# p53 Gene Therapy: A Novel Approach in the Treatment of Cancer

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Outline of Contents

## Introduction

The basic concept of human gene therapy has a history of more than 20 years. An article by Theodore Friedman and Richard Roblin in the March 3, 1972 issue of *Science* reviewed the idea of gene therapy. As many as 1500 genetic diseases had been characterized at that time, which caused physicians and scientists to recognize that human genetic diseases were becoming an increasingly visible and formidable problem. Thus, progress toward biochemical characterization of human genetic diseases and advances in the understanding of DNA led to proposals of replacing "bad" DNA with "good" DNA in those who suffered from genetic diseases.

With advances in recombinant DNA technology, genetic engineering, and molecular techniques, gene therapy has become a well explored scientific area. Although the first clinical use of gene therapy was for marking tumor-infiltrating lymphocytes in patients with malignant melanoma, scientists have since concentrated on the use of gene therapy in the treatment of genetic diseases (2). The first gene therapy clinical trial for a genetic disease was for adenine deaminase deficiency, or ADA, in 1990 (2). In March of 1995 there were 32 clinical trials for gene therapy of genetic diseases such as hemophilia and cystic fibrosis. At this same time, scientists began to translate the ideas of gene therapy to the treatment of cancer. A number of specific genetic mutations had been implicated in cancer and cancer progression. Thus, correcting these mutations through gene therapy seemed to be a rational approach to the treatment of cancer.

However, initial results of the first gene therapy clinical trials for the treatment of inherited and acquired genetic diseases failed to meet initial expectations, and achieved limited success. In the June 1997 issue of *Nature Medicine*, Helen Blau and Paul

Khavari reviewed the progress of gene therapy. According to this article, scientists had changed focus and were now concentrating on developing new technologies for gene delivery, gene regulation, and avoidance of immune responses. They also mentioned that new genes were being characterized and their mutations linked to acquired and inherited genetic diseases. As research in these areas progressed, gene therapy for inherited genetic diseases soon found success in human clinical trials. These successes included gene therapy for ADA deficiency, cystic fibrosis, and hemophilia (40). With these successes, gene therapy for the treatment of cancer began in 1996 with retroviral delivery of the wild type p53 gene into lung cancer patients. Due to the clinical effectiveness of p53 gene therapy, many subsequent clinical studies of gene therapy for cancer have followed. In 1999 the National Institutes of Health Recombinant DNA Advisory Committee reported 277 gene therapy clinical trials, 70% of which were for the treatment of cancer (2). By March of 2000, this number rose to 350 with 67% reported for cancer (2).

The p53 tumor suppressor gene, which is the focus of this paper, has been a major player in numerous gene therapy studies concerning the treatment of cancer. The p53 tumor suppressor has been implicated in more than 50% of human cancers, including colon cancer and breast cancer, and gene therapy based on the p53 tumor suppressor gene has shown potential for the treatment of many cancers. A better understanding of one important gene, such as p53, will make it easier in the future to individualize therapy for particular cancer types and to target the mutant gene involved. In the following sections, I will discuss the current understanding of the p53 gene and protein, its implications in

cancer, and how targeting the p53 gene through the use of gene therapy has shown promise for the treatment of cancer.

## The p53 Tumor Suppressor

It has been estimated that at least half of the human malignancies including breast, ovary, lung, and colorectal carcinomas are associated with a mutation in a specific tumor suppressor gene known as p53. Located on human chromosome 17p13.1, the p53 tumor suppressor gene consists of 20 kb alleles (24). Transcription of this gene produces a pre-mRNA with eleven exons, which can then be spliced to an mRNA between 2 and 2.5 kb in length and containing two promoters (24). Subsequent to translation and oligomerization, the p53 tumor suppressor goes on to regulate cell growth by controlling cell cycle progression at the G1/S and G2/M transitions, or by inducing apoptosis. The p53 protein is DNA-binding and acts as a transcription factor to control the expression of proteins involved in the cell cycle. In response to DNA damage, p53 accumulates in the cell nucleus, which causes cells to undergo cell cycle arrest and DNA repair or apoptosis. It is believed that cancer cells defective in p53 have lost the ability to undergo cell growth by normal cell cycle progression and apoptosis. Therefore, these cells acquire the transformed phenotype of cancer cells.

### Discovery

The discovery of p53 came about when a contaminating simian virus was identified in monkey kidney cultures used to propagate poliovirus vaccines (12). Due to the concern that vaccine recipients may have been introduced to this SV40 simian virus

intensive investigations to assess its pathogenicity were launched. Analysis of this virus led to the identification of a major virally encoded protein known as the large T antigen. In the late 1970's, several groups discovered a protein that coprecipitated with the large T antigen of the SV40 simian virus (12). This protein had an electrophoretic mobility of about 53 kD, and was named p53. Due to the association of the p53 protein and the large T antigen, it was first proposed that p53 functioned as an oncogene. The oncogenic ability of p53 was substantiated due to the fact that overexpression of p53 was associated with tumorigenicity and that p53 taken from tumor cells was tumorigenic to normal cells. However, in the 1980's, the true identity of p53 was revealed when it was found that the overexpressed p53 associated with tumorigenicity was of mutant form and that p53 from normal cells was able to reverse tumorigenicity in tumor cells (8). Therefore, the identity of p53 as a tumor suppressor gene was established.

**Structure**

The p53 protein is a 393 amino acid tetrameric polypeptide. Structure and function analyses have divided the p53 protein into a number of functional domains, as can be seen in figure 1. These include a transcriptional activation domain at the N-terminus, a sequence specific DNA binding domain in the central portion of the protein, and oligomerization and regulation domains at the C-terminus.

The transactivation domain is located within the first 42 amino acids of the acidic N-terminus of the p53 protein (8). This domain interacts with transcriptional machinery such as TATA-binding protein, and is important for the activity of p53 as a transcription

factor. In addition, this domain also plays a key role in the rapid degradation of p53 through the ubiquination pathway (37).

A large DNA binding domain comprising amino acids 102-292 in the central portion of the protein contains a protein:DNA recognition motif (8). Figure 2 shows a 3D representation of the p53 protein and its interactions with DNA as obtained through X-ray Crystallography. The DNA binding motif of the central domain consists of 3 loop-helix structures that bind in the major groove of the DNA (37). This DNA binding motif recognizes and interacts with two DNA sequences (5'PuPuPuC(A/T)(T/A)GPyPyPy3') separated by 0-13 base pairs (8). Residues 248 and 273 contact the DNA directly, and a zinc atom interacts with four metal binding residues to stabilize the loops. This central domain is the hot spot for p53 mutations, which affect core conformation and DNA binding.

The basic C-terminus of the protein contains a domain for the regulation of p53 oligomerization. This domain, consisting of residues 324-335, is responsible for the tetramer structure of the p53 protein. Monomeric p53 proteins first form dimers, and then a dimer of dimers, or tetramer is formed (8). The fact that tetramerization is required for transcription of downstream genes and for inhibition of tumor growth by p53 shows the importance of this domain (37). Several nuclear localization signals are also located at the C-terminus, and are required for p53 translocation to the nucleus where it can then act as a transcription factor (8).

A domain for the repression of DNA binding is also located at the C-terminus, and is capable of binding nonspecifically to various DNAs, especially damaged DNAs (8). Subunits of the transcriptional repair complex also bind to this domain. As a whole,

the C-terminus plays an important role in regulating activity of the entire p53 protein. It has been found that short single stranded DNA activates p53, while longer double stranded DNA inhibits p53 (8). This domain has also been suggested to control the apoptotic activity of the p53 molecule. The SH3 domain near the N-terminal has also been implicated in regulation through cooperation with the C-terminus (37).

The p53 protein has also been found to interact with several cellular and viral proteins. Cellular kinases such as DNA-PK, MAP kinase, JNK1, and JNK2 phosphorylate p53 at the N-terminus, while PKC, Cdc2, and cyclin A phosphorylate p53 at the C-terminus (8). The biological effects of phosphorylations by these kinases are not well understood, but have been suggested to play a role in regulation. Transactivation is inhibited upon binding of the SV40 large T antigen to the central domain, of adenovirus E1B to the N-terminal domain, and of HPV E6 protein the N-terminal domain (8). The Mdm2 oncoprotein binds to the N terminus and inhibits p53 activity as well (8). A redox/repair protein, known as Ref-1, is a potent activator of p53 (8).

**Function**

The p53 protein is activated upon DNA damage in the cell, which can be caused by ionizing radiation, UV radiation, growth factor deprivation, genotoxic stress, hypoxia, chemotherapy, oncogene activation, heat shock, or viral infection (8). The up regulation of p53 occurs at the post-translational level, and is achieved through stabilization of the protein (8). Upon induction, the main outcome of p53 activation is growth suppression, growth arrest at the G1, G2, and M checkpoints. The induction of growth arrest depends

on the ability of p53 to act as sequence specific transcriptional activator. A target gene in the p53 growth arrest pathway is the gene for the p21 protein. The p21 protein binds cyclin dependent kinases and inhibits their action in the cell cycle, thereby blocking cell proliferation. The p21 protein and the Gadd45 also bind proliferating cell nuclear antigen (PCNA), which is a regulatory subunit of DNA polymerase delta (8). This would inhibit DNA replication in S phase of the cell cycle and block cell proliferation.

The p53 protein also plays an important role in cell death through the apoptotic pathway. Bax, a pro-apoptotic protein, is upregulated by p53, while Bcl-2, an anti-apoptotic protein, is down regulated by p53. Bax forms heterodimers with Bcl-2 and prevents its anti-apoptotic activity. Another potential apoptotic mediator is Fas, which is a member of the tumor necrosis factor receptor superfamily. Increased Fas expression on the plasma membrane is induced by p53 (37). In addition to these transactivation pathways toward apoptosis, it has also been suggested that p53 may regulate apoptosis through a transcription-independent pathway. This is substantiated by the fact that both Bax and Fas null mice are only partially resistant to p53-mediated apoptosis (12). Evidence from a cell free assay in the absence of any transcription has connected p53 to the activation of caspases, which are proteins implicated in the apoptotic pathway (12).

While there is still much left to be understood about the p53 protein, one of the most pressing questions concerning p53 is how it “knows” whether to produce apoptosis or arrest. Two models for p53 arrest vs. apoptosis are currently in contention. The first model proposes that p53 protein levels determine the outcome of arrest vs. apoptosis (12). High levels trigger apoptosis, while low levels trigger arrest. Alternatively, it has been proposed that the decision of arrest vs. death is not made at the p53 levels, but is

made downstream of p53 (12). This is supported by the fact that many stress responses disrupt transcriptional and translational machineries, and substantiates the concept of transcription-independent apoptosis.

**Mutations**

While some tumors do not express p53, others express a mutant form of the p53 protein. Mutation hot spots on the p53 protein include codons 117-142, 171-181, 236-258, and 270-286 (24). Fifty percent of p53 mutations affect codons 175, 248, 249, 273, and 282 (24). Mutant forms of p53 are not necessarily inactive, and can carry out several characteristic functions that contribute to tumorigenicity. Most importantly, mutant p53s have been implicated in the multidrug resistance of may tumors through the upregulation of the MDR-1 gene (24).

The activity of mutant p53 depends on the nature of the mutation, the ability to dimerize, and the cellular localization of the protein (24). Dominant negative forms of mutant p53 can interfere with wild type p53 through the formation of hetero-oligomers. These hetero-oligomers will not bind to the normal promoter sites of p53 target genes, and are unable to affect downstream events. Other gain of function mutations cause mutant p53 to bind different promoters and cause the transcription of genes not regularly induced by wild type p53. A set of criteria has been established for identifying mutant forms of p53. These include prolonged half-life, conformational differences that expose different epitopes, lack of binding to viral transforming peptides (SV40 Large T Antigen), inefficient binding to DNA, and inability to regulate wild type p53 responsive promoters (24). Mutant p53s may exhibit various combinations of these characteristics.

**Historical Foundation of Gene Therapy**

One of the strongest correlations in human cancers is preservation of wild type p53 and good prognosis(12). Tumors in this category include pediatric malignancies such as lymphoblastic leukemia, testicular carcinoma, and Wilm's tumor. This is in contrast to the majority of adult malignancies in which p53 is commonly lost or mutated. While it is important to realize that p53 status alone is not sufficient to explain all tumor successes and failures, the role of p53 in cell death and arrest suggests an explanation for tumor-selectivity of cancer therapy. Through simultaneously sensitizing cancer cells and protecting normal cells, p53 may allow certain drugs to produce selective toxicity. Therefore, restoration of p53 function may be valuable on the therapeutic level.

The scientific understanding of p53 protein function combined with new techniques for the efficient transfer and expression of genes *in vivo* may result in the development of useful procedures for the treatment of some human cancers. The fact that the loss of functional p53 can occur by direct genetic mutation has led to many investigations concerning the replacement of mutant p53 with exogenous wild type p53 to determine if this will reverse the cancer phenotype. Two important factors support the idea that reconstitution of the p53 tumor suppressor may be helpful for cancer gene therapy. First, the tumorigenic phenotype of many human cancers is incompatible with continued expression of wild type p53. Second, normal nontransformed cells may tolerate low to moderate levels of extra wild type p53 (24). Therefore, the reconstitution of tumor cells with wild type p53 is potentially anti-tumorigenic, and is not deleterious to cells of the normal phenotype.

With these ideas in mind, several studies have provided a large body of experimental evidence to confirm the potential of p53 as an agent for cancer gene therapy. Table 1 displays the effects of introduction and expression of wild type p53 on the growth and tumorigenicity of several different types of tumor cells. In general, this table shows that wild type p53 suppresses the capability of most of the tested tumors to form colonies and/or to produce tumors in nude mice. This trend did not depend on whether the cells contained mutant p53 or lacked endogenous p53. However, tumors that express mutant p53 (MCF-7 breast cancer cells) are not susceptible to exogenous wild type p53 growth suppression (24). Therefore, it is unlikely that the expression of exogenous wild type p53 will find clinical usefulness for all forms of cancer. However, many clinically important cancers including colon, lung, esophagus, breast, and brain are frequently associated with disruptions in the p53 gene. These cancers are the potential targets for gene therapy with the p53 gene.

## p53 Gene Therapy

### Strategies for Gene Delivery

The area of cancer gene therapy encompasses a number of unique strategies for targeted tumor inhibition and elimination. Some of these include immunotherapies, pro-drug suicide therapies, and therapies based on vector directed cell lysis. In March of 2000, 63% of the gene therapy clinical trials for cancer involved immunotherapies, 15% were based on pro-drug suicide therapies, and only 2% used the approach of vector directed cell lysis (2). Although the number of clinical trials using vector directed cell lysis is small, the first anti-cancer clinical success arose from this approach. This success

involved the ONYX-015 oncolytic virus that could selectively target cancer cells. In 1996 this approach was successful in Phase I clinical trials, and in 2000 was found to be successful in Phase II trails in combination with chemotherapy in patients with cancers of the head and neck (2).

While ONYX-015 has been successful in cancers of the head and neck, it may not be as successful in others types of cancers. Therefore, other gene therapy strategies need to be explored and tested. In addition, as vector technology continues to expand it may become possible to combine a number of these gene therapies to achieve an even greater anti-tumorigenic effect. Thus, although the idea of gene replacement via vector delivery has seemingly been pushed aside due to clinical success in other areas, this idea has been continually explored by a number of research groups (27). From retroviral to liposomal delivery of the correct genetic message, these groups have worked to "perfect" strategies of targeted gene delivery in order to make them safe and efficient for the treatment of cancer.

The following three sections explore the basic ideas behind the major strategies for p53 gene delivery.

*Retroviral Vectors*

Retroviruses are RNA viruses that have the ability to integrate into the host genome following reverse transcription. In nature, retroviruses may carry genes that have been obtained from an infected host, such as oncogenes, and may then transmit these genes to other cells. In gene therapy, the retrovirus most often used to package the gene of interest is the mouse moloney leukemia virus (MMLV) (33). MMLVs have been

specifically modified to maintain the ability to infect cells, but are not capable of replication (33).

As shown in figure 3, once a cell is infected, the viral RNA is reverse transcribed and the viral cDNA is integrated into the host genome. Integration allows for the stability of the viral genome inside host cell and for the viral genome to be passed on to each subsequent daughter cell. However, a number of problems have been shown to be associated with this approach. For example, integration of the viral genome may result in insertional mutagenesis, since the viral genome can randomly integrate into the host genome. Second, high titers of active viral vector are needed to infect cells properly, and the efficiency of this infection has been found to be variable (33). Third, the expression of the desired gene may not be high unless specific promoters and enhancers are present (33). Other potential problems include size constraints on the amount of genetic material (7 to 8 kb) able to be inserted into retroviral vectors, the risk of immune response, and the great chance for the presence of replication competent viruses in vector preparations (33).

*Adenoviral Vectors*

One of the most advanced vectors currently ready for clinical use is the adenovirus-derived vector. Adenoviruses are DNA-containing, non-enveloped viruses with a genome consisting of a linear 36kb double stranded DNA (27). Through the use of electron microscopy, it has been revealed that adenoviruses enter the cell by receptor-mediated endocytosis (27). Figure 4 shows the model for adenoviral entry into a target cell. Once inside the target cell, the adenovirus escapes the endosome by disrupting its membrane. Using the microtubular network, the adenovirus moves toward the nucleus

and releases its genome into the nucleus through nuclear pores (27). Immediately after entry of the adenoviral genome into the nucleus, viral early genes involved in adenoviral DNA replication, late transcription, and immune system evasion are transcribed (27). However, when considering the adenovirus for use in gene therapy, it is desirable to use an adenovirus which is replication deficient. Therefore, the E1 viral sequences required for replication are replaced with foreign cDNA coupled to an appropriate promoter (27). In addition, in order to decrease the immune response that occurs from adenoviral infection, recombinant adenoviruses in which all viral genes have been deleted have been constructed. This has allowed for adenoviral particles containing up to 35 kb of foreign cDNA to be generated (27).

Recently, adenoviral vectors have become very popular for gene therapy. The ease of recombination, high titers, efficient receptor-mediated endocytosis, high level of transgene expression, and large packaging capacity have contributed to this popularity (27). In addition, adenoviruses can infect a variety of cell types, and produce the protein of interest in dividing and non-dividing cells (27). However, adenoviral vectors do not integrate into the host genome, which leaves them susceptible to degradation by the host and results in short term expression of the transgene (27). Also, the host's strong immune response against both the adenovirus and the transgene may also reduce the duration of transgene expression (27). Despite these problems, studies on the use of adenoviral-mediated gene therapy have been underway for several genetic diseases, including cancer.

*Liposomal Vectors*

Liposomes are lipid membranes that have become increasingly popular for their use in drug delivery. Since it is possible to incorporate DNA and RNA into liposomal preparations, the idea of liposomal gene delivery has become increasingly explored (33). Once inside the liposome, DNA and RNA are protected from enzyme degradation in the serum. The DNA/RNA is then delivered to the cell upon liposomal fusion with the cell membrane (33). Through altering the lipid components of the liposome, cell specificity can be conferred (33). Problems in this delivery system arise due to low efficiency of nucleic acid uptake into liposomes and low efficiency of liposomal uptake by target cells (33). In addition, most liposomes are taken up by phagocytic cells when preparations are injected intravenously (33).

While some success has been achieved with retroviral vectors, the size constraints of these vectors has caused many scientists to look for other useful viruses for gene delivery. The most popular and seemingly useful of viral vectors for gene delivery has become the adenoviral vector. However, adenoviral vecotors have disadvantages as well, and scientists continue to look for new and more advantageous systems of gene delivery. Through exploring the advantages and disadvantages of these gene delivery systems, researchers may discover a feasible means of clinical gene delivery that will be safe and effective in clinical trials.

**Colorectal and Breast Cancers: Two Candidates for p53 Gene Therapy**

Colorectal and breast cancers are two cancers in which a high percentage of patients have been found to carry the p53 mutation. Therefore, gene therapy with p53 has the potential to correct an important mutation in the molecular pathway to these cancers. A number of research groups have conducted *in vitro* experiments to determine the effect of introducing exogenous p53 to various colorectal and breast cancer cell lines through the use of vector delivery systems. Evidence from these *in vitro* experiments has provided scientists with information needed to translate p53 gene therapy to *in vivo* studies. Many of these *in vivo* experiments also explored combination therapies with exogenous p53 and chemotherapy or radiation therapy. Through this experimentation, a number of phase I human clinical trials have been approved and conducted.

The following two sections give a background on colorectal and breast cancers, and the current treatments for these cancers. Next, the rationale for using p53 gene therapy for these cancers is discussed. Finally, the research from which scientists have justified the use of p53 gene therapy in human clinical trials is explored.

*Colorectal Cancer*

Together, cancers of the colon and rectum are among the most common cancers found in the United States. In 2002, an estimated 148,300 new cases of colorectal cancer will be diagnosed and 56,600 deaths will occur in the United State (38). Colorectal cancer occurs in both men and women, and is found most often among people over the age of 50. Risk factors associated with colorectal cancer include age, diet, polyps, personal history, family history, and ulcerative colitis. The common signs and symptoms

of colorectal cancer include a change in bowel habits, diarrhea, constipation, blood in the stool, abnormally shaped stool, abdominal discomfort, weight loss, constant tiredness, and vomiting. Treatments for colorectal cancer depend on the size, location, and extent of the tumor, and on the patient's general health. These treatments include surgery, chemotherapy, radiation therapy, and immunotherapy.

The key to surviving colorectal cancer is early detection followed by one of the above treatments. If cancer is diagnosed, the stage of the cancer is determined and treatment is planned. The stages of colorectal cancer are 0 through IV, 0 being very early cancer that is found only in the innermost lining of the colon/rectum and IV being metastatic cancer to the liver, lungs, or other parts of the body. Treatments for colorectal cancers in the early stages are extremely effective, and the five-year survival is approximately 90% (38). Therefore, if caught early colorectal cancer is curable. However, only about 30% of the United States population actually participate in early detection procedures (38). The consequence of this low level of screening is that only 37% of patients are diagnosed when the disease is localized (38). Therefore, many people are still diagnosed with late stage disease. Late stage disease is not curable, and the five-year survival is approximately 8% (38). Thus, effective treatments for late stage, metastatic colorectal cancers are needed. One possible treatment for late stage colorectal cancer may become p53 gene therapy.

*Breast Cancer*

Breast cancer is the most common type of cancer among women in the United States other than skin cancer. More than 180,000 women are diagnosed with breast

cancer each year, and an estimated 203,500 new cases of invasive breast cancer and 54,300 new cases of *in situ* breast cancer are expected to occur among women in the United States in 2002 (38). In addition, about 39,600 deaths are expected in 2002, which places breast cancer as the number two killer of women in the United States (38).

Most breast cancers occur in women over the age of 50, and the risk is especially high for women over the age of 60. Other risk factors for breast cancer include personal history, family history, changes in the breast, genetic alterations, long menstrual history, late childbearing, high breast density, radiation therapy, and alcohol consumption. Symptoms for breast cancer include a lump or thickening in the breast, a change in the size/shape of the breast, nipple discharge or inversion, and a change in the way the skin of the breast/nipple looks or feels. Treatments for breast cancer include surgery, radiation therapy, chemotherapy, hormonal therapy, and immunotherapy.

If breast cancer is diagnosed, the stage of the cancer is determined and treatment is planned. Breast cancers are staged from 0 to IV, 0 being noninvasive carcinoma and IV being metastatic cancer. The five-year survival for early stage breast cancer is 96%, while the five-year survival for late stage breast cancer is only 21% (38). For most cancers, the cancer is considered cured if there is no recurrence after five years. However, survival after diagnosis of breast cancer continues to decline beyond five years, and breast cancer is considered cured only after ten years of a disease free state.

Early detection is the key to surviving breast cancer. However, only about 50% of women in the United States participate in breast cancer screening procedures (38). In addition, while screening mammography has decreased breast cancer mortality by 30%, 15% of breast cancers are not detected by this method (38). Therefore, many women are

still diagnosed with late stage disease, and better treatments for late stage breast cancer are needed. The use of p53 gene therapy in combination with other therapies may provide a useful treatment for late stage breast cancer.

## Evidence for the Use of p53 Gene Therapy in the Treatment of Colorectal and Breast Cancers

### *Colorectal Cancer*

A mutation in p53 is present in 20-69% of colorectal cancer cases (9). This mutation has been found to be a late mutation in colorectal cancer that causes the tumor to progress from an adenoma to a carcinoma, as shown in figure 5. The importance of p53 as a key player in the progression of colorectal cancer suggests that correcting mutations in p53 may suppress tumor growth. Evidence for this has been shown in several studies. In one study, p53-/- colorectal cancer cells were found to be resistant to drugs such as 5-fluorouracil, which is the main adjuvant therapy for colorectal cancer (7). This has become a well-studied phenomenon, due to the fact that chemotherapy and radiation therapy are two of the most commonly used treatments in cancers. Since p53 is an important player in the apoptosis/growth arrest pathways, tumor response to chemotherapy and radiation therapy may be dependent in part on the presence of a normal p53. Therefore, the introduction of exogenous wild type p53 has the potential of increasing chemotherapy and/or radiation therapy responsiveness in tumors with deletions in p53 or tumors containing mutant p53.

In a study by Yang et. al., a mutant colorectal cancer cell line bearing mutant endogenous p53 and exogenous wild type p53 under the control of a Lac repressor

showed reversible growth arrest upon wild type p53 induction with isopropyl beta-D thiogalactoside. In this same study, the induction of wild type p53 was also found to potentiate the cytotoxicity of irradiation, 5-fluorouracil, and camptothecin (36). This study substantiates the role of p53 in the response to chemotherapy and radiation therapy, and provides evidence that p53 deficiency plays a role in drug resistance. Therefore, correcting mutations in p53 through the use of gene therapy is a logical approach in the treatment of colorectal cancers, especially those that have acquired the characteristic of drug-resistance.

*Breast Cancer*

Loss of heterozygosity of p53 is the most common mutation in breast cancer. In contrast to other cancers, such as colon cancer where p53 is associated with tumor progression, mutation in p53 has been found to be an early event in breast cancer and is maintained during tumor progression (3). Evidence from several studies has suggested that correcting p53 mutations suppresses tumor growth. For example, breast cancer cells transfected with wild type p53 exhibited a reduction in growth in soft agar, which is considered an *in vitro* correlate of the transformed phenotype (10). In a different study, breast cancer cells infected with a retrovirus carrying the wild type p53 gene showed decreased tumorigenicity in nude mice (10). Equally important is a study in which specific mutations in p53 were linked to resistance to doxorubicin and early relapse in breast cancer patients (1). These mutations occurred in codons 236-251, and included point mutations at codons 248 and 249, a deletion from codon 217 to 221, and a nonsense mutation to a stop codon at codon 204 (1). Thus, as in colorectal cancer, p53 plays an

important role in drug sensitivity of breast cancer, making gene therapy with p53 a logical approach in the treatment of breast cancer, especially those found to be drug resistant.

**Research Leading to Human Clinical Trials**

The following two sections focus on specific *in vitro* and *in vivo* studies that have led to the approval of p53 gene therapy in human clinical trials for colorectal and breast cancer patients. While the first protocols designed and approved for human trials used retroviral vectors, the present vector of choice is the adenovirus (37). Thus, the majority of the studies discussed explore the use of adenoviral mediated p53 gene therapy in the treatment of colorectal and breast cancers. In addition, studies involving a new approach to p53 gene delivery using liposomes are discussed as well.

*In Vitro* Studies

Several studies have shown that adenoviral vectors carrying p53 are able to efficiently infect colon and breast cancer cells. MDA-MB 231 and MDA-MB 468 breast cancer cells were found to be highly transduced by an adenovirus carrying wild type p53 (p53Ad) at a multiplicity of infection (MOI) of 10 (21). SW620 colorectal cancer cells were 60-80% transduced at a MOI of 50 (29). Table 2 shows the infectivity of various human cancer lines to p53Ad. At a MOI of 30, the p53Ad was also found to have an infectivity of greater than 99% in colorectal cancers such as SW480, SW620, HCT116, RKO and breast cancers such as SkBr3and MCF7 (4). In addition, those cells infected with p53Ad were found to express higher levels of p21 protein as compared to control

cells (4). This provides evidence that wild type p53 is being expressed and working through its normal cellular mechanisms. Therefore, the p53Ad is able to infect cancer cells with a high efficiency, and the expression of p53 has been found in cells transduced by p53Ad.

In addition to adenoviral vectors, liposomal vectors have also been shown to transfer wild type p53 into colon cancer cells. WiDr and COLO320DM colon cancer cell lines were transduced with a wild type p53 cDNA expression vector delivered via an HVJ-cationic liposome (20). Wild type p53 expression was then shown through the use of immunohistochemical staining with anti-wild type p53 antibody (figure 6). The ratio of wild type p53 positive WiDr cells was 73.4%, and the ratio of wild type p53 positive COLO320DM cells was 64% (20). Thus, both viral and non-viral vectors, are also capable of wild type p53 gene transfer into cancer cells.

Growth inhibition and cytotoxicity of exogenous wild type p53 alone or in combination with chemotherapy or radiation therapy has been explored in several breast and colorectal cancer cell lines. Figure 7 shows the visible *in vitro g*rowth inhibition of MDA-MB 231 and 468 breast cancer cells was found upon transduction of a p53Ad alone (21). In combination with cisplatin or 5-fluorouracil, p53Ad had enhanced suppression of MDA-MB 231 cell growth (14) (figure 8). SW620 colorectal cancer cells were found to be increasingly sensitive to radiation therapy in combination with p53Ad (29). This combination also induced a greater amount of apoptosis in SW620 colorectal cancer cells (29) (figure 9). Growth inhibition of WiDr colon cancer cells was also found upon transduction of p53Ad, and the combination of cisplatin and p53Ad had a synergistic effect (22) (figure 10).

The effect of wild type p53 gene transfer via a liposomal vector was able to inhibit cell growth in WiDr and COLO320DM colon cancer cells (20). In addition, the combination of wild type p53 gene transfer and doxorubicin induced a larger reduction in growth than with either treatment alone. Figure 11 shows the results of a terminal deoxynucleotidyl transferase dUTP nick-end labeling assay, or TUNEL assay, performed to determine the efficiency of inducing apoptosis with wild type p53 gene transfer and/or doxorubicin. WiDr cells treated with wild type p53 gene transfer 26.6% apoptosis, while WiDr cells treated with doxorubicin showed only 2% apoptosis (20). WiDr cells treated with the combination of doxorubicin and p53Ad showed 28% apoptosis (20). Similar results were obtained for COLO320DM cells treated with doxorubicin and p53Ad (20), as well as MDA-MB 435 breast cancer cells treated with doxorubicin and p53 delivered via liposomal vectors (17) (figure 11, figure 12). Therefore, both viral and non-viral vectors carrying exogenous wild type p53 are able to induce apoptosis and growth inhibition in human cancer cell lines.

The effect of p53Ad in combination with chemotherapy on drug resistant breast cancer cells has also been explored. The responses of adriamycin resistant MCF7 breast cancer cells to p53Ad alone, adriamycin alone, and both p53Ad and adriamycin were compared to parental MCF7 cells (28). The drug resistant cell line was shown to be more sensitive to killing by p53Ad. Figure 13 shows the pattern of nucleosomal DNA degradation in MCF7 and adriamycin resistant MCF7 cells. Drug resistant cells exhibited nucleosomal DNA degradation, which is a sign of apoptotic death (28). The lack of this same pattern in parental cells indicated that parental MCF7 cells underwent growth arrest (28). As compared to p53Ad alone, a combination of p53Ad and

adriamycin was found to be more toxic to the adriamycin resistant cell line (figure 14). Thus, infection with p53Ad can sensitize drug resistant cells to chemotherapeutic drugs.

Overall, these *in vitro* studies have revealed that both adenoviral vectors and non-viral liposomal vectors are capable of transferring a functional p53 gene into breast cancer and colon cancer cell lines. Most importantly, this exogenous wild type p53 gene is able to induce growth arrest and apoptosis in colorectal and breast cancer cell lines, and this effect is increased in the presence of a chemotherapeutic drugs and radiation. With this evidence in hand, researchers were able to move on to study the effect of p53 gene therapy in animal models.

*In Vivo* Studies

Subcutaneous tumor animal models have provided researchers with evidence for the use of p53 gene therapy in human clinical trials. The p53Ad has been shown to be highly effective against MDA-MB 231 and 468 breast cancer xenografts in nude mice (21) (figure 15). MDA-MB 231 and 468 tumor growth inhibition averaged 86% and 74%, respectively (21). In addition, p53Ad was shown to induce apoptosis in these same xenografts (21). This inhibition of tumor growth by p53Ad alone was found to be enhanced in combination with cisplatin, doxorubicin, 5-fluorouracil, methotrexate, or etoposide (14). As shown in figure 16, MDA-MB 435 breast cancer xenografts growth suppression due to p53Ad was enhanced by doxorubicin as well (17). Most importantly, in a metastatic animal model of MDA-MB 435 breast cancer, systemic administration of p53Ad plus doxorubicin led to significant reduction in the incidence of metastases as compared to either treatment alone (17) (table 3). This same effect on metastasis was

found with the use of a liposome-p53 complex in a nude mouse model using MDA-MB 435 cells, in which the number of metastatic cells to the lung were found to be significantly lower in those mice treated with the liposome-p53 complex (18). Treatment with the liposome-p53 complex alone resulted in greater than 60% reduction in primary tumor as well (18) (figure 17). Growth patterns of individual mice were assessed, and revealed that 8 of 15 mice treated with the liposome-p53 complex showed tumor regression, as compared to 1 of 22 mice in the control group (18). Thus, p53Ad in combination with chemotherapeutic drugs had a growth inhibitory effect in mouse tumor xenografts, and has also been found to have an anti-metastatic effect in breast cancer models. These same anti-tumor and anti-metastatic effects were shown in animal models using a liposome-p53 complex as well.

Similar to its effects in breast cancer animal models, p53Ad has been shown to exhibit growth inhibitory effects in animal models of human colorectal cancer. As shown in table 4, a subcutaneous tumor model in nude mice of WiDr human colon cancer cells was found to be significantly suppressed following treatment with p53Ad and cisplatin, as compared to either treatment alone (22). Figure 18 shows that a significant enhancement of tumor growth suppression was also present in a subcutaneous tumor model of SW620 colorectal cancer cells in nude mice following treatment with p53Ad and radiation therapy (29).

Direct administration of p53Ad into a tumor has been found to at best infect 5 to 10% of cells in the tumor mass, as shown through transgene expression (27). Despite this low transduction efficacy, the above studies have shown that p53Ad gene therapy is able to induce a significant amount of tumor regression in animal models. In addition, it has

been reported that nontransduced cells in the tumor mass have been found to undergo apoptosis (9). Thus, the tumor regression and apoptosis observed in these animal models must be due in part to a bystander effect. While cell-cell interaction clearly plays a role in the bystander effect, other possible explanations include the immune response to viral vectors and the anti-angiogenic effect of the wild type p53 gene. Several studies are currently underway concerning these aspects of the bystander effect.

One study by Yang et.al. has shown that the immune response to p53Ad gene therapy has some specificity. Cells transduced with p53Ad have been shown to overexpress CD95 ligand (Fas ligand) as compared to cells transduced with a control adenovirus (32). This overexpression of CD95 ligand on p53Ad transduced cells may be in part responsible for apoptosis of these cells through T cell mediated cytotoxicity. The overexpression of CD95 ligand was also shown to be followed by a mass infiltration of neutrophils, which would be in part responsible for the bystander effect observed (32). In another study by Bouvet et. al. wild type p53 was shown have an anti-angiogenic activity *in vivo* through a reduction in the expression of vascular endothelial growth factor and increased expression of angiogenesis inhibitors. Several other studies have observed a decrease in the vascular density of the tumor mass in nude mouse xenografts treated with p53Ad (37).

The presence of a bystander effect due to p53Ad treatment has led researchers to explore if the augmentation of this bystander effect may contribute to a greater anti-tumoral effect. MDA-MB 435 xenografts in nude mice were treated with both a liposome-p53 complex and a liposome complexed to an anti-angiogenic peptide of thrombospondin I (35). A decrease in blood vessel density was observed, and the

combination of these liposome complexes achieved a greater anti-tumor effect than either alone (35). A combination of Adp53 and AdIL-2 also showed a greater anti-tumoral effect than either therapy alone in a breast cancer model in nude mice (23). The formation of specific anti-tumor cytotoxic T lymphocytes was reported as well (23).

From these *in vivo* studies with tumor animal models, it has become evident that p53Ad in combination with chemotherapy or radiation therapy has the potential to suppress tumor growth in breast and colorectal cancer patients. In addition, this treatment may also have a potential anti-metastatic effect in these cancer patients, as well as a bystander effect that can augment tumor regression and apoptosis. Therefore, phase I clinical trials in human breast and colorectal cancer patients have been conducted to assess the side effects of adenoviral mediated p53 gene therapy and to determine the level of dosage that can be tolerated.

**Clinical Trials**

In May of 2002, the National Institute of Health reported 1,990 clinical trials for cancer that are currently recruiting patients (39). Of these, approximately 20 involve some form of gene therapeutic approach, and five phase I p53Ad gene therapy clinical trials are recruiting patients (39). The cancers involved in these p53Ad trials include advanced stage ovarian, primary peritoneum, oral cavity, and bladder cancers. The use of p53Ad gene therapy in these cancers has come about due to "successes" in other trials involving cancers such as lung, breast, and colorectal cancers. These studies indicate that p53Ad gene therapy and introduction of wild-type p53 into tumor cells represents a potentially valuable tool for the therapy of many types of human cancers.

The use of p53 gene therapy in a human clinical trial was first reported in 1996 (25). In this phase I trial, a replication defective retrovirus was used to deliver wild type p53 to patients with non-small cell lung carcinoma. The use of retroviral mediated p53 gene therapy resulted in local tumor regression in 3 of the 9 lung cancer patients (25). This phase I trial provided researchers with enough evidence to rationalize further testing of p53 gene therapeutic approaches in human cancer patients.

While the first "successes" with p53 gene therapy in humans involved the use of retroviral vectors, researchers have since abandoned this gene delivery system for use in clinical trials. This was replaced by an adenoviral vector delivery system, which has the advantages of high efficacy of transduction in non-dividing cells, lack of insertional mutagenesis, and the possibility of transferring large amounts of DNA (37). Thus, several clinical trials with p53Ad have been executed or are currently underway for colorectal and breast cancers.

In 1998, Venook and Horowitz reported phase I clinical trials for p53Ad gene therapy of colorectal liver metastases and breast cancer, respectively. In 11 of 16 patients with hepatic metastatic colorectal cancer that were treated with p53Ad and 5-fluorodeoxyuridine, partial responses were observed with tumor shrinkage of 50% or greater (30). The adverse side effects reported included grade 1 and 2 fever, low blood pressure, headaches, myalgia, chills, and nausea. For breast cancer, phase I trials with p53Ad reported toxicities of mild fever, headaches, myalgia, chills, nausea, and abdominal pain (15). Clinical outcomes of breast cancer patients were mixed, with partial response, stable disease, and progressive disease observed. In both of these phase I trials, transgene expression was evident. However, it can not be clear if the clinical

effects observed were due to transgene expression or to immune response to the adenoviral vector itself. Although these clinical effects remain unclear, phase I trials with p53Ad gene therapy have provided evidence that this is a safe and feasible treatment for human cancer patients. In addition, p53Ad gene therapy is also biologically effective with respect to transduction frequency and transgene expression.

Due to the initial clinical effectiveness observed in lung cancer patients treated with p53Ad, a phase II/III clinical trial of non-small cell lung carcinoma patients is currently underway (40). While p53Ad gene therapy has shown "success" in phase I trials of other cancers, such as breast and colorectal cancers, a number of obstacles must be overcome before this type of therapy can move to the next level in the treatment of these cancers. The following section explores the challenges that p53 gene therapy must face before its clinical effects in these cancer patients can be fully assessed.

**The Challenges and Future Directions of p53 Gene therapy**

The most inherent problem that p53 gene therapy currently faces is finding a vector delivery system that is safe and effective in human clinical trials. Although adenoviral vectors have shown some promise in phase I trials, these vectors carry with them several disadvantages. Two of the most prominent disadvantages of adenoviral vectors are its transient gene expression and that it elicits a strong immune response (27). Unlike the retroviral vector, which can integrate into the host genome and maintain stable transfection, adenoviral vectors remain episomal and achieve only transient gene expression for a period of 14-21 days (37). While this is a disadvantage, the establishment of a coordinated timetable for drug administration may provide a means of

overcoming this problem. However, the immune response creates a problem for multiple treatments with adenoviral vectors. There is a high prevalence of neutralizing antibodies against adenoviruses in the human population (37). Thus, intratumoral or local-regional vector administration is required. It is very unlikely that systemic administration with adenoviral vectors will achieve a significant anti-tumoral effect because of the presence of neutralizing antibodies. In addition, since adenoviral vectors only achieve transient gene expression, multiple administrations will incur a stronger immune response that may not be safe for the patient.

Researchers continue to work to improve vector technology in order to find a vector system that will be most effective. As mentioned previously, liposomal vectors have been explored, since they lack the unwanted materials associated with viral vectors and have shown minimal toxicity. However, this method only achieves transient gene expression and has a low transduction efficiency (26). In order to improve liposomal delivery, researchers are looking for tissue specific promoters and enhancers that can achieve stable or inducible transgene expression (26).

Other ideas for improving vector delivery include the construction of chimeric vectors (37), tissue specific promoters (27), and vector targeting (27). Since retroviral vectors and adenoviral vectors each have specific advantages, it is feasible that creating a chimeric retro/adeno-viral vector will help to achieve the goals of high gene transfer, genomic integration, and long term expression. To make this approach tissue specific, expression of a transgene has been explored with an adenovirus carrying the *E.Coli* nitroreductase gene that is driven by a human heat shock protein promoter containing a LacI binding site (19). In this system, if the cell contains wild type p53, another

expression cassette within the vector containing a p53-inducible lac repressor gene, is induced and the expression of nitroreductase is repressed by 70% (19). If the p53 gene is mutated and can not induce the expression of the lac repressor, nitroreductase is expressed, and inhibition of growth is observed in response to a pro-drug (19).

Similar experiments that take advantage of p53 status have been used for vector targeting. The ONXY-015 oncolytic adenovirus selectively replicates and lyses human tumor cells with non-functional p53 (37). In addition, because viral vector systems rely on receptor mediated gene transfer, researchers have looked to exploit this trait in vector targeting. The expression of estrogen receptors on breast cancer cells is a potential target for tumor specific targeting of viral vectors. These approaches have the potential to move p53 gene therapy to the next level in human clinical trials.

While p53 has been the most investigated candidate for gene replacement therapy, not all tumors have responded to p53 therapy. Gene therapy with p53 has been shown to be effective *in vitro* and *in vivo* in cancers of the lung, breast, ovary, and lung (11). However, it has not been found to be effective in pancreatic cancers, glioblastomas, and hepatocarcinomas (11). This can be best explained in that response to p53 gene therapy is best in those cell lines that are defective in p53, while the response is worse in those carrying mutant p53. Restoration of p53 and expression of wild type p53 may not be easily achieved in cancers that carry dominant negative p53 mutants. In order to achieve apoptosis in these tumors as opposed to growth arrest, a certain level of wild type p53 must be achieved.

Several new methods by which p53 expression in tumors can be increased are currently being explored. Sufficient levels of wild type p53 could be effectively achieved

through the use of ribozymes that repair mutant p53 transcripts. In a recent study, cells transduced with a ribozyme expression vector for the repair of p53 mutants showed increased levels of wild type p53 (34). In addition, these cells also showed decreased levels of MDR1 expression, which is associated with drug resistance (34). Another approach to obtain adequate levels of wild type p53 would be to use drugs that block the ubiquination of p53 by Mdm2 (16). This would increase the half-life of exogenous wild type p53 and up regulate wild type p53 activity. In addition, the use of drugs that correct the three dimensional configuration of p53 mutants so as to confer adequate wild type p53 function have also been explored (37). However, due to a lack in the clear understanding of arrest vs. apoptosis, it is unclear as to what this level must actually be and if it can be achieved in mutant p53 expressors. Thus, scientists continue to conduct research in hopes of fully understanding the apoptotic and growth arrest pathways.

Finally, the mechanism or mechanisms through which p53Ad incurs a bystander effect are currently under study. Through a better understanding of this effect, researchers may be able to augment this effect in order to achieve a greater anti-tumoral effect in human cancer patients. In addition, the study of the bystander effect may also lead to a better understanding of the immune response to tumor cells as well as the mechanisms of angiogenesis.

## Conclusion

Correcting specific molecular defects responsible for the aberrant biological behavior of cancer cells is a new and fascinating approach in clinical cancer treatment. Due to a high frequency of p53 alterations in cancers and the central role of p53 in the

regulation of growth and apoptosis, p53 appears to be an appealing target for gene therapy. Initial studies of p53 restoration provided evidence that replacing a defective p53 with a functional wild type p53 has an anti-tumorigenic effect in human cancer cell lines. In addition, *in vitro* and *in vivo* studies with viral vectors carrying wild type p53 provided further evidence for this therapeutic approach. Finally, phase I human clinical trials have shown that p53 gene therapy is a safe and feasible treatment for human cancer patients. However, its overall effectiveness in prolonging survival and causing significant tumor regression in cancer patients still remains to be proven.

**Tables**

| **Table 1: The Effects of Wild Type p53 on Some Tumor Cell Types** (24) | | | | |
|---|---|---|---|---|
| **Cell Type** | **Cell line** | **p53 status** | **method of gene transfer** | **Effects** |
| CML | K562 | none | transfection | growth suppression |
| colorectal carcinoma | SW40 | mutant | transfection | decrease in colony formation |
| hepatocarcinoma | Hep3B | none | transfection | decrease in colony formation |
| lymphoblastic leukemia | Be-13 | none | retroviral infection | growth suppression and decreased colony formation |
| mammary carcinoma | MCF-7 | mutant | transfection | no effect |
| mammary carcinoma | MDA-MB468 | mutant | transfection | growth suppression |
| ovarian adenocarcinoma | SKOV-3 | none | transfection | growth suppression |
| osteosarcoma | Saos-2 | none | retroviral infection | decreased colony formation |
| prostate carcinoma | TSU | none | transfection | growth suppression |

| **Table 2: Adenoviral Infectivity of Human Cancer Cells** (4) | | | | |
|---|---|---|---|---|
| **Cell Line** | **Origin** | **Enodogenous p53** | **B-gal Ad Infectivity** | **p53Ad Infectivity** |
| H460 | NSCLC | wt | +++ | +++ |
| A549 | NSCLC | wt | +++ | +++ |
| SKBr3 | breast | m/m | +++ | +++ |
| MCF7 | breast | wt | +++ | +++ |
| MCF7/Adr | breast, Adr | wt | +++ | +++ |
| Del 4A | Glioblastoma | m/wt | +++ | ND |
| SKOV-3 | ovarian | "-/-" | +++ | +++ |
| OVCAR | ovarian | m/- | +++ | +++ |
| SW480 | colon | m/- | +++ | +++ |
| SW620 | colon | m/- | +++ | +++ |
| SW/AD300 | colon,Adr | m/- | +++ | +++ |
| HCT116 | colon | wt | +++ | +++ |
| RKO | colon | wt | +++ | +++ |
| LNCaP | prostate | wt | +++ | +++ |
| DU145 | prostate | m/m | +++ | +++ |
| HL60 | APL | "-/-" | 0 | 0 |
| ML1 | AML | wt | 0 | 0 |
| RAMOS | Burkitt's | m/- | 0 | 0 |
| CA46 | Burkitt's, Adr | m/- | 0 | 0 |
| "-" indictaes p53 deletion, "m" indicates mutant p53, "wt" indicates wild type p53. "+++" indicates >99% infectivity at 30 MOI or less, "-" indicates < 0.001% infectivity at 150 MOI or greater, "ND" indicates not done. | | | | |

**Table 3: Incidence of Lung Metastases in Nude Mice 16 Weeks After Mammary Fat Pad Implantation of MDA-MB 435 Breast Cancer Cells** (17)

| Animal group | Number of animals | Incidence (%) | p value |
|---|---|---|---|
| no treatment | 8 | 38 | >0.5 |
| doxorubicin | 8 | 50 | >0.5 |
| p53Ad | 8 | 50 | <0.05 |
| p53Ad+doxorubicin | 7 | 0 | <0.05 |
| control (AdLuc) | 8 | 38 | >0.5 |
| control (AdLuc)+doxorubicin) | 6 | 17 | >0.5 |

Treatment was initiated 8 weeks after implantation of tumor cells, and just after exicision of primary tumors. Treatment consisted of 10^9 viral particles of p53Ad or control adminstered 3 times per week by tail vein injection and/or 10mg/kg doxorubicin intravenously on days 1 and 21.

**Table 4: Effects of p53Ad and Cisplatin (CDDP) on *in vivo*Tumor Growth of WiDr Xenografts** (22)

| Treatment | Mean volume (mm^3) | % inhibition |
|---|---|---|
| PBS/saline | 83.5 | ---- |
| PBS/CDDP | 66.7 | 20.1 |
| controlAd/saline | 78.1 | 6.5 |
| controlAd/CDDP | 63.1 | 24.4 |
| p53Ad/saline | 47.5 | 43.1 |
| p53Ad/CDDP | 21 | 74.9 |

WiDr cells were inoculated subcutaneously into nude mice. On days 4, 5, and 6 PBS or Ad vector were injected subcutaneously into the area of the tumor. CDDP at 2mg/kg or saline was administered simultaneously. Tumor formation and volume were evaluated at the end of day 21. Percent inhibition was calculated compared to untreated controls.

**Figures**

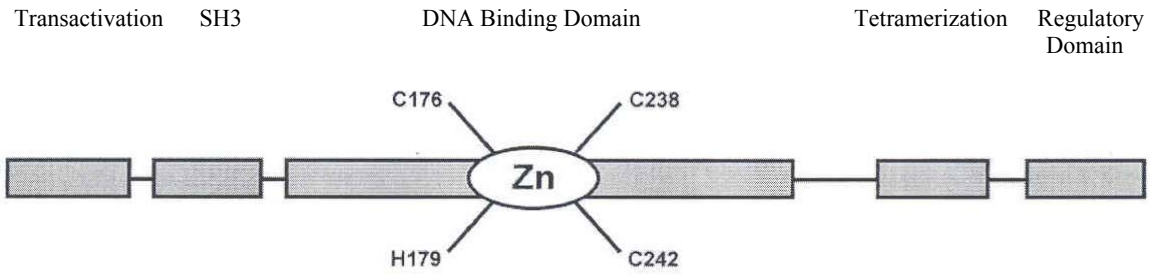



**Figure 1**: A schematic illustration of the functional domains of the p53 protein. (37)

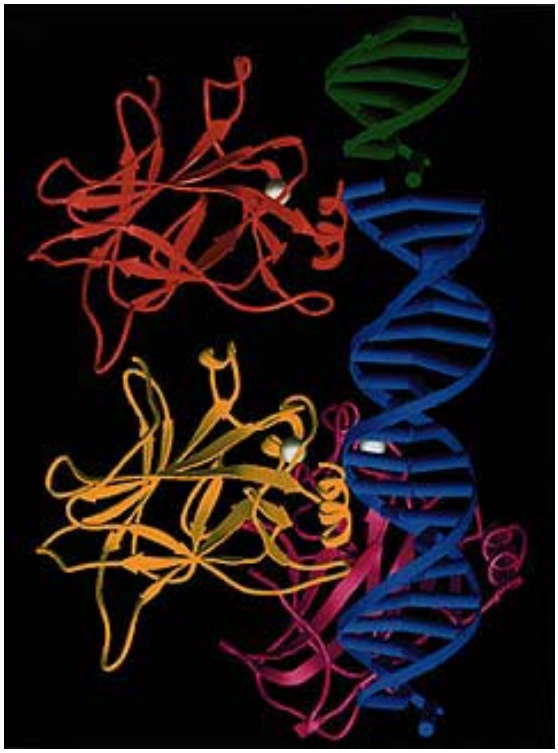


**Figure 2**: A 3-D representation of p53 protein interaction with DNA. Based on X-ray Crystallography by Nikola P. Pavletich. (39)

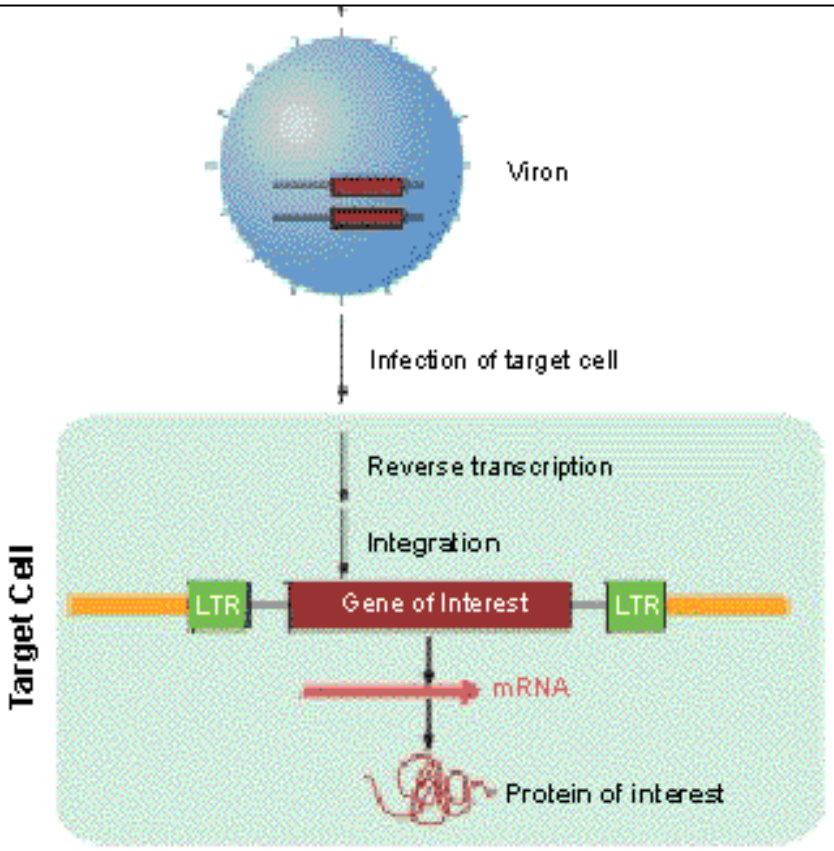



**Figure 3**: Retroviral infection of a target cell and integration of viral cDNA into the host cell genome. (39)

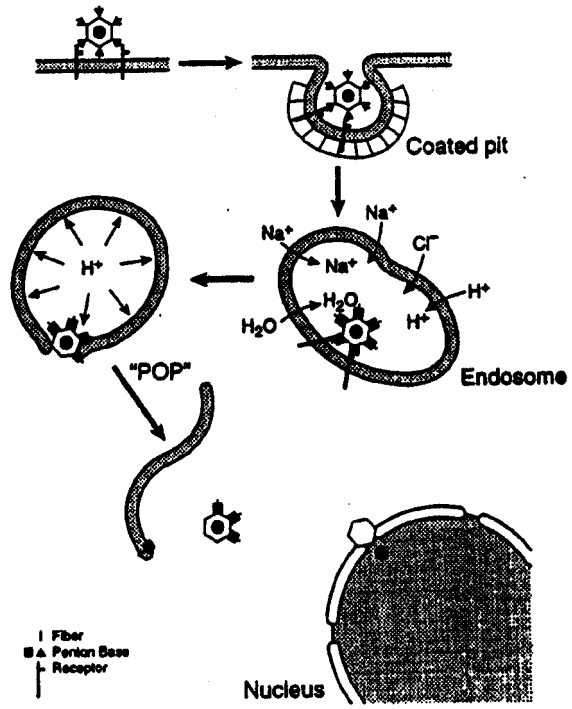



**Figure 4**. A model of adenovirus entry and vesicle disruption. The adenovirus binds to the cell surface and is endocytosed. The adenovirus escapes and moves toward the nucleus. (27)

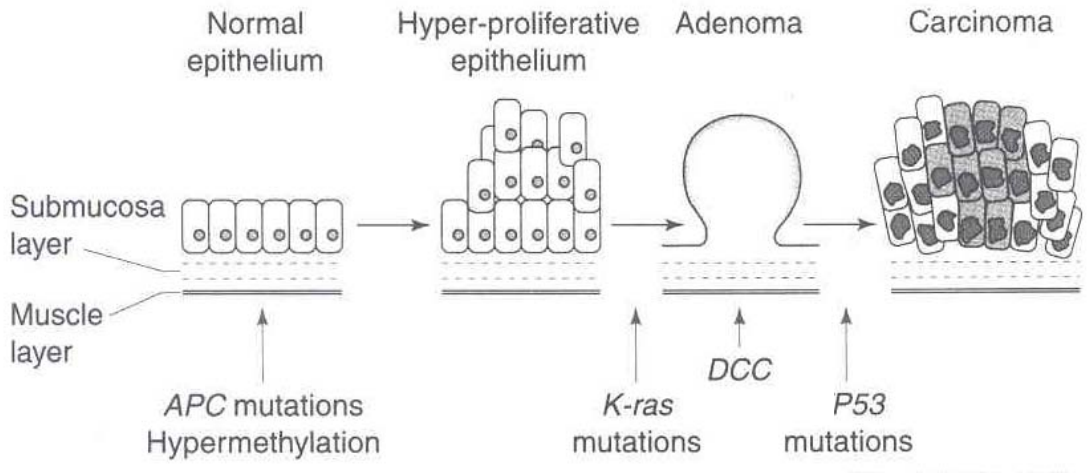



**Figure 5**: The proposed progression of colorectal cancer. Adenomatous polyposis coli (APC) gene mutations and hypermethylation occur early, followed by K-ras mutations. Deleted in colon cancer (DCC) and p53 gene mutations occur later. (9)

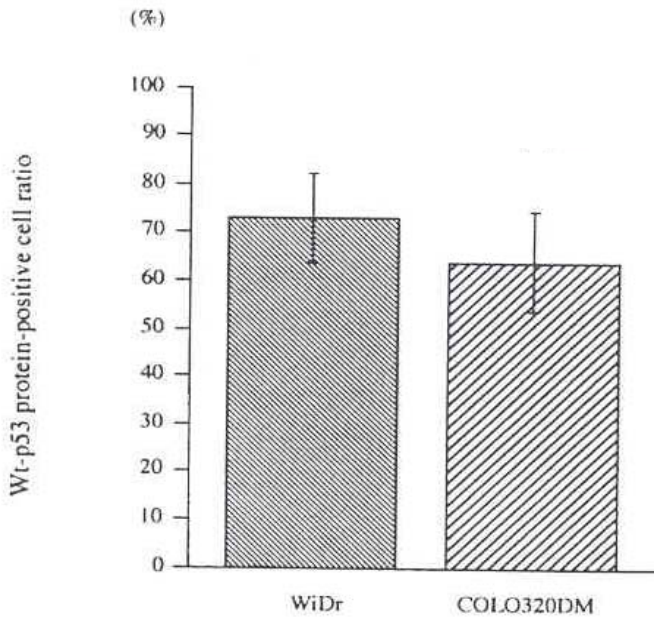



**Figure 6**: The efficiency of immunocytochemical expression of wild type p53 protein in WiDr and COLO320DM cells treated with p53 via HVJ-cationic liposomal vectors. (20)

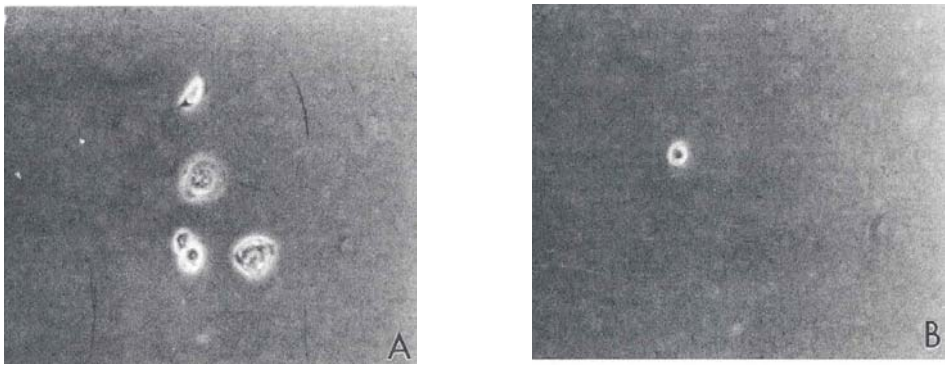



**Figure 7**: Breast cancer cell cultures 72 hours after infection with 50 MOI p53Ad. (A) 231 cells; (B) 468 cells; x320. (21)

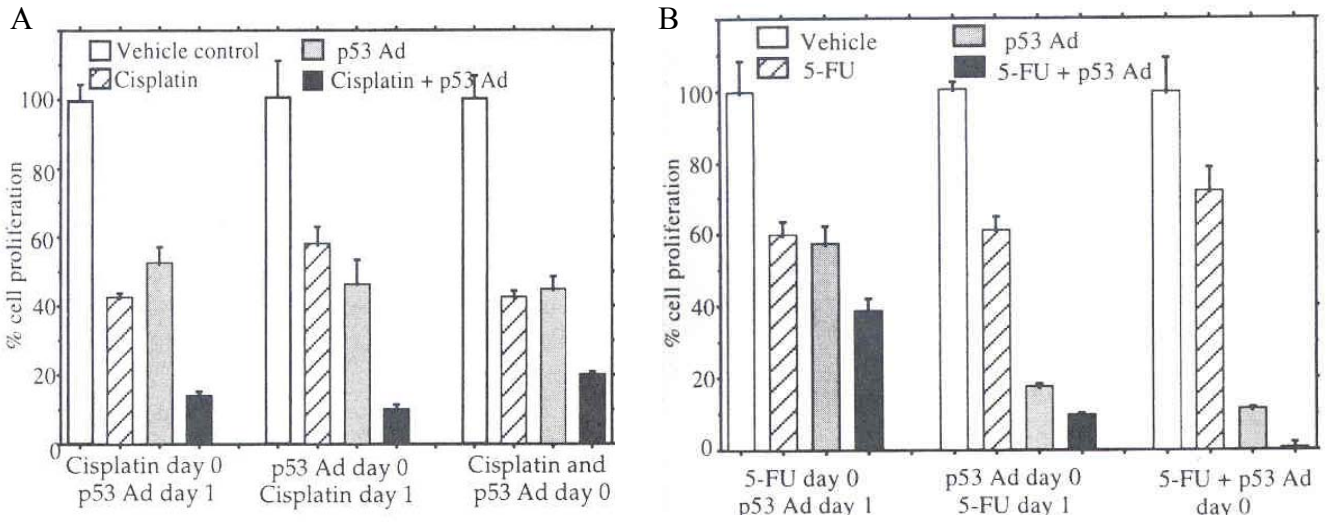



**Figure 8**: Antiproliferative effects of p53Ad in combination with cisplatin or 5-fluorouracil *in vitro.* (A) MDA-MB 231 cells treated with cisplatin. Doses were 17,17,and17 μM cisplatin with 5, 5, and 2.5 MOI p53Ad, respectively. (B) MDA-MB 231 cells treated with 5-fluorouracil. Doses were 2 μM 5-fluorouracil and 5 cellular infectious units per cell of p53Ad. (14)

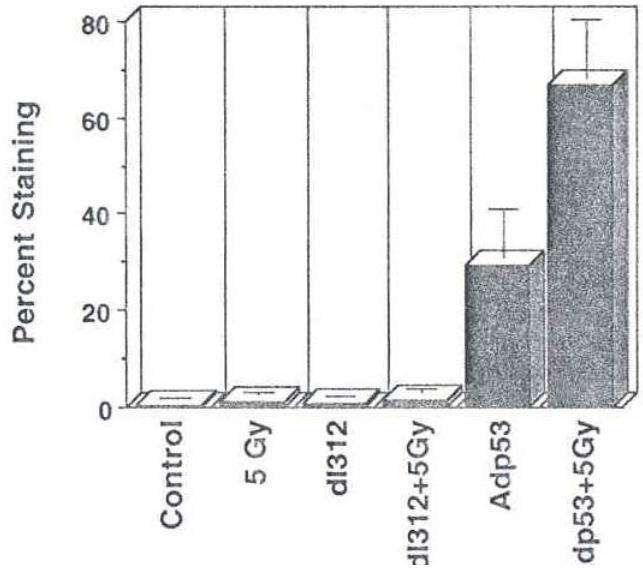



**Figure 9**: The effects of p53Ad and 5 Gy irradiation on *in vitro* TUNEL assay labeling of SW620 cells. Cells were irradiated 48 hours following infection and harvested 24 hours after irradiation. Control is PBS alone; d1312 is a control adenovirus. (29)

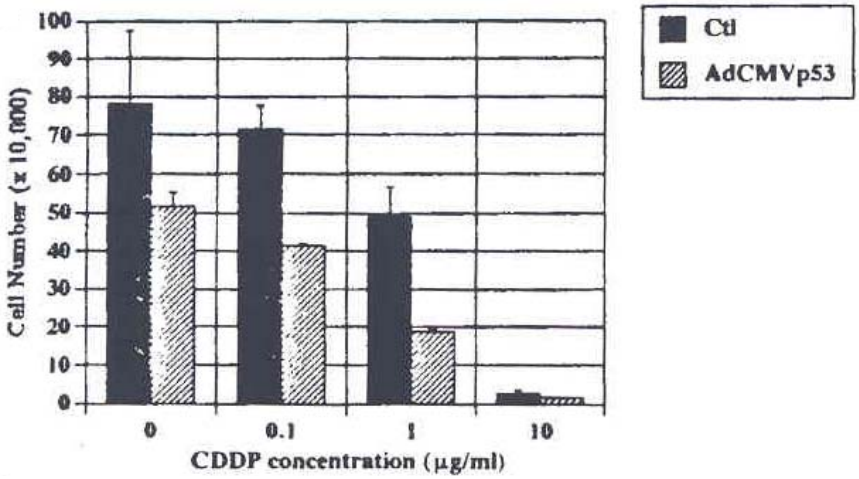



**Figure 10**: Synergistic anti-tumor effects of p53Ad and cisplatin (CDDP) on the growth of WiDr cells *in vitro*. Cells were infected with 50 MOI of p53Ad for 24 hours and then treated with different doses of cisplatin for 4 hours. Cell viability was assessed by Trypan Blue staining on day 6. Cell viability is shown at 0, 0.1, and 10 μg/mL of cisplatin in the presence or absence of p53Ad. (22)

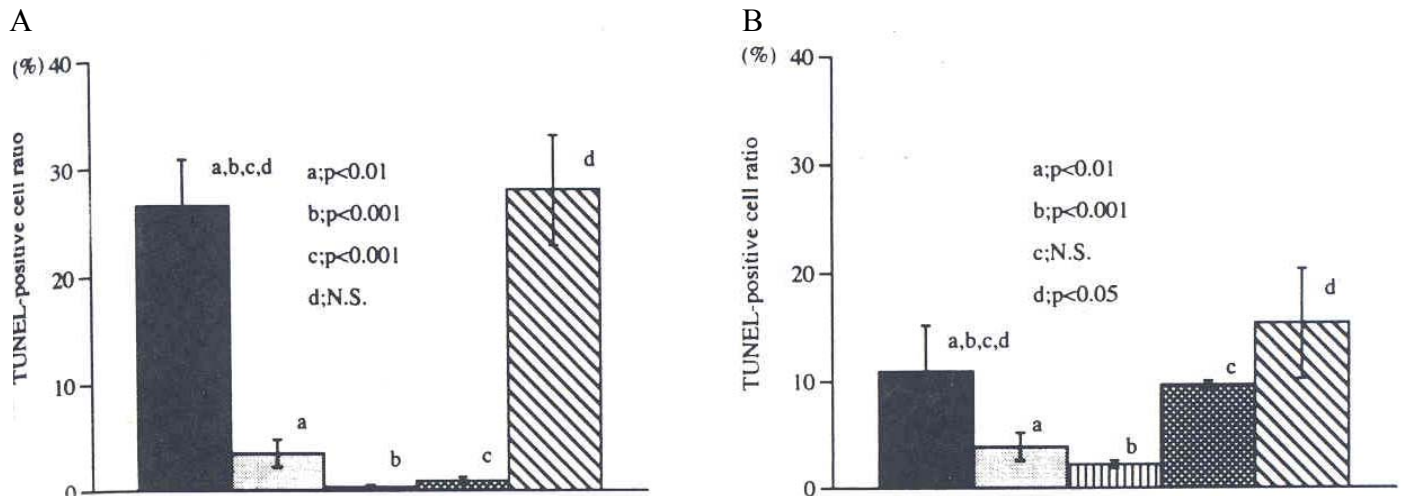



**Figure 11**: TUNEL positive cell ratios in WiDr and COLO320DMells treated with wild type p53 gene transfer and/or doxorubicin. Two days after wild type p53 gene transfer and/or doxorubicin treatment, TUNEL-positive cells were counted and the TUNEL-positive cell ratio of each cell line was estimated. (A) WiDr; (B) COLO320DM. (20)

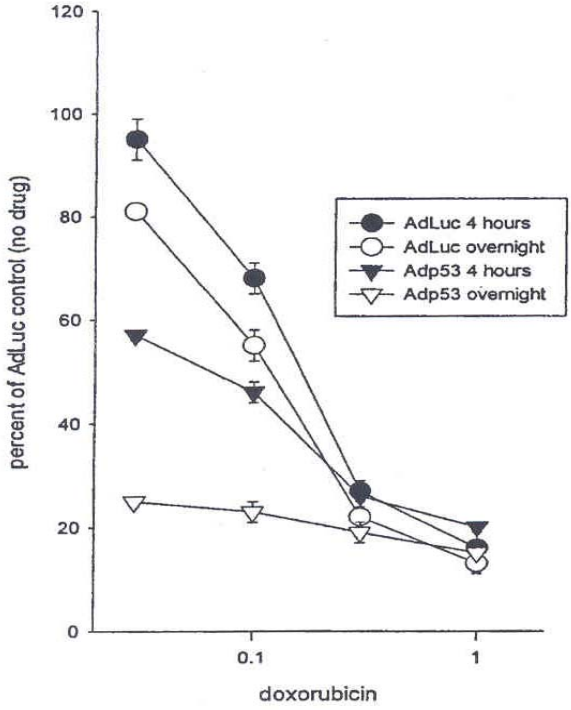



**Figure 12**: Viability assay of MDA-MB 435 cells treated with p53Ad or a control adenovirus (AdLuc) at 100 plaque forming units per cell for 4 hours or overnight, followed by replating and treatment with doxorubicin for 3 hours. Viability was assayed by the MTS assay and is expressed as a percentage of the viability of cells treated with control adenovirus. (17)

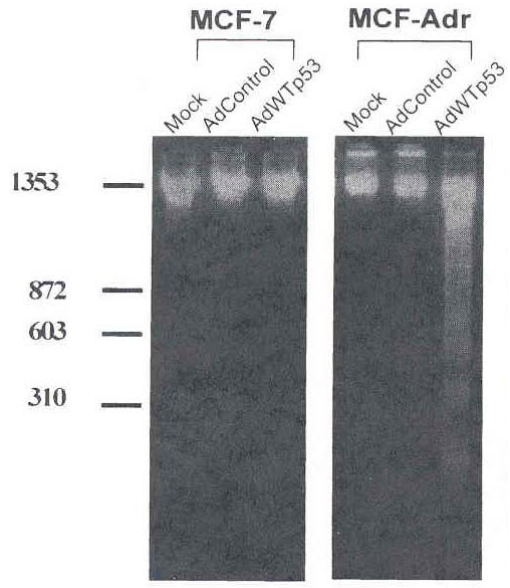



**Figure 13**: Adp53 mediated nucleosomal DNA degradation in Adp53 infected MCF-7 and MCF-Adr cells. Cells were exposed to 100 plaque forming units per cell of p53Ad. One day after infection cells were collected and incubated with lysis buffer. DNA was then isolated and subjected to agarose gel electrophoresis. (28)

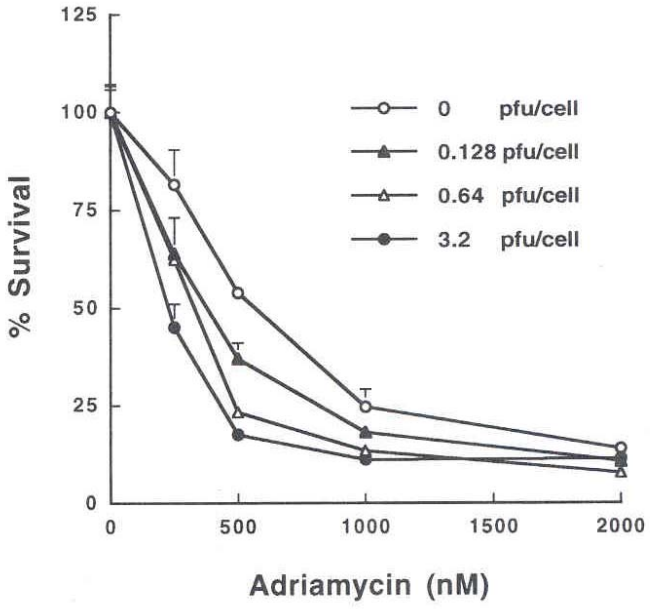



**Figure 14**: The cytotoxicity of p53Ad in combination with adriamycin in MCF-Adr cells. Cells were infected with p53Ad and adriamycin was added 24 hours later. Cell survival was assessed after 7 days using the sulforhodamine assay. (28)

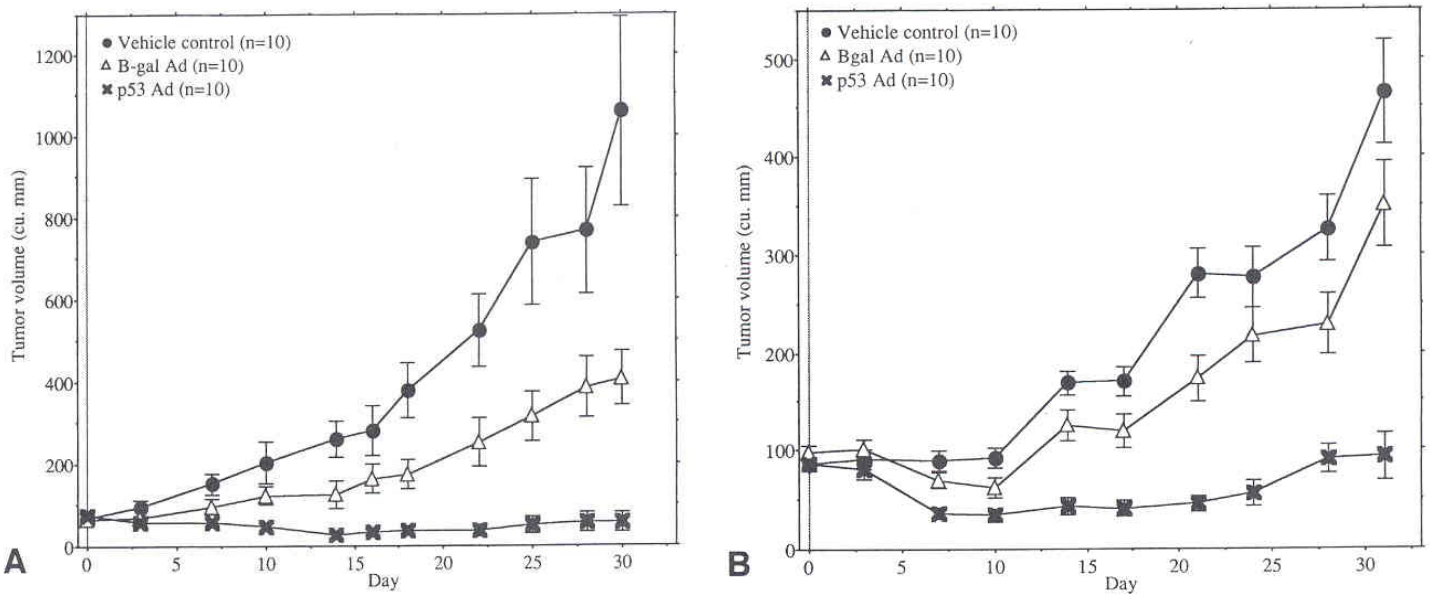
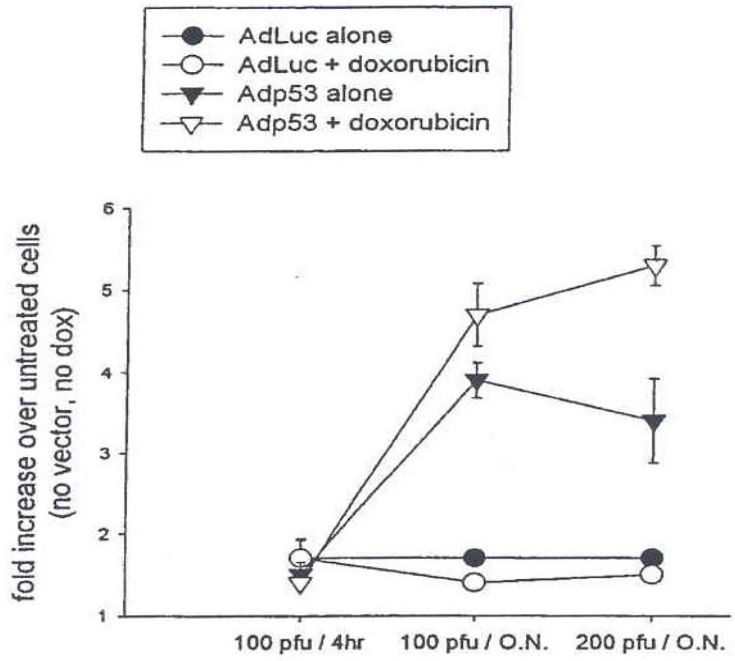



**Figure 15**: Efficacy of p53Ad against human breast xenografts in nude mice. Mice were treated with 2.2 x $10^9$ cellular infectious units per mouse of p53Ad, β-gal Ad, or vehicle control. (A)231 cells; (B) 468 cells; x320. (21)




- **Figure 16**: Apoptosis assay measuring the release of oligonucleosomal fragments from the nucleus. Cells were treated with control or p53Ad and cytoplasmic extracts were prepared 48 hours from the start of treatment and assayed. (17)

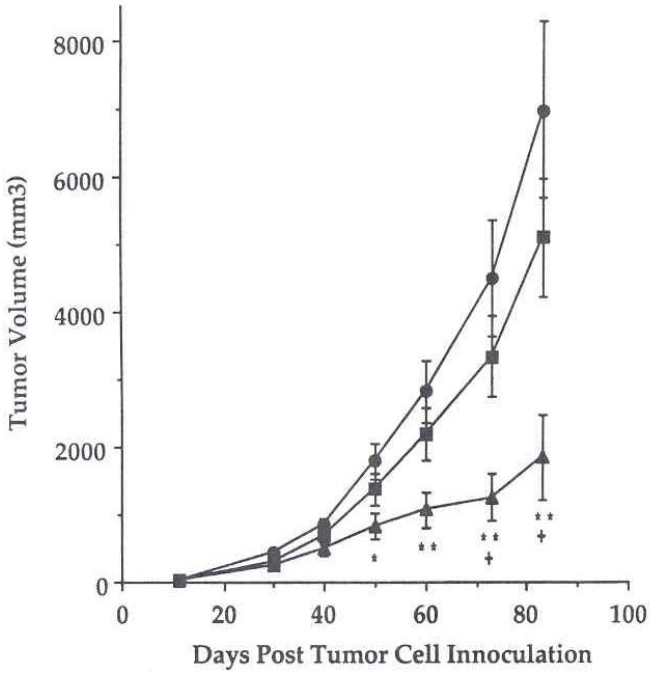



**Figure 17**: Systemic intravenous injection of liposome-vector or liposome-p53 complex into nude mice bearing breast tumor. (•) untreated control mice inoculated with MDA-MB 435 cells; (■) liposome-empty vector treated tumors; (▲) liposome-p53 treated tumors. Significance between vector and p53 treatments is indicated by a + sign ($p < 0.05$). (18)

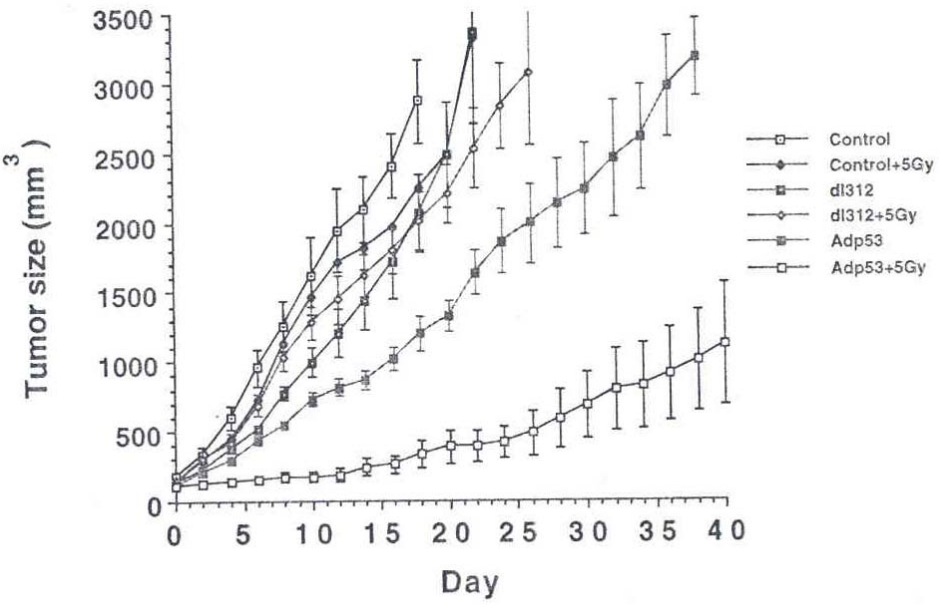



**Figure 18**: Effects of p53Ad and 5Gy irradiation on *in vivo* tumor growth of SW620 subcutaneous xenografts. Tumors were treated after reaching a size of 200 $mm^2$ with three daily injection of PBS or virus, and then treated with 5 Gy on day 4. Tumors were measured every other day and tumor volume was calculated. (29)

## References

1. Aas T, Borresen AL, Geisler S, Smith-Sorensen B, Johnsen H, Varhaug JE, Akslen LA, Lonning PE. Specific p53 mutations are associated with de novo resistance to doxorubicin in breast cancer patients. *Nat Med*. 1996:2:811-4.

2. Anderson WF. Gene therapy scores against cancer. *Nat Med*. 2000:6:862-3.

3. Barnes DM, Camplejohn RS. P53, apoptosis, and breast cancer. *J Mammary Gland Biol Neoplasia*. 1996 :2:163-75.

4. Blagosklonny MV, el-Deiry WS. In vitro evaluation of a p53-expressing adenovirus as an anti-cancer drug. *Int J Cancer*. 1996:67:386-92.

5. Blau H, Khavari P. Gene therapy: progress, problems, prospects. *Nat Med*. 1997:3:612-3.

6. Bouvet M, Ellis LM, Nishizaki M, Fujiwara T, Liu W, Bucana CD, Fang B, Lee JJ, Rth JA. Adenovirus mediated wild-type p53 gene transfer down-regulates vascular endothelial growth factor expression and inhibits angiogenesis in human colon cancer. *Cancer Research*. 1998:58:2288-92.

7. Bunz F, Hwang PM, Torrance C, Waldman T, Zhang Y, Dillehay L, Williams J, Lengauer C, Kinzler KW, Vogelstein B. Disruption of p53 in human cancer cells alters the responses to therapeutic agents. *J Clin Invest*. 1999:104:263-9.

8. Choisy-Rossi C, Reisdorf P, Yonish-Rouach E. The p53 tumor suppressor gene: structure, function and mechanism of action. *Results Probl Cell Differ*. 1999:23:145-72.

9. Chung-Faye GA, Kerr DJ, Young LS, Searle PF. Gene therapy strategies for colon cancer. *Mol Med Today*. 2000:6:82-7.

10. Cox LA, Chen G, Lee EY. Tumor suppressor genes and their roles in breast cancer. *Breast Cancer Res Treat*. 1994:32:19-38.

11. Favrot M, Coll JL, Louis N, Negoescu A. Cell death and cancer: replacement of apoptotic genes and inactivation of death suppressor genes in therapy. *Gene Ther*. 1998:5:728-39.

12. Fisher DE. The p53 tumor suppressor: critical regulator of life & death in cancer. *Apoptosis*. 2001:6:7-15.

13. Friedmann T and Roblin R. Gene therapy for human genetic disease? *Science*. 1972:175: 949-55.

14. Gurnani M, Lipari P, Dell J, Shi B, Nielsen LL. Adenovirus-mediated p53 gene therapy has greater efficacy when combined with chemotherapy against human head and neck, ovarian, prostate, and breast cancer. *Cancer Chemother Pharmacol*. 1999:44:143-51.

15. Horowitz J, Fritz MA, Swanson S. Transgene expression from the phase I pilot program rAd.p53. *Proc Am Accoc Cancer Res*. 1998:39:361.

16. Huang P, Oliff A. Signaling pathways in apoptosis as potential targets for cancer therapy. *Trends Cell Biol*. 2001:11:343-8.

17. Lebedeva S, Bagdasarova S, Tyler T, Mu X, Wilson DR, Gjerset RA. Tumor suppression and therapy sensitization of localized and metastatic breast cancer by adenovirus p53. *Hum Gene Ther*. 2001:12:763-72.

18. Lesson-Wood LA, Kim WH, Kleinman HK, Weintraub B, Mixson AJ. Systemic gene therapy with p53 reduces growth and metastases of a malignant human breast cancer in nude mice. *Hum Gene Ther* 1995:6:395-405.

19. Lipinski KS, Djeha AH, Krausz E, Lane DP, Searle PF, Mountain A, Wrighton CJ. Tumor-specific therapeutic adenovirus vectors: repression of transgene expression in healthy cells by endogenous p53. *Gene Ther*. 2001:8:274-81.

20. Namoto M, Tonemitsu Y, Nakagawa K, Hashimoto S, Kaneda Y, Nawata H, Sueishi K. Heterogeneous induction of apoptosis in colon cancer cells by wild-type p53 gene transfection. *Int J Oncol*. 1998:12:777-84.

21. Nielsen LL, Dell J, Maxwell E, Armstrong L, Maneval D, Catino JJ. Efficacy of p53 adenovirus-mediated gene therapy against human breast cancer xenografts. *Cancer Gene Ther*. 1997:4:129-38.

22. Ogawa N, Fujiwara T, Kagawa S, Nishizaki M, Morimoto Y, Tandia T, Hizuta A, Yasuda T, Roth JA, Tanaka N. Novel combination therapy for human colon cancer with adenovirus-mediated wild-type p53 gene transfer and DNA-damaging chemotherapeutic agent. *Int J Cancer*. 1997:73:367-70.

23. Putzer BM, Bramson JL, Addison CL, Hitt M, Siegel PM, Muller WJ, Graham FL. Combination therapy with interleukin-2 and wild-type p53 expressed by adenoviral vectors potentiates tumor regression in a murine model of breast cancer.*Hum Gene Ther*. 1998:9:707-18.

24. Roemer K, Friedmann T. Mechanisms of action of the p53 tumor suppressor and prospects for cancer gene therapy by reconstitution of p53 function. *Ann NY Acad Sci*. 1994:716:265-80; discussion 280-2.

25. Roth JA, Nguyen D, Lawrence DD. Retrovirus-mediated wild-type p53 gene transfer to tumors of patients with lung cancer. *Nat Med*. 1996:2:985-991.

26. Runnebaum I. Basics of cancer gene therapy. *Anticancer Research*. 1997:17:2887-90.

27. Seth P. Pre-clinical studies with tumor suppressor genes. *Caner Gene Ther: Past Achievement and Future Challenges.* 2000

28. Seth P, Katayose D, Li Z, Kim M, Wersto R, Craig C, Shanmugam N, Ohri E, Mudahar B, Rakkar AN, Kodali P, Cowan K. A recombinant adenovirus expressing wild type p53 induces apoptosis in drug-resistant human breast cancer cells: a gene therapy approach for drug-resistant cancers. *Cancer Gene Ther*. 1997:4:383-90.

29. Spitz FR, Nguyen D, Skibber JM, Meyn RE, Cristiano RJ, Roth JA. Adenoviral-mediated wild-type p53 gene expression sensitizes colorectal cancer cells to ionizing radiation. *Clin Cancer Res*. 1996:2:1665-71.

30. Venook AP, Bergsland EK, Ring E. Gene therapy of colorectal liver metastases using a recombinant adenovirus encoding wt p53 via hepatic artery infusion. *Proc Am Soc Clin Oncol*. 1998:17:431a.

31. Vinyals A, Peinado MA, Gonzalez-Garrigues M, Monzo M, Bonfil RD, Fabra A. Failure of wild-type p53 gene therapy in human cancer cells expressing a mutant p53 protein. *Gene Ther.* 1999:6:22-33.

32. Waku T, Fujiwara T, Shao J, Itoshima T, Murakami T, Kataoka M, Gomi S, Roth JA, Tanaka N. Contribution of CD95 ligand-induced neutrophil infiltration to the bystander effect in p53 gene therapy for human cancer. *J Immunol*. 2000:165:5884-90.

33. Wasil T, Buchbinder A. Gene therapy in human cancer: report of human clinical trials. *Cancer Invest*. 2000:18:740-6.

34. Watanabe T and Sullenger B. Induction of wild-type p53 activity in human cancer cells by ribozymes that repair mutant p53 transcripts. *PNAS*. 2000:15:8490-94.

35. Xu M, Kumar D, Stass S, Mixson A. Gene therapy with p53 and a fragment of thrombospondin I inhibits human breast cancer in vivo. *Mol Gen Met*. 1998:63:103-9.

36. Yang B, Eshleman JR, Berger NA, Markowitz SD. Wild-type p53 protein potentiates cytotoxicity of therapeutic agents in human colon cancer cells. *Clin Cancer Res*. 1996:10:1649-57.

37. Zeimet A, Riha K, Berger J, Widschwendter M, Hermann M, Daxenbichler G, Marth C. New insights into p53 regulation and gene therapy for cancer. *Biochem Pharm*. 2000:8:1153-63.

38. www.cancer.org (website for the American Cancer Society)

39. www.nih.gov (website for the National Institutes of Health)

40. www.uphs.upenn.edu/ihgt/ (website for The Institute for Human Gene Therapy)