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IMMUNOHISTOCHEMICAL EVALUATION OF HUMAN p53 TUMOR SUPPRESSOR PROTEIN CONTENT IN DUCTAL CARCINOMA IN SITU (DCIS) OF THE BREAST

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ABSTRACT

IMMUNOHISTOCHEMICAL EVALUATION OF HUMAN p53 TUMOR SUPPRESSOR PROTEIN CONTENT IN DUCTAL CARCINOMA IN SITU (DCIS) OF THE BREAST

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The p53 content of 100 human breast biopsies which were classified as (DCIS), were evaluated by immunohistochemical method. From this population 15 specimen were immunoreactive. All specimens were microscopically classified into histologic types of DCIS and nuclear grade. Most immunopositive specimens were classified as mixed histologic types. No relationship between nuclear grade and different histologic types of DCIS was noticed. The goal of this study was to establish a data base for long-term patient assessment. Preliminary data indicates that the presence of immunopositive p53 may be a valid predictive indicator of the prognosis of individuals with DCIS.

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I. INTRODUCTION

Breast cancer is among the leading causes of cancer related deaths of women in the USA. According to statistical analysis one in every eight women will develop breast cancer in her life time (1). Early diagnosis and determination of invasiveness of the tumor is important for management and outcome of patients with breast cancer. Nuclear phosphoprotein p53 is among the markers used to evaluate the progression and invasiveness of several types of tumors (2). The focus of this research project is to evaluate the accumulation of the p53 tumor suppressor protein in human ductal carcinoma in situ (DCIS), a type of breast tumor, using a immunohistochemistry method. The goal of this project is to determine if early detection of mutant p53 accumulation, may be an early indicator of tumor aggressiveness and transformation to invasive breast cancer. A population of n=100 of formalin-fixed, paraffin-embedded non-invasive intraductal breast carcinoma tissue samples was used. If a correlation between p53 content and conversion to invasive breast cancer can be established, early forms of aggressive therapy may be directed on the basis of the study.

1.0 Cancer History

Cancer and related words (e.g., canker; cancre, chancre) are derived from the Latin word cancer meaning "crab" (3). The ancient association between the crab and the disease

of cancer developed from the physical resemblance between the legs of a crab and the radiating engorged veins surrounding a cancerous tumor. The word itself derives from two roots: "can" meaning " to surround" and "cer" meaning "hard". It is this image of a hard surrounding that is distilled in the name (3).

The earliest description of cancer appears in the Edwin Smith Papyrus from Egypt in the seventeenth century B.C. After providing the oldest written description of a patient with cancer, the physician advised "There is no treatment" (4). A thousand years later, Hippocrates, the father of Medicine, described cancer as an imbalance between the black humors (from the spleen) and the other three bodily humor: blood, phlegm and bile (5). Although incorrect, the theory was the first (~400 B.C.) to attribute the origin of cancer to natural causes (5). As Europe entered the Age of Reason, Bernardine Ramazzini, an Italian physician, noted the high incidence of breast cancer in nuns and hypothesized that this was in some way related to their celibate life style (3). The age of cancer epidemiology had begun. Another scientific report on cancer epidemiology, from 1775, (6) by Sir Percival Pott an English physician, observed that young men in their twenties who had been chimney sweeps as boys had a high rate of deaths due to cancer of the scrotum (6). He suggested that the causative agent might be chimney soot (now known to be the tar) and recommended frequent changing of clothing that trapped the soot, to reduce exposure to the "carcinogen". Pott's study not only identified a putative carcinogenic agent but also demonstrated that a cancer may develop many years after exposure to the causative agent. In other words, that there may be an extended latent period (6).

The nineteenth century saw the birth of scientific oncology as microscopes of sufficient quality for research on tissues become available. Johannes Muller, applied this

instrument to cancer research and began to correlate cellular pathology with clinical symptoms (7). He established a cellular basis for tumor description. Subsequently, this work was carried on by Virchow, who provided the scientific and microscopic basis for the modern pathologic study of the cancer (7). However, both failed to recognize the cellular nature of metastases. Virchow suggested that metastases could be compared to spread of an infectious agents. Once initiated in one part of the body, a tumor releases certain fluids which cause changes in cells in distance tissue to become tumor like(7). Wilhelm Waldeyer of Berlin did not agree with this idea and he felt embolic transfer through the blood or lymph channels was the mechanism (8). Thus the pathological basis of malignancy began to be understood and pathology began to replace anatomy as the key basic medical science.

With the discovery of the radiation, viral, and chemical carcinogens in the twentieth century the entire focus of cancer research changed. Radiation was recognized as a carcinogen only seven years after the discovery of X rays by Roentgen's (9). In 1911 Peyton Rous, at the Rockefeller Institute, described a sarcoma in chickens cased by what later became known as the Rous Sarcoma Virus (10). In 1915 cancer was induced in laboratory animals for the first time by Yamagiwa and Ichikawa, at Tokyo University (11). They applied coal tar to rabbit skin. This experiment led to the identification of the chemical carcinogenesis with a firm scientific foundation and a research technique (11). In 1935 Peyton Rous reported the differences between what he termed initiation and promotion (12). He defined these terms using two different models of carcinogens; 1) studying the manner in which a benign neoplasm, virus induced rabbit papilloma, transformed into a malignant lesion (12) and 2) Induction of skin tumors by the application of coal tar (13). These classic experiments confirmed by Berenblum and Shubik using corton oil as the promoter and formed the

prototype for the way carcinogenesis was conceptualized (14). In addition, they led to the concept of initiation by one agent, followed by promotion by another and finally progression of the tumor to a more malignant form. The initiator was viewed as able to cause cancer but only after a prolonged time (14). The promoter was considered as an agent not capable alone of causing cancer but able to potentiate the effects of the initiator (14). The term progression was defined by Rous to designate "the process by which tumors go from bad to worse" (12,13).

Fould codified and expanded the concept of multistage carcinogenesis (15). Evidence obtained twenty years after Rous' work indicating that the first stage, initiation, is characterized by damage to DNA while the second stage, promotion, usually does not involve damage to the DNA but rather a stimulation of cellular proliferation. Promotion is reversible and exhibits a distinct dose response and measurable threshold. The third stage, progression, leads to morphological change and increased grades of malignant behavior, such as invasion, metastasis, and drug resistance (12,13). Carcinogenic agents can mutate two types of genes that regulate growth; proto-oncogenes that code for growth factors (16) and tumor suppressor genes which code for growth suppression (17). In humans carcinogenesis can be viewed as a process involving several steps (18), one of which activates an oncogene and another which inactivates a tumor suppressor gene. In the process of carcinogenesis (7), the initial mutation in a cell may confer a survival advantage. If one of the descendants of that cell is hit by a second mutation that also confers additional survival advantage, the next clone may grow even more vigorously. Such a sequence of events leads to the selection of a clone with the characteristics of a neoplasm (7). This allows subclones to progress to even

greater stages of virulence characterized by invasion and metastatic spread that ultimately lead to the death of the host (7).

1.1 Chemical Carcinogens

In spite of the vast array of chemicals discovered to cause cancer in animals, there remains very few chemicals (19), other than the tars associated with tobacco (20), for which there is strong evidence of cancer causation in human. A large amount of work, reaching back to the 1940s, indicated that carcinogenic agents posses a potent ability to damage genes. A key discovery made by Ames (21), found that the carcinogenic potency of a number of chemical compounds is correlated with their mutagenic ability. The conclusion on chemical carcinogens was that cells transformed due to gene mutations that were chemically induced forms of normal cellular genes that become damaged (18). Until recently the nature of these hypothetical target genes was totally unclear.

2.0 Proto Oncogenes Discovery Via Viral Oncogenes (Retroviruses)

In the late 1960 and early 1970s the role of viruses in cancer formation was realized to be a small fraction of the total number and types of cancer formed in this country (18). However, it is ironic that research on animal ribonucleic acid (RNA) tumor viruses (retroviruses), which have no ability to cause human cancer provided the first key to discovering the identity of cellular genes which may be responsible for tumors (18). These retroviruses, which infect chickens, rodents, cats, and monkeys, are potently tumorigenic in infected animals within weeks after initial exposure to the virus (18). One of these viruses, the Rous sarcoma virus of chickens, was found to carry a specific gene that it uses to

transform infected cells from a normal to a malignant state (16). Such a transforming gene was termed a viral oncogene. It clearly possessed great power, since the single oncogene carried into a chicken cell by the Rous sarcoma virus was able to redirect the entire biology of the cell, forcing it to grow in a malignant fashion (16).

Harold Varmus and J. Michael Bishopin in 1976 found that the oncogene in the Rous sarcoma virus was not genuinely a viral gene , instead, it arose from a pre-existing cellular gene that had been captured by an ancestor of the Rous sarcoma virus (16). Once captured, this gene was used by the virus to transform cells. The early ancestor of the Rous sarcoma virus was capable of replicating in infected cells but unable to transform them, it instantly gained tumorigenic potency by kidnapping this normal cellular gene called a proto-oncogene (16).

In the end this work revealed much more about the cell than it did about the Rous sarcoma virus (16). It pointed to the existence of a gene residing in the normal cellular genome that possesses potent transforming ability when appropriately activated, in this case by a retrovirus. It was proposed that nonviral carcinogens such as mutagenic chemical (19) and X-rays (18) might also activate these proto-oncogenes. A cell carrying such a mutant gene might respond by setting a deregulated growth pattern and in this way becoming a malignant cell.

Although retrovirus associated oncogenes clarified the existence of normal cellular genes (proto-oncogenes) in the normal cellular genome, it provided little information about the origin of human cancer. After all, retroviruses like Rous sarcoma virus (16) are not known to cause human infections. Therefore a possible and alternative route through which proto-oncogenes could also be activated is through exposure to chemical mutagenes that

could change the deoxyribonucleic acid (DNA) sequence (19). Mutations induced by these agents in the genome of target cells might be as effective as retroviruses in activating the latent carcinogenic potential of proto-oncogenes (19).

2.1 Oncogenes and The Mechanism of Action

Over the past decade the existence of mutant proto-oncogenes in human tumors has been demonstrated (18). In each case, a change in the sequence structure of a gene was pinpointed as being responsible for the converting of proto-oncogene into an active oncogene. In addition, understanding how normal cells regulate their growth made it possible to explain the mechanism of action of most if not all oncogenes (18,22).

The rate of growth and division of a normal cell, residing within a tissue is largely controlled by its surroundings (18). A normal cell rarely decides on its own to proliferate. Rather, hormone like polypeptide growth factors which are endocrine, paracrine, and/or autocrine signals direct growth and division (22). These factors carry growth-stimulatory (18) or growth-inhibitory signals (18). Each cell possesses complex machinery that enables it to receive these signals, process them, and set a growth program including differentiation (18,22). The presence of growth-activating factors in the extracellular space can be recognized by the specific protein receptors that span the plasma membranes of the cells (18,22). These factors bind to the extracellular domains of specific receptor and activate their cytoplasmic domains to phosphorylate or dephosphorylate the target proteins (22). The change in phosphorylation rate of target proteins are thought to act as intracellular signals which stimulate cell division (18,22). Proto-oncogenes encode many of the proteins in this complex signaling pathway that enable a normal cell to respond to exogenous growth factors

(18). Oncogene proteins participate in activating these signaling pathways even in the absence of stimulation by extracellular growth factors. In doing so, they force a cell to grow, even when its surroundings contain none of the features that are normally required to provoke growth. The proteins encoded by the ras proto-oncogene and the ras oncogene, provide a good example of this. The protein encoded by the ras proto-oncogene sit quietly in the cytoplasm of the cell waiting for stimulation by a growth-factor receptor at the cell surface. When such a receptor binds its growth factor or ligand , it sends a stimulatory signal to the ras protein, which responds by releasing a number of secondary signals into the cell. However, the ras protein specified by a ras oncogene acts much differently. It releases signals into the cell continually, independent of prior stimulation by a growth-factor receptor. Thus the cell is flooded with growth stimulatory factors in its surroundings, when in fact none are present. As an immediate result, proliferation of a ras oncogene bearing cell becomes quite autonomous (18).

3.0 Tumor Suppressor Genes

Since a normal cell requires multiple changes to its genome before it can take on the characteristics associated with a cancer cell (7,18), examining the activated oncogenes (18) in actual tumor cells indicate that activated oncogenes are only part of the picture. Another class of mutated genes, and their protein products, which seem to be equally as important, are the tumor-suppressor genes (17). Indeed, normal cell growth and division appears to be regulated by a finely tuned balance (18) between the action of the gene products of growth-promoting proto-oncogenes (16,18) and the growth-suppressing factors from tumor suppressor genes (17). When the latter are nonfunctional their negative effects on cell growth

are no longer felt and a cell may lose its ability to respond to external growth-inhibitory factors and continues to grow and proliferate. An analogy is commonly used to describe this balance. Activation of an oncogene is like the accelerator stuck to the floor and loss of tumor suppression is like a defective braking system (18). The combination of the two is disastrous with aggressive cell proliferation.

3.1 Mechanism of Action of Tumor Suppressor Genes

A number of tumor-suppressor genes have been discovered over the past five years (17,18). Their role in tumorigenesis appears as important as that of oncogenes. Tumor-suppressor genes, like most all genes, are present in two copies per a cell (17,18). When one of these copies is nonfunctional cell growth is still normal relying on the remaining gene copy (18). Only when the second copy is lost, does abnormal cell proliferation takes place. An example of such a case is retinoblastoma (RB) tumor suppressor gene (23) that require loss of both copies of the gene before the cell behaves abnormally. Another possible genetic mechanism with a similar end result, can be seen with p53 tumor suppressor protein. If one mutated gene copy loses its growth-suppressing function and simultaneously the ability of the remaining intact copy is suppressed by an interfering or blocking constituent (24).

3.2 History of the p53 Tumor Suppressor Protein

The p53 tumor suppressor protein was first identified as a cellular protein in 1979 that coimmunnoprecipitated with the transforming protein of Siman Virus 40 (SV40), the large T antigen in SV40 transformed cells. In these early experiments the purified SV40 large T antigen acquired from tumors, induced by SV40, was used to generate antisera (25).

When this antisera was used in whole cell preparations to immunoprecipitate large T antigen, it was found that p53 also coprecipitated with the large T antigen . It was determined that the coprecipitation of p53 with T antigen was due to binding. Since the large T antigen is needed to maintain the transformed phenotype, it was suggested that this interaction is important for transformation. Thus p53 came to be classified as a tumor antigen (25).

The wild type p53 is known to be expressed at low levels and has a short half-life (20-30 minutes) in normal cells (26). However, in SV40 transformed cells (25) and tumor derived cell lines from both human and nonhuman tumors (27), it was found that p53 was 100-fold higher in concentration than that observed in normal cells and the half-life was increased to over 6 hours. Stable complex formation of p53 with viral oncoproteins like SV40 suggested that this protein plays a role in the transformation of cells by this virus and is critical in the regulation of cell growth (25). Later it became clear that only mutant forms of p53 can contribute to cellular transformation (28,29). These findings were made when the isolated genomic and complementary DNA define as (cDNA) clones of mutant p53 could immortalize cells in culture, thus classified p53 as a dominant oncogene (28,29). This was also demonstrated in a ras transformation assays (28). Several independently derived mutations of p53, which map between amino acids 120 and 230, activate p53 to transform rat embryo fibroblasts (REF) in culture with the ras oncogene (28). Mutant p53 alleles inhibit normal p53 function and enable ras oncogene to transform REF cells (28). Over expression of the wild-type p53 protein suppresses the formation of transformed cells (30,31) and in addition, inhibits the growth of established tumor cells (32,33). However, over expression of the mutant p53 protein in tumor cells inhibit its negative regulatory function (34). These

observations have resulted in the redefinition of the role of p53 to a tumor suppressor gene (35,36).

3.3 The p53 Gene and Protein Structure

The p53 gene encompasses 16 to 20 kilo base (Kb) of DNA on the short arm of human chromosome 17 at position 17p 13.1 (37,38). This gene is composed of 11 exons (37,38). The p53 gene has been conserved during evolution. In cross-species comparison, the p53 protein shows five highly (> 90%) conserved regions within the amino acid residues 13-19, 117-142, 171-181, 234-258, and 270-286 (39). These five evolutionary conserved domains within the coding regions are regarded as essential to the function of the p53 (40).

The product of the p53 gene is a homodimeric 393-amino acid nuclear phosphoprotein (~53 Kilo Dalton in molecular weight) (39). The p53 protein contains; 1) an acidic amino terminal (amino acids 1-75) possessing the transcriptional activation region followed by 2) a central and extended hydrophobic proline rich domain (amino acids 75-150). and 3) a highly charged basic carboxy terminal domain (amino acids 275-390) containing nuclear localization signals (41,42), the oligomerization (43), and DNA binding domains (44) (Fig 1). Oligomerization refers to multiple binding sites on the protein molecules which results in formation of protein complexes (43). The central region is the highly conserved portion of the protein. The majority of oncogenic mutations are located here (39).

3.4 Functions of The p53 Tumor Suppressor Protein

The p53 protein appears to have multiple functions including regulation of DNA

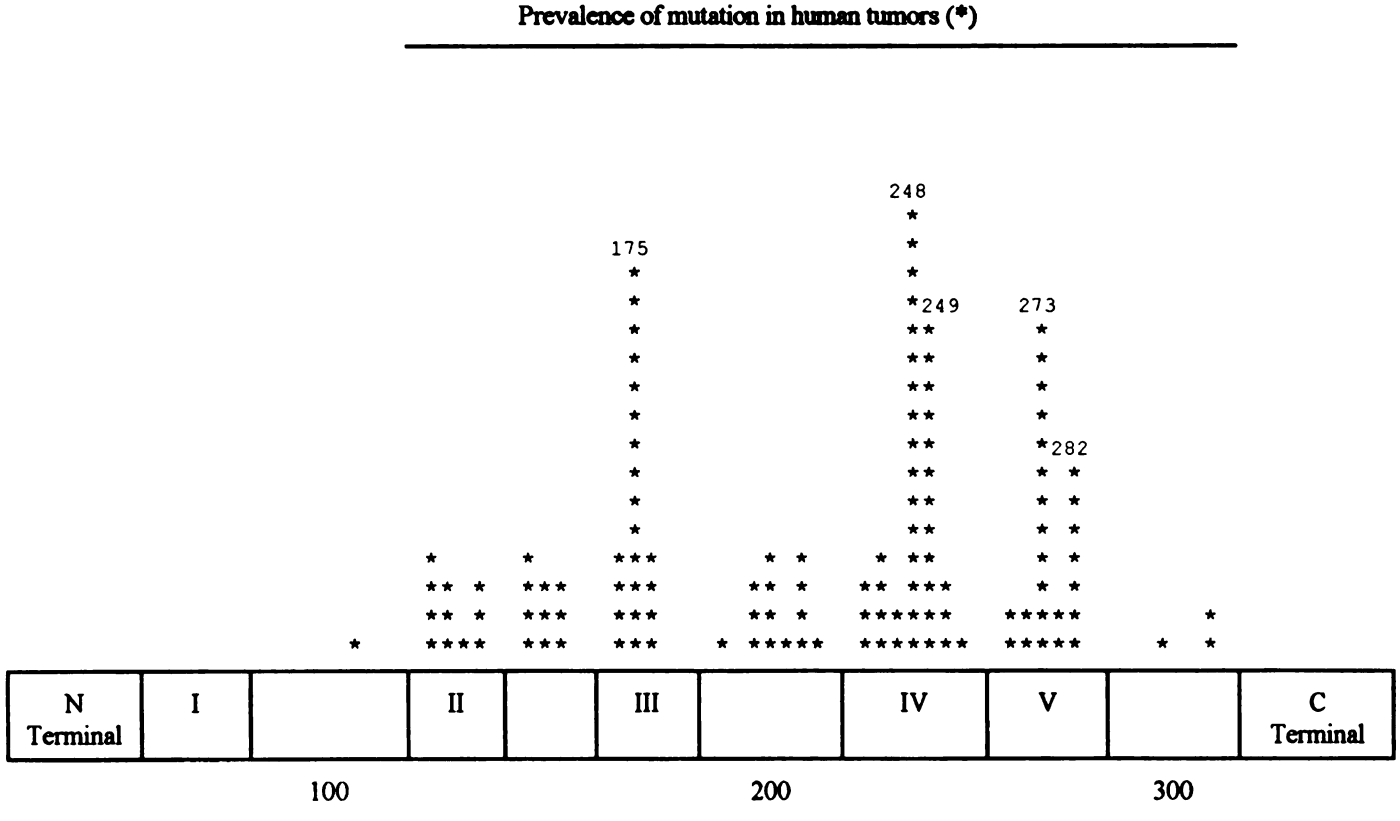

Figure 1: Frequency Histogram of Human p53 Protein Mutations
(Adapted from Science: 262: p. 1980, 1993)
The acidic amino terminal represents area of transcriptional activation. Region I-II represent mdm-2, Eb and E1b binding sites. Region III-V represent the SV40 T antigen binding sites. The basic carboxy terminal is oligomerization and DNA binding site. The asterisks indicate the relative number of substitutions (mutations) that have been found in human tumors in mutational hot spots we found at codons 175 (n=77); 248 (n=112); 249 (n=55); 273 (n=89); 282 (n=42). E1b is the adenovirus E1b p55 protein, Eb is the human papilloma virus and Mdm-2 is cellular protein SV40 is the oncoviral protein.

synthesis (25,45,46), repair (47) and programmed cell death (47,48,49,50), genetic instability(51,52), and as a transactivator of gene expression (53).

3.4.1 The Role of p53 Protein in The Regulation of The DNA Synthesis

The majority of evidence supporting the role of p53 in the regulation of the DNA synthesis comes from the association of wild type p53 with the SV40 large T antigen (25). The SV40 large T antigen is required for the initiation of viral DNA replication. Wild type p53 can block T antigen initiation of DNA polymerase alpha (45). Also wild type, but not mutant, p53 expression can inhibit viral DNA replication in vivo and in vitro (46,54). A simplistic explanation for these observations is that in the normal cell p53 binds a homologue(s) of T antigen involved in the initiation of cellular DNA synthesis (55). The formation of a complex with p53 could prevent the association of this protein with DNA polymerase alpha or another critical cellular protein negatively controlling cell growth (55). The inability of mutant p53 proteins to bind T antigen suggests the p53 mutants are no longer capable of binding the appropriate cellular protein and regulating the cell growth (25,56,57).

Another example of cell growth regulation by p53 involves the p53 mutant for residue 135 (Ala to Val change) which is a temperature sensitive phenotype (58). At 32^{0}C, the protein acts like the wild-type (57) and at 37-39.5^{0}C the protein is primarily in a mutant conformation (59). At 32^{0}C the protein does not cooperate with an activated ras gene in REF in culture transformed by both this temperature sensitive p53 mutant and by ras (58). It can also suppress the development of transformed foci at 32^{0}C (58). In addition, REF stop growing at late G1, failing to enter the S phase when shifted to 32^{0}C (60). This growth

arrest occurs concomitantly with the translocation of p53 (Ala 135, wild-type conformation) protein from the cytoplasm into the nucleus and suggests that p53 must be in the nucleus to exert growth regulatory effect (59,61,62). Whereas at 37-39.5^{0}C this protein is localized in the cell cytoplasm (59,61,62). Two hypotheses have been proposed to account for these results; 1) In cells transformed both by the temperature-sensitive p53 mutant (Val 135) and by ras, the mutant forms a complex with the endogenous rat wild-type p53 and keeps it in the cytoplasm, where p53 is unable to block cell growth (59). A shift to 32^{0}C results in the movement of p53 into the nucleus and a block in cell growth, indicating that some dominant loss-of-function mutations could work by regulating the entry into the nuclear compartment (59,61,62). 2) Introduction of mutant p53 cDNA into a cell line that did not express endogenous p53 produced tumors in animals. So mutant p53 could gain a new function that overcomes the negative regulation by small quantities of wild-type p53 (63).

3.4.2 The p53 Protein Role in Monitoring the Fidelity of the Genome

The p53 gene product is among those proteins that appears to be involved in the monitoring of the fidelity of molecular processes (47,48,49) such as DNA repair (47). In response to DNA damage p53 protein accumulates (47). The mechanism which causes the accumulation of p53 is unclear (47). However, it appears to primarily involve an increase in synthesis (47). This may be induced at the transcriptional (47) and /or translational level (64,65). A variety of agents capable of causing DNA damage (64), increase p53 synthesis. The mechanism of induction appears to be related to general DNA helix distortion (47). The induction of p53, in association with DNA damage, appears to be part of the cellular defense mechanism that monitors genomic fidelity (47,48,49). The wild type p53 protein is a potent

inhibitor of the cell cycle (50, 65). Increase in wild type p53 cause G1 arrest in the cell cycle (47, 50, 65). It has been theorized that this allows DNA repair to occur before the cell divides (47, 50, 65). In this manner, the cell cycle is arrested before DNA synthesis and the cell is given the opportunity repair the damaged DNA (47, 50). In several cancer cells with mutant p53, it has been well documented that these cells fail to arrest in G1 (47, 65).

The mechanism of p53 cell cycle arrest appears to be the inhibition of cyclin dependent kinase (CDK) (50, 66). CDK is a constitutive enzyme to the cell cycle (67). Inhibition of this enzyme results in G1 arrest (66). The p53 inhibition of CDK appears to be mediated by the induction of DNA synthesis at the transcriptional level (50, 68). p53 is known to induce the synthesis of a variety of proteins. One example of this that may explain this effect is p21 (50, 66,69,70). p21 is known to bind to and inhibit CDK activity (66, 69, 70).

An example of this system can be seen in somatic cells with DNA damage (47) caused by radiation (64, 65). This leads to an increase in wild-type p53 protein levels and results in G1 arrest of the cell cycle (Fig2). If DNA repair does not successfully occur, the presence of the wild-type p53 alternatively induces programmed cell death or apoptosis (47, 48, 49, 71, 72) through an unknown mechanism (Fig. 3). Thus wild-type p53 function appears to be a component of the cellular response to DNA damage (47,64) and p53 acts as one of a number of negative regulators of the cell cycle after damage (47). Tumor cells that show p53 mutations or possess p53 protein inactivated by binding to host or viral proteins can not arrest in G1 to allow DNA repair (25, 55). Kastan reported, in an elegant series of experiments a pathway by which p53 may functions (65) (Fig. 4). His group reported that cells bearing no p53 or mutant p53 do not display G1 arrest after DNA damage. However,

p53 null cells, after induction of wild type but not mutant p53 constructs, display DNA damage-induced G1 arrest.

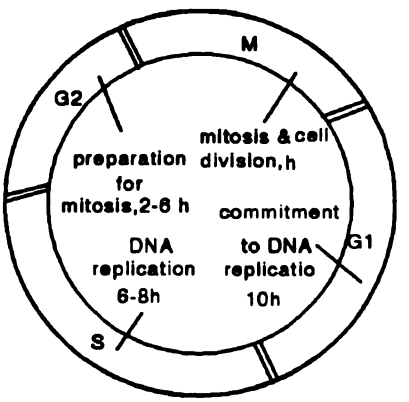

Figure 2: The Eukaryotic Cell Cycle
This diagram is a line drawing depiction of the eukaryotic cell cycle. DNA damage in somatic cells leads to increase in Wild-type p53 protein and results in G_1 arrest.
M (for Mitosis); cell division occurs during relatively brief M phase.
G_1 (for Gap); coverts the longest part of the cell cycle.
S (for synthesis) ; the period when DNA is synthesized.
G_2 (for Gap); the period which tetraploid prepares for mitosis.

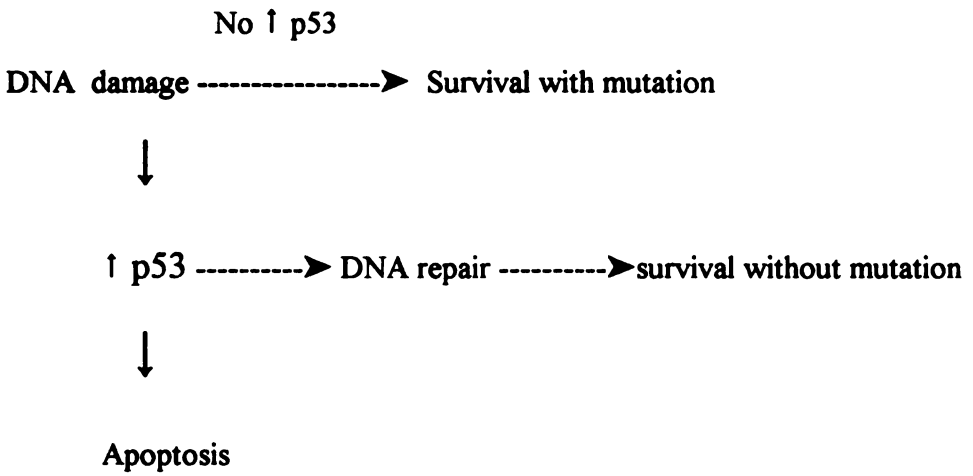

Figure 3: A Pathway Leading to Apoptosis
(Adapted from Nature: 362: p. 786 (1993).
This path is initiated by DNA damage. Increase in wild type p53 concentration may lead to apoptosis if DNA repair is not accomplished. Inactivation of the p53 pathway will result in the survival of cells with mutation/s and may thus have a role in the development of cancer.

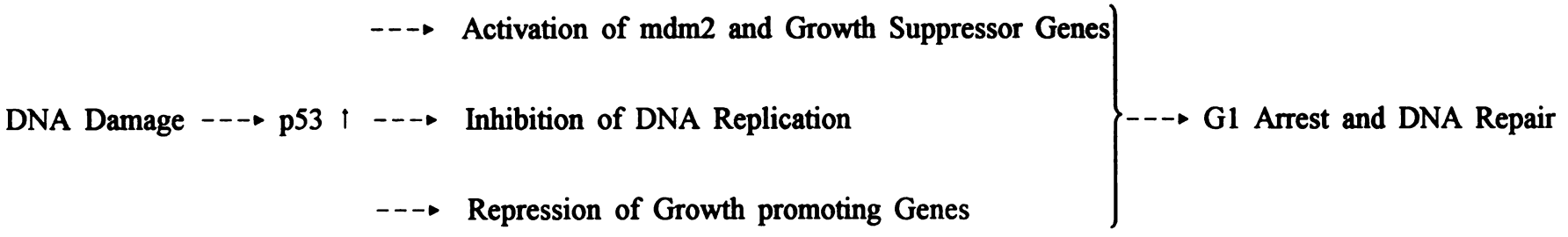

Figure 4: Regulatory Pathway Involving p53 Protein in Response to DNA Damage
(Adapted from Genes & Development: 7: p. 531, 1993).
DNA damaging agents will result in accumulation of high levels of p53 protein, causing suppression and activation of the above mentioned functions and resulting in G_1 arrest.

3.4.3 The p53 Protein Role in Genetic Instability

Another example of p53 function in monitoring the fidelity of the genome is gene amplification; an example of genetic instability (51, 52). Although the genetic basis for gene amplification is not well understood, the available evidence suggests that it is mediated by recombination between large segments of chromosomes, resulting in arrays of amplified genes (73). It has been reported that normal cells in culture have stable karyotype and do not under go gene amplification (51, 52). Whereas, most immortal and transformed cells are unstable and do amplify (51, 52). In addition, the loss of wild-type p53 has been shown to convert normal cells to cells with the ability to undergo gene amplification (74). Tlsty described that p53 null cells are markedly prone to amplification of known specific genes than are cells bearing wild type p53. (75). This finding is consistent with the hypothesis that p53 is a determinant of genetic stability in cells and may function as a cell cycle checkpoint protein following DNA damage (65).

3.4.4 The p53 Protein Role in Transactivation of the Gene Expression

As discussed above, the p53 tumor suppressor gene also appears to be a transcription factor that can induce expression of several different genes containing specific DNA binding sites (53, 76). For example, wild-type p53 binds to a consensus DNA binding sites and activates transcription of the murine double minute 2 oncoprotein (mdm-2) (77, 78, 79). It is likely that p53 exerts its biological activity as a transcription factor and this activity is regulated by modifications and interactions with other suppressor gene products and proto-oncogenes encoding nuclear transcription factors (24). Transcriptional transactivation appears to be responsible for p53 transformation suppression activity too (24). This activity

is lost by the interaction of p53 with the oncogenes of the DNA tumor viruses (80, 81, 82). These viruses synthesize viral DNA that inhibit the ability of p53 to bind DNA and act as a transcription factor. Thus allowing the transformation of cells that are non-permissive for virus replication.

4.0 Inactivation of p53 Tumor Suppressor Protein

Loss of normal p53 function predispose cells to multiple deleterious effects. Inactivation of p53 protein can occur through mutation (60, 83, 84), protein complex formation with viral oncoproteins (80, 82), binding to cellular gene products (77, 78, 79), and dislocation of p53 between the cell compartments (85).

4.1 Mutation of the p53 Gene

Alterations such as small or large allelic deletions (50, 60, 83, 84) structural rearrangements (60, 83, 84) missense mutations (60, 83, 84),and germline mutations (86, 87) in the p53 gene have been reported. Allelic losses in several tumor types, e.g, colorectal neoplasms (50, 60), breast cancers (88, 89), esophageal carcinomas (90), lymphomas (91), and osteosarcomas (92) have been reported. Of these, colon carcinomas (50, 60) have been studied in the most detail (60) and it has been shown that 75-80% of colon cancers have lost both p53 alleles; through deletion and/or, through point mutation (60). The point mutations are usually missense mutations that give rise to an altered protein (93, 94). In the pathogenesis of colon carcinoma, these mutations typically arise during the conversion of a benign adenoma to an invasive adenocarcinoma (95). The molecular mechanisms that underline tumor progression are thought to include the clonal expansion of cells that previously acquired a mutation in cancer

related genes (95). A cell that carries a specific change in a critical gene might acquire a selective growth advantage and become the dominant cell type as the tumor progresses (95). The most common change of p53 in human cancers is a point or missense mutation (73%), substitution of one amino acid for the other, within the coding sequences of the p53 gene resulting in an altered and stable protein (55, 96). The pattern of the p53 missense mutations in human tumors have several features (55, 96). Firstly, the missense mutations do not occur randomly. They are clustered in four regions of the p53 protein that are highly conserved (39,40) from rainbow trout to humans (domains II-V), indicating these regions are of functional importance (table 1). Secondly, there are mutational "hot spots" at amino acids 175, 248-249, 273, and 282 (39). Mutations at these amino acids account for approximately 30% of the total missense mutations observed in human tumors (55). The codon 248 is also a hot spot for germline mutations (97). Thirdly, different "hot spots" are present in different tissues. for example, mutations at amino acid 175 have been found repeatedly in colorectal carcinomas (50, 98) and in lymphoma (92), and have been observed at least once in breast carcinoma (99), glioblastoma (100), and an esophageal

Table 1: Highly Conserved Domains of the p53 Protein
(Adapted from Oncogene: 5: p. 949, 1990).

p53 Domains	Amino Acid Residues
I	13-19
II	117-142
III	171-181
IV	236-258
V	270-286

Missense mutations in p53 protein of transformed cells are clustered in highly conserved domains (II-V) of the protein, indicating these regions are of functional importance.

squamous cell carcinoma (101). An exception is lung carcinomas (55), where a mutation at 175 has not yet been observed.

In addition to the point mutation within the p53 tumor suppressor gene in a variety of sporadic tumors in man, p53 germline mutations have been identified in a familial form of cancer known as Li-Fraumeni syndrome (86, 87); a rare autosomal dominant syndrome. The Li-Fraumeni disorder is characterized by diverse neoplasms at multiple sites, including breast carcinoma, adrenocortical carcinoma, soft tissue sarcoma, brain tumors, osteosarcoma, leukemia, and possibly other tumors. Tumors develop in the family members at unusually early ages, and multiple primary tumors are frequent. Members in these families contain one mutant p53 allele and one wild type allele. The wild-type allele present in the normal tissue from these individuals is generally deleted in the tumor, while the mutant allele is retained. It is therefore thought that Li-Fraumeni syndrome patients are predisposed to cancer because one p53 allele is inactivated in the germline and only the remaining allele needs to be altered by somatic mutation (86, 87).

4.2 Formation of Protein Complexes with Viral Oncoproteins

Complex formation of p53 with viral oncoproteins are highly pertinent to tumor virus-mediated oncoproteins (102). It has been demonstrated that SV40 large T antigen (25) (section; 3.2), adenovirus E1B protein (80), papillomavirus E6 protein (82), hepatitis Bx antigen (HBxAg) (103), Epstein-Barr nuclear antigen-5 (EBNA-5) (104), and Epstein-Barr virus (EBV) immediate-early protein (BZLF1) (105), are capable of binding with p53. While the formation of viral protein complexes generally stabilize the p53 protein, other

possibilities exit the binding of p53 to papillomavirus E6 protein results in accelerated p53 degradation (102, 106, 107).

4.3 Binding to Cellular Gene Products (e.g. mdm-2 gene)

Besides viral oncogenes, p53 protein has also been implicated in the regulation of transcription of many other cellular endogenous genes through its interactions with other cellular proteins. For example, a cellular transcription factor murine double minute 2 (mdm-2), binds to wild type p53 protein and inhibits its transcriptional activity. As discussed earlier (section; 3.4.4), wild type p53 protein induces the expression of the mdm-2 gene product, which contains specific DNA binding sequence (77, 78, 79). In tumor cells overexpressed oncoprotein mdm-2 has been shown to increases the tumorgenic potential of the cells (108). However, over expression of wild type p53 in cells lead to the suppression of cell proliferation (30, 31, 32, 33, 61). Thus it appears that p53 and mdm-2 proteins play a reciprocal roles in regulating each other depending upon their levels or other possible variables such as different spliced forms of mdm-2 (78).

4.4 Dislocation of p53 between the cell compartments

In one third of human breast cancers with wild type p53, the p53 protein failed to translocate into the nucleus (85). Because p53 is a nuclear regulatory transcription factor, only the p53 protein that is transferred into the nucleus can act as a negative regulator of the cell proliferation (85). While the mechanism by which the wild type p53 could be retained in the cytoplasm is not known, an interesting hypothesis has been derived from the fact that one of the nuclear localization signals is located between amino acid residues 316 and 321 (85).

It is therefore possible that a growth regulatory signal could alter p53 protein possibly by phosphorylation or dephosphorylation and exclude it from the nucleus (85).

5.0 p53 Mutations Related to Cancer Etiology and Pathogenesis

Recent laboratory data suggest that some carcinogens also cause point mutation in the p53 gene. For example, exposure to one common carcinogen, ultraviolet (UV) light (109, 110), is correlated with transition mutations at dipyrimidine sites (i.e., CC to TT double base change mutation), or mutations resulting from G:C to T:A transversion occur commonly in hepatocellular carcinomas, as a result of exposure to specific carcinogen (111, 112). This mutation led to a serine substitution at residue 249 of the p53 gene in hepatocellular carcinoma (111, 112). Lastly, exposure to cigarette smoke is correlated with G:C to T:A transversions in lung, esophageal, and head and neck carcinomas (113, 114).

In addition to the exogenous mutagenic factors that lead to the formation of altered p53 protein, endogenous mutagenesis can have the same effect. For example, the high frequency of C to T transitions at CpG dinucleotides in colon carcinomas is consistent with the endogenous mutational mechanism due to deamination of 5-methylcytosine residue found at CpG dinucleotides in mammalian genome (115). Thus analysis of the p53 mutations may provide clues to the etiology and pathogenesis of human cancers.

6.0. p53 as an Independent Tumor Marker in Cancer Progression and Prognosis

A number of clinical studies have reported that mutated forms of p53 are an independent predictive factor of an unfavorable prognosis (68). Although a p53 mutation can occur at the earliest clinically detectable stages of the neoplastic process in some types of

cancer (116, 117), the fraction of tumors with an altered p53 gene is typically higher in late stage tumors (118, 119). The gene product from the aberrant form of the p53, are correlated with more aggressive tumors, early metastasis, and lower survival rates (68, 120). Nuclear accumulation of the mutated p53 has been linked to poor prognosis in breast cancer (116, 117, 120, 121, 122, 123) and some other tumor types (88, 118, 124). In addition, cytoplasmic mutant p53 accumulation has recently found to be an independent prognostic marker for colorectal (125, 126) and breast cancers (127). Thus p53 accumulations appear to be a valuable prognostic marker for certain cancers. The detection of altered p53 in these tumors, may provide additional prognostic information thus serves as an indicator of high risk patients for whom more aggressive therapy is required.

7.0 p53 as a Potential Target for Cancer Treatment

As discussed earlier (sec;3.2), wild type p53 acts as an effective tumor suppressor protein in cells. Inactivation of normal p53 is associated with uncontrolled cell growth (32,33) and development of large number of human tumors (60, 83, 84). Determination of the type and number of mutations in p53 and other cancer related genes in tissues from clinically healthy individuals may allow the identification of those at increased cancer risk and their consequent protection by preventive measures (68). Evaluation of p53 as a prognostic marker for certain cancers also eventually may permit the design of pharmacologic drug that can block uncontrolled cell growth at the site of the defect and lead to more rational and effective treatment for cancer (68).

8.0 The History of Breast Cancer

Probably no disease affecting mankind in the last few years has caused more emotion, confusion, or statistical evaluation than breast cancer. Much of the confusion comes from a lack of knowledge of the disease and the basis for treatment (128). In the first three-quarters, of this century knowledge about breast cancer has been acquired from a combination of epidemiological studies, clinical observations, and practical experience with several types of treatment (129).

The precise description of the earliest stages of human breast carcinoma is currently unavailable. But it has been calculated (130) that in most cases, starting with the first cell which becomes malignant, the tumor mass grows by successive doubling. It takes up to 7-8 years to reach the spherical volume of 1 cm in diameter.

8.1 Epidemiological Studies and Etiology Factors

Breast cancer is the most common cause of death from cancer for women in many countries. Mortality figures vary, ranging from 25-35 per 100,000 in great Britain, Denmark, United States and Canada, to 2-5 per 100,000 in Japan, Mexico, and Venezuela. Breast cancer also affects males, but the incidence is about 1% of the rate of females (130).

Familial history of breast cancer is important. It has been observed that female relatives of women (mother, daughters, and especially sisters) with breast cancer are subject to greater risk than the general population (130). The highest risk occurs in women whose mothers had bilateral breast cancer prior to menopause (130). These women have a risk factor nine times the average. Over 50% of these women will get breast cancer (130).

In 1915 T.H.C. Stevenson drew attention to the fact that after age of 45, the mortality rate for breast cancer was markedly higher for single than for married women (131). This confirmed several observations made in the seventeenth and eighteenth century, when it was noticed that relatively large number of nuns were breast cancer patients (3).

In 1926 Elizabeth Lane-Clypon compared a series of breast cancer patients with a group of healthy women (132). Her conclusion was that a significant number of breast cancer patients had married late, were of a lower parity, had breast fed their babies less often, and on average entered menopause at a later age. She also hypothesized familial relationships associated with the condition. Her first report in which she presented a retrospective study on the result of operative treatment will be discussed latter.

Subsequently, in 1929 Clemens Von Pirquet reported a bi-modal age distribution of patients with breast cancer (129). One peak represented the patients who were 45 to 49 years of age at the time of diagnosis. The second peak was at about 65 years. This observation has since been confirmed by several reports in Europe and in the United States.

Furthermore, in 1960 and 1964 F.de Waad and his associates also found that ovarian estrogen and alternatively, adrenocortical estrogen disturbance may play an important part in the formation of breast cancer (133). He divided breast cancer population into two groups on the basis of the presence or absence of certain signs, such as obesity, hypertension, and decreased glucose tolerance. These signs were suggestive of adrenal estrogen production. The conclusion was that the second statistical age peak (65 years in previous study) was made up of patients showing signs of imbalance in adrenal estrogen. In addition, they found that a heredity tendency existed in this group.

8.2 Normal Breast Anatomy

The normal anatomy of the breast has been studied in detail and has been shown that each breast consist of numerous lobes (134). These lobes are formed at sixth week of embryonic life from the epithelial cells (135). They are separated by layers of connective tissue and surrounded by abundant adipose tissue (135, 136). Each lobe is networked with lactiferous duct and lined by columnar epithelium (135). Beneath the areola each of the ducts has a local dilation called the lactiferous sinus. Furthermore, each lobe is subdivided into lobules. The smallest lobules consist of a cluster of rounded alveoli, which opens into the smallest branches of the lactiferous ducts. These ducts unit to form larger ducts and end in a single canal (136) (Fig. 5). The epithelium of the breast differs according to the state of activity of the organ (136). In the gland of a woman who is not pregnant, the alveoli are very small and solid, and are filled with a mass of granular polyhedral cells (136). During pregnancy the alveoli enlarge, and the cells undergo rapid multiplication. At the commencement of lactation, the cells in the center of alveolus undergo fatty degeneration. The peripheral cells of the alveolus remain, and forms a single layer of granular, short columnar cells with spherical nuclei, lining the basement membrane (136).

It is generally agreed that histologically and ultrastructurally, the lining of the normal nonlactating ducts (136, 137, 138) is composed of two fundamental types of cells; 1) the epithelial cells forming the inner luminal layer and 2) myoepithelial cells that are arranged between epithelial cells and basal lamina. In addition to these typical category of cells, are the indeterminate cells. These are believed to represent either a progenitor from which epithelial and myoepithelial cells can originate independently, or a transition of state between the two.

The epithelial cells are columnar with the long axis perpendicular to the lumen. The nuclei of these cells are mostly oval with occasional indentations and have evenly distributed

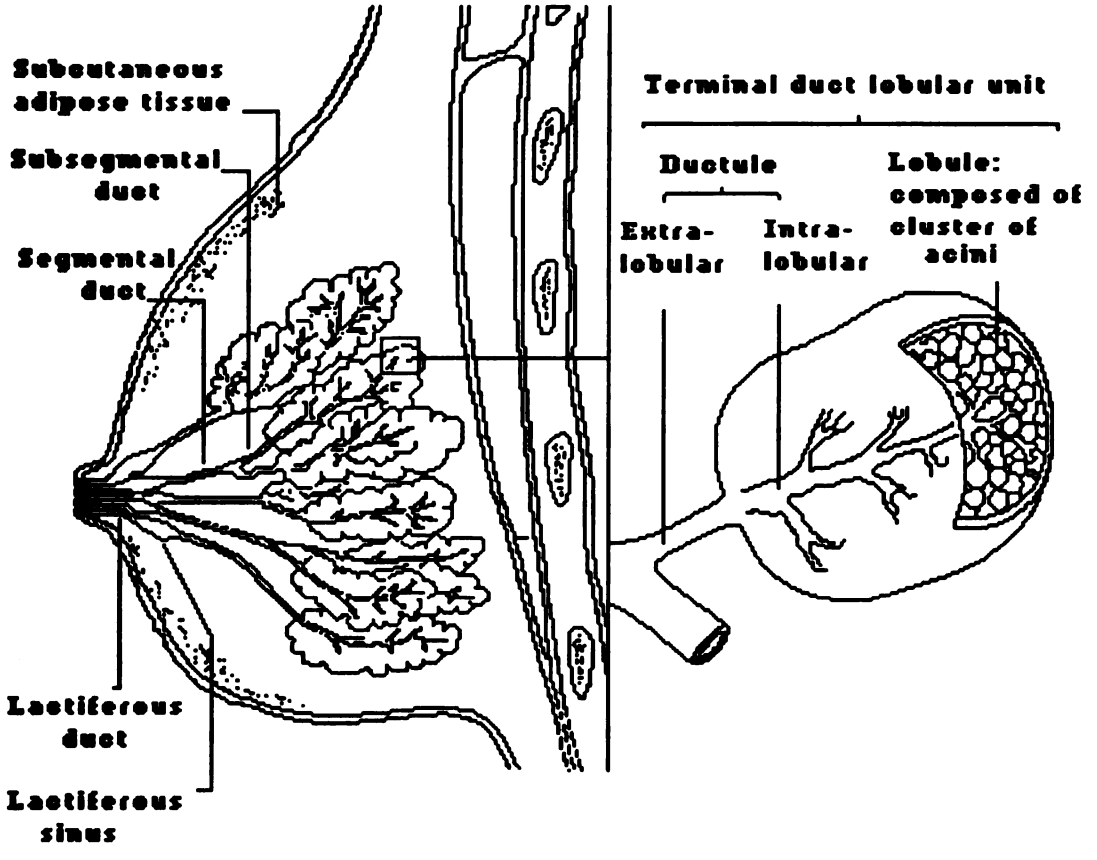

Figure 5: The Anatomy of the Breast
(Adapted from Clinical Oncology: p. 1652, Edt: Abeloff A, et al,1995)
The anatomy of the breast, demonstrating the organization of the element comprising the terminal duct lobular unit.

chromatin. The nucleoli are frequent and large in size. The endoplasmic reticulum is rough-surfaced and golgi complex shows the usual parallel array of cisternae. Mitochondria are ordinarily few and irregularly distributed.

Most myoepithelial cells are flat and have numerous fine filaments in their cytoplasm. The other cytoplasmic organelles are scanty and occupy a limited portion of the cell. Continuous with the intercellular spaces, is an area between epithelial and myoepithelial cells, called the lamina lucida which is frequently traversed by fine filamentous densities. The basal lamina follows the contour of ducts and ductules as a single layered structure. Beneath it there is a layer of connective tissue fibers that varies in thickness and is made up of collagen fibers among which are a few bundles of elastic fibers. Peripheral to the connective tissue fibers is a layer of fibroblasts that seems to constitute a boundary between the structures described and the remainder of the stroma. Examining these structures and their relationship to each other shows that ducts and ductules are enclosed by a continuous envelope representing a complex structure whose components are contributed in part by the epithelium and in part by the stroma (139). Stroma consists of plasma membranes of epithelial and myoepithelial cells, intercellular spaces, lamina lucida, basal lamina, layer of connective tissue fibers, and fibroblast. The change in morphology associated with mammary dysplasia is the result of a functional interplay affecting, in varying degrees, both epithelial and stromal structures.

8.3 Clinical Overview of Breast Cancer

The majority of breast cancers arise from the epithelium of the lobules and ducts of the gland (130). Sarcomas of the breast are rare. Both lobular and ductal carcinomas may

be non-invasive (in situ) or invasive (130).

Gallagher and Martin (1968-69) in a series of breast tumors with less than 5.0 cm in diameter, showed that considerable alterations in the duct epithelium and supporting connective tissue were present (128, 140). Their hypothesis was that there is a continuous development from the normal mammary epithelium to hyperplasia then to noninvasive intraductal disease and finally to invasive carcinoma (140). Based on their observations the earliest change in the duct was a thickening of the epithelial layer due to an increase in the number of cells present. This could occur at irregular intervals along the duct with patches of normal duct membrane intervening. As the changes progressed, the duct lining become several cells deep accompanied by varying degrees of disarrangement and anaplasia (128). These disarrangements in the duct lining finally range from duct hyperplasia to intraductal non-invasive carcinoma more commonly known as ductal carcinoma in situ (DCIS) (128, 140). Moreover, the changes in the duct epithelium could be seen in a single or multiple foci.

Alterations in the basal laminae, such as gaps may lead to the progression of intraductal carcinoma to an invasive breast cancer (138, 141, 142). The presence of these gaps appear to initiate the invasive phase of the disease by helping groups of malignant cells protrude into the stroma without losing continuity with the cells inside the duct. The size of the gaps and associated cell protrusions may be limited and present only in a few sections. Under these conditions the foci of invasion is too small as to be seen by light microscopy. Thus the lesions of intraductal carcinomas may be markers for a breast likely to develop to an invasive carcinoma (140).

Once the invasive carcinoma have been established, spread takes place via lymphatic and the blood stream (130). The lymph nodes act as the main filter of the cells that have

escaped from the primary tumor. The tumor cells, once past the axillary filters, invade the supraclavicular lymph nodes and then enter venous circulation. Through circulation metastasis occur in other body organs such as the lung, the liver, and the brain (130).

Another type of non-invasive breast carcinomas is the lobular carcinoma in situ (130). This form of breast cancer is probably more frequent and may remain silent for years. In addition, it has been shown that in about 35% of the cases lobular carcinoma may eventually become invasive (130). The invasion is as likely to occur in the opposite breast or elsewhere in the same breast.

Another major lesion of the breast, which includes an in situ component, is Paget's disease of the nipple (130). This type spreads very slowly in the epidermis of the nipple, the areola, and surrounding skin. At a latter stage, the tumor become invasive and develops as a common mammary carcinoma (130).

8.3.1 Detection and Diagnosis of Breast Cancer

Since early diagnosis of breast cancer is directly related to prognosis, attempts to detect small tumors in asymptomatic women deserve special attention. About 80% of breast tumors are first noted by the patients themselves and less are often discovered during general clinical examination or by mammography (130). Tumors are rarely detectable by palpation if they are less than 5 mm diameter. The mammogram can pick up most lesions before they reach a palpable size (1 cm in diameter) and thus provide for earlier detection (130). Once detected, tumors are biopsied. This procedure initially uses a fine needle to remove fluid or a small amount of tissue from the breast lump. This is important in discriminating between lumps which are fluid-filled cyst or a solid mass. If a solid mass, part or all of a tumor is taken

out for histological examination which will classify the tumor by type and stage of development (130).

8.3.2 Types of Treatment of Breast Cancer

Different types of therapy have contributed to the present knowledge and to the practical approach to the breast cancer. Below is a brief discussion of the development of surgery, radiation therapy, hormonal treatment, and chemotherapy in the present century (129). The use of surgery has been well established indicating that the postoperative prognosis after a complete operation is related to the stage of the disease at the time of operation. In 1924 Lane-Claypon presented an important statistical surveys of breast cancer. Her finding was that for those patients who underwent a radical mastectomy (removing breast, supraclavicular and the internal mammary nodes alone) there was a survival of 43.2% after three years and 33.1% after five years. In addition, women who had been treated with a non-radical operation had a three year survival rate of only 29.2% (143).

Many hospitals in Europe and United States started to use postoperative irradiation (129) as an adjunct to surgery with the hope that cancer cells that may have been left behind after operation, would be killed. Thus radical mastectomy followed by X-ray became the standard treatment for breast cancer in the first half of twentieth century and is still commonly used today (129).

The modern era of endocrine therapy started with the work of Huggins in 1953 (144). He introduced surgical ovariectomy to remove the main source of endogenous estrogens. Since the adrenals appeared to be an additional source of steroid hormones not eliminated by removal of the gonads, bilateral adrenalectomy was also instituted (144). Available

corticosteroids, were given for substitution for cases of metastic breast cancers unresponsive to ovariectomy.

Modern chemotherapy started with the introduction of alkylating agents and antimetabolites (129). In the beginning when single chemotherapeutic agent were employed, the results were disappointing. Such drugs often had undesirable side effects which reduced their value. Ever since drugs of different types have been used in succession, and chemotherapy appears to be a valuable adjuvant in the treatment of disseminated breast cancer (129). The success of all forms of therapy are dependent on early intervention and appropriate identification of invasive versus non-invasive form of tumors which the type of therapy applied.

9.0 Ductal Carcinoma in Situ (DCIS)

Ductal carcinoma in situ has become clinically important only since the advent of routine, high quality mammography (145, 146). Historically, DCIS represented only 3 to 5 percent of newly detected cases of breast carcinoma (147) and is generally referred to as tumors arising from duct epithelium that are confined within the lumen of the ducts or lobules of the breast (147).

In the last decade numerous reports have dealt with the ultrastructural of the neoplastic cells of noninvasive DCIS. Morphologically the epithelial cells (138) in DCIS is characterized with a moderate density of cytoplasm that varies from cells to cells. There is also a variable density in the distribution of the organelles. Mitochondria may show considerable variations in size, shape, and internal organization. Occasional giant mitochondria are seen, some with peculiar dense bodies of unexplained origin. Frequently,

the cells contain a large amount of glycogen distributed either diffusely or randomly throughout the cytoplasm. Cytoplasmic filaments are found in many of the neoplastic cells are thick and irregularly clustered in groups. The nuclei show variations in size and shape and nucleoli are frequent and prominent. The chromatin distribution may be uneven. The myoepithelial cells (138) are inconstantly present at the periphery of ducts harboring an intraductal carcinoma. Even though they are commonly flattened out and somewhat distorted by the increasing size of the ducts, they maintain their fundamental ultrastructural characteristics and can be distinguished from the adjacent neoplastic cells. The intercellular spaces vary from the usual width to greatly dilated. In DCIS most of the ducts are surrounded by basal laminae that are straight and vary from irregularly thickened to markedly attenuated (138). The continuity of the basal laminae is occasionally interrupted by gaps of varying length (138, 141, 142). Where these gaps occur, a tumor cell or small groups of cells protrude through the gaps into the stroma without losing continuity with the cells inside the ducts initiating the invasive phase.

Ductal carcinoma in situ, also known as intraductal carcinoma in situ or noninvasive ductal carcinoma, and lobular carcinoma in situ (LCIS) (130, 148) are both characterized by a proliferation of malignant epithelial cells confined to the mammary ducts or lobules, without light microscopic evidence of invasion though the basement membrane into the surrounding stroma.

The cells that proliferate in lobular carcinoma in situ (LCIS) evolve from the epithelial component and fill the lumen so as to form solid rounded units (148). These new neoplastic cells are slightly larger than the normal cells lining the ducts and their cytoplasm is paler. As

the cells multiply, they fill and distend the normal ductal structures making up a breast lobule. Although (LCIS) involves the mammary lobules, the ducts frequently are also affected (148).

9.1 Types of DCIS

Ductal carcinoma in situ is heterogeneous in terms of its histopathologic growth pattern, cell type, extent of growth, and biologic behavior (149). A recent classification categorizes DCIS into cribriform, micropapillary, papillary, and solid forms (149). All types of DCIS may be found as "pure" or "mixed" forms (149). This classification is based on the histologic growth pattern and considers comedo necrosis to be an independent feature of DCIS rather than a specific histologic type (149). Necrosis refers to death of a cell or a group of cells within the central portion of affected ductules (149). Furthermore all forms of DCIS can have significant necrosis.

The Cribriform of DCIS is characterized with holes and oval spaces. In addition, the lumina has smooth borders and nuclei are small, dark, and of relatively uniform ovoid shape (149).

Micropapillary forms of DCIS consist of thin, elongated layers of cellular elements extending as papillae into the ductal lumina. They also exhibit bridging pattern with other papillations and have blunted tips. As is typical with micropapillary carcinoma, the nuclei are large, and in some cases exhibit prominent nucleoli. Furthermore, budding of individual papillae and aggregates of tumor cells lying free in lumens can be found (149).

Papillary forms of DCIS are characterized by a relatively high density of papillae. They are most often comprised of delicate fibrovascular stalks supporting epithelial but not myoepithelial cells (149).

Finally, the solid type is represented by luminal obliteration of involved ducts by tumor cells. Ducts are also distended by uniform cellular elements. Histologically, it appears to represent an advanced form of clinging (149, 150) pattern which is considered to be an early indicator of solid type (149). This pattern has been described as one, or several layers, of ductal epithelial cells with overtly atypical nuclei (149).

10.0 Breast Cancer and the p53 Tumor Suppressor Protein

The p53 tumor suppressor protein was first associated with human mammary neoplasia in 1982 where it was shown that 90% of patients with breast cancer, particularly those with visceral metastases, had circulating antibodies to human p53 protein (151). This data led to the hypothesis that " p53 is altered in amount, and type in breast tumors" (151).

In another study the accumulation of p53 in breast carcinoma was associated with point mutations within highly conserved regions of the p53 gene (96). These altered genes encode stable proteins that can be detected by standard immunohistochemical techniques which detects both wild type and mutant form of the p53 protein. Since the wild type p53 has a short half life (20-30 minutes) what is being detected by this method is considered to be the mutant form of the p53 protein. In addition, a significant association between high levels of p53 and late stages of the disease with metastatic tumor spread was found (116, 120). Results of these and other studies (121, 122, 152), clearly indicate that immunohistologic expression of p53 is associated with a clinically advanced and highly aggressive forms of breast tumors.

There have been two studies in which accumulation of p53 tumor suppressor protein in DCIS has been evaluated by immunohistochemistry methods (116, 117). In one, nuclear

p53 protein staining was observed in 16% of the 31 DCIS cases (116). In the other study, immunohistochemical expression of p53 protein is presented in approximately 25% of 143 DCIS cases and is confined almost exclusively to the comedo form of DCIS, a morphologic subtype of in situ breast carcinoma thought to be more biologically aggressive (117).

11.0 Objective of the Current Study

The role of p53 in initiation and progression of cancer is unclear. However, a number of studies have provided information indicating that it indeed plays a role by promoting proliferation in some types of tumors (50, 116, 117, 119, 125, 126). Of specific interest to this project is the possible use of p53 protein accumulation as a predictive marker of DCIS progression to invasive breast carcinoma. Currently about 5% of DCIS convert to invasive forms of breast cancers.

The objective of this study is to screen tumor samples from 100 patients diagnosed with DCIS for p53 content. These cases will be followed up to determine what percentage of the DCIS samples convert to invasive form of breast carcinoma and correlate this to the initial p53 content.

Similar studies have been performed in other tumor types. In early non-small-cell lung cancer (NSCLC) presents with localized tumors about 30% of the time (124). Successful surgical management with long term disease control is restricted to this early stage of the disease. p53 point mutations are found in approximately 45% of NSCLC (124). It has been shown that the presence of p53 gene mutation in NSCLC patients may be independently associated with a shortened survival. In addition, p53 gene mutation and thus accumulation of the mutated p53 protein are by far the most common molecular changes detected in

NSCLC and this correlates with the spread of tumor into mediastinal and hilar lymph nodes (124). Since the latter are well established prognostic factors for NSCLC, p53 alterations may also be a useful predictor of tumor aggressiveness (124).

In low grade brain tumors only one p53 allele has been shown to be mutated (118). Whereas, both alleles are mutated in high grade advanced form of the brain tumors. Moreover, it has been suggested that mutation of p53 in brain tumors leads to a selective growth advantage in vivo that seems to be a critical step in transformation from low grade to high grade tumors (118).

It appears that p53 gene mutation, which produces mutated proteins, in squamous cell carcinoma of the head and neck occur in low frequency in noninvasive lesions and the frequency increases as the invasive carcinomas progress (153). The significant frequency of p53 mutations in early lesions of head and neck squamous cell carcinoma has been used to predict the rate of progression (153).

It has been found that p53 mutations were associated with poor prognostic factors, low response rates to chemotherapy, and shortened survival in three types of hematologic malignancies including acute myeloid leukemia (AML), myelodysplastic syndromes (MDS), and chronic lymphocytic leukemia (CLL) (154). Unexpectedly, mutations were less frequent in patients with Burkitt's lymphoma (BL) and Burkitt's acute lymphoblastic leukemia (ALL). The difference between these two cases has no obvious explanation (154).

This study will qualitatively evaluate p53 tumor suppressor protein expression in breast tissue samples with ductal carcinoma in situ (DCIS). There are many different methods currently used for the detection of p53 alterations. From the clinical stand point, p53 immunohistochemistry appears to be the most practical and useful method for the qualitative

evaluation of p53 alterations in precancer lesions and carcinomas (68). This technique allows precise localization and identification of the cells that exhibit p53 alterations (68). Presence of missense mutation/s in the p53 gene structure, lead to the prolong half life of the protein (from 6 to 20 minutes for normal to up to 6 hours for transformed cells) (60). The increase in half life is associated with stabilization of the protein. As discussed earlier (Sec.4.0) many mutations in p53 gene are correlated not only with an increase in the half life but also with a lack of function (60, 83, 84).

Many monoclonal antibodies have been developed to epitopes on both wild type and mutant p53 species (123, 155). The use of these monoclonal antibodies for immunohistochemical detection and localization of the p53 protein has been widely applied in both clinical and research laboratories. Depending on which monoclonal antibody is used different aspects of p53 can be evaluated (155). The majority of mutations to p53 are associated with amino acid substitution in the middle section and carboxy terminus of the protein (39). Most of these mutations are associated with intracellular accumulation of this protein (Sec. 4.1 & 4.4). Immunohistochemical analysis will only detect those mutations associated with accumulation (55, 96). In normal cells, p53 is not detectable by immunohistochemical evaluation (155). However, increases may occur in the absence of mutation due to viral infection which increases the concentration of the binding proteins (Sec. 4.2). In most instances these mutations are associated with changes in epitope recognition in the middle section and carboxy terminus. Thus the application of monoclonal antibody to these sections of the protein in random samples may not detect p53 (155, 156). However, the amino terminus appears to be conserved in most types of p53. Thus,

immunohistochemical evaluation of p53 using monoclonal antibody to the amino terminus provides the best method for evaluation of this protein in tissue samples (155).

For this study monoclonal antibody PAb1801-Ab2 was chosen. This antibody is a mouse monoclonal antibody generated by immunizing Balb/c mice with p53 protein and fusing with NS-1 mouse myeloma cells (157). The p53 Ab-2 reacts with a denatured stable determinant of p53 (156). This antibody also reacts equally on frozen and formalin-fixed, paraffin embedded sections (156). Furthermore, the p53 Ab-2 recognizes the p53 of human cellular origin only (158) and reacts with an epitope located near the amino end of all known forms of p53 (158). Thus allowing for the detection of accumulation of most forms of this protein (158). The p53 antigen is not affected by normal processing. However, it has been recently reported that long term storage of paraffin-embedded specimens at room temperature may decrease the binding (159).

II. OBJECTIVES

1. Establish a valid immunohistochemical assay for the detection of p53 in breast tissues.
2. Evaluate 100 cases of DCIS for the presence of p53 accumulation.
3. Correlate p53 immunohistochemical staining with the subtypes of DCIS.
4. Determine if a correlation exists between p53 immunohistochemical staining and nuclear grade.
5. Generate a data base for the long term follow up of these patients which will complete the project.

III. MATERIALS AND METHODS

1.0 Source of Tissue

Samples were acquired from women whom had had surgical removal of breast tumor/lumps for pathologic evaluation. These samples were processed by routine procedures after immediate immersion in a 10% formal saline. Preserved samples were then subjected to processing; dehydration and embedding in paraffin blocks. Five micron (5μ) sections were cut and fixed on slides pretreated with poly-L-lysine to prevent detachment of tissues from the glass slides. Sections were first evaluated microscopically, by a pathologist, after standard hematoxylin and eosin staining (160). Tissue sections on glass slides were forwarded to this laboratory on specimens which had been classified as DCIS by initial pathologic evaluation. Along with these samples data on tumor histology and patients characteristics were obtained from the pathology reports.

2.0 Immunohistochemistry Overview

The immunohistochemistry technique used was an enzyme linked method. Deparaffinized sections were first incubated with monoclonal antibody (Ab 1801,Ab-2,Oncogenescience) (157). After binding non bound antibody was removed by washing. Secondary biotinylated anti-mouse was added. Non-bound secondary antibody was removed by washing and followed by incubation with Avidin-Biotinylated horseradish

peroxidase micro molecular complex (ABC). After binding has occurred non-bound ABC was removed by washing. Diaminobenzidine (DAB) substrate was added and product was visualized under light microscopy as a brown precipitate (161) (Fig. 6).

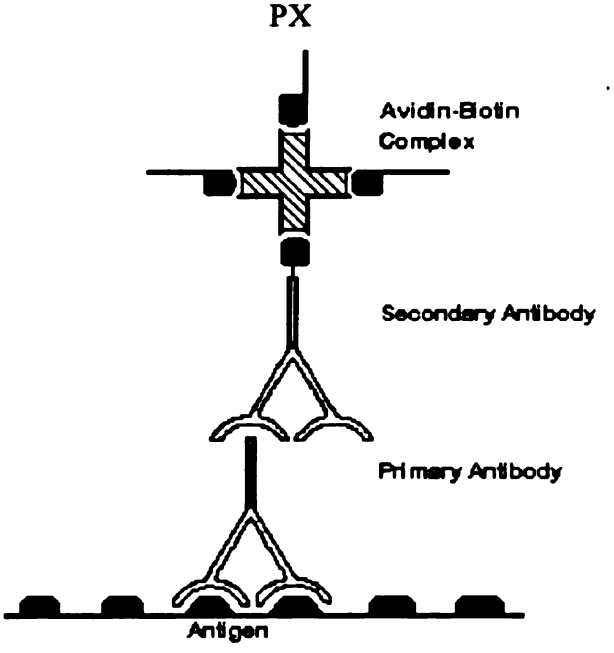

Figure 6: Avidin-Biotin immunoenzymatic technique
(Adapted from Arch Pathol Lab Med: 107: p. 108, 1993)
This is a schematic drawing of immunohistochemistry method used. PX indicates Peroxidase. Square indicates Biotin. Shaded open + indicates Avidin. Solid semi circle indicates Antigen. Formalin-fixed and paraffin -embedded tissue sections were first incubated with a primary monoclonal antibody to p53 antigen. Bound antibody is then visualized by incubation with a biotinylated secondary antibody which was followed by incubation with ABC horseradish peroxidase and eventually substrate.

3.0 Immunohistochemical Procedure

Formalin-fixed, paraffin-embedded slides(5μ) were cleared in xylene (see Appendix) (3x for 5 mins each) and absolute alcohol (2x for 2 mins each). Slides were treated with 0.3% hydrogen peroxide-methanol (10mins) to remove endogenous peroxidase activity. Following a distilled H_2O (D-H_2o) rinse, the slides were pretreated with a 1 mg/ml solution of trypsin (see Appendix) for 5 minutes. Slides were treated for 30 minutes with phosphate buffer solution (PBS,PH=7.4) (see Appendix) containing 0.15% bovine serum albumin. To reduce background staining, slides were incubated in normal horse serum (5% in PBS) for 30 minutes. The normal horse serum was allowed to run off, and the slides were incubated overnight with the primary antibody (see Appendix), from Oncogene Science, at 4^0C in a moist chamber (162). The monoclonal p53 (Ab-2) recognizes both wild-type and mutant p53 forms. A dilution of 1:200 in PBS (0.5 ug/ml) was used for this antibody as well as the negative control antibody. The negative control antibody was mouse myeloma IgG(see Appendix) . After washing with PBS for 10 minutes at room temperature (RT),the slides were incubated with the secondary biotinylated anti-mouse IgG antibody (Vector Laboratories, BA-2000) for 30 minutes at RT. A dilution of 1:250 in PBS (0.6 ug/ml) for secondary antibody was used. Following PBS wash, the slides were incubated with the avidin-biotin-peroxidase reagent, (Vector Elite) for 60 minutes. After PBS wash for 10 minutes the antigen-antibody complex was visualized using 0.05% solution of diaminobenzidine(DAB)tetrahydrochloride tablets in tris solution (pH=7.6) for 8 minutes (see Appendix). The slides were counterstained with Mayer's hematoxylin for 5 minutes, blued with Scotts tap water for another 5 minutes, dehydrated, and coverslipped (162).

4.0 Positive and Negative Controls

For each assay, formalin-fixed, paraffin-embedded tissue section of human colon cancer was used as positive control to ensure interassay consistency. Colon carcinoma tissue sections are known to have mutations of the p53 protein (60, 95). For the negative control PBS (without antibody) and (purified mouse myeloma IgG,1:200) were used instead of the primary antibody. Benign breast tissues were also studied for p53 immunoreactivity to role out detection of the wild-type p53 by the primary antibody.

5.0 Grading System

The grading system was based on binding of primary anti p53 antibody and subsequent evaluation of horse radish peroxidase activity which generates a brown precipitate. The presence of brown precipitated in the nucleus of tumor cells was scored semiquantitatively for the proportion of the cells stained that stained darkly within the tumor (163). This staining was given scores from 0 to 3; (0, no staining, 1-30% =1+ , 31-60%=2+, and >60% =3+).

6.0 Tumor Grading

Each specimen was classified based on histologic presentation. Tumors were classified according to the predominant cellular architectural pattern: solid, cribriform, micropapillary, and papillary types. This classification was only applied when such a pattern corresponded to more than 75% of the tumor. When no pattern predominated, the tumor was classified as mixed.

Nuclear grade was defined as grades 1-3 in order of increasing pleomorphism with regard to the invasive carcinoma (164). Pleomorphism of the nucleus and the presence of nucleoli wee used as the criteria for grading. A nuclear grade was assigned in a semiquantitative manner by comparative evaluation of nuclei in adjacent normal breast duct as small (1-2X size), intermediate size (3-4x size), or large (5x size or more). Grade 1 nuclei was characterized by monomorphism, small or intermediate size, and inconspicuous nucleoli defined. Grade 2 nucleus showed moderate pleomorphism, small or intermediate size, and was characterized by evident nucleoli. Grades 3 nuclei showed marked pleomorphism, intermediate or large in size, and frequently contained multiple and conspicuous nucleoli (164).

IV. RESULTS

1.0 Dilution of the monoclonal antibody Pb1801, Ab-2

The dilution of IgG monoclonal antibody to human p53 protein (Pb1801,Ab-2) used in the immunohistochemical procedure was a 1/200 dilution of the stock protein produced by the manufacture. This dilution, of the primary antibody, produce the highest degree of sensitivity and specificity, as determined by experiments using checkerboard analysis. When 1:50 dilution of antibody was used, cytoplasmic staining in negative normal breast tissue sections was noticed which made the evaluation of nuclear reactivity difficult to interpret. Positive results were only considered in conjunction with nuclear staining (Table 2).

Table 2: Checkerboard Analysis for Determination of Primary Antibody Concentration

Primary Ab Dilution	1:50	1:150	1:200	1:250	1:300
Positive breast tissue	3+	1+	2+	0.00	0.00
Negative breast tissue	cytoplasmic	cytoplasmic	0.00	0.00	0.00

This table contains the results of the titration experiment used to determine the optimal titer of primary antibody to p53 antigen. A 1/200 dilution of primary antibody produced highest degree of sensitivity and specificity. A 1/50 dilution of this antibody produced cytoplasmic staining in negative normal breast tissue sections.

2.0 Positive and negative control

Sections of colon carcinoma were used as the positive control (60, 95). These

sections showed intense p53 staining (Fig 7). Two negative controls were also done with each assay. One was section of colon carcinoma which had not been treated with primary antibody to the p53. Instead, had been treated with mouse myeloma IgG then secondary anti mouse IgG and ABC reagent This control was always negative, indicating no nonspecific binding of the secondary antibody (Fig 8). A second negative control was benign breast tissue section which had been treated with standard staining procedure. This control also was always negative, indicating no nonspecific binding of primary antibody (Fig 9).

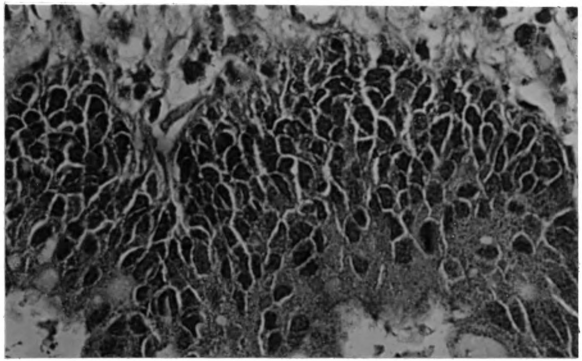

Figure 7: p53 Immunopositivity in Colon Tissue Section
This section of tissue was stained using the avidin-biotin immunoperoxidase staining methodology. The primary antibody (Pb1801, Ab-2) dilution was 1:200. There is a deposition of brown pigment indicating a reactive positive result. Magnification 100x oil.

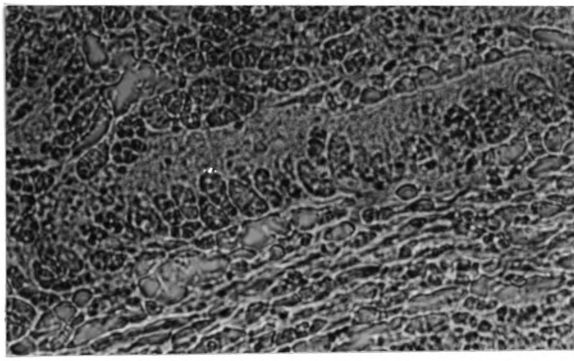

Figure 8: p53 Immunonegativity in Colon Tissue Section
Mouse Myeloma IgG was used instead of the Primary Antibody (Pb1801, Ab-2). There was no deposition of brown pigment indicating a non-reactive result. Magnification 100x oil.

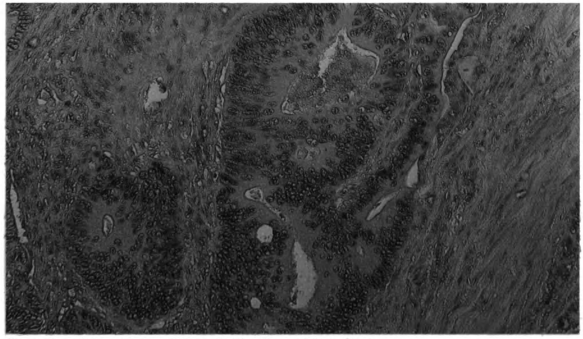

Figure 9: p53 Immunonegativity in Benign Breast Tissue Section
This section of tissue was stained using the avidin-biotin immunoperoxidase staining methodology. The primary antibody (Pb1801, Ab-2) dilution was 1:200. There is no deposition of brown pigment indicating a non-reactive result. Magnification 40x.

3.0 Histologic Classification, Nuclear Grade, and p53 Content

A total of a 15 of 100 DCIS cases exhibited nuclear immunostaining for p53. As previously mentioned only nuclear staining was considered a positive. Analysis of DCIS with regard to frequency of the predominant histologic pattern showed that mixed type was the most common pattern occurring in 54% of the cases. This was followed by solid pattern occurring in 23%, cribriform occurring in 13%, papillary occurring in 8%, and micropapillary occurring in 2% of the cases. The majority of positive results were found in the mixed type of DCIS. Approximately 23% of all mixed forms of DCIS were p53 positive. In mixed lesions, a solid pattern was the predominant, being followed by cribriform pattern. In addition comedo necrosis was present in all p53 positive DCIS cases.

Nuclear grade 3 was seen in 1 (13%) of positive DCIS cases. Nuclear grade 2 was seen in 4 (27%), and nuclear grade 1 was seen in 10 (67%) of the positive DCIS cases (Table 3&4).

Table 3: Histologic Types of DCIS & p53 Immunostaining

Histologic Pattern	p53 (+) Cases	p53(-) Cases
Total	15	85
Mixed	10	44
Cribriform	2	11
Papillary	1	7
Solid	2	21
Micropapillary	0	2

This table displays the results of the project. A total of a 15 of 100 DCIS were p53 immunopositive. Of all DCIS tissue samples Predominant histologic pattern was mixed type(54%). Of all p53 immunopositive cases of DCIS 67% had the mixed histologic pattern.

Table 4: Nuclear Grading of Immunopositive p53 DCIS Samples

Histologic Pattern	p53 Grade	Nuclear Grade
Mixed:		
Case #1	3+	1
Case #2	1+	2
Case #3	1+	3
Case #4	1+	1
Case #5	1+	1
Case #6	1+	1
Case #7	1+	1
Case #8	1+	1
Case #9	1+	1
Case #10	1+	1
Solid:		
Case #1	2+	1
Case #2	1+	2
Cribriform:		
Case #1	3+	1
Case #2	2+	2
Papillary:		
Case #1	1+	2

This is a table showing distribution of p53 immunopositive samples by histologic pattern, p53 grade, and nuclear grade. Nuclear grade 1 is characterized by monomorphism, small size, and inconspicuous nucleoli. Nuclear grade 2 nuclei is characterized by moderate pleomorphism, intermediate size, & evident nucleoli. Nuclear grade 3 nuclei is characterized by marked pleomorphism, large size, & multiple nucleoli.
Nuclear grade 3 was noticed in one mixed pattern of the positive cases.
Nuclear grade 2 was noticed in four of the positive cases; one in mixed, one in solid, One in cribriform, and one in papillary patterns. The rest of the cases showed nuclear grade of 1.

V. DISCUSSION

Despite the fact that a number of molecular and cellular markers have been proposed as prognostic indicators for breast cancer, p53 tumor suppressor protein appears to be valuable prognostic marker for many types of carcinomas including breast tumors. Previous studies have found a concordance of monoclonal antibody pb1801 immunohistochemical nuclear pp53 protein accumulation and point mutations within highly conserved regions of the p53 gene. These altered genes encode for p53 proteins with a higher level of stability (27, 55, 96) that allows for accumulation of the protein and can be detected by standard immunohistochemical techniques. From a clinical standpoint (68) this technique is a useful and practical method in detection of mutant forms of the p53 protein. Since the wild type p53 protein has an extremely short half life and thus intercellular concentration, what is being detected by this method is the mutant form of the p53 protein.

The role of p53 protein in initiation and progression of cancer is unclear. However, studies provided information indicating a relationship between high level of p53 and clinically advanced, highly aggressive forms of the breast tumors (2, 116, 117, 120). In breast carcinoma, as it has been previously proposed (2), p53 mutation may occur relatively early at the development of ductal carcinoma in situ (DCIS). These cells with mutated p53 then may have a growth advantage that would result in clonal expansion of the neoplastic population (2).

Ductal carcinoma in situ (DCIS) of the breast is generally referred to as tumors arising from duct epithelium that is confined within the lumen of the ducts or lobules of the breast (147). DCIS is heterogenous in terms of its histopathologic growth pattern, and behavior (147). Histologically, DCIS is classified into cribriform, micropapillary, papillary, ans solid patterns. Each of the DCIS patterns may be found as "pure" or "mixed" forms. In addition, all forms of DCIS may have an area of linear comedo necrosis with in the central portion of affected ductule (147).

Objective of this study was to screen tumor samples from 100 patient diagnosed with DCIS for p53 content. Then follow up these cases to determine what percentage of the DCIS cases convert to invasive form of breast carcinoma and correlate this to the initial p53 content. Data presented here indicates that accumulation of p53 protein , as assessed by immunohistochemistry using monoclonal antibody Pb1801, (Ab-2), occurs at low frequency (15%) in ductal carcinoma in situ of the breast (DCIS). The p53 immunostaining often was seen in mixed histologic type of the DCIS. These findings are in accordance with the result of Lennington et al (164) who provided evidence regarding the frequency of mixed patterns in DCIS. In this study, the predominant pattern seen in p53 immunopositive mixed cases was solid followed by cribriform type of DCIS. Furthermore, all p53 immunopositive DCIS specimens exhibits comedo necrosis features. This is especially true in specimens with pure cribriform and papillary types of DCIS.

The significance of nuclear grade for classifying DCIS into high and low nuclear grade of malignancy has been emphasized by Bellamy et al (165). In their series of experiments they found that conversion to the invasive form followed only in high nuclear grade DCIS, regardless of the histologic pattern. This study showed no relationship between DCIS

histologic patterns, p53 immunostaining, and nuclear grade was found. Preliminary data, related to the long term evaluation of the patient population is available on a limited basis. At present no specimen with immunonegative staining for p53 has converted to invasive form. However, of the fifteen immunopositive results, six have converted to more invasive form. Interestingly, one case presented with a cribriform pattern and a nuclear grade of 2. This may be indication of clinically more aggressiveness of this type. It should be noted that most of the specimens evaluated were from samples acquired within the last four years. However, a few specimens were up to nine years old. The time line for patients which have converted to invasive form is shown in figure 10.

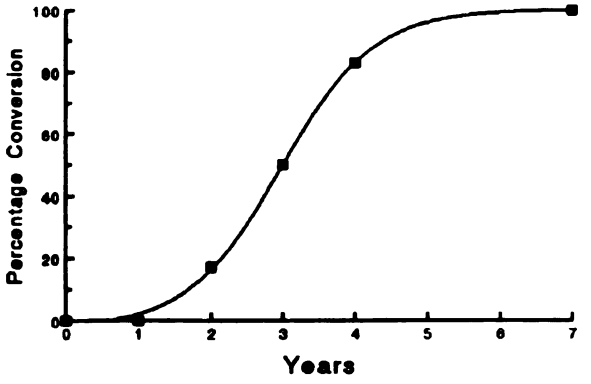

Figure 10: The Results of % Conversion to Time in Years. Preliminary data (n=6) DCIS p53 immunopositive cases, indicates that by 7 years after evaluation all converted to the invasive form.

The purpose of this project is to establish a database for the long-term evaluation of patients which will convert to invasive carcinoma. This will require long-term evaluation of data in terms of outcome assessment. To refine this database further, studies on the

population are needed using molecular techniques such as DNA sequencing and/or probe analysis. These techniques may identify correlations between mutations and immunopositive p53 accumulations that eventually will rule out occurrence of false positive and false negative results. Using these techniques may provide a correlation between a given mutation and prognosis.

APPENDIX

APPENDIX

Reagents

1) Phosphate Buffered Saline (PBS), PH=7.4, 12mM total phosphates.

2) Analytical Xylene, (MSU Chemical Store).

3) Absolute Ethyl Alcohol, 200 proof, (MSU chemical Store).
Dehydrated Alcohol, (punctilious)

4) 0.30% H_2O_2 tincture
Composed of: 0.3 ml 30% H_2O_2, (Sigma, Cat# H 1009, St.Louis, MO, USA) in 100 ml Methanol.

5) Methyl Alcohol
Chrom AR HPLC Grade, (MSU Chemical Store).

6) Trypsin solution
Composed of: 0.1 gr Trypsin Type III from Bovine Pancreac, Activity 11,100 IU/mg protein, (Sigma, Cat# T8253, St. Louis, MO, USA) in 1 ml 12mM PBS .

7) 0.15% Albumin Solution
Composed of: 0.15 gm Albumin, Bovine, Crystallized Lyophilized, Nitrogen 14.7%, (Sigma, Cat# A4378, St. Louis, MO, USA) in 100 ml 12mM PBS.

8) 5% Normal Horse Serum (NHS)
Composed of: 10 ml Stock NHS (100%), (Gibco, Cat # 200-6050, Grand Island, NY, USA) in 90 ml 12mM PBS.

9) Mouse Monoclonal Antibody
Pab1801 p53(ab-2), (Oncogene Science, Unlondale, NY, USA).

10) Mouse IgG_1 (Myeloma) Purified, (ICN Biomedical Inc, Cat # 64-335, Costa Mesa, Ca, USA).

11) Biotinylated Anti-Mouse IgG Antibody
BA-2000, (Vector Laboratories, Burlingame, CA, USA).

12) Avidin-Biotin-Peroxidase Complex Kit(ABC), (Vector Elite, Burlingame, CA, USA)
Contains: Reagent A: Avidin DH Solution
Reagent B: Biotinylated Horseradish Peroxidase.

13) Tris Buffer, PH=7.6, 0.2M Ttis base

14) Substrate solution
Composed of: One Diaminobenzidine Tetrahydrochloride (DAB)tablet, (Sigma, Cat# D-5905, St.Louis, MO, USA).
(10 mg substrate content), dissolved in 15 ml of Tris buffered saline & 12μ of fresh 30% hydrogen peroxide that is added prior to use .

15) Mayer's Hematoxylin Solution, (Sigma, Cat# MHS-32, St.Louis, MO, USA).

16) Scott's Tap Water (bluing agent), (Sigma, Cat # 55134, St. Louis, MO, USA).

17) Histological Mounting Medium, (Fisher Scientific, Cat # SP 15-100, Livonia, MI, USA).

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