatin. Evidence from the p63 immunoprecipitation study suggests that miR-510 overexpression allows for increased dissociation of TAp73 from ΔNp63 upon cisplatin treatment.

It has been previously shown that cisplatin induced activation of c-Abl leads to increased TAp73 protein levels, most likely through increasing the half-life of the protein [90]. Other studies has demonstrated that phosphorylation of TAp73 by c-Abl is required for the stabilization of the protein, thus increasing its accumulation in cells after cisplatin treatment [89, 91]. Since we have shown a higher level of phosphorylated

TAp73 and total TAp73 in the 510 cells upon cisplatin treatment, it suggests that increase in TAp73 accumulation is attributed to p73 protein phosphorylation and stabilization. Although increase in total TAp73 could allow for higher phosphorylation as well, previous studies showing that phosphorylation of tyrosine-99 leading to stabilization suggests that phosphorylation occurs prior to accumulation, not vice versa. The tyrosine residue could be mutated to an alanine to further determine if stabilization and accumulation of TAp73 is dependent on its phosphorylation. Acetylation of TAp73 by acetyltransferase p300 has also been shown as an alternative mechanism of p73 stabilization [89]. Increase in total TAp73 protein expression could also be attributed to various mechanism besides protein stabilization, including: increased transcription, mRNA stabilization, and increased translation. Since TP73 mRNA does not increase in 510 cells treated with cisplatin for 6 hrs (Figure 23-C), it suggests that the mechanism of TAp73 protein expression upregulation may be through protein stabilization. The increase in protein expression could also be explained by increase translation of TAp73 mRNA in the 510 overexpressing cells. This could be further tested with assays such as polysome profiling or pulsed SILAC. Treatment of cisplatin for 24 hours leads to significant increase in TP73 mRNA in both the 510 and SCR cells (Figure 23-C), although it is significantly higher in the 510 cells (2.5 fold vs 1.5). This upregulation of TP73 mRNA could be through a parallel pathway or downstream of the TAp73 pro-apoptotic pathway. To rule out the possibility that TAp73 upregulation is due to mRNA upregulation, the cells could be treated with a transcription inhibitor to see whether

TAp73 protein expression is dependent on increased transcription. The increase in TAp73 protein levels may indicate increase protein activity, but will have to be further investigated. While kinase activity of c-Abl is required for the cisplatin-induced cell death by p73, phosphorylation of tyrosine residue (Tyr-99) has mainly been shown to increase stabilization of p73 protein [89-91]. Phosphorylation of Ser-289 and Ser-47 on p73 by PKCδ and Chk1, respectively, has been shown to modulate the transcriptional and pro-apoptotic activity of p73 [89]. This suggests that overexpression of miR-510 allows for the increase kinase activity of c-Abl, upon cisplatin treatment, by phosphorylating p73 to stabilize its expression and therefore allowing increased phosphorylation of its serine residues to upregulate its downstream, pro-apoptotic functions.

PUMA and NOXA are downstream apoptotic effectors of the c-Abl:TAp73 pathway and are transcriptionally regulated by TAp73 in response to cisplatin. Upon their transcriptional upregulation, PUMA and NOXA promote mitochondrial translocation of pro-apoptic BAX and mitochondrial dysfunction, leading to release of apoptogenic proteins and cell death [92]. While upregulation of mRNA is not directly indicative of increase transcription of these genes, previous data has shown increase TAp73 phosphorylation, dissociation of the TAp73-repressor ΔNp63 and accumulation of total TAp73 suggests that the increase of PUMA and NOXA mRNA, in miR-510 overexpressing cells, is most likely attributed to increased transcriptional activity of TAp73 upon cisplatin treatment. To distinguish this mechanism from stabilization of

PUMA and NOXA mRNA, further studies with nuclear run on assays are required. However, PUMA and NOXA transcription could also be attributed to other transcription factors besides TAp73. Due to the high homology between p53 family members (TAp53, TAp63 and TAp73) they are predicted to target transcription of many similar genes, including PUMA and NOXA [38, 93]. Since MDA-MB-231 has a pro-survival form of mutant p53 [94], PUMA and NOXA are unlikely transcribed by p53 in these cell lines. Although TAp63 may or may not play a role, further evidence is needed to rule out role of TAp63 in PUMA and NOXA induction. Knockout of TAp73 in these cells will determine if PUMA and NOXA are being transcribed by TAp73. Once again, the previous data showing increased p73 phosphorylation, dissociation of the TAp73-repressor ΔNp63 and accumalation of total TAp73 indicate that PUMA and NOXA transcription is attributed to increased transcriptional activity of TAp73 upon cisplatin treatment.

The cumulative data supports the hypothesis that elevated miR-510 levels leads to upregulation of the proposed TAp73 apoptotic pathway. These experiments have demonstrated that miR-510 mediated negative regulation of Prdx1 is required for cisplatin sensitivity, leading to increased phosphorylation of p73, dissociation of ΔNp63 from p73, p73 protein accumulation and increased expression of pro-apoptotic genes PUMA and NOXA upon cisplatin treatment. However further experiments will have to be completed to fully characterize the mechanism of miR-510 mediated, cisplatin induced cell death, including c-Abl knockdown experiments (imatinib or c-Abl shRNA)

and p73 knockdown experiments (p73 shRNA). Despite this evidence, it is unlikely that it is the only mechanism of miR-510 mediated cisplatin sensitivity. The Prdx1 rescue experiment indicate that miR-510 mediated cisplatin sensitivity is mainly through negative regulation of Prdx1. Cisplatin induced DNA damage is known to activate various pathways besides c-Abl, including MAPK pathways (p38, JNK and ERK). Prdx1 is also known to play a regulatory role in these pathways, suggesting a parallel apoptotic signaling pathway that could be independent of, or acting synergistically with, c-Abl signaling. It should also be noted that TAp73 could be subjected to various post-translational modifications that affects its function. Most notable of these modifications is phosphorylation of p73 by various kinases, including c-Abl, PKCδ, Chk1 and JNK [88, 89]. Phosphorylation of each residue by different kinases play a different role in p73-mediated apoptosis. Further exploration of these other pathways will help uncover the complex mechanism of miR-510 mediated cisplatin sensitivity in TNBC. The limitation of these mechanistic studies is that they were performed in only one TNBC cell line. While we provide evidence of TAp73 pro-apoptotic pathway activation upon overexpression of miR-510 in MDA-MB-231 cells, this pathway may be specific to MDA-MB-231 cells. Further mechanistic studies are required in other TNBC cell lines to demonstrate that this pathway is relevant in all of TNBC.

Conclusion

Breast cancer is recognized not as single entity but as a heterogeneous group of diseases. As conventional methods are still employed to categorize and treat these diseases, it is becoming more evident that there is still high heterogeneity within these subtypes. Although the emphasis on early detection and advances in treatment has improved breast cancer survival, the triple negative subtype of breast cancer is still associated with the poorest prognosis of all breast cancer subtypes. TNBC is less likely to be detected early through mammograms, when a cure is more likely and tumors are most responsive to treatment. Due to lack of receptors, effective targeted therapies are not available for TNBC and cytotoxic chemotherapy remains the standard of care. Therefore, there is currently extensive research on development of targeted therapies for TNBC. This would help prevent progression into late stage disease where TNBC has the poorest survival rates compared to all other subtypes of breast cancer.

Platinum agents, such as cisplatin, have emerged as a novel therapy to treat TNBC, due to its aberrant DNA repair mechanisms. However, the heterogeneity of TNBC was once again demonstrated as cisplatin was highly effective in treating only a subset of TNBC patients. Discovery of the c-Abl/TAp73 mechanism of cisplatin induced cell death in TNBC lead to the assessment of ΔNp63/TAp73 as a biomarker of cisplatin sensitivity in TNBC. However, this marker was not sufficient to identify sensitive patients, suggesting a need for more specific biomarkers. Our lab has identified miR-510 to be overexpressed in human breast tissue samples, suggesting a role in breast cancer.

We have also shown that miR-510 plays an oncogenic role in breast cancer by upregulating proliferation, migration, invasion and colony formation. Our lab has validated Prdx1 to be a direct target of miR-510. Prdx1 has been shown to protect cells from cisplatin and also inhibit the tyrosine kinase c-Abl. Therefore we wanted to determine the efficacy of miR-510 as a biomarker of cisplatin sensitivity and elucidate the mechanism of this sensitivity.

Screening cisplatin cytotoxicity and miR-510 expression in TNBC cells lines reveals a positive correlation between miR-510 expression and cisplatin sensitivity. The GOF study shows that overexpression of miR-510 could sensitize resistance TNBC cell lines to cisplatin. On the other hand, LOF study demonstrates that inhibition of miR-510 of sensitive cell lines leads to increased resistance. This data suggests that miR-510 directly modulates cisplatin sensitivity in TNBC and may serve as a potential biomarker. MicroRNAs are ideal biomarkers in cancer as they regulate many processes in cancer development and progression and are also stable in biological fluids. Therefore, we wanted to explore the potential of miR-510 as a non-invasive biomarker. Analysis of serum samples from the in vivo study reveals that miR-510 could be detected by qPCR. Since serum expression of miR-510 is not indicative of its activity in the tumor, we then quantified miR-510 expression of tumor samples and compared them to miR-510 expression of matched serum samples. A positive correlation of miR-510 levels in matched tumor and serum samples suggests that serum expression of miR-510 is indicative of its expression in tumor samples. To determine whether serum expression

of miR-510 is relevant in human breast cancer, we quantified miR-510 in serum samples of human breast cancer patients. The data demonstrates that varying levels of miR-510 can be detected in human samples as well.

We then went on to explore the mechanism of miR-510 mediated cisplatin sensitivity. IHC analysis of in vivo tumors suggests that miR-510 mediated sensitivity may not be through decreased proliferation or increased cleaved-caspase 3 dependent apoptosis. Prdx1 IHC analysis, along with matched tumor miR-510 expression, shows that miR-510 negatively regulates Prdx1 in an in vivo setting. This also suggests that negative regulation of Prdx1 may play a role in miR-510 mediated cisplatin sensitivity. The Prdx1 rescue experiments found that the mechanism of miR-510 mediated sensitivity is dependent upon the negative regulation of Prdx1. To explore further downstream of this pathway, we then explored TAp73 activity. We found that overexpression of miR-510 leads to increased phosphorylation of TAp73 upon cisplatin treatment, which possibly leads to the stabilization and accumulation of TAp73. We have also shown that this leads to dissociation of TAp73 from its repressor ΔNp63. To provide further evidence of activation of TAp73 pathway, our qPCR analysis reveals a higher level of downstream pro-apoptotic genes, PUMA and NOXA in miR-510 cells treated with cisplatin. Cumulatively, our data demonstrates that miR-510 is a potential, non-invasive biomarker of cisplatin sensitivity in TNBC. We also show this mechanism of sensitivity is attributed to, at least in part, the negative regulation of Prdx1 and the subsequent activation of TAp73 apoptotic pathway.

Future Directions

Next steps in this project will be to continue to explore the efficacy of miR-510 as a biomarker of cisplatin sensitivity in TNBC. Screening additional TNBC cell lines will provide a comprehensive overview of miR-510 role in this heterogeneous subtype of breast cancer. Screening cell lines harboring BRCA1 mutations, like SUM149, will help determine if miR-510 could mediate cisplatin sensitivity independent of BRCA1 status. Repeating GOF and LOF studies in additional cell lines will also strengthen the data to support our hypothesis. Further mechanistic studies are required to continue elucidating the mechanism behind miR-510 mediated cisplatin sensitivity. Although we believe miR-510 mediated activation of TAp73 is through c-Abl, there still lacks definitive evidence. c-Abl inhibition studies with imatinib (tyrosine kinases inhibitor) or c-Abl shRNA will determine whether miR-510 mediated cisplatin sensitivity is dependent on the kinase activity of c-Abl. Various studies have demonstrated the role of MAPK, particularly JNK, to interact with proteins in this pathway. Uncovering the roles of these proteins in this pathway as potential synergistic signaling pathways or feedback mechanisms will help in revealing the complex process of miR-510 mediated cisplatin sensitivity. Understanding the mechanism of this sensitivity will allow for more effective utilization of miR-510 as a biomarker. In a collaboration with Amelia Zelnack (EMORY University) using clinical trial (NCT01194869) samples, we also want to demonstrate that miR-510 is an effective marker of cisplatin sensitivity in human TNBC patients. This clinical trial is investigating the efficacy of preoperative sorafenib (tyrosine kinase

inhibitor) and cisplatin, followed by paclitaxel (taxane chemotherapy) for early stage TNBC. Analysis of the patient serum samples and their treatment response will allow us to determine if miR-510 has potential utility as a biomarker of response to platinum chemotherapy agents. There are limitations utilizing this trial as proof of concept, as the combination therapy will not provide as clear evidence as a trial with single agent cisplatin. However, it could provide partial evidence that miR-510 is a feasible biomarker in the clinic and possibly lead to single-agent cisplatin trials here at MUSC in the future. To test the efficacy of miR-510 as a non-invasive biomarker, more studies are required to standardize serum collection, processing, normalization and interpretation; which are problems that hamper the clinical application of miRNA biomarker studies [76].

Study Significance

The goal of this investigation is to assess the utility of miR-510 expression as a potential treatment choice marker in TNBC. Our lab has previous demonstrated the oncogenic role of miR-510 in breast cancer, suggesting that miR-510 is better suited as a biomarker rather than a therapeutic target. This will aid in identifying patients who would be responsive to platinum chemotherapy agents and spare those patients who would be non-responders from unnecessary treatment; and as a direct consequence, increase the number of patients who respond well to first line treatment. Maximizing response to first-line chemotherapy is crucial as it is associated with increased overall survival in TNBC. TNBC is known to relapse early and improving treatment outcome is

important, as TNBC that do not recur in 5 years are considered to be cured. Utilization of miR-510 as a clinical biomarker is feasible as miRNA diagnostics test are commercially available (Prometheus Laboratories Inc). This research has great translational significance, potentially providing a means to improved survival disparity of the aggressive subtype of triple negative breast cancer.

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