THE EFFECTS OF IONIZING RADIATION AND P53 MUTATION ON CANCER CELL MIGRATION AND EPITHELIAL-MESENCHYMAL TRANSITION (EMT)

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By

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INTRODUCTION

Cancer is a growing problem because in 2012 alone there were about 14 million new cases and 8.2 million cancer-related deaths worldwide, and these numbers are likely to increase in the upcoming years[1]. It occurs when cells in the body begin dividing without control causing a tumor to grow and invade other tissue[2]. Metastasis, the spread of cancer to other parts of the body, is especially deadly as it contributes to 90% of cancer related deaths[3]. According to the American Cancer Society, stage III breast cancer patients, who have not experienced metastasis, have a 72% five-year survival rate while stage IV breast cancer patients, who have experienced metastasis, have a significantly lower five-year survival rate of 22%[4]. Decreased survival after metastasis is not limited to breast cancer either. The most common cancer to cause death among men and women, lung cancer, which has an estimated 222,520 new cases per year in the United States, becomes significantly more deadly once it has metastasized[5]. Therefore, preventing metastasis is important to improve the outcomes for cancer patients.

Cancer Treatments

Cancer treatments have developed immensely from their beginnings. The first treatment was surgical resection, or removal of the tumor by surgery. This is still a common treatment for cancer, but today it is used in conjunction with three other main categories of treatment: chemotherapy, immunotherapy, and radiotherapy.

Chemotherapy

Chemotherapy involves the use of chemicals to treat cancer. Chemotherapy can be used in different ways. In some situations, it is used alone to fight a tumor on its own. In other situations, it is neoadjuvant chemotherapy, meaning that it is used to shrink a tumor before surgical resection. The opposite of neoadjuvant chemotherapy is adjuvant chemotherapy, which is the use of the chemotherapeutic agent after surgical resection to kill any cancerous cells that may remain. The final use of chemotherapy is in palliative care, which helps alleviate some symptoms, but does not cure the cancer; it is used to prolong a cancer patient's life, but will not cure the patient[6].

The majority of chemotherapy is administered intravenously, so it is a systemic treatment, meaning that it reaches the entire body. This is good because it can reach both the primary tumor and cancer cells that have left the primary tumor site, but it also means that healthy tissue throughout the body is also exposed to the toxic chemicals. Most chemotherapeutic agents are cytotoxic or cytostatic, which means they either kill cells or halt their growth, respectively. Because cancer cells grow quickly, most chemotherapeutic agents target mechanisms involved in proliferation and cell growth. This prevents the drugs from having large effects on healthy, slow growing tissues, but healthy tissues that grow quickly, such as hair cells, mucous membrane cells, and blood-producing cells, are more affected by the treatments. This results in common side effects, such as hair loss, anemia, gastrointestinal dysfunctions, and more[6].

One of the first effective chemotherapeutic agents was 5-fluorouracil (5-FU). The name of 5-FU is derived from the molecule's atomic structure, which is the same as uracil except the hydrogen at Carbon-5 is replaced with a fluorine atom. Using the same intake mechanism as uracil, 5-FU easily enters cells. Once it is in the cell, it can be metabolized in several ways, including being converted into FUTP and FdUTP, which can be incorporated into RNA and DNA, respectively. Incorporation of FdUTP into DNA is aided by the inhibition of thymidilate synthase (TS) by FdUMP. TS catalyzes the conversion of dUMP to dTMP, so because FdUMP blocks this conversion, DTTP for DNA replication and repair becomes depleted, forcing the cell to incorporate FdUTP in its DNA. The incorporation of the fluorouracil metabolite into RNA and DNA causes damage and blocks protein synthesis and cellular proliferation (Fig. 1)[7].

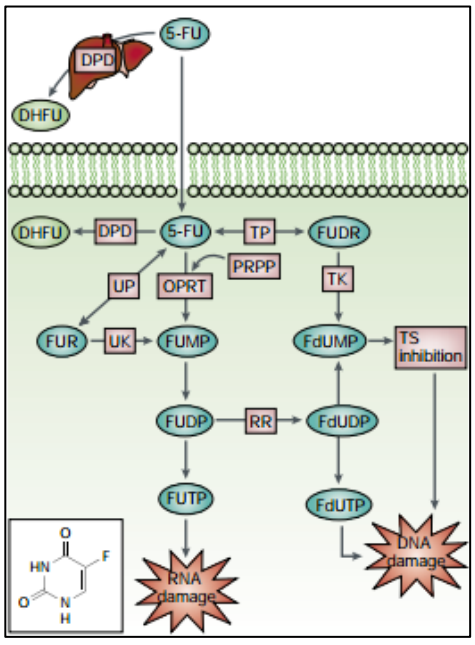

Figure 1. The metabolism of 5-Fluorouracil in a cell.[7]

The idea of using chemicals to treat cancer started during the beginning of the 1900s. However, it started with a slow process requiring developments in multiple fields, such as mouse models and drug screening and development. They struggled with screening a vast array of chemicals, and they also struggled with finding an appropriate way to test the chemicals' efficacy. During World War II, several research programs started yielding results to support further investigation. One of those studies noticed that soldiers who were exposed to mustard gas during World War I had decreased bone marrow, so mustard gas was experimentally used to treat lymphomas. The treatment worked at combating the cancer, but its effects were short-term and remission was short-lived. It simultaneously provided excitement and pessimism for chemotherapy research. There were several other advances in the chemotherapeutic treatment of hematologic cancers, also known as cancers of the blood. However, it was not until the middle of the 1950s that there was a development in treating tumors that did not affect blood. That development came in the form of 5-FU, which is still used to fight colorectal cancer[8].

During the 1960s and 1970s more chemotherapeutic agents were developed and researchers discovered that combining drugs provided better outcomes. Thus, combination chemotherapy was formed. By 1970, complete remission for Hodgkin's disease increased from 0% to 80% because of the use of combination chemotherapy. Other types of cancer have experienced similar progress due to chemotherapy development[8].

Starting in the 1970s, chemotherapy began being used as an adjuvant therapy to other treatments such as surgical resection and radiotherapy to increase the overall effectiveness of treatments. By 1990 the overall incidence of and mortality from cancer began to decrease and has decreased every year since very much thanks to the development of chemotherapeutic agents[8].

Immunotherapy

Immunotherapy is a modem of treatment that up until recently did not have much success in treating cancer. Essentially the goal of immunotherapy is to activate a patient's immune system to prevent cancer or to fight it once it has formed. One strategy in immunotherapy is the use of vaccines, which come in two types: prophylactic and therapeutic. Prophylactic vaccines aim to prevent cancer and have had success recently in the form of vaccines for hepatitis B virus and human papilloma virus (HPV). However, therapeutic vaccines aimed at fighting tumors that have already formed have not had much success until recently. Researchers have found that by treating patients with interleukin (IL)-2 to activate the immune response in addition to administering peptide vaccines they can increase survival[9].

There are many other strategies for cancer immunotherapy in addition to vaccines. One other strategy is using viral vectors to express tumor antigens, which takes advantage of the immune system's naturally strong response to viruses. Another strategy uses antibodies that are targeted to dendritic immune cells to deliver antigens

that are coupled to the antibody. Some research focuses on using actual tumor cells to isolate antigens for use in creating vaccines. Finally, research has also been exploring isolating dendritic cells from patients and activating them to react against tumor antigens *ex vivo* before reinserting them into the patient[9].

There are a few main difficulties in the field of creating effective cancer immunotherapies. First, researchers struggle to identify good antigenic targets. It is difficult to identify good antigenic markers because first they must only be found on cancerous cells and not on healthy cells. If an antigen is determined to be only located on cancerous cells, then it must also be immunogenic enough to elicit an immune response. Second, it has been difficult to optimize the treatments to fully activate $CD8^+$ T-cell responses that are still safe. Finally, many tumors create microenvironments to prevent successful immune responses[9].

Radiotherapy

Radiotherapy usually comes in the form of ionizing radiation (IR). IR can come in two forms: particles and waves. Particle radiation comes in the form of neutrons, electrons, protons, and heavy ions. IR in the wave type is part of the electromagnetic radiation spectrum. This is the same spectrum onto which ultraviolet light, visible light, infrared, microwaves, and radio waves fall. These types of electromagnetic radiation have lower frequencies, longer wavelengths, and less energy compared to ionizing radiation, which consists of X rays and gamma rays. When an X

ray or a gamma ray hits an atom, it can eject an electron from the atom's orbit causing it to become charged ion, hence the name ionizing[10].

The mechanism for radiotherapy killing cancer cells is two-fold. IR causes direct damage to DNA by causing electrons to be ejected, but it also causes indirect damage by creating reactive oxygen species (ROS), also known as free radicals, from the ejection of electrons from water in the cell (Fig. 2). ROS then react with DNA[10, 11]. The types of damage can range from nucleotide base damage to single- and double-strand breaks[12]. DNA damage causes genome instability, ultimately leading to cell death.

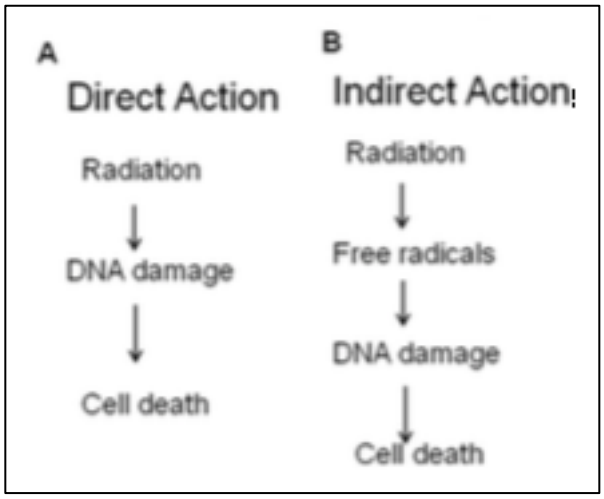

Figure 2. Mechanisms of cell death following exposure to ionizing radiation.[13]

Radiotherapy can cause several types of cell death. The cell death pathways are interrelated and most involve p53. One type of cell death is apoptosis, which is programmed cell death. Apoptosis occurs when damaged DNA activates ATM or ATR and the p53 pathway is activated[14]. Another type is mitotic cell death/

catastrophe, in which chromosomes do not segregate properly during mitosis causing giant cells to form with multiple nuclei, which also involves p53[13]. Apoptosis and mitotic cell death/catastrophe are responsible for most cell death in response to IR. A third type of cell death is necrosis, in which cells swell and the cell membrane degrades. Cell death also occurs by autophagy, which causes a cell to die by digesting itself. Finally, senescence is when the cells remain viable, but stop proliferating; senescent cells will eventually undergo apoptosis. Senescence is also triggered by a p53 pathway[13, 14].

Cell death prevents the tumor from growing, making it an effective treatment. However, some tumors are resistant to IR[10]. By understanding the pathways that lead to cell death following radiotherapy, we can potentially harness the power of IR even more to combat radioresistance. Building our understanding of what causes radioresistance is of utmost importance to create more effective treatments.

In order to use IR in a clinical setting, a measure for how much radiation is absorbed is necessary. The gray (Gy) was created and defined as 1 joule of energy per 1 kilogram of mass. However, the biological effects are not only dependent on the amount of energy absorbed, but also on the intensity of the ionization in the organism depending on the source of the radiation. Therefore, the Sievert (Sv) was made to measure equivalent dosage[15]. Particle radiation is more intense than x-rays and gamma rays, so 1 Gy of particle radiation will have more biological effects than 1 Gy of wave radiation. For x-rays and gamma rays, the equivalent dose (Sv) and the absorbed dose (Gy) are the same; essentially 1 Gy is equivalent to 1 Sv[16].

The specifications for each cancer treatment plan depend on the location of the tumor, the type of tumor, and the tissue surrounding the tumor, so individual radiation therapy treatment plans vary. The location of the tumor and the surrounding tissue are important because radiation must travel through the surrounding tissue to reach the tumor. Some tissue is more sensitive to radiation, so a physician and a medical physicist must work together to design a treatment plan that uses multiple fields to deliver the dosage without directly hitting critical tissue. Because the treatment plan is so individualized, it is important to set up a system to ensure that the patient is in the exact same position for every single administration[17].

In addition to creating a treatment plan for how to deliver the dosage, the physician must choose a dosage to be delivered over multiple fractions. Different areas of the body can tolerate different dosages. For carcinomas of the lung, it is recommended to deliver a total of 65 to 75 Gy to the primary tumor over the course of six to seven weeks in fractions. For lung carcinoma metastases, 30 to 40 Gy to be delivered over the course of two to three weeks is suggested[18]. In research, the recommended protocol for lung carcinoma metastases was used in a phase II clinical trial testing erlotinib as a treatment in conjunction with radiotherapy for non-small cell lung cancer (NSCLC) brain metastases. The researchers used 2.5 Gy fractionations five days per week until 35 Gy was reached (14 fractions)[19]. To put the intensity of radiotherapy in perspective, one posterior to anterior diagnostic chest x-ray has a dose of 0.02 mGy, or 0.00002 Gy, which is more than 100,000 times weaker[20].

In addition to causing cell death in many cancer cells, previous research has shown that IR has effects on multiple aspects of metastasis via several mechanisms in cells that survive. Migration, invasion, and epithelial-mesenchymal transition (EMT) have all been altered by IR in previous research. These alterations have been made by inducing cell adhesion molecule (CAM) expression[21,22,23] and via cell signaling pathways such as TGF-β, HGF, EGF, microRNA (miR-30e), Wnt/β-catenin, and more[24,25,26,27,28]. Even with all of this information, the mechanisms behind IR-induced changes in CAM expression remain unclear in many situations.

Metastasis

Mechanism of metastasis

Metastasis is a complicated, multistep process, in which cancer cells invade local tissue, enter the bloodstream via intravasation, disseminate via the circulatory system or lymphatic system, exit the bloodstream via extravasation, and colonize a new location in the body (Fig. 3)[29, 30]. Basically, they have to find a way to move from the original tumor into the bloodstream or lymphatic system, which can bring them to distant parts of the body where they can escape and form a new tumor. The mechanisms involved in metastasis are multifaceted and interrelated, but include cell motility, cell-cell and cell-matrix adhesion, angiogenesis, and extracellular matrix (ECM) degradation[31].

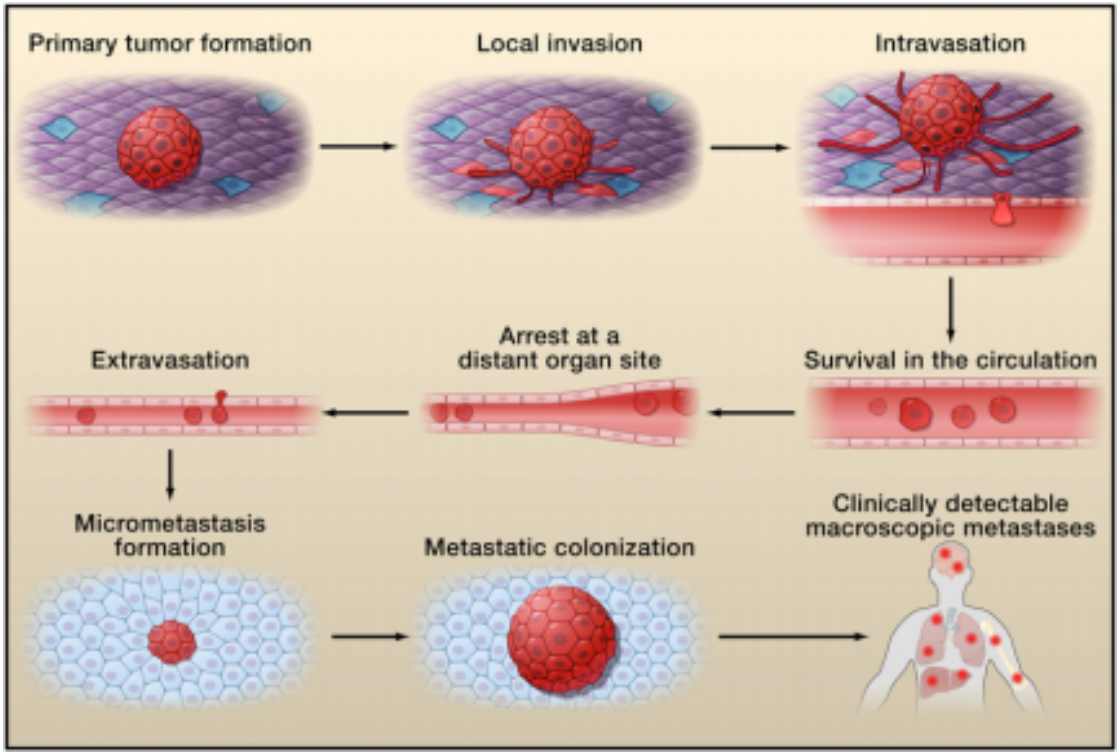

Figure 3. Steps of metastatic tumor formation.[30]

Several mechanisms involved in invasion, such as cell motility and cell-cell and cell-matrix adhesion, are highly dependent on cell adhesion molecules (CAMs) expressed by the cancer cell and the endothelial cells lining blood vessels. The mechanisms are also dependent on the ECM, which provides structural support to tissue invaded by the cancer cell[32]. In order for a cancer cell to migrate and leave the primary tumor, its cell-cell and cell-matrix adhesion must be altered to allow the cell to escape, but in order for it to migrate into the circulatory system and eventually exit it, its motility mechanisms must be activated and its adhesive potential must be strong enough to adhere to the blood vessel endothelium and roll to a stop[33]. Therefore, any

stimulus that can affect the expression and activation of CAMs is very important for metastasis.

Angiogenesis, the production of new blood vessels, also plays an important role in tumor development and metastasis. Angiogenesis is typically found in embryogenesis and in wound healing and the female reproductive cycle in adults. However, tumors use angiogenesis to create vasculature that provides direct blood flow to the tumor. This provides the tumor with all of the nutrients it needs to grow. Furthermore, it decreases the distance that a metastasizing cell must travel to reach vasculature. By decreasing the distance the metastasizing cell must travel to find vasculature, it increases the likelihood that the cell will intravasate and eventually form a secondary tumor. Once the metastasizing cell has reached the vascular system, it can be transported to distant places in the body where it can form a secondary tumor[31].

The secretion of certain molecules by cancer cells can increase invasive potential too. Matrix metalloproteinases (MMPs) are a type of these secreted proteins that can degrade ECM. There are 21 proteins in the MMP family that can digest a diverse set of ECM proteins, including fibronectin, collagen, laminin, and proteoglycans. They can also break down cell-cell and cell-matrix interactions[31]. The degradation of ECM breaks down tissue, which allows cancer cells to invade more successfully in several steps of the metastatic process. MMP expression has been implicated in other aspects of tumorigenesis recently as well[34].

Cell Adhesion Molecules

CAMs play an integral role in multiple steps in metastasis[32]. Therefore, their expression and activation are important for determining the metastatic potential of cancer cells. There are several groups of CAMs, including integrins, cadherins, selectins, and the immunoglobulin superfamily. Within each group there are numerous varieties[31].

Integrins, one group of CAM, are heterodimers that consist of an alpha subunit and a beta subunit. There are many signals that regulate their expression[35], and they are highly expressed in cancer cells, where they play an important role in cancer cell survival and metastasis[33]. For example, doses of 2 Gy and 6 Gy, which are similar to and higher than a clinical radiotherapy dose respectively, induced the expression of the β1 integrin subunit 48 hours after exposure in the human lung cancer cell line A549. This increased their adhesion to fibronectin and laminin. Adhesion to ECM proteins like fibronectin and laminin also caused increased radioresistance compared to cells plated on plastic or bovine serum albumin (BSA)[36]. The researchers have not elucidated a mechanism yet, but it is likely due to some extracellular membrane contact dependent signaling. Further research showed that IR induces sialylation of the β1 integrin subunit, which was correlated with increased migration[37]. Research on the α5β1 integrin showed that its expression was increased after IR in pancreatic cancer cell lines, which caused increased invasion[38].

There are several studies that have investigated the expression of intercellular cell adhesion molecule-1 (ICAM-1) after ionizing radiation. Across several cell lines,

ICAM-1 expression increased after treatment with IR. ICAM-1 is the ligand for LFA-1, which is an integrin (αLβ2)[21, 22, 23]. The findings that CAM expression can be altered by IR and can have effects on the adhesion and migration of cancer cells are important because of the potential to cause metastasis in a cancer patient receiving radiotherapy.

The cadherin family of proteins contains many proteins that are expressed in various tissues at various times during development. In adults, E-cadherin is very important for cell-cell adhesion and for maintaining polarity[39]. Blocking with anti-E-cadherin antibody has increased invasiveness because of the loss of cell-cell adhesion mediated by E-cadherin[40]. Decreased E-cadherin expression has also been linked to the poor prognosis for patients with NSCLC and to increased metastasis[41,42,43,44,45,46]. Similarly to the integrin family, the cadherin family plays an important role in cancer metastasis.

Epithelial-Mesenchymal Transition

In addition to migration mediated by CAMs, there are other aspects that contribute to metastasis. One is epithelial-mesenchymal transition (EMT), which can be induced by reactive oxygen species (ROS) among many other signals[47]. Cells in the epithelial state are characterized by being polarized and associated with a basement membrane. Epithelial cells maintain their structure by cell-cell interactions, including tight junctions, adherens junctions that are connected to the actin cytoskeleton and require cadherins, gap junctions to allow chemicals to travel freely between connected

cells, and desmosomes that are connected to the intermediate filaments in the cell. Epithelial cells adhere to the ECM via integrins and other CAMs[47]. Epithelial cells usually express E-cadherin (epithelial cadherin), which is a Ca^{2+} -dependent cell-cell adhesion molecule that can play a role in tumor suppression[48].

In EMT, epithelial cells lose their polarity, their cell-cell connections, and their connection to the basement membrane and gain mesenchymal characteristics (Fig. 4)[49,52]. Mesenchymal cells have increased motility, lack cell-cell junctions, produce extracellular matrix, and survive better in liquid suspension[50]. In addition to being more mobile, cells in the mesenchymal state are able to remodel ECM by both degrading it using matrix metalloproteinases (MMPs) and by producing the ECM protein, fibronectin, which regulates integrin-mediated mesenchymal stem cell migration[47,51]. Interestingly, EMT is reversible in a process called Mesenchymal-Epithelial Transition (MET). In this process cells lose their mesenchymal characteristics and gain epithelial characteristics[52]. EMT is important during development, but after development, EMT can aid metastasis by changing the cell to a state in which it is more able to migrate, does not require cell-cell contact, and can invade other tissue with the help of MMPs.

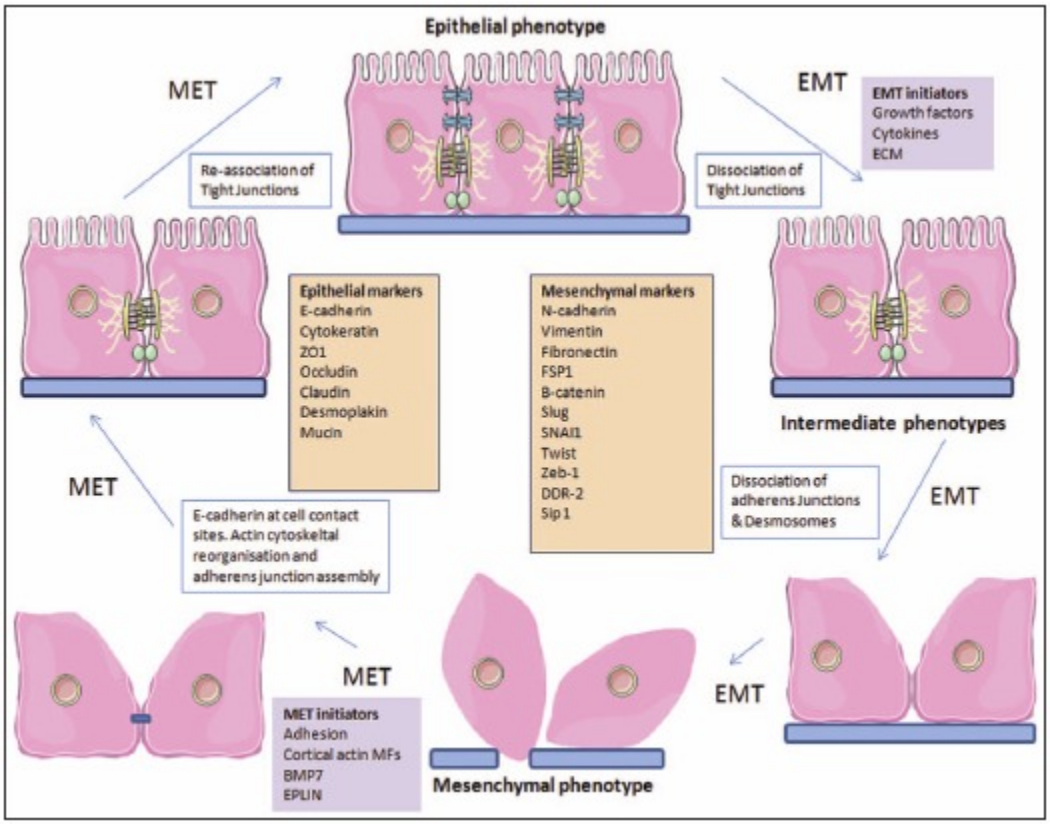

Figure 4. Protein expression and phenotypic changes in epithelial-mesenchymal transition and mesenchymal-epithelial transition.[52]

In order for EMT to occur, the cell must produce a specific set of transcription factors[47]. Several protein expression changes occur that aid in the detection of EMT. In EMT, epithelial proteins, such as E-cadherin, α-catenin, and γ-catenin are no longer expressed[50]. E-cadherin is the epithelial cadherin, which is involved in cell-cell connections. α-catenin and γ-catenin connect E-cadherin to the actin microfilament network in epithelial cells[53]. After EMT occurs, mesenchymal cells express markers such as vimentin, fibronectin, and N-cadherin[50]. Vimentin is an intermediate filament that is first expressed on embryonic day 8.5, but it is not expressed in many cells in

adults other than in connective tissue mesenchymal cells, in the central nervous system, and in muscle. Epithelial cells usually only express keratin, but upon EMT, they also express vimentin[54]. Fibronectin is also produced because attachment to it by mesenchymal cells can stimulate more EMT[47]. N-cadherin is also known as neural cadherin. N-cadherin usually functions in embryogenesis during gastrulation and neural crest development, and it also acts as an invasion promoter in cancer cells[55].

Emerging data support that cancer progression and metastasis can be altered by IR-induced regulation of cell adhesion molecules on tumor cells[56,57]. IR has been shown to induce integrin expression and modulate its binding capacity to matrix proteins[37,58]. However, we know little about the mechanism of the effects of IR on the metastatic potential of survivor cells, cells that are resistant to IR-induced death. The radiation survivor cells of three prostate cancer cell lines (PC3, DU145, and LNCaP) have been previously analyzed for phenotypic changes. The researchers found that multiple fractions of IR could induce changes in mRNA, miRNA, and the phosphoproteome. Pathways that showed altered expression included immune response, DNA damage, cell-cycle arrest, TGF-β, survival, and apoptosis. Furthermore, p53 was heavily involved in these changes[59]. Therefore, understanding the effects on CAM expression, migration, and EMT is germane to providing the best treatments possible.

p53

p53 is a tumor suppressor protein. It is a transcription factor that controls proteins involved in the cellular response to DNA damage. If it is not functioning properly, somatic mutations are less likely to be repaired or apoptosis will not occur[60].

When DNA damage occurs, it activates ATM or ATR, which will phosphorylate p53 to stabilize it. ATM and ATR also activate Chk2 and Chk1, respectively. Chk2 and Chk1 can phosphorylate p53, in addition to being able to cause cell cycle arrest on their own via Cdc25 and Cdk1/Cdk2. However, once p53 is stabilized, it accumulates and is a transcription factor for p21. p21 inhibits the Cdk2/cyclin E complex that is required to progress through the G1 phase, which causes the cell cycle to arrest in the G1 phase[14].

If the DNA damage is severe enough, the cell will undergo apoptosis instead of cell cycle arrest. This pathway starts similarly to the cell cycle arrest pathway, but instead of p53 activating transcription of p21, it will activate the transcription of PUMA and Noxa, which are BH3-only proapoptotic members of the Bcl-2 protein family. PUMA and Noxa activate Bax and Bak, which allow the release of cytochrome *c* from the mitochondria. Cytochrome *c* activates caspase-9, which then activates caspase-3 to cause cell death[14].

In approximately 50% of cancers, p53 mutation occurs[61]. The majority of these mutations are point mutations in the central DNA-binding domain, which is the part of the protein that would usually bind to promoter regions of target genes. However, p53 mutations usually make the protein less stable and less able to bind the DNA. There

are several mutation "hotspots" within the central DNA-binding domain at R175, G245, R248, R249, R273, and R282[62]. Although p53 mutation causes a loss of tumor suppressor functions, it can aid in growth, multi-drug resistance, invasion, migration, scattering, angiogenesis, stem cell expansion, survival, and tissue remodeling[63,62]. Therefore, p53 mutation not only causes the loss of tumor suppression, but it also can cause the gain of malignant characteristics, all within a single cancer cell[62].

Project Rationale

Research has shown increased expression of several integrins after IR, but the mechanism behind the increase is still unknown[56]. Other research has shown that p53 mutants may be involved in pathways that support tumor growth and progression after IR[64]. A previous study in Dr. Wu's lab showed that the expression of several integrins and cell adhesion is differentially regulated in human cancer cell lines MCF-7 (p53-WT) and MDA-MB-231 (p53-R280K) upon treatment with a single dose of 8 Gy. This is interesting because, among other differences, each cell line has a different genomic background in regard to p53[58]. This prompted us to investigate how p53 mutation can affect EMT and migration, which are closely related.

Since adhesive molecules play such an important role in metastasis and their expression is altered after IR in multiple cell lines, this project aimed to elucidate the role of p53 mutation in a cell's response to IR. In order to investigate the effect of specific p53 mutants and wild type p53 on a cancer cell's response to IR, H1299 cells,

a non-small cell lung cancer line that does not express p53, were transfected to stably express one of the following: p53-WT, p53-R175H, p53-R280K, or the empty vector, PCDNA6. These mutants were chosen because p53-R175H is a "hotspot" mutation within p53's DNA-binding domain and because p53-R280K, another DNA-binding domain mutation, is the mutant expressed in my lab's previous research using MDA-MB-231[62,58].

In previous experiments, cells were treated with one fraction of radiation and allowed to recover 24 hours before testing[58]. For this project, single dose radiation and fractionated radiation treatments will be used. Cells treated with one dose will have 24 hours to recover before testing, while fractionally treated cells will be tested one week after their first day of treatment. Having these two treatment conditions will allow us to investigate the cells' short-term and long-term responses to IR.

In clinical treatments, fractionated treatments are usually used. In a fractionated treatment, a person usually receives a treatment every weekday approximately 24 hours apart. Because there is a rest period between each treatment, there is a chance for temporary phenotypic changes to occur. Therefore, cells analyzed 24 hours after a single treatment are representative of the short-term phenotypic changes that can occur. Assaying this time point is important because the time between each treatment could pose an opportunity for metastasis. The fractionated IR treatment was also assayed for in order to understand phenotypic changes that occur long-term. This helps us understand more permanent changes that fractionated IR can have on cells that survive treatment. The effect of multiple treatments of IR is

important to understand too because cells that survive are likely to experience phenotypic changes, which could include increased metastatic potential.

I hypothesized that single dose radiation and fractionated radiation would cause an increase in α5β1 integrin expression in H1299 p53-WT cells and an even greater increase in α5β1 integrin expression in H1299 p53-R175H and H1299 p53-R280K cells because previous research shows CAM expression to be upregulated after IR, including α5β1 in my lab's previous work. I also hypothesized that migration would increase in H1299 p53-R175H cells and in H1299 p53-R280K cells after radiation to match the change in α5β1 integrin expression. Although increased integrin expression has been reported in metastases, a delicate balance in expression must be maintained in order for metastasis to occur. Integrin expression must not be too high or too low because metastasis requires the ability to migrate, detach, and reattach[33].

This work aims to elucidate how IR alters the metastatic potential of cancer cells expressing WT-p53, p53-R175H, or p53-R280K. By understanding how p53 mutation and IR affect metastasis, we can potentially characterize tumors as candidates for radiotherapy with lower or higher risks of metastasis based on the p53 genotype. This is one step toward achieving personalized medicine. This research could also lead to the development of chemotherapeutic targets in conjunction with radiotherapy. Ultimately, this research has the potential to be a foundation for future work that could save lives from succumbing to cancer metastasis.

METHODS

Cell lines and cell culture: These experiments will use H1299 cells, a non-small cell lung carcinoma line. H1299 cells do not express p53, so multiple stable lines expressing WT-p53, p53-R175H, p53-R280K, and the empty plasmid, PCDNA6 have been created. All cells will be maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 2 mM glutamine. They will grow at 37°C, 5% CO_2, and 95% humidified air. They will grow until confluent, at which point they will be passaged them into new plates using trypsin-EDTA.

Ionizing Radiation: Dr. Shinhee Lee, a research scientist in Dr. Wu's lab, is licensed to use the ^{137}Cs irradiator (J. L. Shepherd Associates, San Fernando, CA) located in the Konneker Research Labs at The Ridges. She will irradiate cells with one dose of 4 Gy or 8 Gy, or four fractions of 2 Gy on four consecutive days. The fractionated treatment will mimic a clinically relevant treatment as outlined in *Principles and Practice of Radiation Oncology*[18], whereas the single treatments are common in *in vitro* research. All fractionated IR experiments were conducted one week from the first radiation treatment, while cells were given 24 hours to recover after a single dose before experimentation. See Figure 5 for the radiation and experimental schedule.

	Day 0	1	2	3	4	5	6	7	8
Western Blot Fractionated IR								Make Lysate	
Wound Healing Fractionated IR							0.5% FBS + Act. D Medium	Scratch	24 Hour Picture
Flow Cytometry Fractionated IR								Fix and analyze	
Western Blot Single Dose IR		Make Lysate							
Wound Healing Single Dose IR	 0.5% FBS + Act. D Medium	Scratch	24 Hour Picture						

Figure 5. Ionizing radiation and experimental schedule.

Wound Healing Assay: Cells were plated in 6-well plates to reach confluence by one week after the first IR treatment. Six days after the initial IR treatment, regular growth medium was replaced with wound healing assay medium containing DMEM with 0.5% FBS, 1% penicillin/ streptomycin, 2 mM glutamine, and 2 ng/mL actinomycin D. Twenty four hours after the medium was changed, the medium was removed and scratches were made using sterile 200 µl pipet tips. Each well was then washed with 2 mL of PBS and 2 mL of wound healing assay medium was added to each well. Pictures were then taken under 40x total magnification at 0, 8, and 24 hours for fractionated IR treatment and at 0 and 24 hours for single dose IR treatment.

Wound healing assays were analyzed using ImageJ. To more easily view the scratch boundaries, the background was subtracted and the contrast was increased. The area of the scratch was measured using the freehand drawing option, which gave the area in terms of pixels. The number of pixels in the area of the scratch was then divided by the total number of pixels in the image. These proportions were then normalized to the non-irradiated H1299 control cells at 0 hours. Using these normalized proportions, the normalized proportions at 8 hours or 24 hours were subtracted from their respective normalized proportion at 0 hours. These normalized differences were then tested for significance.

Western Blot: Western blotting was used to measure protein expression. Cells were harvested one week after the initial radiation treatment. Cells were harvested by scraping them off of the plate into one mL of PBS. After harvest, they were centrifuged for two minutes at 4,000 rpm and the supernatant was removed. The pelleted cells were then washed with one mL PBS and centrifugation and aspiration were repeated. The remaining pellet was resuspended in 100 to 200 μl of 2% NP-40 buffer containing protease cocktail set III depending on the size of the pellet. This mixture was then incubated on ice for 15 minutes to allow cell lysis to occur. After this incubation, the mixtures were centrifuged at 13,200 rpm for 10 minutes at 4°C. The remaining supernatant was collected and moved to new eppendorf tubes. Using BioRad's DC Protein Assay, the concentration of protein in each sample was determined. Samples containing 20 μg protein were prepared and mixed with 4x

NuPAGE Sample Buffer (Invitrogen) and heated at 99°C for five minutes. They were then loaded into wells in an 8% SDS-PAGE gel and electrophoresed at 150 V for one hour. The extra protein lysates were then stored at -80°C to save for future experiments if necessary. After electrophoresis, gels were electroblotted onto nitrocellulose membranes at 25 V overnight in the 4°C room in Towbin buffer (25 mM Tris, 192 mM glycine, and 20% methanol by volume). The following morning the nitrocellulose membrane was blocked in 5% skim milk in Tris-Buffered Saline/Tween 20 (TBS-T) for one hour on a shaker at room temperature. The membrane was then washed for five minutes in TBS-T three times. The membrane was then incubated in the primary antibody diluted in TBS-T overnight on a shaker in the 4°C room. The primary antibodies used were anti-integrin α5 (Santa Cruz, 1:1000), anti-vimentin (Cell Signaling, 1:1000), anti-N-cadherin (Cell Signaling, 1:1000), anti-β-catenin (Cell Signaling, 1:1000), and anti- β-actin (Santa Cruz, 1:2000). The next morning the membranes were washed three times in TBS-T as before, and then incubated at room temperature for one hour on the shaker in the proper secondary antibody: anti-mouse (Santa Cruz, 1:1000) or anti-rabbit (Santa Cruz, 1:1000). The secondary antibodies were diluted 1:1000 in 5% skim milk in TBS-T. Following secondary antibody incubation, the membranes were washed in TBS-T three more times before being developed with the West Pico Supersignal Chemiluminescent substrate.[58] HyBlot CL Autoradiography film was exposed to the membrane in a dark room and then developed by hand by bathing the film in T2 Automatic X-Ray Developer followed by T2 Automatic X-Ray Fixer (White Mountain Imaging). The membrane was then

rinsed with water and allowed to dry. Using an Epson scanner, films were converted into computer images. ImageJ was used to measure the percentage of the total darkness that each band contributed. The percentage for each band was then divided by the percentage of the non-irradiated H1299 control sample to get the relative darkness of each band. To control for loading errors, the relative darkness of the band was divided by the relative darkness of the corresponding β-actin band to get the adjusted darkness. The adjusted darkness values were then used for statistical analysis[65].

Flow Cytometry: Flow cytometry was used to measure the surface expression of integrin α5β1 and integrin α5. Cells were harvested one week after the first IR treatment. After being harvested using 0.25% Trypsin-EDTA, cells were centrifuged for two minutes at 4,000 rpm and then washed twice in one mL of PBS. Each sample was then fixed in 500 µl of 4% formaldehyde in PBS for 10 minutes at 37°C. The samples were then incubated for one minute at 4°C. The samples were then centrifuged and washed two more times as before. After washing, the control sample was divided into three equal parts. The three control samples and the other samples were resuspended in 100 µl of incubation buffer (0.5% BSA in PBS) and incubated for 10 minutes at room temperature. After this incubation, 1 µg of anti-integrin α5β1 (Millipore) was added to every sample, except for two control samples. One of the other two control samples was not incubated with any antibody and the last control sample was incubated with 1 µg of FITC-anti-Mouse IgG2b, κ (BD Pharmigen),

which is the isotype control. This incubation lasted one hour at room temperature in the dark to protect the FITC conjugated to the isotype control antibody. Following the primary antibody incubation, samples were washed twice using 1 mL incubation buffer. The samples incubated in the primary antibody were resuspended in 100 µl of incubation buffer containing 2 µl anti-mouse-FITC (Santa Cruz Biotechnology) and incubated for 30 minutes at room temperature in the dark, while the other two control samples were incubated in plain incubation buffer. After this incubation, the samples were washed two more times in incubation buffer and then resuspended in PBS for analysis.[58] Analysis was done using the FACSAria machine located in the Academic and Research Center on Ohio University's campus.

Statistical Analysis: Experiments were repeated three to five times. Student's t test will be used to determine significance with $p<0.05$. Microsoft Excel was used for statistical analysis.

RESULTS

EMT marker proteins and integrin α5 expression after fractionated IR

In order to determine the effect of fractionated IR and expression of p53-WT, p53-R175H, and p53-R280K on EMT and integrin expression, Western blot analysis was used. The total expression of the mesenchymal marker proteins β-catenin, N-cadherin, and vimentin and the CAM, integrin α5 was measured (Fig. 6). One set of H1299 p53-WT blots was lost, so statistical analysis could not be run on those samples.

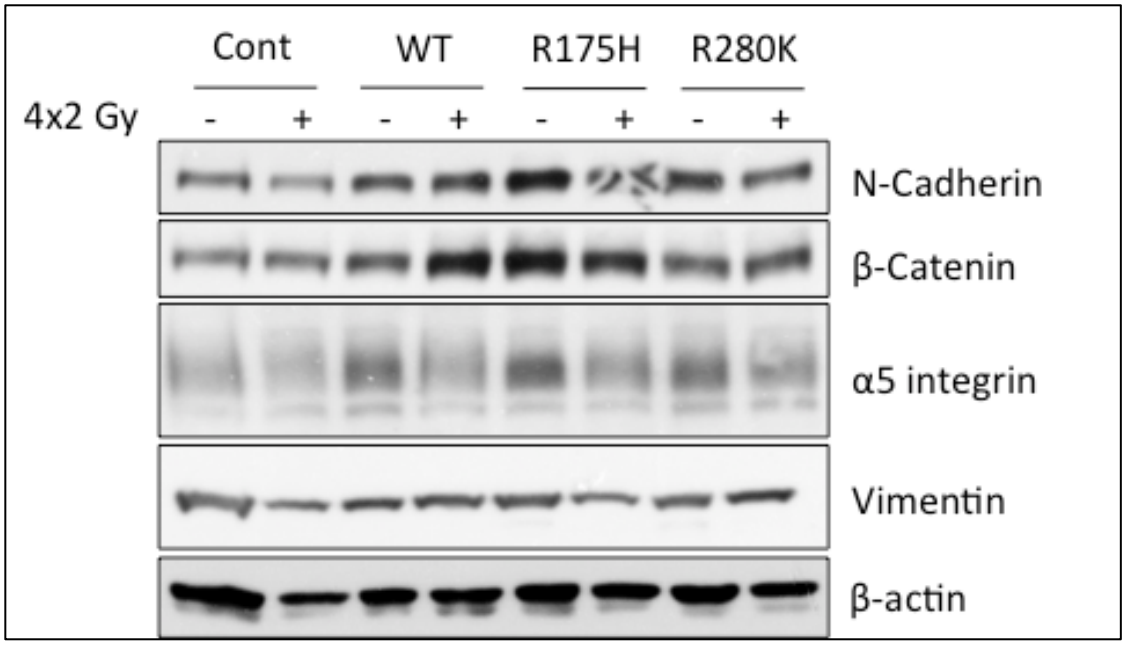

Figure 6. EMT protein marker and integrin α5 expression in H1299 cells after fractionated IR treatment.

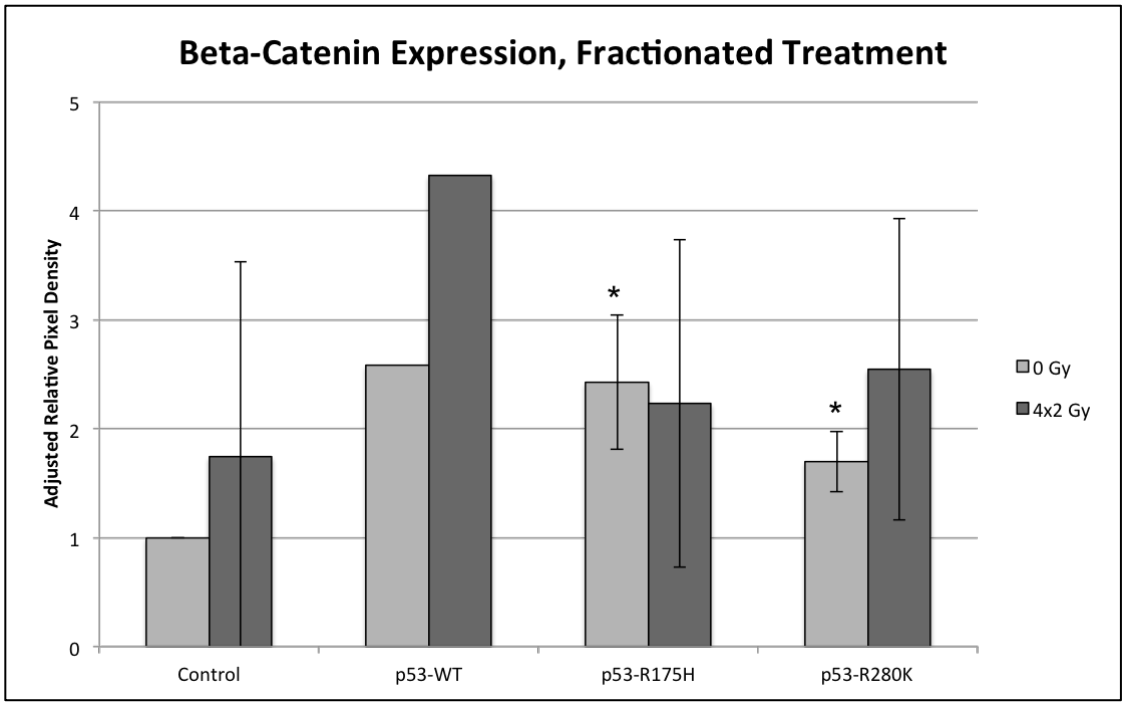

Figure 7. β-catenin expression in H1299 cells after fractionated IR treatment. Significance is noted by *$p<0.05$ compared to 0 Gy H1299 Control sample.

The expression of β-catenin is quantified and graphed in Figure 7. H1299 p53-WT, H1299 p53-R175H, and H1299 p53-R280K cells all had higher baseline β-catenin expression compared to H1299 control cells (p53 null). Compared to non-irradiated H1299 control cells, β-catenin expression is significantly increased in non-irradiated H1299 p53-R175H and non-irradiated H1299 p53-R280K cells. However, there are no other significant differences in expression of β-catenin. Fractionated radiation treatment has no significant effect on β-catenin expression regardless of p53 genotype. Similar to the p53 mutants, H1299 p53-WT trends toward increased β-catenin expression both with and without fractionated radiation compared to the non-irradiated H1299 control cells, but statistical significance cannot be determined.

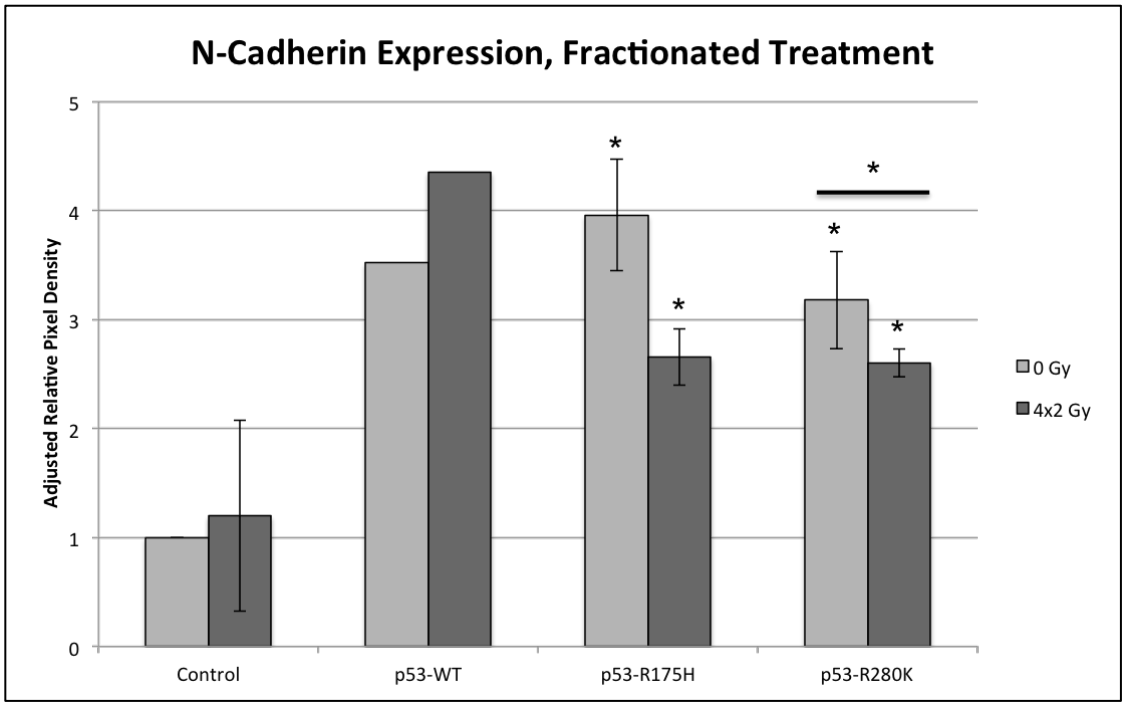

Figure 8. N-Cadherin expression in H1299 cells after fractionated IR treatment. Significance is noted by *p<0.05 compared to 0 Gy H1299 Control sample or between two samples under a bar with a star.

N-cadherin expression was quantified and graphed in Figure 8. Baseline expression of N-cadherin was significantly increased in H1299 p53-R175H and H1299 p53-R280K cells both with and without fractionated radiation compared to non-irradiated H1299 control cells. H1299 p53-WT cells also trend toward increased expression of N-cadherin both with and without fractionated radiation. Fractionated radiation treatment caused a significant decrease in N-cadherin expression in H1299 p53-R280K cells. H1299 p53-R175H cells also trended toward decreased N-cadherin expression following fractionated radiation treatment but did not achieve statistical significance. H1299 control cells and H1299 p53-WT cells do not have altered expression of N-cadherin following fractionated radiation treatment.

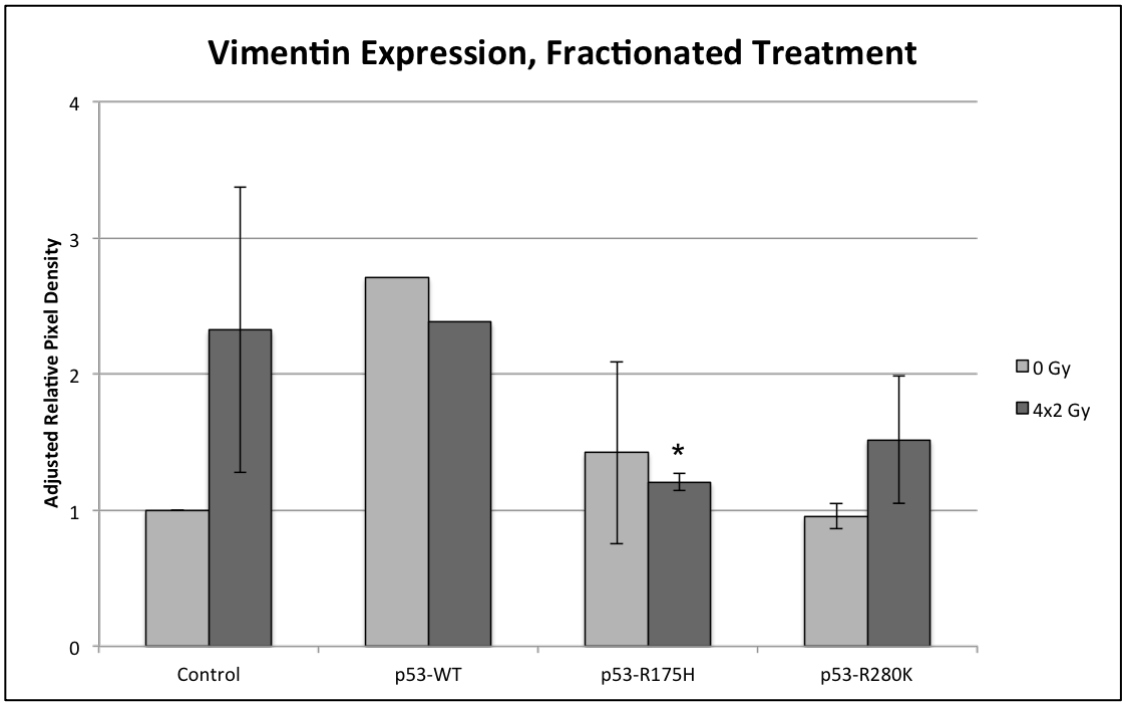

Figure 9. Vimentin expression in H1299 cells after fractionated IR treatment. Significance is noted by *p<0.05 compared to 0 Gy H1299 Control sample.

In Figure 9, vimentin expression was quantified and graphed. Vimentin expression was significantly increased in H1299 p53-R175H cells treated with fractionated radiation compared to non-irradiated H1299 control cells. However, there are no other significant differences in vimentin expression. H1299 p53-WT cells trend toward increased vimentin expression. Although the expression of vimentin in H1299 control cells trends up after fractionated radiation treatment, there is no significant change in vimentin expression regardless of p53 genotype following fractionated radiation treatment.

The results for mesenchymal marker proteins suggest that fractionated radiation treatment does not cause an increase in mesenchymal characteristics regardless of p53 genotype. If anything, EMT decreases after fractionated radiation

treatment in H1299 p53-R280K cells causing the cell to gain a slightly more epithelial phenotype. Although significance could not be determined for H1299 p53-WT cells, the presence of p53-WT appears to have increased the expression of all three mesenchymal markers compared to H1299 control cells.

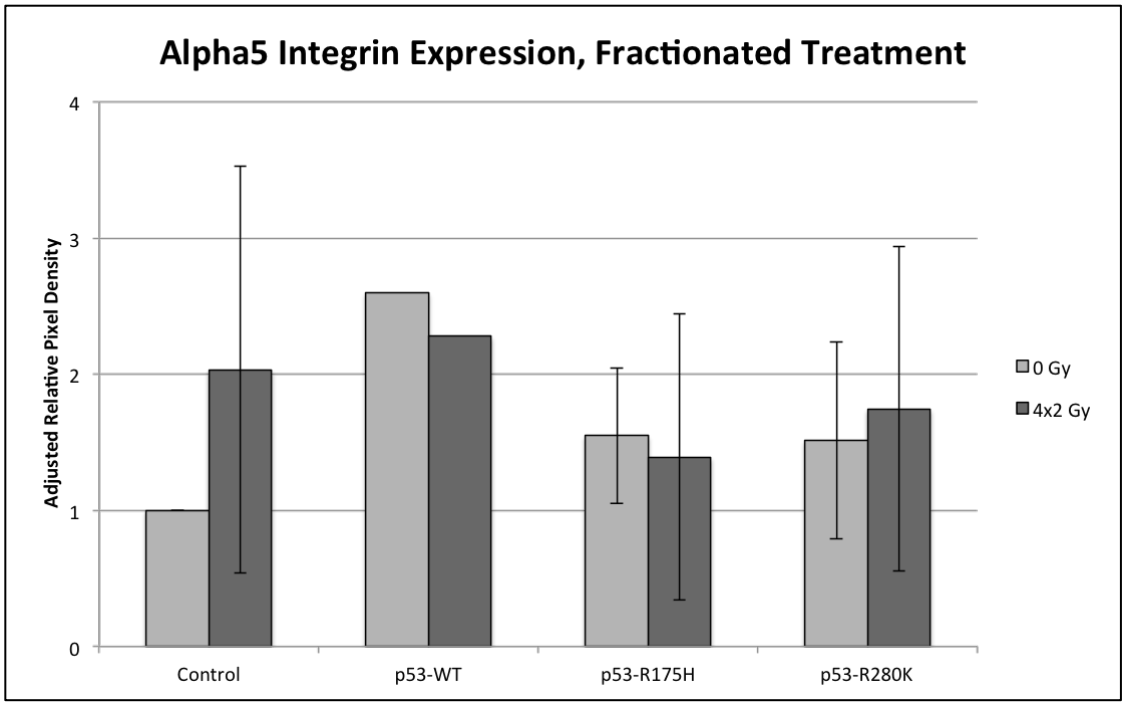

Figure 10. Integrin α5 expression in H1299 cells after fractionated IR treatment.

Integrin α5 expression was tested because of its importance in adhesion and migration in many cancer types. Its expression was quantified and graphed in Figure 10. Although integrin α5 expression trends up following fractionated radiation treatment in H1299 control cells, there were no significant changes in the expression of integrin α5 regardless of p53 genotype or fractionated radiation treatment. The expression of integrin α5 in H1299 p53-WT cells appears to increase compared to

non-irradiated H1299 control cells, but statistical significance could not be determined.

Fractionated IR alters α5β1 surface expression in H1299 cells expressing p53 and p53 mutants

While Western blot analysis can measure the overall expression of adhesive molecules, such as integrin α5, it cannot determine where proteins are located in the cell. In order for integrin α5 to directly affect cell adhesion and migration, it must be in the cell membrane. Therefore, flow cytometry was used to determine the surface expression of integrin α5β1, which binds to the extracellular matrix protein, fibronectin. Determining the surface expression of integrin α5 was also attempted; however, the isotype control antibody showed non-specific binding, so these data have not been included.

The surface expression of α5β1 is shown in Figure 11. The baseline surface expression of integrin α5β1 significantly increased in H1299 cells expressing p53-WT compared to H1299 control cells. There was no significant difference between the baseline levels of integrin α5β1 in mock irradiated H1299 cells expressing p53-R175H or p53-R280K compared the non-irradiated H1299 control cells. However, there was a significant increase in integrin α5β1 expression in H1299 p53-WT, H1299 p53-R175H, and H1299 p53-R280K cells following fractionated IR compared to the mock irradiated H1299 control cells while there was no significant difference between the mock irradiated and fractionally irradiated H1299 control cells. Fractionated IR

also increased the surface expression of integrin α5β1 in H1299 cells expressing p53-WT or p53-R280K compared to mock irradiated cells expressing p53-WT or p53-R280K, respectively. H1299 cells expressing p53-R175H also appear to follow this trend, but just slightly miss statistical significance (p=0.06). Finally, fractionally irradiated H1299 p53-WT cells had significantly increased surface expression of integrin α5β1 compared to fractionally irradiated H1299 control cells.

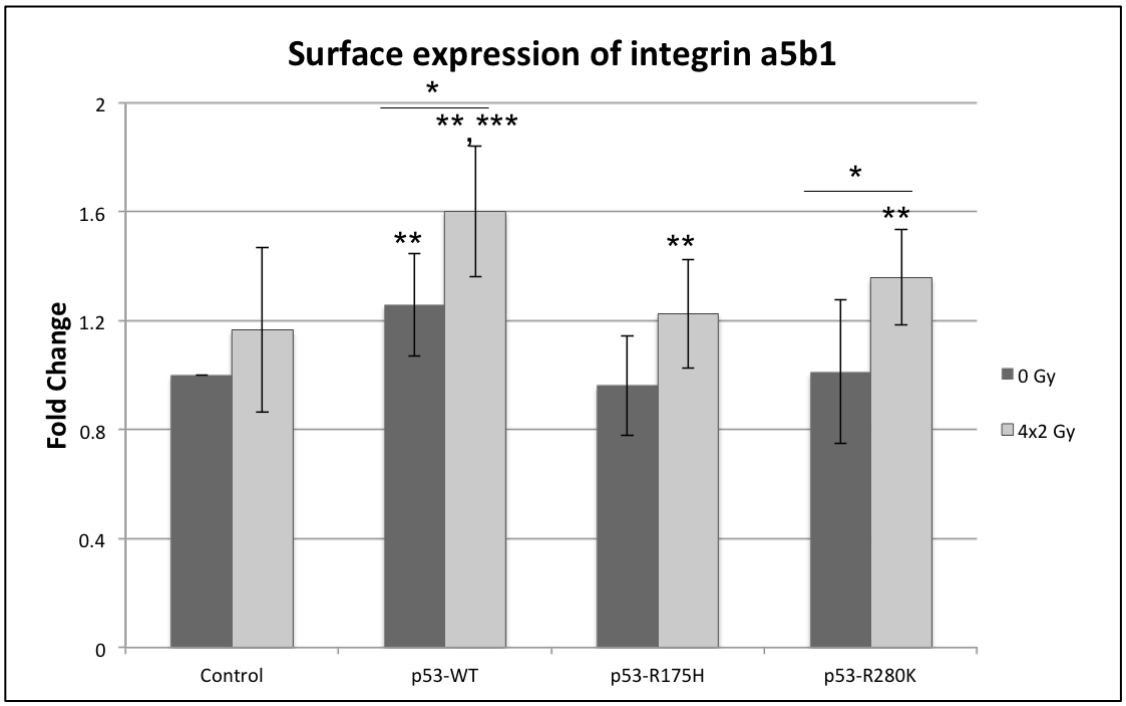

Figure 11. Surface expression of integrin α5β1 after fractionated IR. The bars represent the fold change in the surface expression compared to the non-irradiated control. Significance is noted by *p<0.05 between 0 Gy and 4x2 Gy of same p53 characteristic, **p<0.05 compared to 0 Gy H1299 control cells, or ***p<0.05 compared to 4x2 Gy H1299 control cells.

These results show that p53-WT expression can increase the surface expression of integrin α5β1. Furthermore, p53-WT and p53-R280K appear to play a

role in increasing the expression of integrin α5β1 in response to fractionated IR. Expression of p53-R175H may be involved in increasing the expression of integrin α5β1 in response to fractionated IR, but further exploration is needed to confirm that.

Western blot data show that fractionated radiation treatment had no significant effect the overall expression of integrin α5, which does not match these results. Therefore it is possible that the increase in expression of surface integrin α5β1 is due to integrin β1 expression being induced by fractionated radiation treatment.

Migration after fractionated IR treatment

Wound healing assays were performed to test cell migration one week following the first treatment of four fractionations of 2 Gy administered for four consecutive days. Representative photos are shown below (Fig. 12).

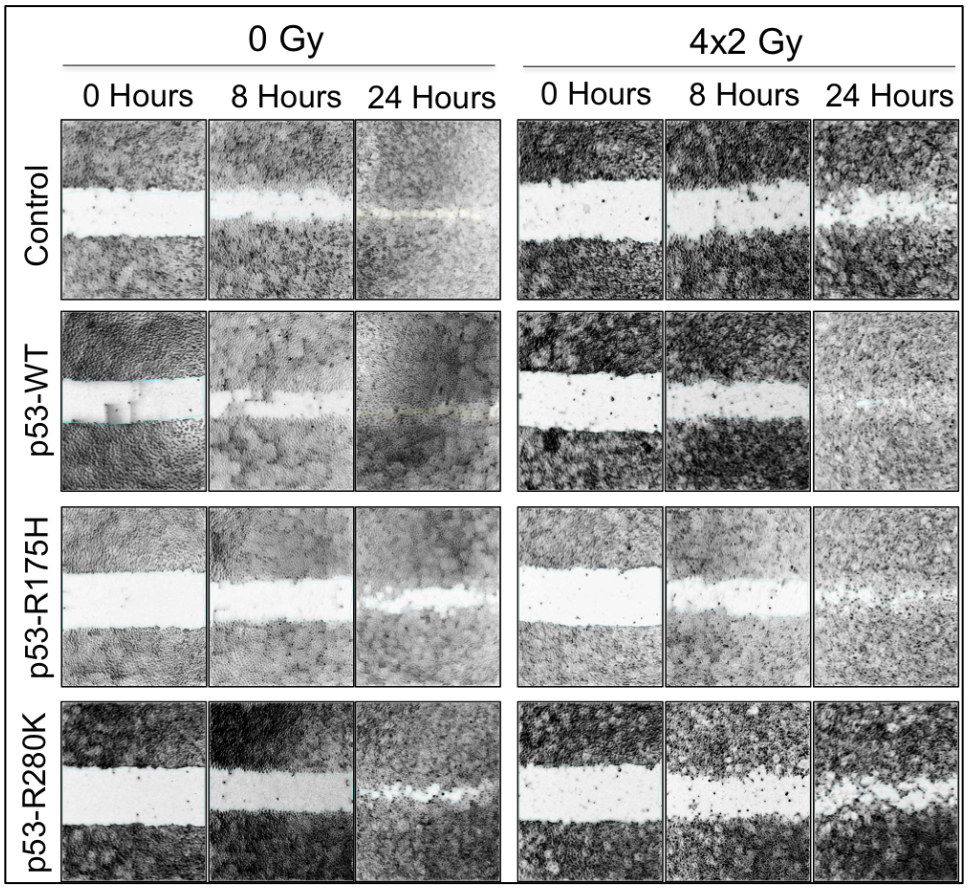

Figure 12. Migration of H1299 cells after fractionated IR treatment.

The results show that at 8 hours after the scratch was made, migration is significantly increased in H1299 p53-WT and H1299 p3-R175H cells treated with fractionated radiation compared to non-irradiated H1299 control cells. However, there

are no other significant differences in migration at 8 hours. Fractionated radiation treatment does not significantly affect migration within each p53 genotype (Fig. 13).

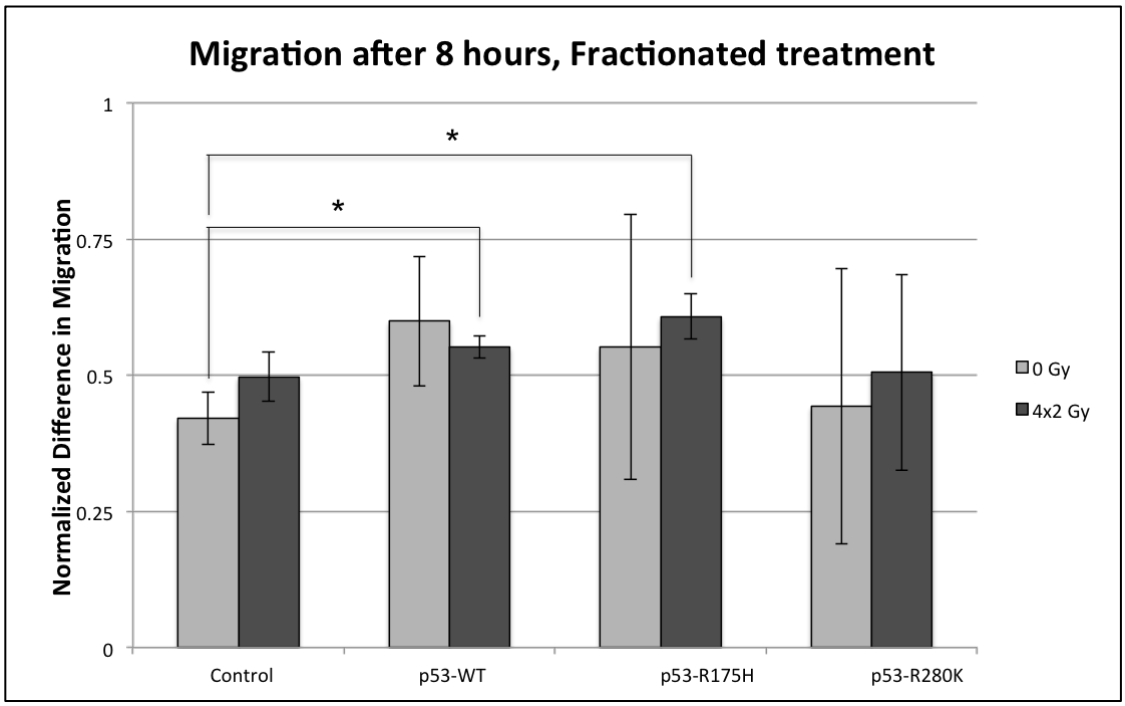

Figure 13. Migration of H1299 cells treated with fractionated radiation after 8 hours of healing. Significance is noted by *$p<0.05$.

At 24 hours after the scratch was made, migration remained significantly increased in H1299 p53-WT and H1299 p53-R175H cells treated with fractionated radiation compared to non-irradiated H1299 control cells. Furthermore, migration in fractionated radiation treated H1299 control cells increased compared to non-irradiated H1299 control cells at 24 hours. Although fractionated radiation significantly increases migration in H1299 control cells after 24 hours, migration is

not significantly affected by fractionated radiation treatment in any other p53 genotype after 24 hours (Fig. 14).

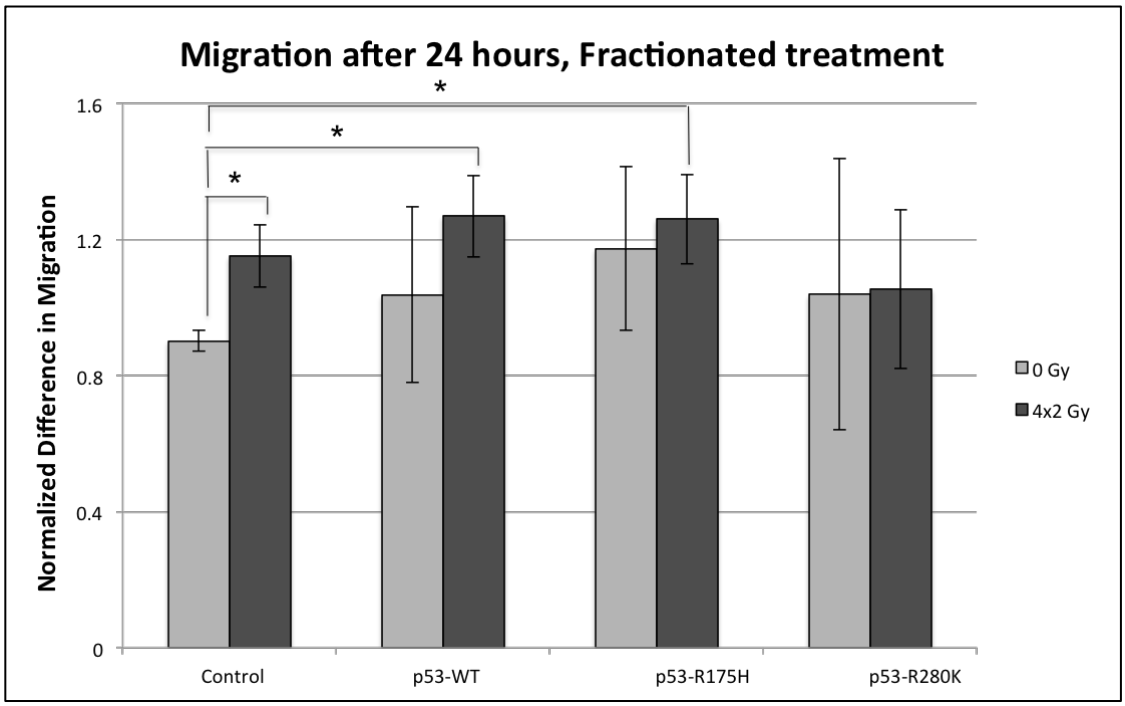

Figure 14. Migration of H1299 cells treated with fractionated radiation after 24 hours of healing. Significance is noted by *$p<0.05$.

The data show that fractionated IR has very little, if any, effect on migration. Furthermore, only p53-WT expression showed a small increase in migration, and p53-R175H showed a small decrease in migration compared to the control, suggesting that p53-WT, p53-R175H, and p53-R280K do not play a role in the cells' motility or their migratory response to fractionated IR (Fig. 14).

Although there were significant increases in the surface expression of integrin $\alpha5\beta1$ following fractionated IR treatment on H1299 p53-WT, p53-R175H, and p53-

R280K cells, there were not complementary results in migration experiments. One could hypothesize that p53-WT expression, which induced expression of integrin α5β1, resulted in increased migration, but more support would be necessary.

Wound healing results show that fractionated IR does not largely induce migration, which corroborates with Western blot data. Since there was little evidence for EMT being induced in the Western blot data, one would not expect increased migration, which is a characteristic of the mesenchymal phenotype.

EMT marker protein and α5 integrin expression after single IR dose

The mesenchymal marker protein N-cadherin and integrin α5 expression were probed by Western blot after 24 hours treatment with a single dose of 4 Gy or 8 Gy. N-cadherin was used to approximate EMT, while integrin α5 was probed for its role in migration (Fig. 15).

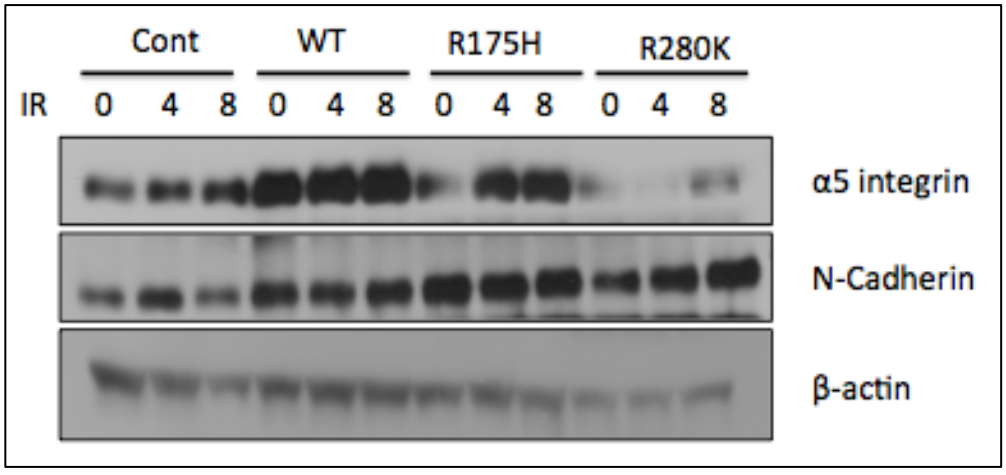

Figure 15. EMT protein marker and integrin α5 expression in H1299 cells after a single dose of 0 Gy, 4 Gy, or 8 Gy.

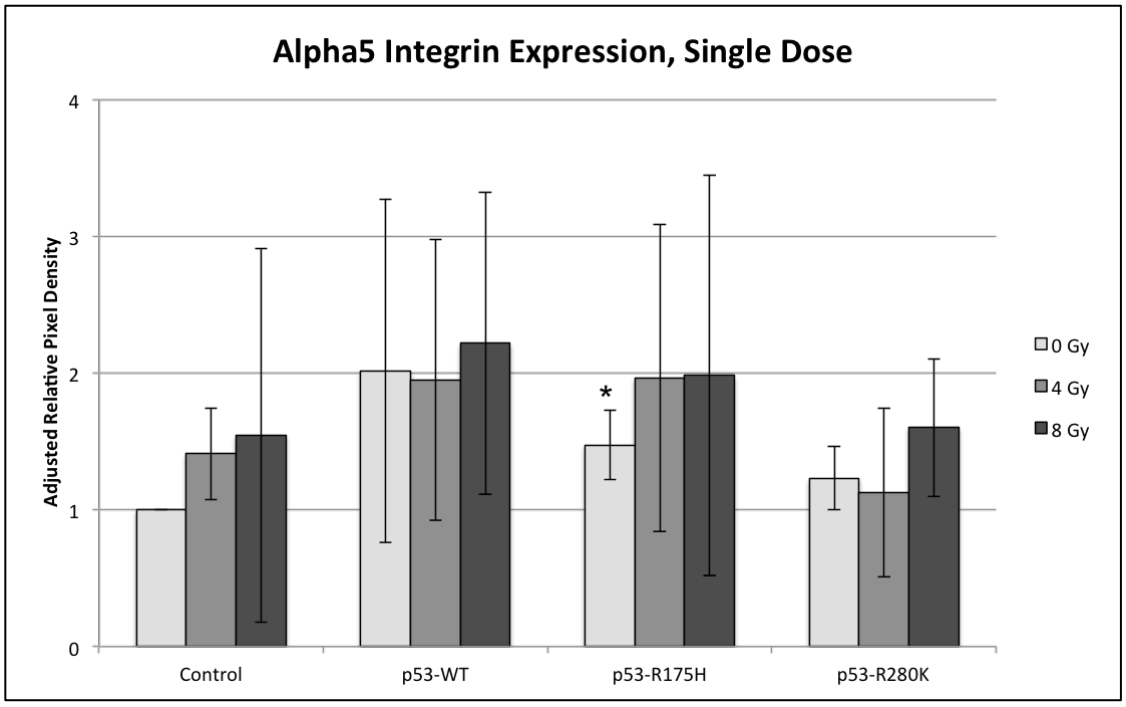

Figure 16. Integrin α5 expression in H1299 cells after treatment with 0 Gy, 4 Gy, or 8 Gy. Significance is noted by *$p<0.05$ compared to 0 Gy H1299 Control sample.

Figure 16 shows the relative expression of integrin α5. Integrin α5 expression was significantly increased in non-irradiated H1299 p53-R175H cells compared to non-irradiated H1299 control cells. Although there were no other significant differences in integrin α5 expression, the expression trends up after 4 Gy and 8 Gy in H1299 control and H1299 p53-R175H cells and after 8 Gy in H1299 p53-R280K cells. There does not appear to be any change in integrin α5 expression after 4 Gy or 8 Gy in H1299 p53-WT cells. Additionally, surface expression of integrin α5β1 was increased in preliminary experiments in p53-mutant cells (data not shown).

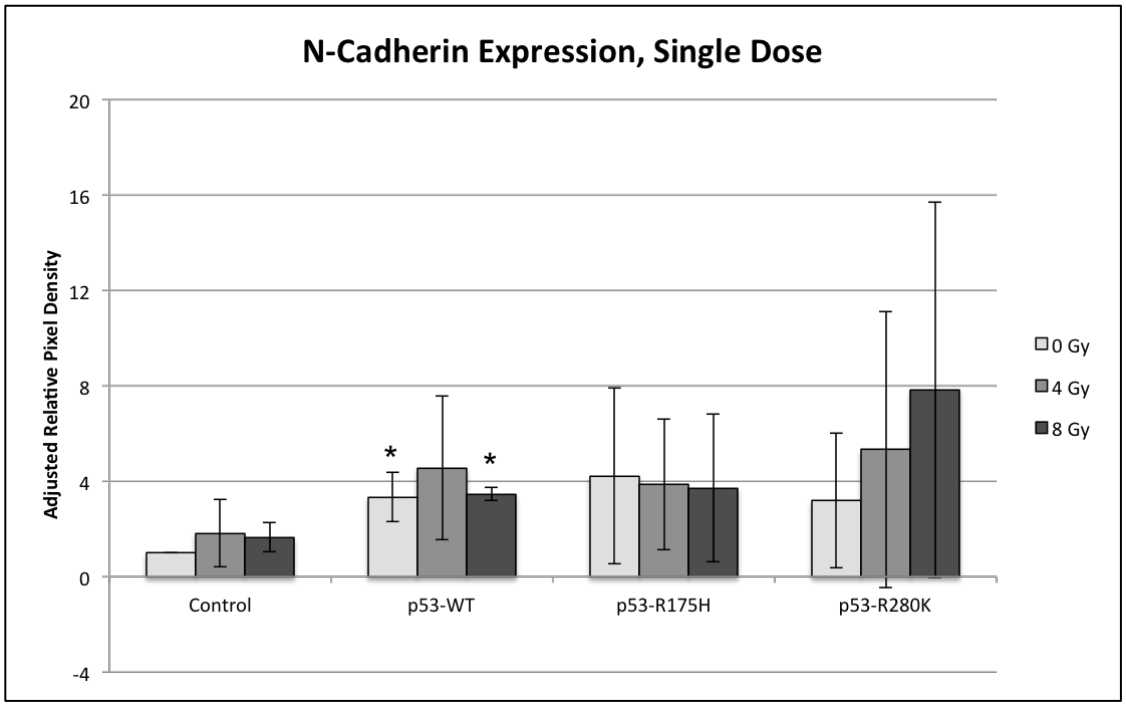

Figure 17. N-Cadherin expression in H1299 cells after treatment with 0 Gy, 4 Gy, or 8 Gy. Significance is noted by *$p<0.05$ compared to 0 Gy H1299 Control sample.

H1299 p53-WT cells that were not treated with radiation and that were treated with 8 Gy showed significantly increased expression of N-cadherin compared to the non-irradiated H1299 control cells. The expression of N-cadherin in H1299 p53-WT cells treated with 4 Gy also appeared to be increased compared to the non-irradiated H1299 control cells, but the increase was not statistically significant, likely due to a large standard deviation. There was no effect on N-cadherin expression by 4 Gy or 8 Gy radiation treatments within H1299 control cells, H1299 p53-WT cells, or H1299 p53-R175H cells. Although there was not a significant increase in N-cadherin expression in H1299 p53-R280K cells after 4 Gy or 8 Gy radiation treatment, there was an upward trend in expression as the dose increased, but large standard deviations definitely contributed to there being no significant difference.

These data show that a single dose of 4 Gy or 8 Gy, followed by 24 hours to recover, does not significantly alter EMT marker protein expression or the expression of integrin α5. However, although there were no significant differences in integrin α5 expression or N-cadherin expression after treatment with 4 Gy or 8 Gy, there appears to be an upward trend in expression of integrin α5 in H1299 control cells and H1299 p53-R175H cells. There also appears to be an upward trend in expression of N-cadherin in H1299 p53-R280K cells following treatment with 4 Gy or 8 Gy.

Migration increases in H1299 p53-R280K cells after single IR dose

Twenty-four hours after treatment with a single dose of 4 Gy or 8 Gy, migration in H1299 control and H1299 p53-WT cells was not altered. Representative pictures are shown in Figure 18.

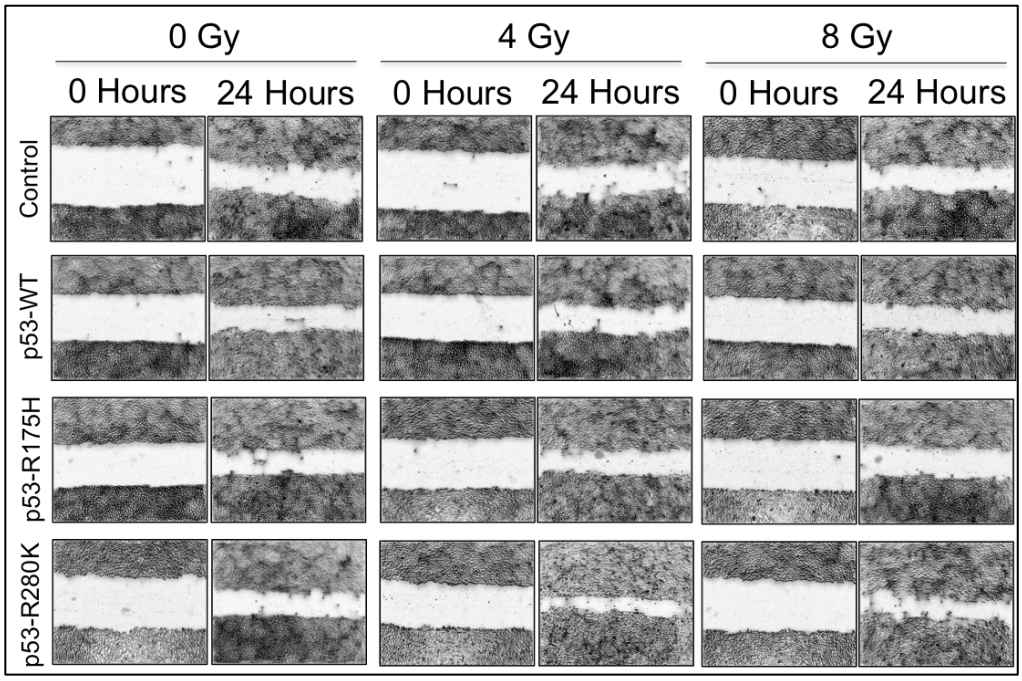

Figure 18. Migration of H1299 cells after a single dose of 0 Gy, 4 Gy, or 8 Gy.

Migration appeared to increase in H1299 p53-R175H cells after treatment with 4 Gy and 8 Gy compared to non-irradiated H1299 p53-R175H cells, but there was no statistically significant increase. However, treatment with 8 Gy caused a significant increase in H1299 p53-R280K cells compared to non-irradiated H1299 p53-R280K cells. Treatment with 4 Gy also appeared to increase migration in H1299 p53-R280K

cells compared to non-irradiated H1299 p53-R280K cells, but the change was not statistically significant (Fig. 19).

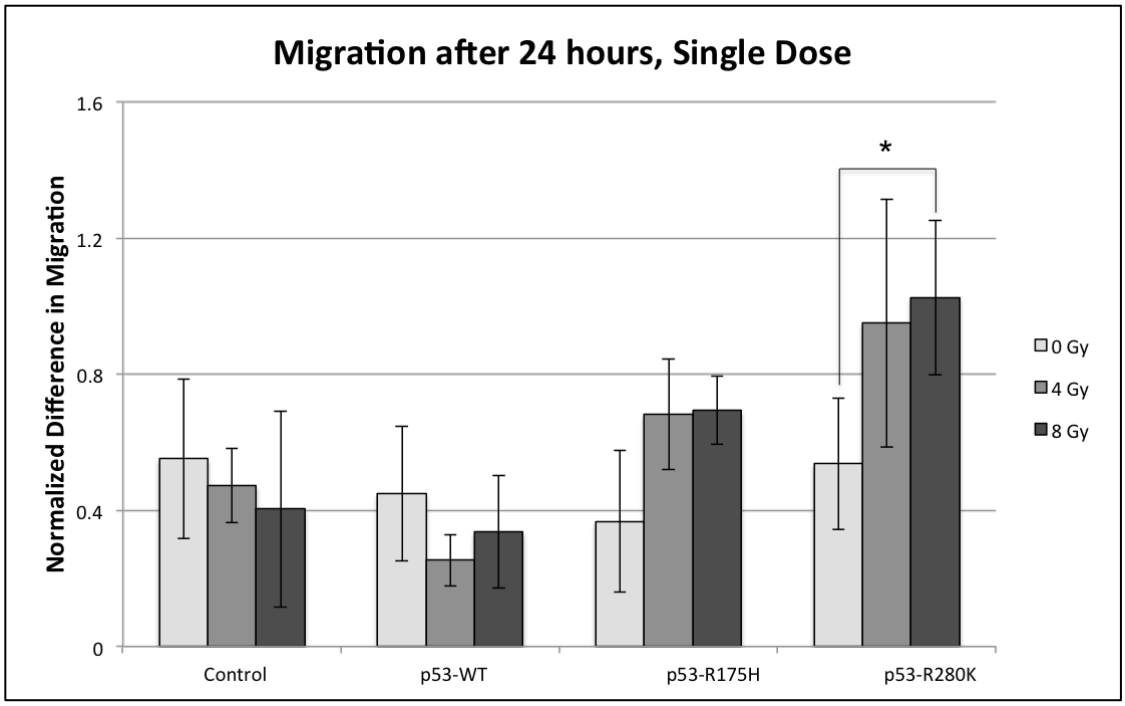

Figure 19. Migration of H1299 cells treated with 0 Gy, 4 Gy, or 8 Gy after 24 hours of healing. Significance is noted by *$p<0.05$.

Although statistical significance did not occur in Western blots after treatment with 4 Gy or 8 Gy, the patterns observed in N-cadherin expression in H1299 p53-R280K cells matched the increase in migration. The pattern of increased expression of integrin α5 after treatment with 4 Gy and 8 Gy in H1299 p53-R175H cells also matched the pattern of increased migration, but statistical significance did not occur for either.

DISCUSSION

One of the initial goals of this project was to investigate the short term and long term effects of IR on cells expressing common p53 mutants. As has been shown in previous research, IR can alter the expression of adhesive molecules and migration in some cancer cells[36,37,38,21,22,23,56,57,58]. Prior research in our lab also suggested that p53 mutation could alter the response to IR, so we aimed to elucidate the role of specific p53 mutants (p53-R175H and p53-R280K) in response to IR compared to p53-WT and p53-null cells[50]. Both p53-R175H and p53-R280K have a point mutation in the DNA-binding domain, and p53-R175H is considered a "hotspot" mutation because of its increased prevalence in cancers[62]. While p53-R175H is a common hotspot mutation, p53-R280K is the mutant expressed in the MDA-MB-231 breast cancer cells used in my lab's previous research[58]. Nevertheless, both p53 mutants were good candidates for preliminary investigations of their effect on a cancer cell's EMT and migratory response to IR.

In order to investigate long-term effects, we planned to create a survivor cell line from cells that survived fractionated IR treatment and to assay the phenotypic changes that occur in the cells. However, the growth rate of the majority of the survivor cells was too low to maintain those cells as a separate cell line, so we had to change our course of action. The dose was likely too high to produce a maintainable cell line, so instead we used the same fractionated IR treatment, but assayed them one week after the first treatment.

On the other hand, in order to explore how single doses of IR affect the short-term phenotype of our cells, we treated the cells with a single dose of 4 Gy or 8 Gy IR and assayed them 24 hours after treatment. Previous research had shown short-term changes in migration following IR, so we expected that short- and long-term changes would occur, especially in cells expressing a p53 mutant.

Our data show that N-cadherin expression was not significantly increased by a dose of 4 Gy or 8 Gy regardless of p53 genotype, so we cannot conclude that EMT occurred in any cell line. If EMT would have clearly occurred according to mesenchymal protein marker expression, there would have been concurrent loss of epithelial characteristics, such as being polarized, being associated with a basement membrane, and having cell-cell junctions. In addition to losing these characteristics, they would have gained mesenchymal characteristics such as the ability to survive in liquid suspension, the lack of cell-cell junctions, an increase in motility, and an increase in the ability of cells to remodel ECM by degradation and rebuilding[47].

Although there was not any significant increase in N-cadherin expression after treatment with 4 Gy or 8 Gy, the expression of N-cadherin appeared to increase in H1299 p53-R280K cells following a single dose of 4 Gy or 8 Gy, but significance was not achieved. There was a significant increase in migration in H1299 p53-R280K cells treated with 8 Gy compared to non-irradiated H1299 p53-R280K cells though. While a wound healing assay does not directly tell anything about EMT, increased migration is a characteristic phenotype of cells in a mesenchymal state.

These results support that p53-R280K expression can increase migration in response to treatment with 8 Gy. Both the p53-R175H and p53-R280K alleles are dominant negatives, showing the ability to inhibit p53-WT function when expressed together[66], so even in the case of heterozygous cancer with a p53-WT allele and a p53-R280K allele, one would expect increased migration after treatment with 8 Gy. Other research has shown gains of function from p53 mutation that contribute to cancer promotion and metastasis[62,63], so this adds a new function of p53-R280K in response to 8 Gy. There were no significant increases in migration in H1299 p53-R175H after 4 Gy or 8 Gy, but a trend toward increased migration exists. If standard deviations were lower, significance may have been achieved. The standard deviations are high likely due to the difficulty of creating equally sized scratches by hand and to the difficulty of performing the experiments at the exact same cell density each time.

One aspect of EMT that was not explored much yet is the secretion of proteins that can modify ECM, such as MMPs, which degrade ECM. Using an ELISA assay, we could quantify the effect of IR and p53 mutation on MMP secretion. If MMP secretion is increased after IR treatment, it could suggest that there is increased EMT and increased invasive potential, especially in H1299 p53-R280K cells that already showed significantly increased migration after 8 Gy and also trended toward increased EMT marker protein expression.

EMT is associated with increased metastatic potential, so the fact that single dose IR may induce EMT in H1299 p53-R280K cells is important for understanding the wide range of potential effects of radiotherapy. In the future it will be important to

research if the potential induction of EMT correlates to increased invasive potential. Invasive potential can be partially explored using basement membrane extract (BME) invasion assays[67]. These experiments would test the cumulative effect of factors of EMT, such as migration and ECM degradation, to give an overall estimation of the invasive potential of the cells following treatment with IR.

We planned to do flow cytometry on samples treated with 4 Gy or 8 Gy IR 24 hours after treatment, but we did not have enough time to repeat it more than once. However, we have preliminary data showing that α5β1 integrin surface expression increases in response to a single dose of IR in H1299 p53-R175H and H1299 p53-R280K cells. If the results are confirmed after repeating the experiment, it would be worthwhile to investigate the role of α5β1 integrin in IR-induced migration by using a α5β1 integrin function-blocking antibody and assaying for migration. If migration caused by IR in H1299 p53-R280K cells is inhibited by the function-blocking antibody, it would suggest that IR-induced migration is mediated by α5β1 integrin in cells expressing p53-R175H or p53-R280K.

In addition to investigating p53-R175H and p53-R280K more in depth in future research, it would be pertinent to investigate other hotspot p53 mutations. For example, these experiments could be expanded to other hotspot mutants, such as p53-R248W and p53-R273H. By characterizing the role of these other mutants in addition to the ones that we have already begun to explore, our breadth of knowledge would expand and could help guide us to individualized cancer treatments.

We hypothesized that since there are phenotypic changes following a single dose of IR in H1299 p53-R280K, there would be long lasting, or even permanent, changes following fractionated IR that would reflect those changes. However, none of such changes occurred. EMT marker protein expression decreased, if anything, following fractionated IR. Migration in wound healing assays was not altered by fractionated IR in H1299 cells expressing WT or mutant p53, but was increased in H1299 control cells.

We believe that our fractionated IR treatment may have caused cells too much damage to *in vitro*, forcing them to senesce and border on cell death because growth was inhibited following treatment and because our results did not reflect the short-term treatment. It is possible that fractionated IR does not affect EMT and migration, but the increase in the surface expression of integrin α5β1 suggests that something else, such as altered trafficking or recycling of the subunits, may be involved too since its expression has been associated with migration. Furthermore, in a normal tumor, many cells undergo apoptosis or experience growth arrest in response to radiotherapy, but very rarely does the entire tumor die; it usually leaves a population of radioresistant cells. Understanding this population of radioresistant cells is paramount to prevent the regrowth of the primary tumor or the metastasis of these cells to distant sites.

The differing results between single treatment and fractionated treatment warrant further investigation too. At some point between the single dose and the fractionated treatment, the EMT induced by IR 24 hours after a single dose was

reversed and returned to the non-irradiated level in cells treated with fractionated IR. Understanding this timing could bring insights into the mechanism behind a cell's response to IR. Eventually understanding the time course could improve the efficacy of radiotherapy by altering the treatment schedule or by informing us when to prescribe a drug that can prevent EMT.

The only result from the fractionated treatment that showed a significant increase was in the surface expression of integrin α5β1. However, overall expression of integrin subunit α5 was decreased following fractionated treatment. There are several possibly explanations for this phenomenon. One possibility is that integrin β1 subunit expression increased after fractionated IR treatment, which could have caused the increase in α5β1 integrin surface expression. To test this, we could assay the overall expression of the integrin β1 subunit by Western blot and the surface expression by flow cytometry. Another possibility lies in the trafficking and recycling of the integrin subunits. Immunofluorescence could be used to visualize where the α5 and β1 integrin subunits are localized in the cell.

Overall, these results suggest that further research is warranted in the role of p53 mutants in a cancer cell's response to IR. A single dose appears to induce EMT and migration in H1299 p53-R175H and H1299 p53-R280K cells in response to a single dose of IR after 24 hours, but a fractionated treatment can inhibit EMT and has no effect on migration. Understanding how EMT is induced after a single dose, but inhibited by fractionated treatment is imperative to improving the efficacy of radiotherapy as a cancer treatment. This study is a building block from which future

research can further elucidate these mechanisms. Additionally, research involving p53 mutation and IR will provide direction for individualized cancer treatment for patients with p53 mutation undergoing radiotherapy. Moreover, further investigation of the detailed mechanism induced by p53 mutation and IR might provide strong clinical improvements in the success rate of radiotherapy.

References

1. "Cancer." World Health Organization; Updated 2015 Feb. [Accessed 2015 April]. http://www.who.int/mediacentre/factsheets/fs297/en/.
2. "What Is Cancer?" National Cancer Institute. Updated 9 Feb. 2015. [Accessed 2 Sept. 2015]. http://www.cancer.gov/about-cancer/what-is-cancer.
3. Sporn MB. The war on cancer. *Lancet* (1996); 347: 1377–1381.
4. Breast Cancer. American Cancer Society. [Accessed 2015 April]. http://www.cancer.org/cancer/breastcancer/detailedguide/index.
5. Jemal A, Siegel R, Xu J, and Ward E. "Cancer Statistics, 2010." *CA: A Cancer Journal for Clinicians* 2010; 60: 277-300.
6. "How does chemotherapy work?" Informed Health Online; Updated January 14, 2016. [Accessed 2016 April]. http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0072611/.
7. Longley DB, Harkin DP, and Johnston PG. 5-Fluorouracil: Mechanisms of action and clinical strategies. *Nat Rev Cancer* 2003; 3: 330-338.
8. DeVita Jr. VT and Chu E. A History of Cancer Chemotherapy. *Cancer Res* 2008; 68: 8643-8653.
9. Mellman I, Coukos G, and Dranoff G. Cancer immunotherapy comes of age. *Nature* 2011; 480: 480-489.
10. Radiation Therapy Principles. American Cancer Society. [Accessed 2015 April]. http://www.cancer.org/treatment/treatmentsandsideeffects/treatmenttypes/radiation/index.
11. Ward JF."DNA Damage Produced by Ionizing Radiation in Mammalian Cells: Identities, Mechanisms of Formation, and Reparability." *Progress in Nucleic Acid Research and Molecular Biology.* San Diego: Academic, 1988.
12. Urbano, Kevin V. "Ionizing Radiation, DNA Double Strand Break and Mutation." *Advances in Genetics Research.* Hauppauge, NY: Nova Science, 2010.
13. Basker R, Lee KA, Yeo R, Yeoh KW. Cancer and Radiation Therapy: Current Advances and Future Directions. *Int J Med Sci* 2012; 9(3): 193-199. Doi:10.7150/ijms.3635.
14. Cooper, Geoffrey M., and Robert E. Hausman. *The Cell: A Molecular Approach.* Washington, D.C.: ASM, 2009.
15. "What Is Ionizing Radiation?" World Health Organization. [Accessed 23 Sept. 2015]. http://www.who.int/ionizing_radiation/about/what_is_ir/en/index2.html.
16. "X-rays, Gamma Rays, and Cancer Risk." American Cancer Society. [Accessed 23 Sept. 2015]. http://www.cancer.org/acs/groups/cid/documents/webcontent/acspc-038756-pdf.pdf.
17. Leaver, Dennis T., and Charles M. Washington. *Principles and Practice of Radiation Therapy*. St. Louis: Mosby, 1996.
18. Perez, Carlos A., and Luther W. Brady. *Principles and Practice of Radiation Oncology*. Philadelphia: Lippincott, 1987.
19. Welsh JW, Komaki R, Amini A, et al. Phase II trial of erlotinib plus concurrent whole-brain radiation therapy for patients with brain metastases from non–small-cell lung cancer. *J Clin Oncol* 2013; 31: 895-902.

20. "Doses from Medical X-Ray Procedures." Health Physics Society. [Accessed 23 Sept. 2015]. http://hps.org/physicians/documents/Doses_from_Medical_X-Ray_Procedures.pdf.
21. Gaugler MH, Squiban C, van der Meeren A, Bertho JM, Vandamme M, Mouthon MA. Late and persistent up-regulation of intercellular adhesion molecule-1 (ICAM-1) expression by ionizing radiation in human endothelial cells in vitro. *Int J Radiat Biol* 1997; 72: 201-209.
22. Hallahan D, Kuchibhotla J, Wyble C. Cell adhesion molecules mediate radiation-induced leukocyte adhesion to the vascular endothelium. Cancer Res 1996; 56: 5150-5.
23. Heckmann M, Douwes K, Peter R, Degitz K. Vascular activation of adhesion molecule mRNA and cell surface expression by ionizing radiation. *Exp Cell Res* 1998; 238: 148-154.
24. Dong Z, Zhou L, Han N, Zhang M, Lyu X. Wnt/beta-catenin pathway involvement in ionizing radiation-induced invasion of U87 glioblastoma cells. *Strahlenther Onkol* 2015.
25. Kwak SY, Kim BY, Ahn HJ, et al. Ionizing radiation-inducible miR-30e promotes glioma cell invasion through EGFR stabilization by directly targeting CBL-B. *FEBS J* 2015; 282: 1512-1525.
26. Park CM, Park MJ, Kwak HJ, et al. Ionizing radiation enhances matrix metalloproteinase-2 secretion and invasion of glioma cells through Src/epidermal growth factor receptor-mediated p38/Akt and phosphatidylinositol 3-kinase/Akt signaling pathways. *Cancer Res* 2006; 66: 8511-8519.
27. Patel ZS, Grugan KD, Rustgi AK, Cucinotta FA, Huff JL. Ionizing radiation enhances esophageal epithelial cell migration and invasion through a paracrine mechanism involving stromal-derived hepatocyte growth factor. *Radiat Res* 2012; 177: 200-208.
28. Zhou YC, Liu JY, Li J, et al. Ionizing radiation promotes migration and invasion of cancer cells through transforming growth factor-beta-mediated epithelial-mesenchymal transition. *Int J Radiat Oncol Biol Phys* 2011; 81: 1530-1537.
29. Wan L, Pantel K, Kang Y. Tumor metastasis: moving new biological insights into the clinic. *Nature Medicine* 2013; 19(11): 1450-1464.
30. Valastyan, Scott and Robert A. Weinberg. "Tumor Metastasis: Molecular Insights and Evolving Paradigms." *Cell* 2011; 147(2): 275-292.
31. Perlikos F, Harrington KJ, and Syrigos KN. "Key molecular mechanisms in lung cancer invasion and metastasis: A comprehensive review." *Crit Reviews in Onc/Hem* 2013; 87: 1-11.
32. Berrier AL and Yamada KM. Cell-Matrix Adhesion. *J Cell Physiol* 2007; 213: 565-573.
33. Holly SP, Larson MK, and Parise LV. Multiple roles of integrins in cell motility. *Exp Cell Res* 2000; 261: 69-74.
34. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. *Nature Rev Cancer* 2002; 2(3): 163-174.

35. Margadant C, Monsuur HN, Norman JC, and Sonnenberg A. Mechanisms of integrin activation and trafficking. *Curr Opin Cell Biol* 2011; 23: 607-614.
36. Cordes N, Blaese MA, Meineke V, Van Beuningen D. Ionizing radiation induces up-regulation of functional beta1-integrin in human lung tumour cell lines in vitro. *Int J Radiat Biol* 2002; 78: 347-357.
37. Lee M, Lee HJ, Seo WD, Park KH, Lee YS. Sialylation of integrin beta1 is involved in radiation-induced adhesion and migration in human colon cancer cells. *Int J Radiat Oncol Biol Phys* 2010; 76: 1528-1536.
38. Yao H, Zeng ZZ, Fay KS, et al. Role of alpha(5)beta(1) Integrin Up-regulation in Radiation-Induced Invasion by Human Pancreatic Cancer Cells. *Trans Oncol* 2011; 4: 282-292.
39. Halbleib, Jennifer M. and W. James Nelson. Cadherins in development: cell adhesion, sorting, and tissue morphogenesis. *Genes Dev* 2006; 20: 3199-3214.
40. Behrens J, Mareel MM, Van Roy FM, Birchmeier W (1989) Dissecting tumor cell invasion: epithelial cells acquire invasive properties after the loss of uvomorulin-mediated cell–cell adhesion. *J Cell Biol* 108(6): 2435–2447.
41. Wu Y, Liu HB, Ding M, Liu JN, Zhan P, Fu XS, and Lu G. The impact of E-cadherin expression on non-small cell lung cancer survival: a meta-analysis. *Mol Biol Rep* 2012; 39: 9621-9628.
42. Shiozaki H, Tahara H, Oka H, Miyata M, Kobayashi K, Tamura S, Iihara K, Doki Y, Hirano S, Takeichi M et al. Expression of immunoreactive E-cadherin adhesion molecules in human cancers. *Am J Pathol* 1991; 139(1): 17–23.
43. Shiozaki H, Oka H, Inoue M, Tamura S, Monden M. E-cadherin mediated adhesion system in cancer cells. *Cancer* 1996; 77(8 Suppl): 1605–1613.
44. Siitonen SM, Kononen JT, Helin HJ, Rantala IS, Holli KA, Isola JJ. Reduced E-cadherin expression is associated with invasiveness and unfavorable prognosis in breast cancer. *Am J Clin Pathol* 1996; 105(4): 394–402.
45. Cheng L, Nagabhushan M, Pretlow TP, Amini SB, Pretlow TG. Expression of E-cadherin in primary and metastatic prostate cancer. *Am J Pathol* 1996; 148(5): 1375–1380.
46. Sato F, Shimada Y, Watanabe G, Uchida S, Makino T, Imamura M. Expression of vascular endothelial growth factor, matrix metalloproteinase-9 and E-cadherin in the process of lymph node metastasis in oesophageal cancer. *Br J Cancer* 1999; 80(9): 1366–1372.
47. Radisky DC. "Epithelial-mesenchymal Transition." *Cell Science* 2005; 118: 4325-326.
48. Roy FV and Berx G. "The Cell-cell Adhesion Molecule E-cadherin." *Cell Mol Life Sci* 2008; 65(23): 3756-3788.
49. Kalluri R and Weinberg RA. "The Basics of Epithelial-mesenchymal Transition." *J Clin Invest* 2009; 119(6): 1420-428.
50. Kang Y and Massagué J. "Epithelial-Mesenchymal Transitions." *Cell* 2004; 118: 277-79.

51. Veevers-Lowe J, Ball SG, Shuttleworth A, and Kielty CM. "Mesenchymal Stem Cell Migration Is Regulated by Fibronectin through alpha5beta1-integrin-mediated Activation of PDGFR and Potentiation of Growth Factor Signals." *J Cell Sci* 2011; 124(8): 1288-300.
52. Martin TA, Ye L, Sanders AJ, et al. Cancer Invasion and Metastasis: Molecular and Cellular Perspective. In: Madame Curie Bioscience Database [Internet]. Austin (TX): Landes Bioscience; 2000-2013. Available from: http://www.ncbi.nlm.nih.gov/books/NBK164700/.
53. Kim YT, Choi EK, Kim JW, Kim DK, Kim SH, and Yang WI. "Expression of E-Cadherin and α-, β-, γ-Catenin Proteins in Endometrial Carcinoma." *Yonsei Med J* 2002; 43(6): 701.
54. Satelli A and Li S. "Vimentin in Cancer and Its Potential as a Molecular Target for Cancer Therapy." *Cell Mol Life Sci* 2011; 68(18): 3033-3046.
55. Derycke LD and Bracke ME. "N-cadherin in the Spotlight of Cell-cell Adhesion, Differentiation, Embryogenesis, Invasion and Signalling." *Int J Dev Biol* 2004; 48(5-6): 463-476.
56. Baluna RG, Eng TY, and Thomas CR. Adhesion molecules in Radiotherapy. *Radiation Research* 2006; 166: 819-831.
57. Calaf GM, et al. "Differential expression of cell adhesion molecules in an ionizing radiation-induced breast cancer model system." *Oncol Rep* 2013; 30(1): 285-291.
58. Lee SH, Cheng H, Yuan Y, Wu S. Regulation of ionizing radiation-induced adhesion of breast cancer cells to fibronectin by alpha5beta1 integrin. *Radiat Res* 2014; 181: 650-8.
59. Makinde AY, John-Aryankalayil M, Palayoor ST, Cerna D, and Coleman CN. "Radiation Survivors: Understanding and Exploiting the Phenotype following Fractionated Radiation Therapy." *Mol Cancer Res* 2012; 11(1): 5-12.
60. Prives C and Hall PA. "The P53 Pathway." *J Pathol* 1999; 187(1): 112-126.
61. Rivlin N, Brosh R, Oren M, and Rotter V. "Mutations in the P53 Tumor Suppressor Gene: Important Milestones at the Various Steps of Tumorigenesis." *Genes & Cancer* 2011; 2(4): 466-474.
62. Muller PAJ and Vousden KH. "P53 Mutations in Cancer." *Nat Cell Biol* 2013; 15(1): 2-8.
63. Dittmer D, Pati S, Zambetti G, Chu S, Teresky AK, Moore M, Finlay C, and Levine AJ. "Gain of Function Mutations in P53." *Nat Genet* 1993; 4(1): 42-46.
64. Mirzayan R, Andrais B, Scott A, and Murray D. New insights into p53 signaling and cancer cell response to DNA damage: implications for cancer therapy. *Journal of Biomedicine and Biotechnology* 2012; 2012: 170325.
65. Miller L. "Analyzing gels and Western blots with ImageJ." Lukemiller.org; Updated Novermber 10, 2010. [Accessed April 2016]. http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/.
66. Junk DJ, Vrba L, Watts GS, Oshiro MM, Martinez JD, and Futscher BW. Different Mutant/Wild-Type p53 Combinations Cause a Spectrum of Increased

Invasive Potential in Nonmalignant Immortalized Human Mammary Epithelial Cells. *Neoplasia* 2008; 10(5): 450-461.

67. Benton G, Kleinman HK, George J, Arnaoutova. Multiple uses of basement membrane-like matrix (BME/Matrigel) in vitro and in vivo with cancer cells. *Int J Cancer* 2011; 128(8): 1751-1757.