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Expression of Tumor Suppressor Genes during Breast Cancer Progression in the Rat

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Expression of Tumor Suppressor Genes During Breast Cancer Progression in the Rat

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I dedicate this work to the soul of my late uncle...

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Wafa Saqer Aldhaheri

ABSTRACT

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Breast cancer is the most common type of cancer and the leading cause of cancer deaths among women in the United Arab Emirates and all over the world. Although many factors contribute to the high incidence of breast cancer, a considerable number of cases are related to environmental factors. In this study, breast cancer was induced in female rats using a single dose, 80 mg/kg body wt, of the environmental carcinogen 7,12-dimethylbenz[a]anthracene (DMBA). The aim was to characterize some of the early cellular and molecular changes that occur during breast cancer development in the DMBA-treated rat model. Mammary gland tissues of control and DMBA-treated rats were processed for: i) routine histological examination, to define the initial histopathological changes that occur in mammary gland tissues, ii) immunohistochemical probing using anti-PCNA and anti-BRCA1 antibodies to characterize and correlate the localization of these cell cycle proteins during progression to cancer, iii) Western blotting, to analyze the alteration of p53 protein expression in pre-neoplastic and neoplastic lesions of the mammary glands, iv) polymerase chain reactions using primers specific for PCNA, BRCA1, and p53 genes followed by single stranded conformational polymorphism (SSCP) or restriction fragment length polymorphism (RFLP) assays to detect possible mutations in these genes during development of breast cancer.

Microscopic examination revealed a wide range of pre-neoplastic and neoplastic lesions in the mammary glands of DMBA-treated rats. They provided a sequence representing the multistep process of breast cancer formation. The earliest morphological change observed was moderate dilatation of the terminal ducts and accumulation of extruded dead cells in their lumens. These changes were referred to as the stage of "cell death". It was followed by stages of hyperplasia, dysplasia, and then *in situ* cribriform carcinoma. Finally, the invasive cribriform and papillary carcinomas developed. In

addition, some DMBA-treated rats developed benign tumors: lactating adenoma and squamous cell papilloma.

Immunohistochemical localization of PCNA revealed an initial down regulation during the stage of cell death followed by a gradual increase in the number of PCNA-labeled cells during the following stages of breast cancer development. Probing for BRCA1 protein suggested a gradual defect in its translocation from the cytoplasm to the nucleus during breast cancer progression. In control rats, BRCA1 was present in the nuclei of terminal duct epithelial cells. However, in the pre-neoplastic lesions, BRCA1 was localized in both the cytoplasm and nuclei of the epithelial duct cells. In all malignant lesions, BRCA1 was mainly found in the cytoplasm.

Western blotting revealed that the expression of p53 protein during breast cancer development was initially down regulated. However, with progression toward malignancy, upregulation of the mutant form of p53 was observed. These changes were associated with polymorphism in p53 gene, which was detected in exon 5 using SSCP assay. However, no polymorphisms were detected in the PCNA and BRCA1 genes.

Because some recent studies suggested that the use of multiple alkaloids may have some synergistic inhibitory influence on cancer cell growth, the effect of crude extract of *Vinca* alkaloids on the progression of breast cancer was tested in DMBA-treated rats. A daily treatment of these rats with 200 mg/kg body wt of *Vinca* alkaloids for 21 days starting 1 week after DMBA administration induced about 50% decrease in the incidence of mammary gland neoplasia. When these tumors were compared with those of DMBA-treated rats, the former were smaller in size, exhibited a smaller number of PCNA-labeled cells, and showed down regulation in the expression of both PCNA and mutant p53 proteins.

In conclusion, characterization of the early changes that occur during breast cancer development may provide some clues for better understanding of its pathogenesis and would hopefully improve modalities for its prevention and/or early detection.

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INTRODUCTION & LITERATURE REVIEW

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Breast cancer is the most frequent type of cancer and the leading cause of cancer deaths in women worldwide (Smymiotis et al., 2005). In the United Arab Emirates (UAE), breast cancer is also the most frequent cancer among national females. According to UAE National Cancer Registry in Tawam Hospital, 175 breast cancer cases out of 538 female cancer cases (32.5%) were recorded from 1998 to 2002.

It is well known that the etiology of breast cancer is multifactorial. Many hormonal and nonhormonal factors contribute to the development of breast cancer (Martin and Weber, 2000). Risk factors include both endogenous and environmental estrogens, diet and lifestyle, geographic area of residence, and age at menarche or menopause. However, in most cases there are no obvious causes of breast cancer, supporting the view that a variety of environmental carcinogens play a major role in initiation of the disease (Steinetz et al., 2006).

Another important risk factor for the development of breast cancer is the positive family history and genetic influences (Martin and Weber, 2000). The expression of some genes could be altered by mutations or amplification which therefore affect the cell cycle and the normal control process of cell proliferation (Hayes, 2000). Even though much has been revealed regarding these genes, but several questions remains to be answered regarding their expression in the commonly used animal models of breast cancer and their possible mutation.

I.1. Animal models of breast cancer

Due to complexity of the factors involved in breast cancer, many studies have been conducted to elucidate its pathogenesis in the hope of providing some clues towards its early detection, prevention and/or treatment. It is generally agreed that animal models are the most useful tools to study breast cancer. These models have demonstrated the development of several types of breast neoplasms in animals comparable to those that occur in humans. In these animals, breast cancer was induced by chemicals, radiation, viruses or genetic manipulations. Then, the pathogenesis of the disease including its initiation, promotion and progression was examined (Russo and Russo, 2000).

Several animal models have been used to study breast cancer. The rat model has been commonly used because: (i) its mammary tumors, like those of humans, lack a major viral etiology, (ii) tumors in the rat can be rapidly induced by chemicals, and (iii) the rat mammary tumors resemble those of the human. Accordingly, rat animal models have been considered as useful tools to examine preneoplastic, neoplastic and metastatic breast tissues. Furthermore, rat model can also be used to study genetic alterations that occur early during tumor development (Sukumar et al., 1995; Russo et al., 1990c).

It is well known that mammary glands are sensitive to exposure to X-rays. Based on this fact many investigators used radiation to induce mammary carcinogenesis in the rat. However, the hormonal status of the rat is very important to determine the outcome of irradiation. It has been shown that ovariectomy prevents radiation-induced mammary tumors (Russo et al., 1990c).

The xenograft model has been developed by growing breast cancer cells in nude mice and found useful to test potential inhibitors of breast cancer cell growth (Long et al., 2004). In addition, genetically engineered animals, such as transgenic and knockout mice, have been found invaluable in defining and characterizing genes involved in breast

cancer. Introducing a foreign gene or deleting a normal gene in the mice has been useful to examine the role of specific genes in mammary tumor development and also to develop strategy for drug target validation (Matulka and Wagner, 2005).

For many mammary gland cell biologists, the chemically induced breast cancer by polycyclic aromatic hydrocarbons, such as benzo(a)pyrene (B(a)P) and 7,12-dimethylbenz[a]anthracene (DMBA), has been the preferable model due to several reasons: 1) the reliability of tumor induction, 2) organ target specificity, 3) tumors developed are mostly ductal (epithelial in origin), 4) the slowly developing tumor provides the potential to examine the early scenario that occur during tumor development (Liska et al., 2000), 5) the possible mutagenic effects of DMBA in the rat can alter some oncogenes (Sukumar at al., 1995), and 6) DMBA-induced mammary tumors in rats have been considered as a good representation of those occurring in humans (Russo et al., 1990b).

I.2. The structure of rat mammary gland

In female rats, six pairs of mammary glands are aligned ventrolaterally along the mammary or milk lines. The first pair of mammary glands is located in the cervical region. The second and third pairs are found in the thoracic region, and the three remaining pairs, in the abdomino-inguinal region. Each mammary gland extends dorsolaterally in a flat subcutaneous sheet of fibroadipose tissue (Russo and Russo, 1978).

Microscopically, the mammary gland of adult virgin rats is composed of parenchyma and stroma. The latter includes a small amount of fibrous connective tissue surrounded by much adipose tissue. The parenchyma is made of a branching duct system which forms a tree-like structure. Close to the nipple, one or two major lactiferous ducts

branch into smaller ducts that again branch and finally terminate deep in the gland (Russo et al., 1990c).

The lining epithelium of the walls of the major ducts is variable. Some areas are lined by pseudostratified epithelium and others by simple columnar epithelial cells. The walls of smaller ducts and their terminal buds contain two main cell types: internal or luminal secretory cells and external or peripheral myoepithelial cells. While the luminal cells are joined by epithelial junctions to form a continuous layer, myoepithelial cells are discontinuous (Russo and Russo, 1996).

I.3. Tumors of rat mammary gland induced by DMBA

DMBA-induced tumors do not occur randomly in the six pairs of rat mammary glands. Virgin animals treated with DMBA between the ages of 45 and 55 days develop a greater number of tumors in mammary glands located in the thoracic region than glands located in the abdomino-inguinal or cervical regions. This has been explained by the asynchronism of the development of the thoracic mammary glands (Russo et al., 1990b). In these animals, the high susceptibility of the small and terminal ducts to neoplastic transformation is ascribed to the high proliferative activity of their lining cells, as determined by mitotic and DNA-labeling indices. These indices are maximal at the terminal small ducts and decrease toward the major ducts of the gland (Russo and Russo, 1987).

Mammary gland tumors induced in rats by a single dose of DMBA comprise various benign and malignant forms (Russo et al., 1990c). It has been shown that each tumor could be composed of a single histologic type or a combination of several patterns. Russo and Russo (2000) have classified rat mammary tumors into different types based on their microscopic features.

The earliest histological change observed in the mammary parenchyma after carcinogen administration is progressive increase in the intraductal cell proliferation leading to enlargement of small terminal ducts. Later, carcinoma *in situ* develops and forms micropapillae and/or cribriform patterns. In the micropapillae type of ductal carcinoma *in situ*, the lining epithelium of the dilated ducts grows inward, forming epithelial papillae with or without a thin fibrovascular core. Cells in this lesion have uniform size and shape. But they are characterized by an increased nucleus/cytoplasm ratio and prominent nucleoli. The cribriform type of ductal carcinoma *in situ* is characterized by accumulation of cells in dilated ducts with secondary lumens. Cytologically, it appears to be similar to the papillary type (Russo and Russo, 2000).

Invasive papillary and cribriform carcinomas arise from *in situ* lesions, in which they disrupt the normal architecture of the gland, and invade the surrounding structures. In case of papillary carcinoma, the main features are secondary papillary projections including pleomorphic cells and serrated lumens. Cribriform carcinoma exhibits haphazardly arranged, finger-like epithelial projections into the surrounding stroma with moderate to marked pleomorphic cells (Russo and Russo, 2000).

Another form of malignant lesions is ductal comedocarcinoma. It is characterized by intraductal growth of epithelial lining. So, the ducts appear distended and lined by a multilayered epithelium surrounding necrotic debris (Russo and Russo, 2000).

Benign tumors also develop in DMBA-treated rats. Lactating adenoma is the most common type. This tumor varies in size and is characterized by numerous glandular profiles with serrated lumens due to decapitation or supranuclear vacuolization of the lining epithelium. These glandular lumens are usually distended with much secretion inside, which may accumulate and lead to the formation of large cysts lined with flattened epithelium (Russo and Russo, 2000).

I.4. Mechanism of DMBA-induced breast cancer

It is well established that a single dose of DMBA results in a high yield of mammary tumors in rats. DMBA is most effective in the induction of tumors when administered to rats at 50–56 days of age (Sinha et al., 1983). This optimal window of susceptibility to tumors is probably due to the active proliferation of the terminal ducts during this period (around 7–8 weeks of age). Therefore, mammary glands of virgin rat are more susceptible to tumorigenesis by chemical carcinogens than mature and differentiated glands (Ariazi et al., 2005).

DMBA is a synthetic, polycyclic aromatic hydrocarbon. Following its administration, the concentration of DMBA in the whole mammary gland is 110-fold higher than that obtained from collagenase-dissociated mammary epithelial cells. Therefore, it seems that mammary fat pad serves as a reservoir for sustained release of the procarcinogen into the parenchymal tissues. This phenomenon explains the great susceptibility of the mammary epithelial cells to carcinogenesis by DMBA (Menon et al., 1987).

Radioautographic studies using [^{3}H]-DMBA have demonstrated that within the mammary epithelium the highest uptake of DMBA occurs in the nuclei of cells lining the small terminal ducts. This indicates that DMBA binds to the structures of the gland with the highest proliferative activity. Following its binding, DMBA causes up-regulation of the cellular cytosolic receptor, the aryl hydrocarbon receptor (Trombino et al., 2000). Then, aryl receptor-dependent up-regulation of cytochrome P450 occurs. Finally, DMBA gives rise to polar and phenolic metabolites as well as the active compound, 3,4-diol-1,2-oxide. The latter interacts with cellular DNA and forms different adducts that cause mutations and tumor initiation (Rundle et al., 2000).

I.5. Genes involved in breast cancer

Advances in cellular and molecular biology techniques have made it possible to clarify much of the basic cellular and molecular events leading to cancer. Many genes have been found to play a role in the development of cancer. They are classified into three major categories: tumor suppressors, oncogenes and DNA repair genes (Jorde et al., 2000). In case of breast cancer, tumor suppressor genes, such as P53 and BRCA1, are found to be frequently involved in its development (Ingvarsson, 1999).

I.5.1. P53

In humans, P53 is located on chromosome 17p (Levine et. al., 1991). The normal allele of this autosomal gene encodes a 53 kD nuclear protein (Hollstein et al., 1991), which consists of at least three domains: a transactivation domain at the NH_2 terminus; a central specific DNA binding domain; and an oligomerization domain at the COOH terminus (Zhang et al., 2000). In addition to the main function of P53 as a guardian of genome, it also appears to function in the regulation of various cell biological processes: cell cycle and growth, cell death, signal transduction, and gene expression (Hulla and Schneider, 1993).

While the wild-type p53 protein suppresses cell growth, the mutated form acts as an oncogene. Mutations in the P53 gene usually result in stabilization and accumulation of its expressed protein (Sarbia et al., 1994). Increased levels of p53 protein were found in a high percentage of malignant tumors but only rarely in benign tumors and normal tissues (Porter et al., 1992).

It has been estimated that up to 58 % of breast cancer patients have mutated P53 gene with accumulation of altered p53 protein, which can be detected by immunological assays (Lipponen et al., 1993; Elledge and Allred, 1994).

The mutational spectrum of p53 differs according to the type of cancer. In breast cancer, G to T transversion is more commonly seen. Induction of such a single point mutation results in a change of conformation, increased protein stability, and marked changes in its function. Most mutant forms of p53 lose the ability to bind to DNA and cause abnormal cell growth (Bargonetti et al., 1991; Raycroft et al., 1991; Dittmer et al., 1993). Moreover, it has been shown that the p53 abnormalities correlated with the grade of cancer and advanced with tumor growth (Teramoto et al., 1994).

Using a panel of human breast cancer cell lines, it has been shown that the large amounts of the mutant p53 proteins are due to their much longer half-life than that of the wild-type protein (Levine et al., 1991). Mutant forms of p53 was also found to bind to the endogenous wild-type p53, forming tetrameric complexes, which caused stabilization of the rapidly degraded wild-type protein and inactivation of its suppressor function by altering its confirmation (Milner and Medcalf, 1991; Kaklamanis et al., 1993).

In the rat, P53 gene has 10 exons and 9 introns, whereas mouse or human P53 gene contains 11 exons. In addition, rat P53 gene lacks sequence corresponding to intron 6, indicating that this sequence is nonfunctional in species that retain it (Hulla and Schneider, 1993). However, P53 gene conservation has been found to be similar in size of the exons, introns and locations of splice junctions. The majority of the missense mutations are located at codons corresponding to amino acids conserved in human, monkey, rat, mouse, chicken, xenopus, and fish (Hollstein et al., 1991).

1.5.2. BRCA1

In humans, BRCA1 gene is located on chromosomes 17q12-21 and produces protein of 1863 amino acid (≈220 kDa). It contains 24 exons (22 coding and 2 non-coding) and covers a span of ≈ 100 kb of genomic DNA. The BRCA1 coding region

comprises ≈5.5 kb. In addition to the full length of 8 kb mRNA transcript has been identified, various smaller BRCA1 transcripts with tissue specific expression pattern have also been described (Rosen et al., 2003).

BRCA1 protein interacts with other nuclear proteins, such as Rad51 and BRCA2 (Scully et al., 1997; Chen et al., 1998), and consequently plays several critical functions in the cell. Its amino terminal ring finger domain is involved in: repression of estrogen receptor-α signaling, modulation of DNA repair, and apoptosis. The carboxyl terminal acidic domain of BRCA1 acts as transcriptional activation domain when linked to DNA binding domain. Moreover, BRCA1 plays a role in cell cycle checkpoints and proper replication and functioning of centromeres (Rosen et al., 2003).

Individuals carrying mutations in the BRCA1 gene have an increased risk of developing breast and ovarian tumors (Smith et al., 1992). Mutations in BRCA1 alone account for approximately 45% of families with high incidence of breast cancer and up to 80% of families with both breast and ovarian cancer (Easton et al., 1993). It has been shown that BRCA1 knockout mice are hypersensitive to γ-irradiation which induces chromosomal aberrations (Shen et al., 1998). Therefore, loss of transcription activation by BRCA1 is an important factor in oncogenesis.

BRCA1 germline mutations account for most hereditary breast cancers. However, BRCA1 is mostly related to tumors diagnosed before age 40 years (Ottini et al., 2000). No somatic mutations have been detected in sporadic breast cancer, but other alterations were reported such as loss of heterozygosity, decreased levels of the BRCA1 mRNA, and methylation of the BRCA1 promotor region. Accordingly, BRCA1 is involved in breast neoplasias of sporadic origin and the magnitude of its decreased expression correlates with disease progression (Yoshikawa et al., 1999; Mueller and Roskelley, 2002; Fraser et al., 2003).

In rats, it has been shown that BRCA1 sequence is highly homologous to that of mouse (88%) and human (81%) with the highest homology in the region (nucleotide 51-173) encoding the ring-finger domain (Chen et al.,1996). This domain is a zinc binding motif that facilitates interaction between BRCA1 and other proteins to perform several functions such as DNA repair and ubiquitination (Fang et al., 2003). The ring-finger domain is 94% identical to the mouse sequence and 89% to the human. In addition to the homology in ring-finger domain among mammalian species, nuclear localization signals in rat are also identical to those of human and mouse. Analysis of BRCA1 rat promoter revealed that it shares 90% and 68% identity with the mouse and human sequences, respectively (Bennett et al., 1999). In some rat models of breast cancer, it has been shown that while BRCA1 mRNA level does not change, allelic imbalance of BRCA1 may occur (Chen et al., 1996).

There is a controversy regarding the localization of BRCA1 protein in the mammary gland. Some investigators reported that it is found in the nuclei of normal epithelial cells, but is aberrantly located in the cytoplasm of malignant mammary cells (Chen et al., 1995). However, the expression of BRCA1 protein in the nuclei of both normal and malignant cells was demonstrated (Scully et al., 1996). Also, the localization of BRCA1 in cytoplasm and the cell membrane was reported (Jensen et al., 1996). The localization of BRCA1 protein in cytoplasmic tube-like invaginations in the nucleus was also reported (Coene et al., 1997). These contrarieties on the location of BRCA1 protein were interpreted as differences in the specificity of the antibodies or fixation protocols used, or as evidence for the presence of splice variant isoforms of the BRCA1 protein in tumors (Perez-Valles et al., 2001).

I.6. Potential breast cancer preventive agents

It has been reported that some factors may influence the development of breast cancer. Early full-term pregnancy significantly lowers a woman's risk of developing breast cancer (Peck et al., 2002). This association between pregnancy and reduced breast cancer risk has been validated in experimental studies which have shown that pregnancy protects rats against carcinogen-induced mammary cancer. Therefore, it seems that pre-neoplastic cells present in the mammary glands prior to pregnancy are either destroyed or altered during pregnancy by hormone induced differentiation of the glands (Thordarson et al., 1995).

Dietary factors have been an appealing approach for the prevention of breast cancer. Naturally occurring coumarins represent one of the largest classes of phytochemicals. These compounds are found in many plants and had a greater inhibitory effect on the formation of DMBA-DNA adduct in mouse mammary glands (Prince et al., 2005). In addition, a natural isoflavonoid phytoestrogen found in soy products, "genistein", is thought to possess breast cancer preventive properties. It has been shown to provide some protection against DMBA-induced mammary tumors in rats (Lamartiniere et al., 1998).

Recently, the anti-malarial compound called "artemisinin", which was isolated from the sweet wormwood *Artemisia annua L.*, has been shown to have a significant preventive effect on breast cancer development in DMBA-treated rats (Lai and Singh, 2006).

Vinca alkaloids are natural compounds which are present in the plant called *Cathalanthus roseus G. Don* (or *Vinca rosea Lin*, or periwinkle; Fig. 1). Catharanthus roseus contains about 90 different alkaloids, some are indole monomeric while others are dimeric alkaloids. Some of these alkaloids, such as vincristine, are commonly prescribed

Fig. 1: *Catharanthus roseus* or *Vinca rosea* whole plant.

for cancer chemotherapy (Rowinsky and Donehower, 1991; Ali, 1998; Murata and DeLuca, 2005).

Vinca alkaloids molecule is composed of two parts: a large multiple ring structure called catharanthine (the upper half of the *Vinca* structure) and lower multiple ring domain called vindoline. It has been shown that the catharanthine ring is responsible for the binding of the *Vinca* alkaloids to tubulin molecules and the vindoline ring enhances the binding process and interferes with tubulin polymerization (Budman, 1992). In addition to their blocking effect of microtubule assembly and cell cycle progression in the G2-M phase, *Vinca* alkaloids has been recently shown to induce apoptosis via its effect on API transcription factor (Fan et al., 2001).

In India and some other countries, leaves and flowers of *Vinca rosea* are being used traditionally by some patients. There have been some experimental trials on the use crude extract of *Vinca rosea* as a potential anti-cancer cell agent and promising results were reported. Mice carrying the Ehrlich ascites carcinoma cells and treated with the crude *roseus* extract showed a 46% reduction in cancer cells (Rana et. al., 2004). Studies have shown that such crude extracts are not toxic in rats (Singh et al., 2001). Other studies on the extracts of leaves and twigs of *Vinca rosea* also indicate very high margin of safety (Chattopadhyay et al., 1992; Chattopadhyay, 1991).

AIMS OF THE STUDY

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The overall objectives of this study was to characterize the early changes that occur during breast cancer progression in DMBA-treated rats and to test whether treatment with a crude extract of *Vinca* alkaloids could alter these changes. Therefore, the specific aims of the present study were to utilize the DMBA-treated rat model to:

1. characterize the basic microscopic changes that occur during breast cancer development, with special emphasis on the early stages, using routine histological techniques.
2. correlate the histological changes that occur during breast cancer development with the changes in cell proliferation by using antibody specific for the proliferating cell nuclear antigen (PCNA) as a marker for dividing cells.
3. analyze the expression of protein products of two tumor suppressor genes, P53 and BRCA1, in the mammary glands during the different stages of breast cancer development by using immuohistochemistry and western blotting techniques.
4. test whether mutations occur in P53, BRCA1, and PCNA genes during breast cancer development by using single strand conformation polymorphism (SSCP) and restriction fragment length polymorphism (RFLP) assays.
5. study the effect of the crude extract of alkaloids of *Vinca rosea* on the incidence of pre-neoplastic and neoplastic lesions in the mammary glands by using morphological techniques.
6. examine whether *Vinca* alkaloids has an effect on the P53, BRCA1, and PCNA at the level of proteins and DNA during breast cancer development by using immunohistochemistry, western blotting, SSCP, and RFLP assays.

CHAPTER II

MATERIALS AND METHODS

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II.1. Extraction of crude alkaloids from *Vinca rosea*

The crude extract of alkaloids was prepared according to the protocol published in the textbook of Physicochemical Standards of Unani Formulations (Central Council For Research In Unani Medicine, 1987). All chemicals used for extraction of alkaloids were purchased from Sigma Company (USA). The aerial parts of virgin *Vinca rosea* (which has no flowers yet) were collected from the nursery of Al-Ain municipality between September and November, 2003. Collected plants were washed thoroughly with tap water followed with distilled water and were shade dried. About 100 gm of dried plant material was incubated with 2 liters of 0.5N H_2SO_4 for 20 min at room temperature. After filtration, acid treatment was repeated 3 times. The pH of the acid extract was raised to about 12 by adding 50% NH_4OH with shaking in separating funnel containing a mix of chloroform and ether (2:1). All chloroform-ether extract was pooled out and then shaked with different volumes (100, 50, 50, 25 ml) of 0.5N H_2SO_4 for complete extraction. Combined acid extract was filtered and its pH was checked alkaline to litmus with the ammonium hydroxide solution. The liberated alkaloids were extracted with successive portions (100, 50, 50, and 25ml) of chloroform to ensure complete extraction. The combined chloroform extract was washed with 50 ml of water twice. Chloroform was distilled off on a water bath and removed completely in vacuum desiccator. Five millilitres of 90% alcohol was added to the residue and the solvent was again removed. Evaporation with alcohol was repeated, and the residue was dried in vacuum desiccator and the weight was taken as total alkaloids. Figure 2 shows the extracted crude alkaloids.

Fig. 2: Crude extract of alkaloids prepared from the aerial parts of virgin *Vinca rosea.*

II.2. Animals

Female virgin Wistar rats (43-50 days old) were used in this study. Rats were supplied by the Animal House of the Faculty of Medicine and Health Sciences, UAE University, UAE. All rats were kept in standard conditions with regimen light-dark: 12-12 hrs and received food and water *ad libitum*.

II.3. Preparation of DMBA solution

For induction of breast cancer, freshly prepared solution of DMBA (Sigma, USA) was used. About 0.3 gm of DMBA was suspended in 40 ml corn oil, and then heated in a water bath at ~95°C for 30 minutes with mixing by a glass rod. When the solution became clear, with no visible particles of DMBA, it was cooled to room temperature and immediately administered into rats.

II.4. Experimental design

Three groups of rats were used in this study. The first group contained 21 rats which were used to induce breast cancer by intragastric treatment with DMBA solution as a single dose of 80 mg/kg body weight (Russo and Russo, 1996). The second group included 20 rats which were treated with DMBA as in the first group, but one week later, they received a daily oral dose of crude alkaloid extract (200 mg/kg body weight) suspended in 50% polyethylene glycol 600 (Fluka, Switzerland) for three weeks. The third group, included 9 rats and was served as age-matched control and received corn oil only.

All rats were examined weekly to detect any changes in the general physical activity and body weights. In addition, the mammary glands were gently palpated to detect early development of any abnormal mass.

Rats in each group were chosen randomly and killed by an overdose of anesthetic after 25, 30, 35 or 40 weeks from the beginning of treatments. For each rat, the mammary glands of one side were immediately dissected and stored at -80°C for DNA extraction and protein analysis. The opposite line of mammary glands were dissected along with skin pelt, immediately immersed overnight (12-24 hr) in Bouin's solution. The Bouin's fixative was made of 70% picric acid solution, 10% formaldehyde, and 5% acetic acid. Fixed tissues were then processed for dehydration in ascending grades of alcohol, clearing in xylene and paraffin infiltration and embedding. Paraffin tissue blocks were cut at 5 μm thickness, mounted on gelatin-coated glass slides and then used for histopathological and immunohistochemical studies.

In case that a mammary gland had a mass or tumor, it was weighed and divided into two equal parts. One part was processed for histopathological and immunohistochemical studies, and other part, for DNA and protein analyses.

II.5. Histopathological studies

Mammary gland tissue sections of control and treated rats were processed for routine hematoxylin and eosin (H&E) staining as follows. Sections were dewaxed in xylene twice (5 min each), then rehydrated with descending grades of alcohol. Sections were then stained with Harris hematoxylin for 30 min, washed in tap water for a few seconds, and dipped in 1% hydrochloric acid in alcohol. Sections were then kept in tap water for 5 min followed by staining with eosin Y for 25 second. Finally, sections were dehydrated, cleared in xylene, and mounted with DPX medium.

Stained tissue sections were examined with Olympus light microscope to define the histopathological changes in the mammary glands, and to classify tumors according to previously established criteria (Russo et. al, 1990b; Russo and Russo, 2000).

II.6. Immunohistochemical studies

Paraffin tissue sections obtained from control rats and their age-matched DMBA-treated and DMBA-plus-alkaloids-treated rats were mounted on the same slides. Tissue sections were deparaffinized in xylene, rehydrated in descending grades of alcohol, and washed in phosphate buffered saline (PBS). To inhibit endogenous peroxidase activity, sections were incubated in 1% hydrogen peroxide in methanol for 30 min. The slides were placed horizontally in a humid chamber. To ensure equal conditions on all tissue sections to be probed, slides were drained off, area around sections were wiped dry, and circled with a thin film using PAP-pen (Dako, Denmark). Nonspecific binding was blocked by incubating sections in 1% bovine serum albumin containing 0.5% Tween-20 in PBS for 45 min. Then, sections were incubated with the primary antibody for overnight at 4°C.

The first primary antibody used was mouse monoclonal anti-PCNA antibody (clone 5A10, dilution 1:100, Medical and Biological Laboratories Co., Japan). Sections were washed 3 times with PBS, 3min each, and then incubated with biotinylated donkey-anti-mouse immunoglobulin IgG (dilution 1:800, Jackson ImmunoResearch Laboratories Inc., USA) for 30 min. Following three PBS washes, sections were incubated in extravidin/peroxidase conjugate (dilution 1:1000, Sigma, USA) for 1 hour. The antigen-antibody binding sites were revealed by incubating tissue sections with 3, 3`-diaminobenzidine tetrahydrochloride (DAB, Sigma, USA). Development of the colour was stopped after 35 min by washing the sections twice with distilled water (5min each).

Tissue sections were counterstained with periodic acid Schiff (PAS) technique. Briefly, periodic acid (0.5%) was added to the tissue sections for 10 min, followed by washing in tap water. Then, Schiff's reagent (BDH Laboratory supplies, UK) was added for 20 min. Sections were washed using tap water for 15 min. Finally, sections were

dehydrated through graded alcohol, cleared in xylene and mounted using DPX. Negative control slides were prepared in parallel without adding the primary antibody.

To measure the intensity of PCNA immunolabeling of the cells, image analysis was performed using Scion Image Beta 4.02 software for Windows (http://www.scionimage.com/). To minimize possible differences among tissues, all sections used for measurements were placed on the same slide, and subjected to the same staining procedures. Probed sections were examined at high magnification (x640) and 2-3 fields were photographed with digital camera at the same conditions and used for measurement. Cells cut through the center of their nuclei were only considered for measurement. Following measurement of staining intensity of the various nuclei in one section, the background intensity was measured and subtracted. Quantitative results of the optical density were reported in arbitrary units corresponding to DAB staining intensity which was taken as an indication of the amount of PCNA in the sectioned cells. The measurements were presented as means of arbitrary units ± standard error of the mean (SEM).

To estimate the labeling index (LI) of PCNA immunostaining, for each mammary gland, images of at least three fields at x640 magnification were randomly selected from 2 or more rats. The total number of PCNA-labeled cells per high power field was counted. Data were expressed as mean ± SEM.

The student *t* test (SigmaPlot for Windows, version 9.0) was used to examine the differences in labeling intensity and labeling index in control and treated rats. $P<0.05$ was considered to be statistically significant.

The second primary antibody used was to study cellular expression of BRCA1. The rabbit polyclonal anti-BRCA1 antibody (clone I-20, Santa Cruz Biotechnology Inc., USA) specific for the C-terminal region between codons 1823-1842 (Schofied et al..

2000) was used. In addition, the catalyzed signal amplification kit (CSA kit, Dako, Denmark) was used. Staining procedures were carried out according to the manufacturer's instructions. Briefly, sections were first incubated for 5 min with 3% hydrogen peroxide to suppress endogenous peroxidase activity. Then, protein block was added for 5 min to prevent nonspecific binding. Anti-BRCA1 antibody was diluted at 1:50 and added for overnight at 4°C. Sections were washed with TBST (50mM Tris-HCl PH 7.6, 300mM NaCl, 0.1% Tween-20) three times, 3 min each. Biotinylated donkey-anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., USA) was then added at dilution 1:500 for 15 min. Sections were washed with TBST and streptavidin-biotin solution was added for 15 min. After washing, amplification reagent was added for 30 min, followed by washing and applying streptavidin-peroxidase reagent for 15 min. Finally, substrate-chromogen solution was added, and the development of the brown colour was monitored by microscope. Colour development was stopped by adding distilled water (twice, 5 min each). Sections were counterstained with Harris hematoxylin for 5 min and then washed in tap water and differentiated in acidic alcohol. Tissue sections were then kept in water for 5 min. Finally, sections were dehydrated through ascending grades of alcohol, cleared and finally mounted on glass slides using DPX. Negative control slides were prepared in parallel without adding the primary antibody.

II.7. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

The whole mammary gland tissues of control and treated rats were homogenized into a powder under liquid nitrogen temperature and then lysed with 1 ml buffer containing: 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, PH 7.5), 10% sucrose, 10 mM 1,4-Dithio-DL-threitol (DTT), 0.1% 3-[(3-

cholamidopropyl)dimethyl-ammoniol]-1-propanesulfonate (CHAPS), 150 mM NaCl, and protein inhibitors: 100 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% leupeptin and aprotenin. Tissue lysates were centrifuged at 14,000 rpm for 30 min at 4°C. Protein concentrations in the supernatants were determined according to the method of Bradford (1976) using the BioRad protein assay kit (BioRad Laboratories, USA).

Equal amounts of protein (30 μg) from each sample were mixed with 5x sample buffer containing Tris HCl (PH 6.8), 10% glycerol, 2% sodium dodecyl sulphate, 2-mercaptoethanol, and bromophenol blue. Samples were boiled for 5 min and electrophoresed on 8% polyacrylamide gel at 80 volts for 2 hours. To check the expected size of the protein, prestained protein marker (BioRad Laboratories, USA) was used.

After electrophoresis, proteins in the gels were transblotted onto nitrocellulose membrane. Non-specific binding was blocked by 5% non-fat dry milk in PBS containing 0.1% Tween-20 (PBST) for 1 hour. Following washing in PBST two times 10 min each, blots were incubated with mouse monoclonal anti-p53 antibody (clone PAb240, dilution 1:1000, Dakocytomation, USA) for overnight at 4°C. After rinsing with PBST (two times, 10 min each), blots were incubated with horseradish peroxidase-conjugated goat-anti-mouse IgG (Cell Signalling Technology, USA) at dilution 1:1000 for 2 hours at room temperature. Blots were then washed with PBST three times for 10 min each, and immunoreactive proteins in the blot were detected using enhanced chemiluminescence (ECL) western blotting detection reagents (Pierce Biotechnology, USA) on Fuji medical X-Ray film (Japan). To confirm equal loading of proteins, same blot was immunoprobed with rabbit polyclonal anti-actin antibody (dilution 1:1000, clone AC40, Sigma, USA). Films were scanned using BioDocAnalyze system (Biometra, Germany), and the intensity of bands was measured.

II.8. Extraction of DNA

Genomic DNA was obtained from mammary glands of control and treated animals using DNA extraction kit (QIAGEN, Canada). Tissues were quickly washed in cold PBS, weighed, and then homogenised in a mortar at liquid nitrogen temperature. The fine powder was mixed with sterile/cold PBS (100 µl PBS for each 20 mg of the tissue). Homoginized tissue was mixed with equal amount of digestion buffer provided by the kit. QIAamp column containing DNA was transferred to clean eppendorf tube. and 50 µl elution buffer was added. The extracted DNA was stored at -20 °C until used.

DNA concentration was determined by measuring the optical density (OD) at 260 nm by using spectrophotometer (WPA Cambridge, UK).

II.9. The polymerase chain reaction (PCR)

PCR was performed by using PuReTaq Ready-to-Go PCR beads (Amersham Pharmacia Biotech, Sweden). The procedure was carried out according to the manufacturer's instructions.

Primers of P53 and PCNA genes were obtained from Pharmacia Company, USA. BRCA1 primers were obtained from Operon Biotechnologies, Sweden. Sequences of all primers used are listed in table 1.

For PCR amplification of PCNA, the reaction was performed with 2.5µl of each primer at 10 pmol/µl, and diethylpyrocarbonate-treated water (DEPC, ResGen. Invitrogen, USA) was added to a total volume of 25 µl. The cycling program was as follows: predenaturation (95°C, 5min), 40 cycles of amplification through denaturation (95°C, 1min), annealing (55°C, 1min), and extension (72°C, 1min). A final 5 minutes extension step was added in TECHNE/GENIUS thermal cycler.

Table 1: Primers used in PCR reaction.

Primers	**Sequences**	**Annealing temperature (°C)**
PCNA/E1	Forward: 5'-CTG GGA CAA CTT AAT GTT CCC-3' Reverse: 5'-TAG TGC AGC TTA CTC TGC GC-3'	55
BRCA1/E11	Forward: 5'-TTT CAC CCA TAC ACA TTT G-3' Reverse: 5'-CCT TTG CCA ATA TTA CCT G-3'	48
P53/E5	Forward: 5´-GAC CTT TGA TTC TTT CTC CTC TCC-3´ Reverse: 5´-GGG AGA CCC TGG ACA ACC AG-3´	64
P53/E6-7	Forward: 5´-CTG GTT GTC CAG GGT TCT CC-3´ Reverse: 5´-CCC AAC CTG GCA CAC AGC TT-3´	64
P53/E8-9	Forward: 5´-CTT ACT GCC TTG TGC TGT GC-3´ Reverse: 5´-CTT AAG GGT GAA ATA TTC TCC-3´	58
P53/E10	Forward: 5'-GTA CTG TGA ATA TAC TTA CTT CTC C-3' Reverse: 5'-GGG CTG AGG TCA CTC ACC-3'	60

The PCR amplification of P53 exons was performed using 40 cycles of amplification through denaturation (95°C, 1min); annealing for 1min (see table 1), and extension (72°C, 1min). A final 5 minutes extension step was added. The sequences of synthetic primers used in the enzymatic amplification of p53 were according to Vancutsem et al. (1994).

PCR amplification of BRCA1 exon 11 was performed with genomic DNA, 2.5µl of each primers at 10 pmol/µl, and DEPC water was added to a total volume of 25 µl. For amplification, each sample was initially denatured at 95°C for 5 min, and then subjected to 40 cycles, each included denaturation at 95°C for 1min, annealing at 48°C for 1min, and then extension at 72°C for 1min. An additional 5 min was added for more extension at 72°C.

II.10. Agarose gel electrophoresis

From each PCR product 5 µl were added to 3 µl loading buffer containing 0.25% bromophenol blue, 0.25% xylene cyanol, 20% glycerol and 1X TBE buffer (90mM Tris base, 90mM Boric acid, 2mM ethylenedimethyltetra acetic acid, PH 8.4) and loaded into 1.5% agarose gel. The gel was run at 100 volts for 1 hour at room temperature. To determine the expected size of PCR products, 100 base pair ladder marker (Amersham Biosciences, USA) was used. The bands were visualized by staining the gel with ethidium bromide (10 mg/ml) and exposing it to the UV trans-illuminator (Lifetechnology, USA). The gel image was captured using the Polaroid camera (Polaroid, UK).

II.11. Single-strand conformation polymorphism (SSCP)

To detect possible alterations in the nucleotides sequence of PCNA and p53 genes, the migration pattern of their PCR product on polyacrylamide gel was analyzed by using the SSCP method. A 5 µl of the PCNA or p53 PCR-products was denatured by adding 5 µl loading buffer containing formamide, xylene cyanol, and bromophenol blue and incubating at 96°C for 10 minutes. The mixture was immediately chilled on ice. Then, the denatured PCR products were loaded onto a 10% nondenaturating polyacrylamide gel and run at 60 volts for 4 hr at 4°C, using vertical electrophoresis cell (BioRad, USA).

The silver staining kit (Amersham Pharmacia, Biotech, Sweden) was used to stain DNA bands in the polyacrylamide gels. The procedure was carried out according to the manufacturer's instructions. Briefly, DNA in the gels was fixed overnight by 0.6% benzene sulphonic acid. Gels were incubated in silver nitrate solution for 30 min. DNA bands were visualized by using a solution containing sodium carbonate, formaldehyde and sodium thiosulphate. The developed colour was stopped by using a mixture of 1% acetic acid, 5% sodium acetate and 10% glycerol. Finally, the stained polyacrylamide gels were sealed and dried using cellophane membrane (Promega, USA). Computer scanning was performed by hp office jet 5510 all-in-one (Hp image zone program).

II.12. Restriction fragment length polymorphism (RFLP)

RFLP analysis was used to identify changes in the nucleotide sequence of the PCR product of BRCA1 gene at the site where a restriction enzyme cuts. BamHI was used to cut the PCR product of BRCA1. The enzymatic reaction included 3 µl of 10X enzyme buffer E (Promega, USA), 0.3 µl 1% bovine serum albumin (Promega, USA), 30U BamHI enzyme (Promega, USA) and 10 µl BRCA1 PCR product. The reaction

mixture was brought up to 30µl with DEPC-treated water. All samples were incubated overnight at 37°C in block heater (Stuart Scientific, U.K.). The digested products were loaded in nondenaturing 10% polyacrylamide gel for 2 hr at 110V at room temperature. Gels were stained in the running buffer (1X TBE) containing ethidium bromide (10 mg/ml) with continuous shaking for 2-3 hrs. Bands were visualized by using the UV trans-illuminator and photographed.

CHAPTER III

RESULTS

CHAPTER III
RESULTS

Throughout this study, no change was detected in the general activity and body weights of DMBA- and DMBA-plus-alkaloid-treated rats as compared with control ones. The body weights of all rats were estimated weekly and were within the normal range of the corresponding age. No significant difference was observed between the control and the treated rats. However, 35% (7/20) of the 32- and 37-week-old rats treated with the crude extract of *Vinca* alkaloids showed some alteration in their genital tracts manifested by the presence of vaginal blood. Such blood was not detected in control and DMBA-treated rats.

III.1. Morphological features of the mammary glands in DMBA-treated rats

Gross observation and gentle palpation of the mammary glands of control and DMBA-treated rats revealed the development of variable masses in about 29% of the rats. These masses were of variable sizes, and the covering skin appeared normal. They were provisionally considered to be tumors (Fig. 3). They developed in different topographical locations of the mammary glands; some were observed in the cervical region, others in the thoracic and abdomino-inguinal regions. Nevertheless, these tumors were more frequent in the cervical region than in other regions (Table 2).

To confirm the nature of these tumors and to detect the early microscopic changes that occur during development of breast cancer, H&E stained tissue sections of all the

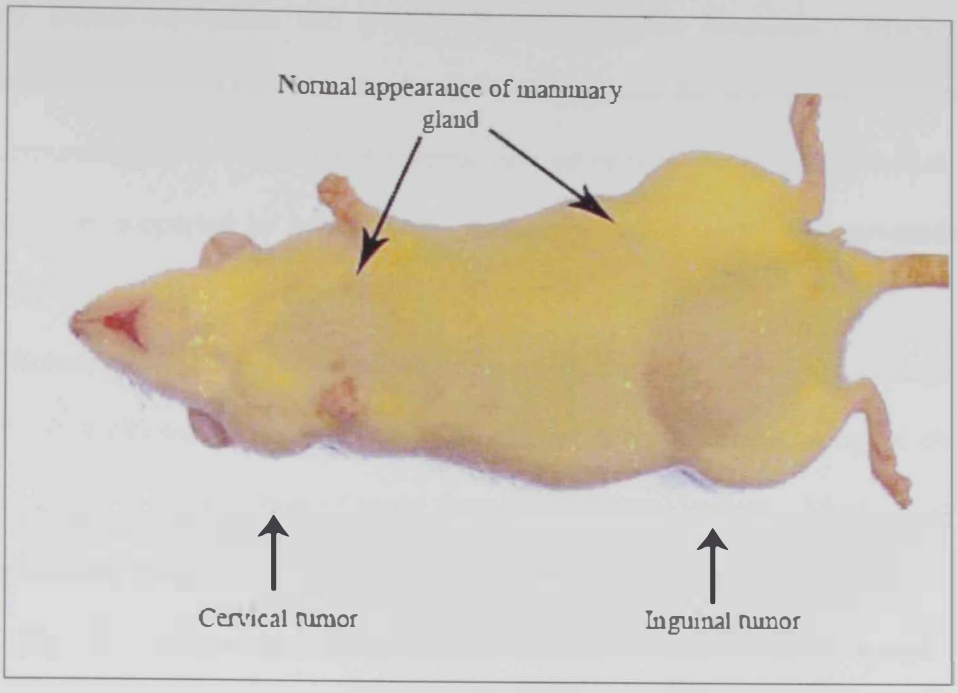

Fig. 3: DMBA-treated 42-week-old rat. Two tumors developed in cervical and abdomino-inguinal regions (lower arrows).

Table 2: Tumors developed in DMBA-treated rats.

	Age groups of rats			
	32 weeks	**37 weeks**	**42 weeks**	**47 weeks**
No. of rats	6	5	5	5
Rats with tumor	1	1	3	1
Tumor location	Cervical	- (*in situ*)	Cervical, thoracic and abdomino-inguinal	Cervical
Weight of tumor (gms)	15	No mass (*in situ*)	- No mass in 2 rats (*in situ*) - 3.2; 13.8; 3; 3.1	3
Type of tumor	▸ Ductal solid and cribriform carcinoma ▸ *In situ* cribriform carcinoma	▸ *In situ* cribriform carcinoma	▸ Lactating adenoma ▸ Ductal solid and cribriform carcinoma ▸ Papillary carcinoma ▸ *In situ* cribriform carcinoma	▸ Squamous cell papilloma

mammary glands of control and DMBA-treated rats were examined. Microscopic examination of the mammary glands of control rats showed the normal ductal epithelial lining surrounding narrow lumen and external discontinuous layer of myoepithelial cells. The latter were supported by some fibrous connective tissue which was surrounded by massive amount of adipocytes (Fig. 4).

Microscopic examination of all mammary glands of rats treated with a single oral dose of DMBA (80 mg/kg body wt) revealed various degrees of morphological changes which is usually a combination of more than one abnormal pattern. Most of DMBA-treated mammary glands had moderately dilated ducts with groups of small cells in their lumens (Fig. 5). These free luminal cells had eosinophilic cytoplasm and small hyperchromatic nuclei. These cells were considered to be dead. In some other ducts, a sign of increased secretory activity was evident with the accumulation of homogeneous secretory material in their lumens. Therefore, these mammary glands seemed to have unbalanced cell dynamics and enhanced secretory activity. All DMBA-treated rats had such changes in some of their mammary glands. Of the total number of DMBA-treated mammary glands examined (n=78), 46% were with altered cellular dynamics.

The second type of lesion observed in the mammary glands of DMBA-treated rats was characterized by an increase in the cell number which led to an apparent increase in the density of the cells forming the terminal ducts (Fig. 6). Mammary glands with these changes were referred to as hyperplastic. However, those showing the previous lesions described in the former paragraph with altered cell dynamics were referred to as mammary glands in the stage of cell death.

Hyperplastic mammary glands also showed other small ducts with signs of cell death and other pre-neoplastic lesions. These mammary glands with mixed lesions represented about 39% of all DMBA-treated mammary glands examined (Table 3).

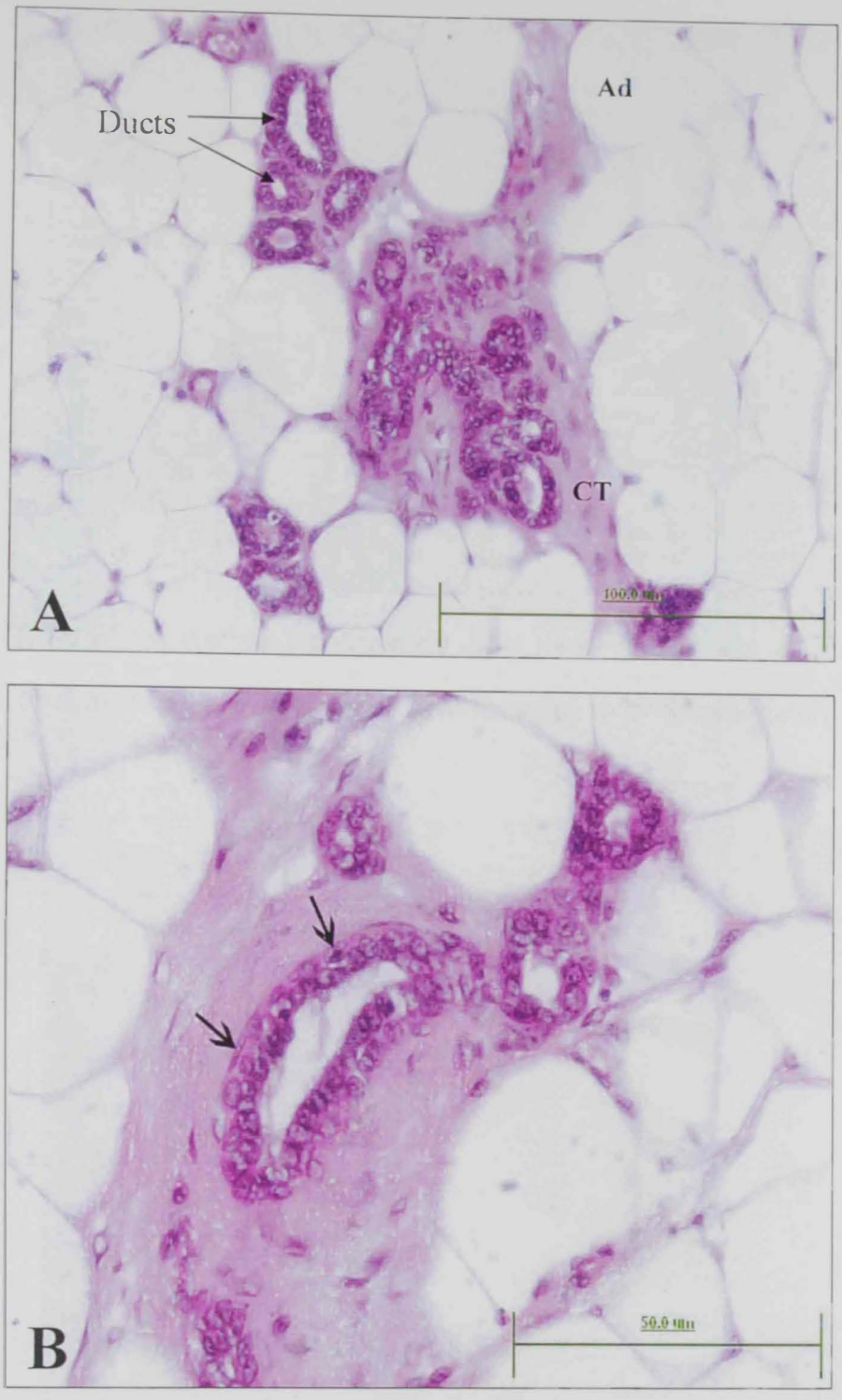

Fig. 4: Normal mammary gland of a control rat as it appear in a paraffin section stained with H&E. (A) The gland shows small ducts surrounded by fibrous connective tissue (CT) and much adipose tissue (Ad). (B) Higher magnification of mammary gland duct showing discontinuous layer of myoepithelial cells (arrows). Magnification: x400 (A); x640 (B).

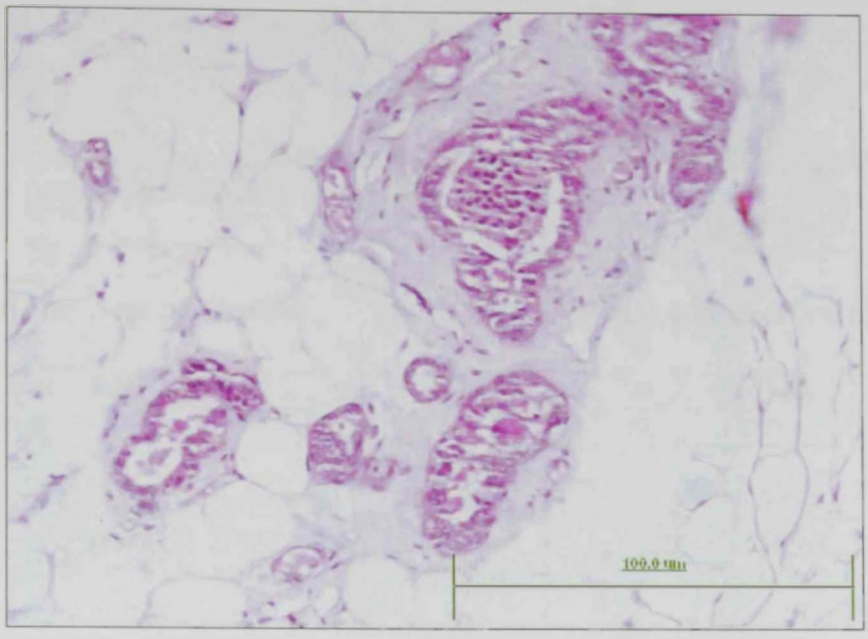

Fig. 5: Stage of cell death in the mammary gland showing early sign of morphological change. Notice the moderate dilatation of the ducts and presence of dead cells in their lumen. Two ducts in the lower third of the micrograph show some secretory material in their lumens. Magnification: x400.

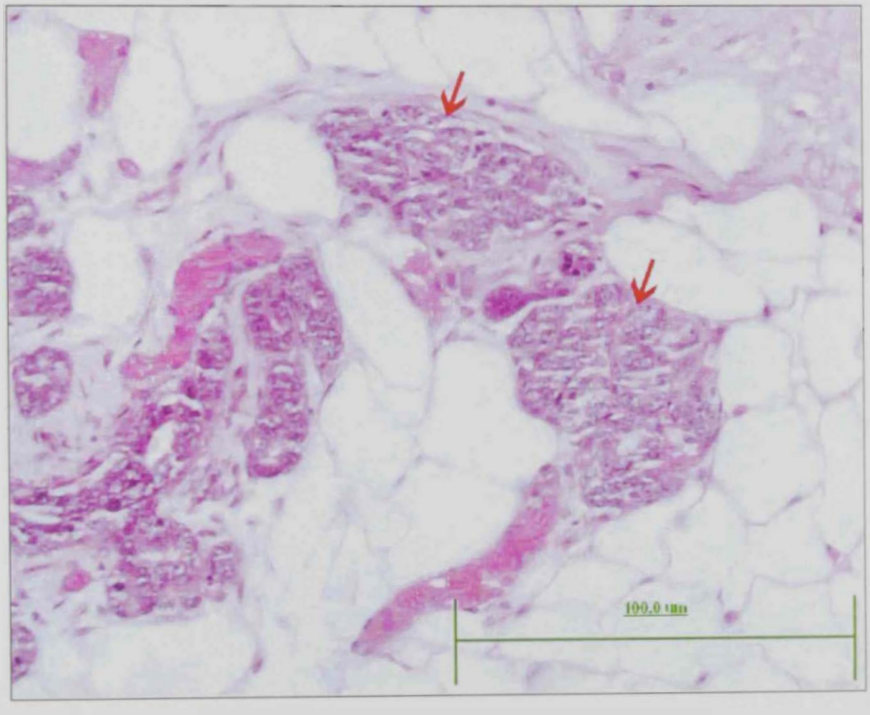

Fig. 6: Hyperplasia as seen in a DMBA-treated rat. Note the increased number of terminal small ducts (arrows). Magnification: x400.

Table 3: Numbers of pre-neoplastic and neoplastic lesions developed in the DMBA-treated and DMBA-plus-alkaloids treated mammary glands.

Mammary gland lesions	DMBA*	DMBA-plus-alkaloids**
Stage of cell death	36 (46%)	48(45%)
Hyperplasia	-	1 (1%)
Dysplasia	1 (1.2%)	-
Stage of cell death + Hyperplasia	21 (27%)	21 (20%)
Stage of cell death + Dysplasia	4 (5%)	16 (15%)
Stage of cell death + Hyperplasia + Dysplasia	8 (10%)	14 (13%)
In situ carcinoma + Stage of cell death + Hyperplasia + Dysplasia	1 (1.2%)	1 (1%)
In situ carcinoma + Stage of cell death + Hyperplasia	1 (1.2%)	-
Tumors	6 (8%)	3 (3%)

*Total number of DMBA-treated mammary glands examined = 78
**Total number of DMBA-plus-alkaloids-treated mammary glands examined = 107

The third kind of lesion observed was characterized by increased number of epithelial duct cells which was manifested with an increase in the number of small ducts and many of them showed an apparent increase in the secretory activity and accumulation of secretory material. In the epithelial cells of these ducts, many of the nucleoli became prominent. Mammary glands with these features were referred to as dysplastic (Fig. 7). Dysplastic mammary glands were developed in about 17% of all examined DMBA-treated mammary glands. A few of these glands (1.2%) showed only signs of dysplasia. The others were mixed with signs of cell death and hyperplasia (Table 3).

Two of the DMBA-treated rats (9.5%) developed large microscopic lesions made of dilated ducts which were mostly filled with cells. The nuclei of these cells were variable in density and were frequently seen in mitosis. There were some spaces in between the cells that formed secondary lumens. This lesion was typical of the cribriform type of carcinoma *in situ* (Fig. 8). It was estimated that 2.4% of DMBA-treated mammary glands showed *in situ* cribriform carcinoma. These mammary glands also showed signs of cell death, hyperplasia, and dysplasia (Table 3).

Advanced palpable pathological lesions were developed in 5 different rats and showed different histopathological features. These lesions were either benign or malignant. Benign lesions were developed in rats sacrificed at 42 and 47-weeks-old, with incidence of 14% of the total number of tumors. The first type, lactating adenoma, was characterized by numerous round profiles. Lumens of these alveolar structures had serrated appearance due to decapitation or supranuclear vacuolization of the lining epithelium. The size of these alveoli may increase due to accumulation of secreted material inside the lumens (Fig. 9). The other benign lesion, squamous cell papilloma (Fig. 10), was developed in the 47-week-old rat. Ductal structures were maintained with multilayered epithelial cells surrounding the lumen, which was full of dead cells. The

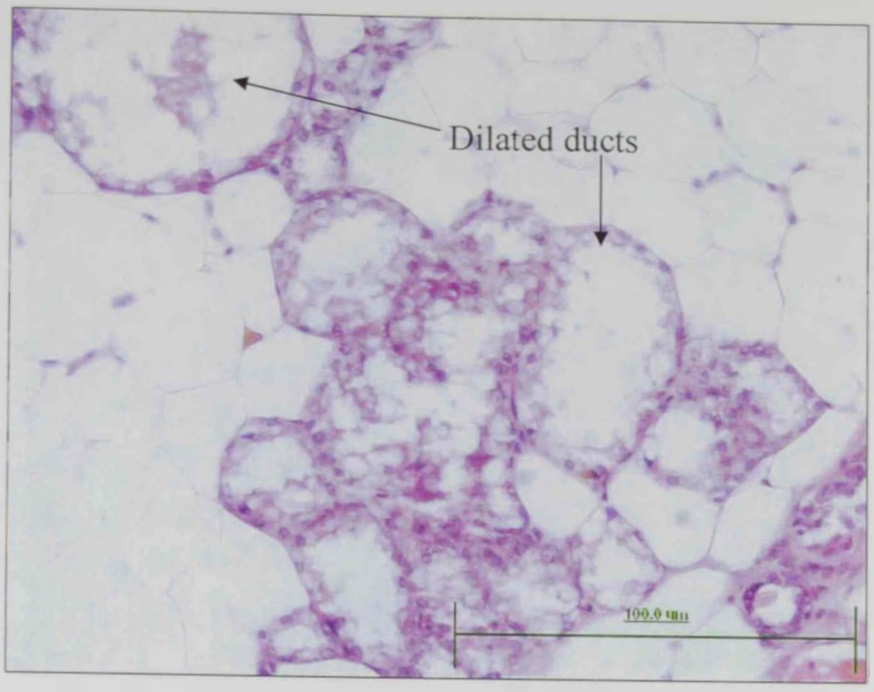

Fig. 7: Dysplastic DMBA-treated mammary gland. Note the dilated ducts (arrows) and decreased amount of connective tissue. Magnification: x400.

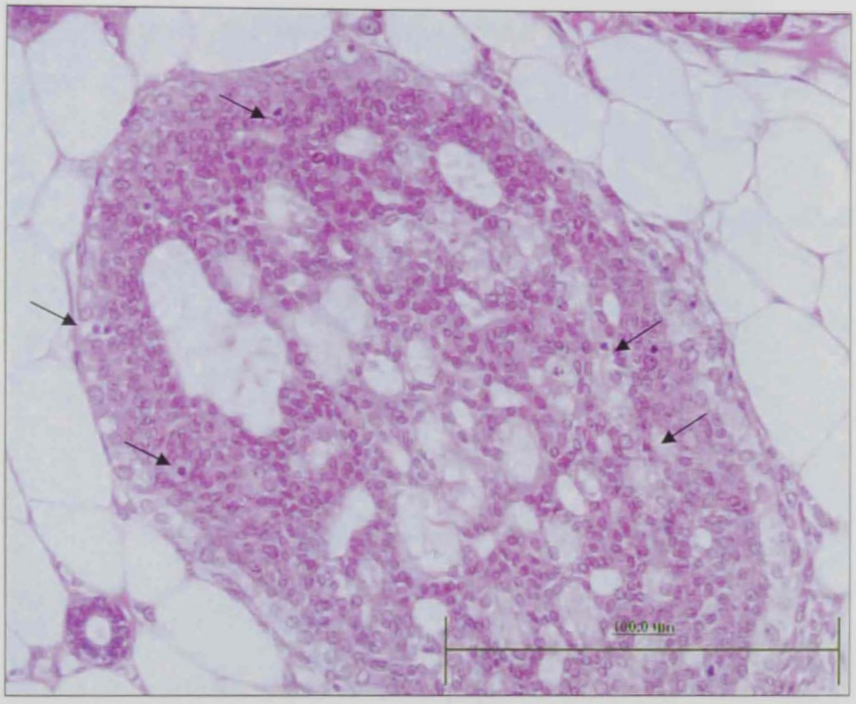

Fig. 8: *In situ* cribriform carcinoma in DMBA-treated rat showing localized area of dilated ducts partially filled with tumor cells. Note the numerous peripheral mitotic figures (arrows). Magnification: x400.

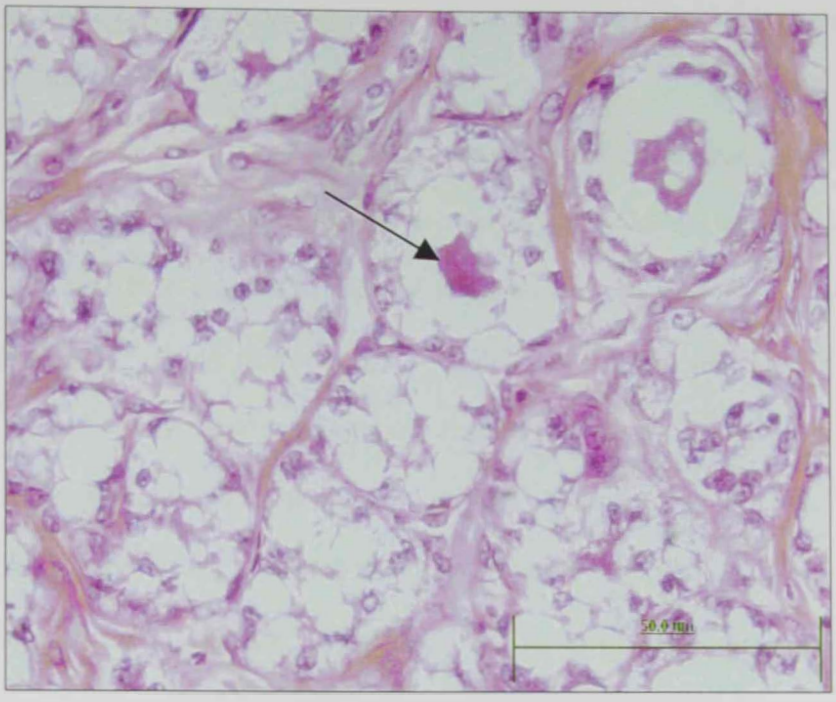

Fig. 9: Lactating adenoma in DMBA-treated mammary gland showing numerous round profiles which may contain secretory material (arrow). Magnification: x640.

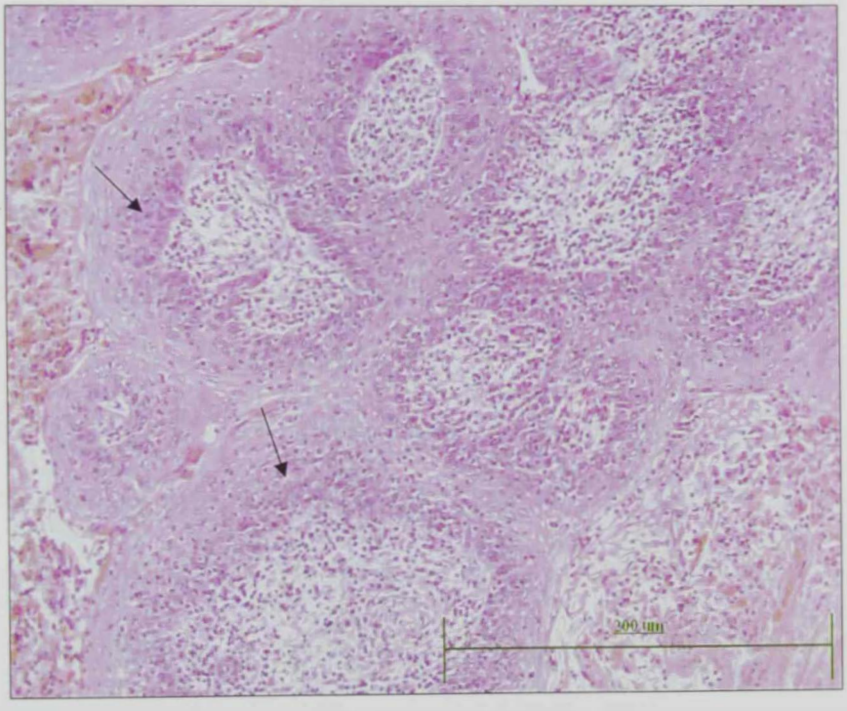

Fig. 10: Squamous cell papilloma in DMBA-treated rat showing multiple layers of epithelial cells (arrows). Magnification: x200.

presence of keratin and the decrease in the amount of connective tissue were typical features of this tumor.

In the DMBA-treated rats, malignant lesions were more frequent (19%) than benign lesions and were developed in one of the 32-week-old-rats and two of the 42-week-old rats. The first type of malignant lesions observed was typical of invasive cribriform carcinoma. It was characterized by solid sheets of neoplastic epithelial cells which were interrupted by round or irregularly shaped secondary lumens of variable sizes (Fig. 11). Individual neoplastic cells were moderately pleomorphic.

The second malignant tumor observed was invasive papillary carcinoma. It was characterized by numerous papillary projections with thinner fibrovascular core. Papillae were lined by columnar cells that were continuous with a multilayered and pleomorphic epithelium. Mitotic figures were frequently observed in this tumor. Nucleoli appeared prominent and often multiple (Fig. 12).

III.2. Effects of *Vinca* alkaloids on the pre-neoplastic and neoplastic changes of the mammary glands of DMBA-treated rats

To test whether the crude extract of *Vinca* alkaloids affects the morphology of normal mammary glands, some tissue sections of the mammary glands of alkaloid-treated rats were stained with H&E and examined with the light microscope. No change was observed in the appearance of the mammary glands. The mammary duct system of alkaloid-treated rats appeared similar to that of the control rats shown in figure 4.

When the extract of *Vinca* alkaloids was given into DMBA-treated rats the incidence of tumors was reduced to 15% compared with the 29% incidence in rats treated only with DMBA (Fig. 13). These tumors were developed in the thoracic and abdomino-inguinal regions of the 32- and 37-week-old rats (Table 4). No tumors were developed in

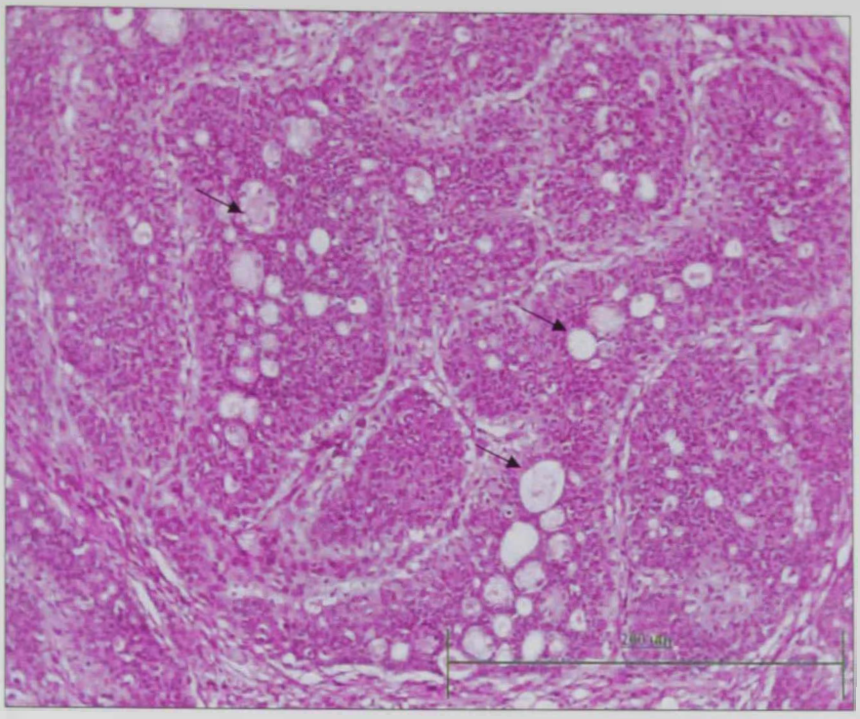

Fig. 11: Invasive cribriform carcinoma developed in DMBA-treated rat. Note the solid sheets of cells interrupted by secondary lumina (arrows). Magnification: x200.

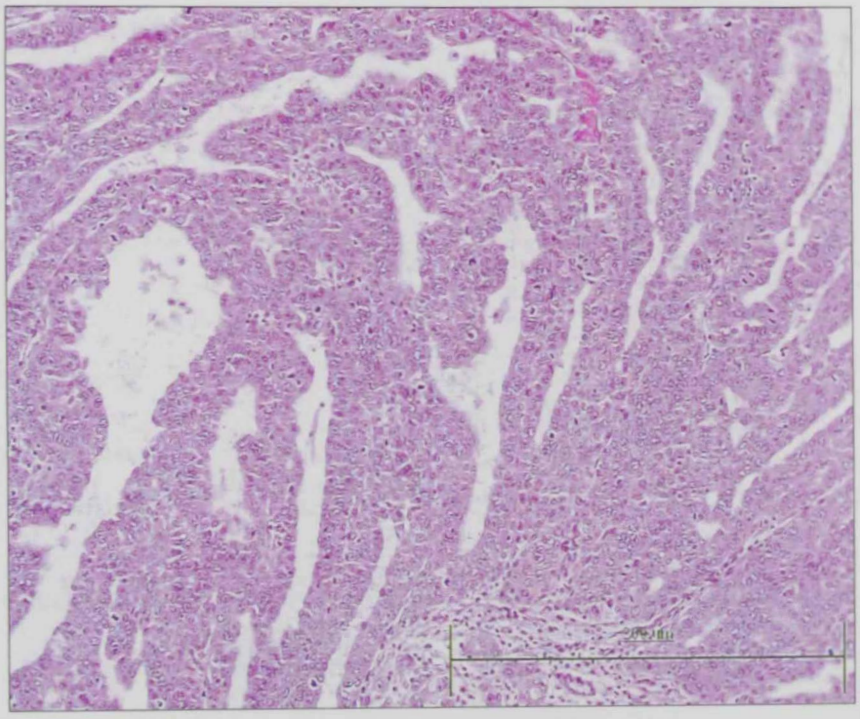

Fig. 12: Papillary carcinoma as it appear in a DMBA-treated rat. Notice the epithelial papillary growth with scanty connective tissue. Magnification: x200.

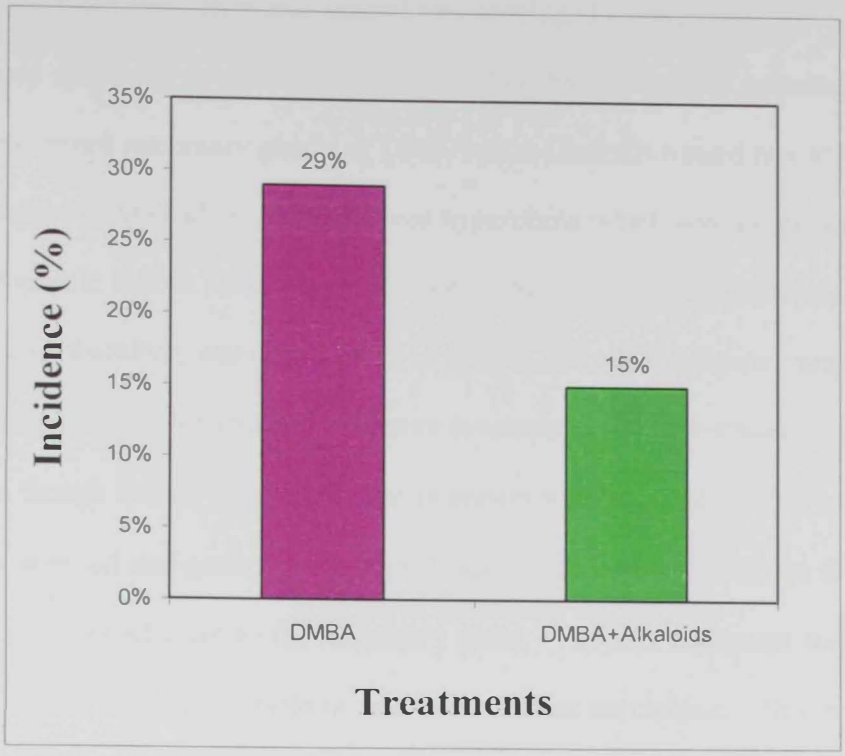

Fig. 13: Tumor incidence of DMBA-treated and DMBA-plus-alkaloids-treated rats. Note that treatment with *Vinca* alkaloids decreases tumor incidence by about 50%.

Table 4: Effects of *Vinca* alkaloids on the development of DMBA-induced mammary gland tumors.

	Age groups			
	32-week	**37-week**	**42-week**	**47-week**
No. of rats	5	5	5	5
Rats with tumor	2	1	0	0
Tumor location	- Thoracic - Abdomino-inguinal	Thoracic	-	-
Weight of tumor (gm)	- 2.5 - 5.6	0.14	-	-
Type of tumor	▸ *In situ* cribriform carcinoma ▸ Cribriform carcinoma ▸ Ductal solid, Cribriform & comedoform carcinoma	▸ Cribriform carcinoma	-	-

42- and 47-week-old rats. However several rats developed hyperplastic (n=13 out of 19 rats; 68%) and dysplastic (n=12 out of 19 rats; 63%) changes in their mammary glands. Among all examined mammary glands of DMBA-plus-alkaloids treated rats 45% showed prominent signs of cell death and 37% showed hyperplasia which was usually mixed with other pre-neoplastic lesions (Table 3). Only one of the 32-week-old rats developed about 5 gm tumor and, therefore, was sacrificed at 15 weeks. All other mammary tumor masses developed were very small compared to tumors developed in DMBA-treated rats.

Even though tumors developed after treatment with alkaloids tended to be small in size, they were all malignant. No benign lesions were developed except for the one appeared in the skin adjacent to the mammary gland. The first malignant lesion was a mix of two types: invasive cribriform and comedoform carcinoma. This tumor was characterized by multilayered neoplastic epithelial cell growth interrupted by secondary lumens with variable shapes and sizes in addition to large central areas with necrotic debris (Fig. 14A). The other tumors developed in the 32- and 37-week-old alkaloid-treated rats exhibited the same histological features of cribriform carcinoma which appeared in DMBA-treated rats (Fig. 14B and C).

It should be mentioned that some mammary glands of the DMBA-plus-alkaloid-treated 42- and 47-week-old rats showed an increase in the amount of fibrous tissues in the stroma. Also, 2 weeks before the planed date of sacrifice, one death was recorded in the 32-week-old group. Autopsy revealed no tumors and no obvious reason for death.

An additional group containing 4-rats was treated orally with the same dose of *Vinca* alkaloids as in the second group for three weeks in order to check any sign of toxicity by observing the general behavior and by examining mammary gland tissue sections. All mammary glands examined appeared normal compared to the control mammary glands.

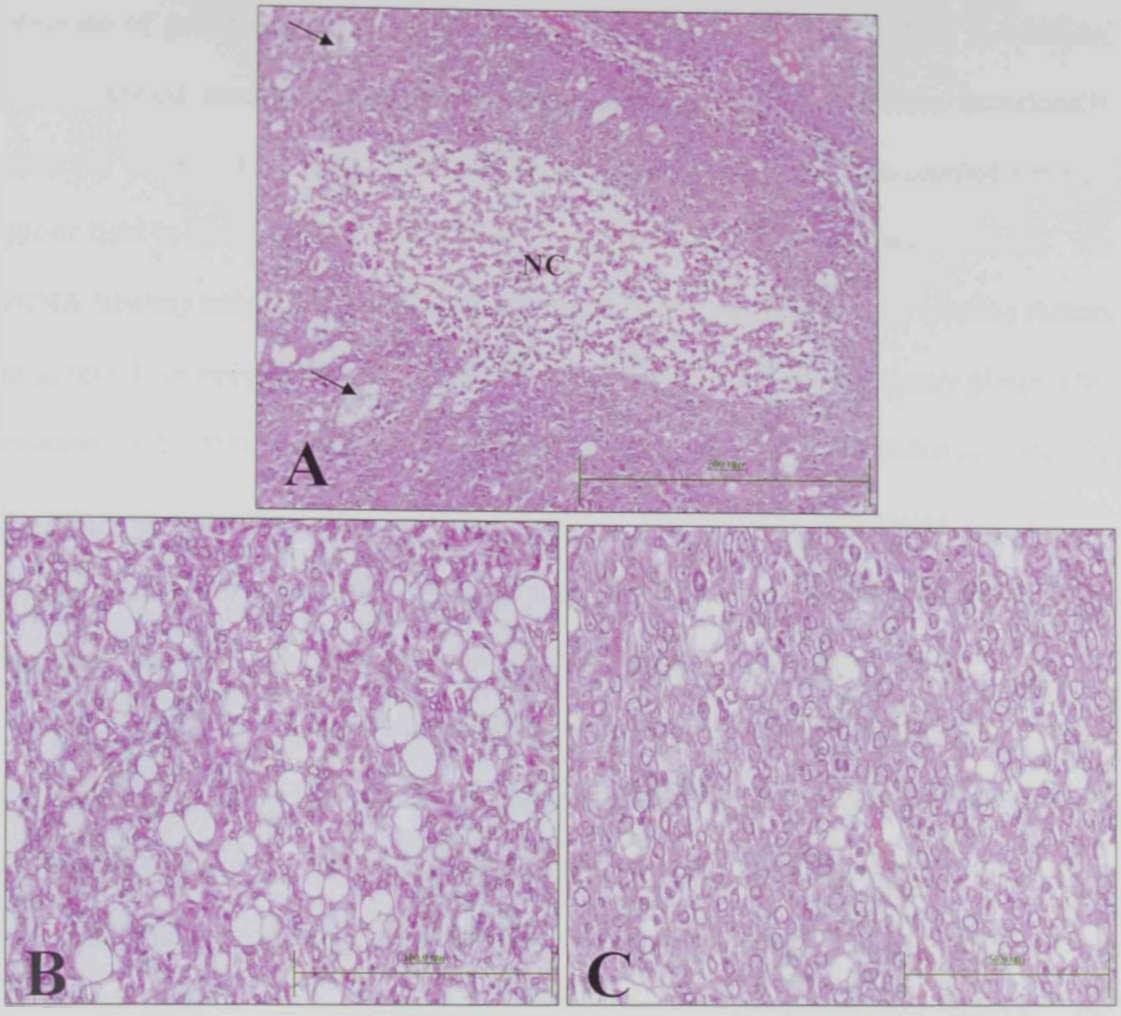

Fig. 14: Light micrographs of tissue sections obtained from rats treated with DMBA-plus-alkaloids. **(A)** Ductal solid, cribriform and comedoform carcinoma. Necrotic cellular debris (NC) and secondary lumens (arrows) are typical features of this tumor. **(B and C)** Cribriform carcinoma developed in 32- and 37-week-old rats, respectively. Magnification: x200 (A), x400 (B), x640 (C).

III.3. Immunohistochemical localization of PCNA and BRCA1

Immunolocalization of PCNA in the mammary glands of control rats revealed the presence of proliferating cells in some small terminal ducts (Fig. 15A). In addition, PCNA-labeled nuclei of myoepithelial cells in these control rats were occasionally observed (inset in Fig. 15A). The number of PCNA–labeled cells was counted per high power field in the mammary glands of all control and DMBA-treated rats examined. The PCNA labeling index was then calculated, and averaged. Comparing the labeling indices in different pre-neoplastic and neoplastic lesions developed in the mammary glands after treatment with DMBA revealed an increase in the number of PCNA-labeled cells starting from hyperplasia, then dysplasia (Fig. 15C and D, respectively) progressing to malignant lesions (Fig. 16A and C). However, during stage of cell death, the PCNA-labeling index was lower than that of control mammary glands (Fig. 15B; Table 5).

In mammary glands with palpable benign and malignant tumors, the PCNA-labeling indices were higher than those in the control and other nonpalpable lesions. The PCNA labeling was highest in the case of cribriform carcinoma (Table 5).

In rats treated with DMBA-plus-alkaloids, a decrease in PCNA labeling was noted during the stage of cell death similar to that of the DMBA-treated rats. However, the labeling index of PCNA showed an increasing trend during progression from hyperplasia to cancer (Table 5). When the labeling indices for each type of lesion in DMBA-treated rats were compared with those of DMBA-plus-alkaloids treated rats, a significant reduction in the PCNA labeling of the latter group was observed ($P<0.05$).

When PCNA immunolabeling was examined in various mammary gland tissues of rats treated with DMBA or DMBA-plus-alkaloids and compared with labeling in tissues of control rats, some variation in the labeling intensities were observed. These tissue

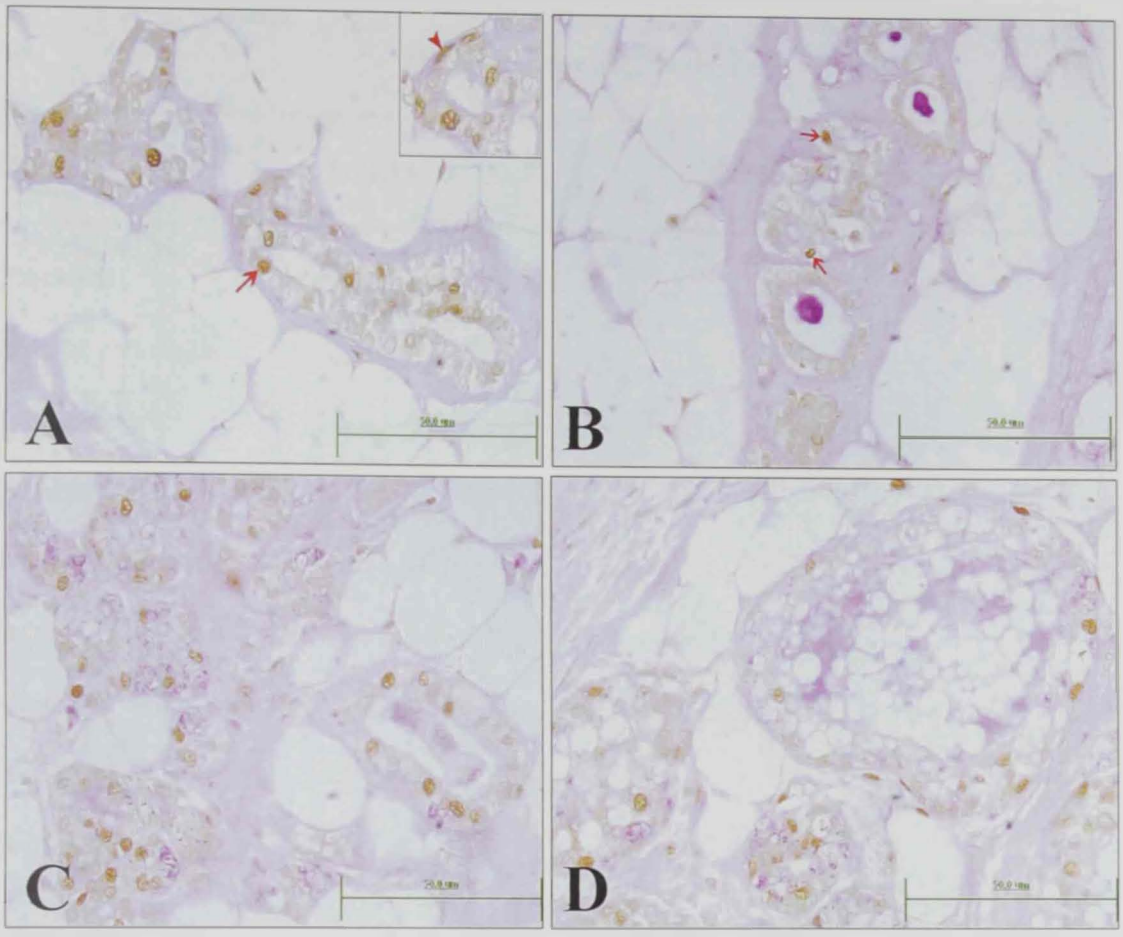

Fig. 15: PCNA labeling in control mammary gland (A) and in early pre-neoplastic lesions developed in DMBA-treated rats (B-D). **(A)** PCNA-labeled nuclei are seen in some epithelial duct cells of control mammary gland (arrow). In the inset, the nucleus of a myoepithelial cell expresses PCNA (arrow head). **(B)** Stage of "cell death" lesion after DMBA treatment showing a few PCNA-labeled cells (arrows). Notice increased secretory activity of the cells with accumulation PAS-stained material in the lumen. **(C)** Severe hyperplasia showing an increased number of PCNA-labeled cells. **(D)** Dysplastic mammary gland of a DMBA-treated rat. Notice the increased number of PCNA-labeled cells and accumulation of secretory material in the dilated lumen. Magnification: x640.

Table 5: PCNA-labeling indices (means ± SEM) estimated in normal tissues of control rats as well as in various pre-neoplastic and neoplastic lesions developed in rats treated with DMBA or with DMBA-plus-alkaloids.

	PCNA-labeling indices	
Tissues	**DMBA**	**DMBA-plus-alkaloids**
Control	12.3 ± 4.1*	7.5 ± 2.3**
Stage of cell death	2.3 ± 0.3	4.7 ± 0.3
Hyperplasia	12.7 ± 4.2	5 ± 1.5
Dysplasia	42.7 ± 13.9	23.7 ± 10.7
In situ cribriform	64.5 ± 27.6	44.7 ± 11.3
Lactating adenoma	25.3 ± 6.9	ND
Squamous cell papilloma	121.3 ± 20.3	ND
Papillary carcinoma	120 ± 5.3	ND
Cribriform carcinoma	179.7 ± 15.1	215.3 ± 18.2 91.7 ± 9.1 49.7 ± 4.5

*Treated with corn oil only.
**Treated with alkaloids only.
ND = not detected

sections were processed, cut, mounted, and immunoprobed together. So, variation in PCNA labeling intensity was taken to reflect changes in the expression of PCNA protein.

Measurements using Scion computer program revealed no significant difference in PCNA labeling intensity in the lesions developed in DMBA-treated rats when compared with those of control rats. However, by comparing the intensity of PCNA protein expression in the tumors developed in DMBA-treated rats with tumors developed after treatment with DMBA-plus-alkaloids, it seemed that the intensity of PCNA immunostaining was decreased (Fig. 16; A *vs* B and C *vs* D). Measurements showed a statistically significant difference ($P < 0.05$) between labeling intensity in DMBA-treated tissues and intensity in DMBA-plus-alkaloids treated tissues (Fig. 17).

Immunohistochemical localization of BRCA1 in control rats showed that this protein was present mainly in the nuclei of some luminal epithelial duct cells (Fig. 18A). Faint cytoplasmic stain was also observed in some small ducts, which was more apparent in the large ducts. Early microscopic lesions (pre-hyperplasia, hyperplasia and dysplasia) developed in DMBA- and alkaloids-treated rats showed both nuclear and cytoplasmic localization of BRCA1 (Fig. 18B). The nuclear and cytoplasmic localization of BRCA1 was also noted in lactating adenoma and squamous cell papilloma. However, in case of localized and invasive carcinomas (*in situ* cribriform, cribriform and papillary carcinoma), BRCA1 protein became mostly cytoplasmic (Fig. 18C).

III.4. Expression of p53 protein

Homogenized mammary gland tissue samples obtained from all control and treated rats were analyzed by Western blotting to study the expression of p53 protein during breast cancer progression and to test whether *Vinca* alkaloids has an effect on its expression. Representative results of three different experiments are shown in figure 19.

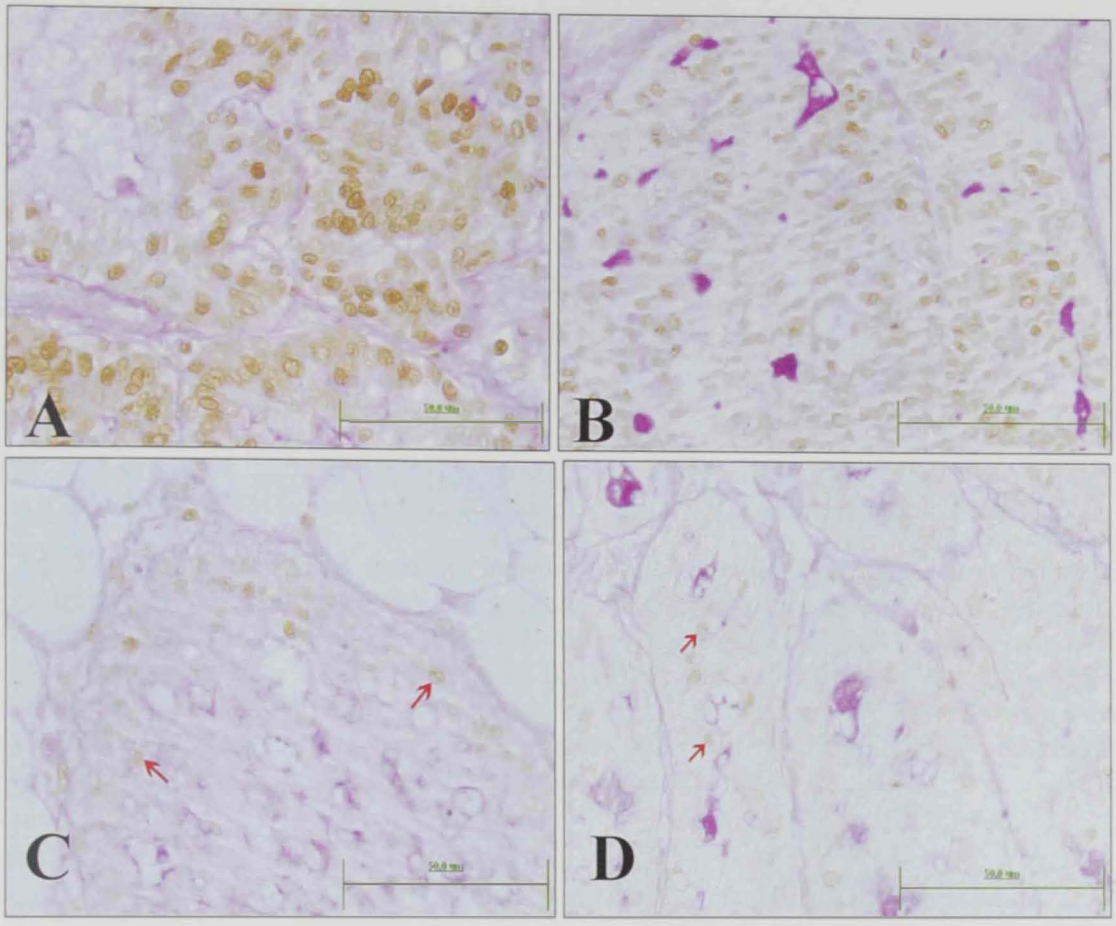

Fig. 16: PCNA expression in DMBA-treated (A and C) and DMBA-plus-alkaloids-treated (B, D) rats. **(A)** Cribriform carcinoma treated with DMBA shows higher number of cells expressing PCNA compared with control. **(B)** In cribriform carcinoma after treatment with alkaloids, the expression of PCNA is down regulated. **(C)** *In situ* cribriform carcinoma showing PCNA labeling in the periphery of dilated duct (arrows). **(D)** Expression of PCNA in cribriform carcinoma of alkaloids-treated rat showing low intensity of PCNA labeling compared with C (arrows). Magnification: x640.

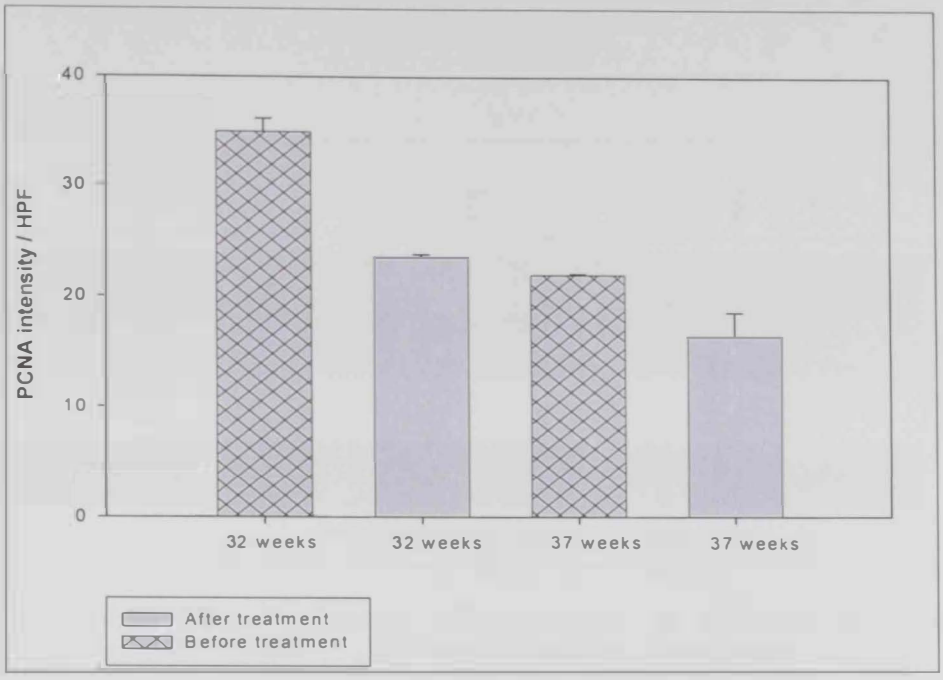

Fig. 17: Bar plot of PCNA intensity measured in four mammary gland lesions similar to those shown in figure 16 (A-D) and plotted in the same order from the left: cribriform carcinoma of DMBA-treated rat (32-week-old rat) and in DMBA-plus-alkaloid-treated rat (32-week-old rat), *in situ* cribriform in DMBA-treated (37-week-old rat) and then in DMBA-plus-alkaloid-treated (37-week-old rat) rats. Analysis of PCNA intensity using *student-t-test* showed significant decrease in the intensity ($P<0.05$) of the lesions developed in 32-week-old rats after treatment with alkaloids.

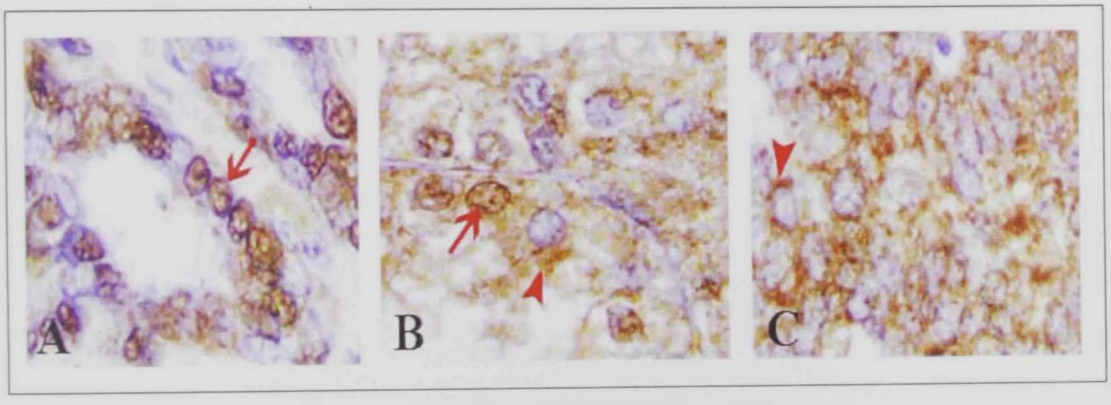

Fig. 18: Immunoreactivity of BRCA1 in mammary glands of control (A) and DMBA-treated rats (B and C). **(A)** Normal mammary gland of control rat expresses BRCA1 mainly in the nuclei of duct epithelial cells (arrow). **(B)** Dysplastic mammary gland shows BRCA1 in both the nucleus (arrow) and cytoplasm (arrow head) of epithelial cells. **(C)** Cribriform carcinoma showing mostly cytoplasmic expression of BRCA1 (arrow head). Magnification: x1000.

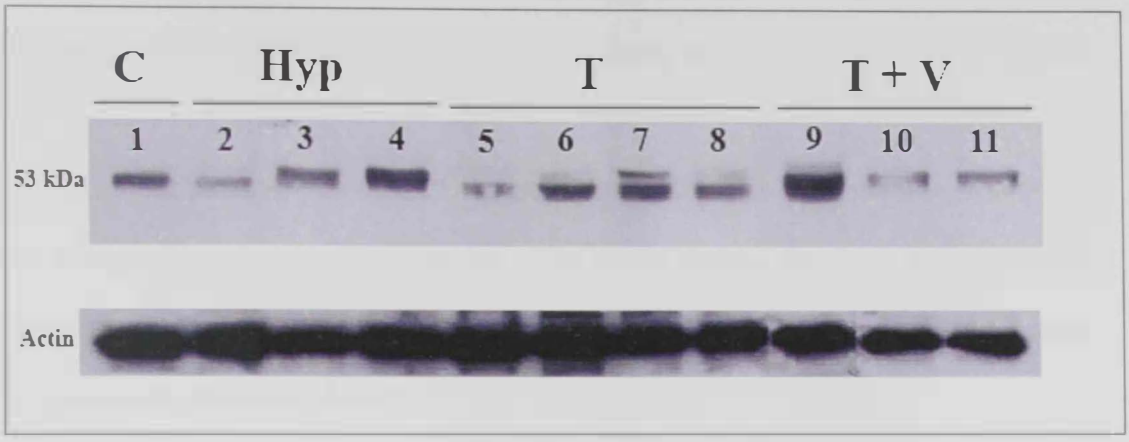

Fig. 19: Immunoblot analysis showing p53 expression in mammary gland tissues obtained from control rat (C; lane 1) and DMBA-treated rats with hyperplasia (Hyp; lanes 2-4) and tumors (T; lanes 5-8). Lanes 9-11 shows results from rats treated with DMBA-plus-*Vinca* alkaloids (T+V). The bottom panel shows immunoblotting for the housekeeping protein, actin, to reflect the amount of proteins loaded in each lane.

Comparing to control tissue (Fig. 19; lane 1), initial down regulation in some hyperplastic and benign lesions was observed (Fig. 19; lanes 2 and 5, respectively). As the mammary gland progresses toward malignancy, the amount of p53 was increased. This was probably the mutant form detected by using anti-p53 antibody clone PAb240 (Fig. 19, lanes 3, 4 and 6-8).

Tumors obtained from DMBA-treated rats which also received *Vinca* alkaloids were analyzed for p53 protein expression. The results showed that there was a tendency to detect only a small amount of p53 (Fig. 19; lanes 10 and 11). Only one tumor from rats treated with alkaloids showed high level of p53 (Fig. 19; lane 9).

III.5. Effects of DMBA or DMBA-plus-alkaloids on PCNA, P53 and BRCA1 genes in rat mammary glands

To test whether DMBA-treated rats with various pre-neoplastic and neoplastic mammary gland lesions developed mutations in PCNA, P53 and BRCA1 genes, PCR-SSCP and PCR-RFLP assays were used.

Amplification of PCNA exon 1 yielded 230 base pair (bp) product (Fig. 20), which was analyzed for base change-induced mobility shifts under undenatured condition of SSCP. Control animals displayed the normal banding pattern of SSCP analysis, which appeared as four main bands (Fig. 21). Administration of DMBA alone or with alkaloids did not induce any abnormalities in the banding pattern of PCNA exon 1.

PCR products of P53 exon 5 revealed 270 bp amplicon for all samples when compared to the standard DNA molecular marker (Fig. 20). SSCP analysis of the 270 bp product of P53 exon 5 of control mammary glands showed two bands as indicated in figures 22 and 23. Out of 9 tumors, two of them (22%) exhibited band shift (three extra bands) with electrophoresis in addition to the two bands appeared in control sample.

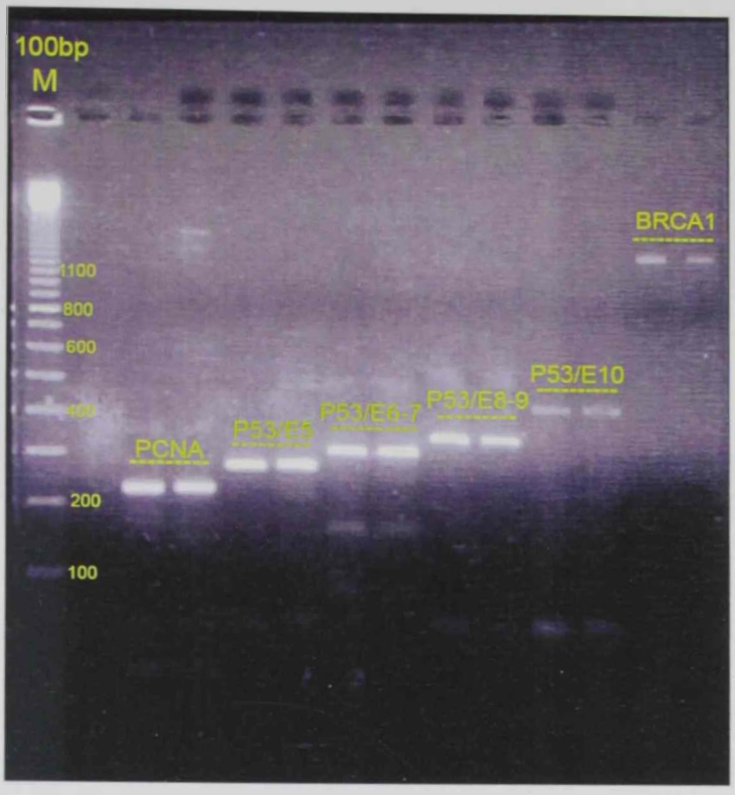

Fig. 20: PCR products of PCNA exon 1, various exons of P53 and BRCA1 exon 11 analyzed by electrophoresis in 1.5% agarose gel stained with ethidium bromide. The first lane on the left (M) shows the 100 base pair ladder marker.

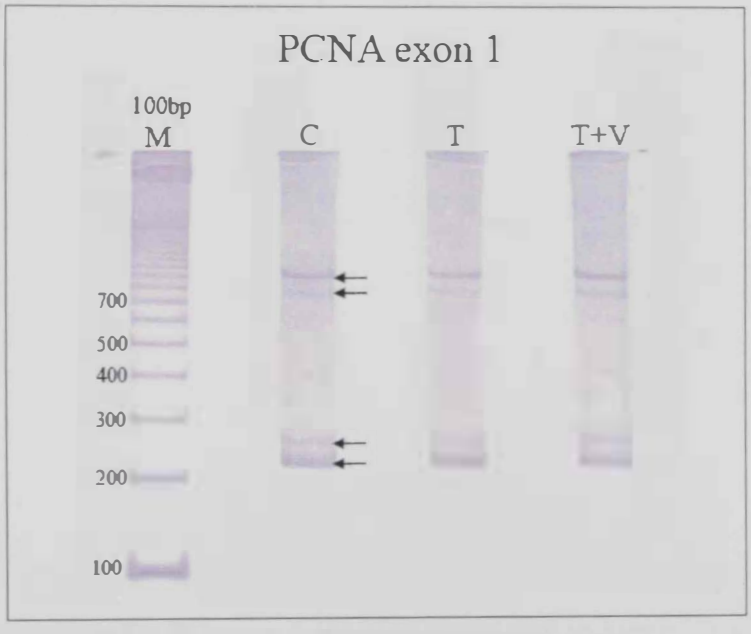

Fig. 21: SSCP analysis of the PCR products of PCNA exon 1 for control tissues (C), and tumors obtained from DMBA-treated (T) and DMBA-plus-alkaloids-treated (T+V) rats. Arrows indicate main bands of PCNA exon 1 under SSCP. The first lane on the left (M) shows the 100 base pair ladder marker.

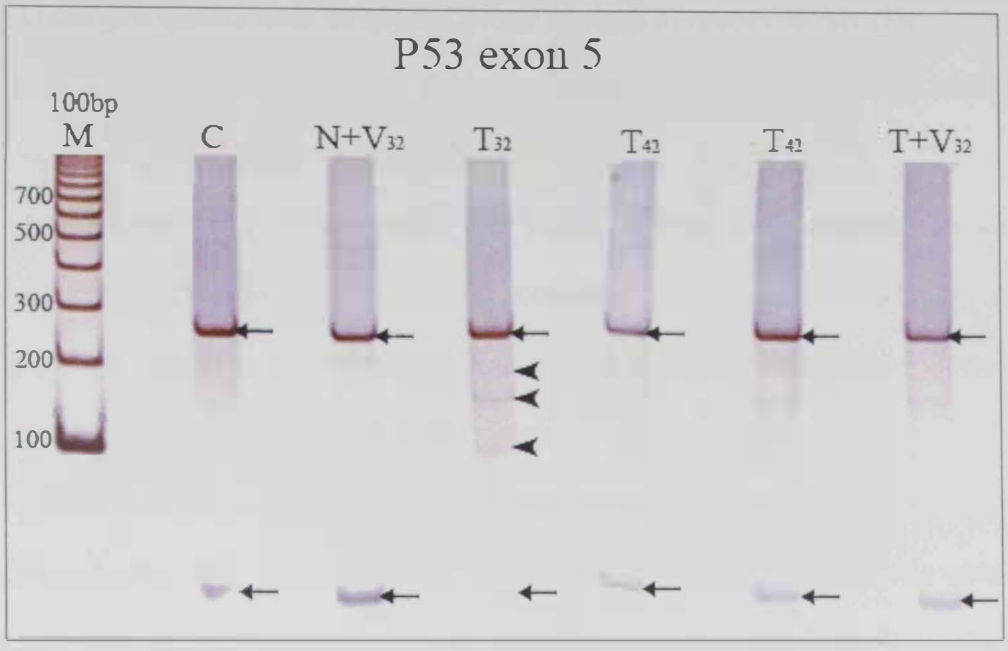

Fig. 22: SSCP analysis of the PCR products of P53 exon 5 in control (C), DMBA-treated (T) and DMBA-plus-*Vinca* alkaloids-treated (N+V and T+V) rats. Notice the loss of normal banding pattern in one DMBA-treated tumor (T32). The bands of the hyperplastic (N+V) and tumor (T+V) samples of the DMBA-plus-alkaloids-treated rats are similar to those of the control.

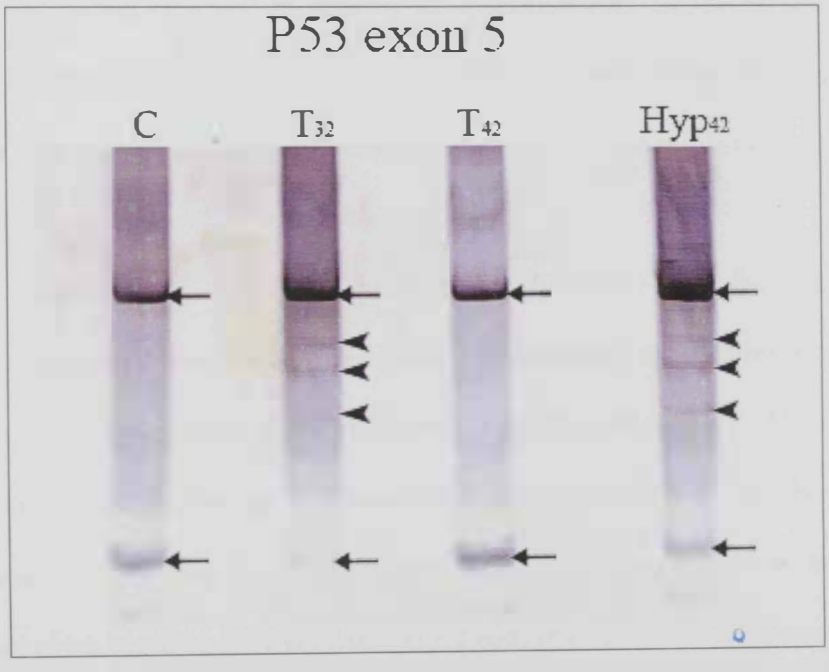

Fig. 23: SSCP analysis for P53 exon 5 showing bands shift in hyperplasia (arrow heads in lane Hyp42) and tumor samples (arrow heads in lane T32 and T42) compared to control pattern. Arrows indicate main SSCP bands.

These two tumors were developed in the 32- and 37-week-old rats (Fig. 22 and 23). In addition, some hyperplastic mammary glands showed similar band shifting similar to that of tumor samples shown in figure 23.

Tumor samples of DMBA-treated rats which received *Vinca* alkaloids showed normal banding pattern with no evidence of abnormality. The remaining samples showed banding pattern similar to that of control (Fig. 22 and 23). No hyperplastic mammary glands exhibited abnormal band shift in this group.

PCR amplification of exon 6-7 of P53 gene produced 300 bp band for all samples in agarose gel (Fig. 20). SSCP analysis displayed three bands representing the normal pattern of P53 exon 6-7. Analysis of DMBA-treated samples revealed bands similar to those of control with no shift detected in the polyacrylamide gels (Fig. 24). Normal banding pattern was also detected in rats treated with DMBA-plus-alkaloids.

All PCR products of P53 exon 8-9 from control and treated mammary glands showed single amplicon indicated by agarose gel electrophoresis at 350 bp (Fig. 20). In Figure 25, SSCP analysis of P53 exon 8-9 showed two bands similar to the pattern of exon 5. This fragment showed no difference in the mobility among tumors and control before and after treatment with *Vinca* alkaloids.

SSCP analysis of the 410 bp fragment of exon 10 of P53 gene showed two main bands in the control mammary gland. Band shift was observed in two tumors out of nine (22%), which were all from rats treated with alkaloids (Fig. 26 and 27). The number of bands and their position in the gel indicated DNA sequence variation. In figure 26, the sample in lane $T+V_{32}$ shows two more extra bands, while the sample in lane $T+V_{37}$ produced three extra bands as compared to control pattern of corresponding exon.

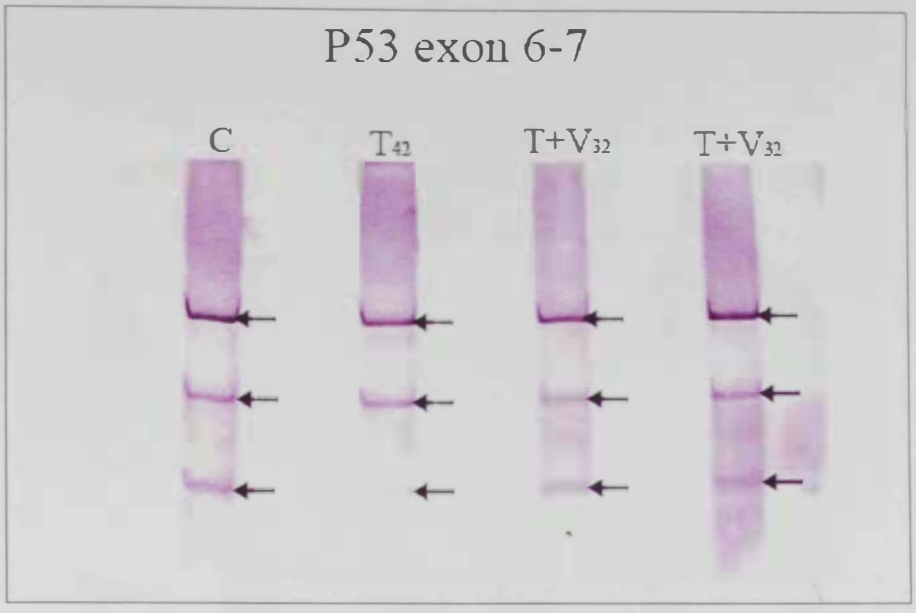

Fig. 24: The SSCP analysis of the P53 exon 6-7 PCR products of control tissue (C), and tumors of DMBA-treated (T42) and DMBA-plus-alkaloids-treated rats (T+V32 and 42). Notice that there is no difference in the SSCP pattern in all samples obtained from treated rats compared with control pattern in lane C.

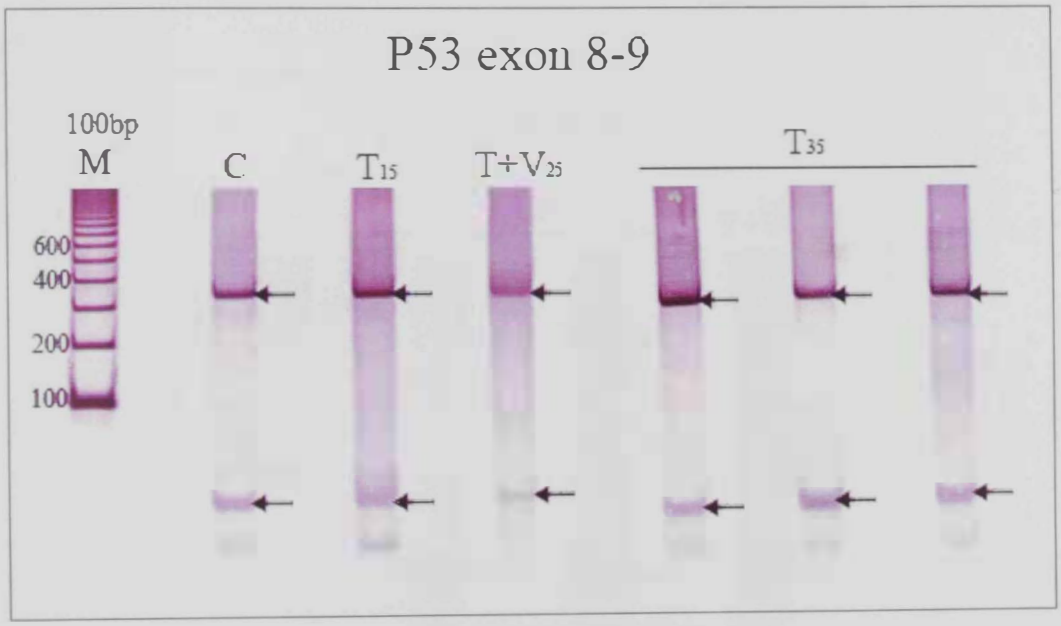

Fig. 25: The SSCP analysis of the PCR products from P53 exon 8-9 of control tissue (C), and tumors obtained from DMBA-treated (T) and DMBA-plus-alkaloids-treated rats (T+V).

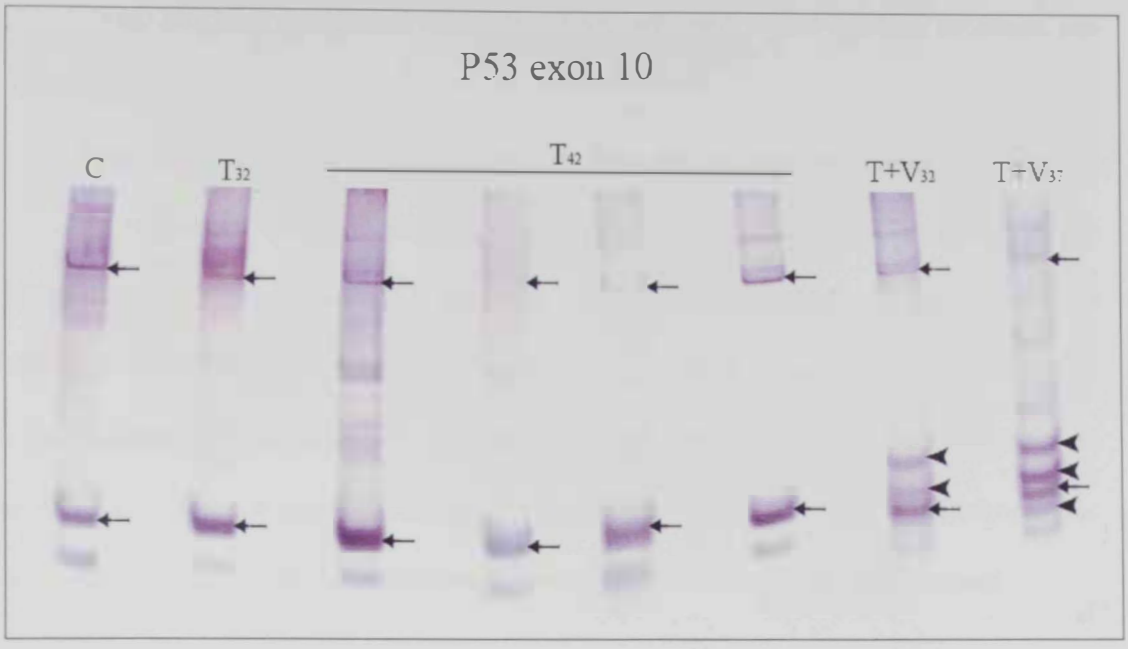

Fig. 26: The SSCP analysis of the PCR products of p53 gene exon 10 of control tissue (C), and tumors obtained from DMBA-treated (T) and DMBA-plus-alkaloids-treated rats (T+V). Notice clear bands shift in T+V lanes.

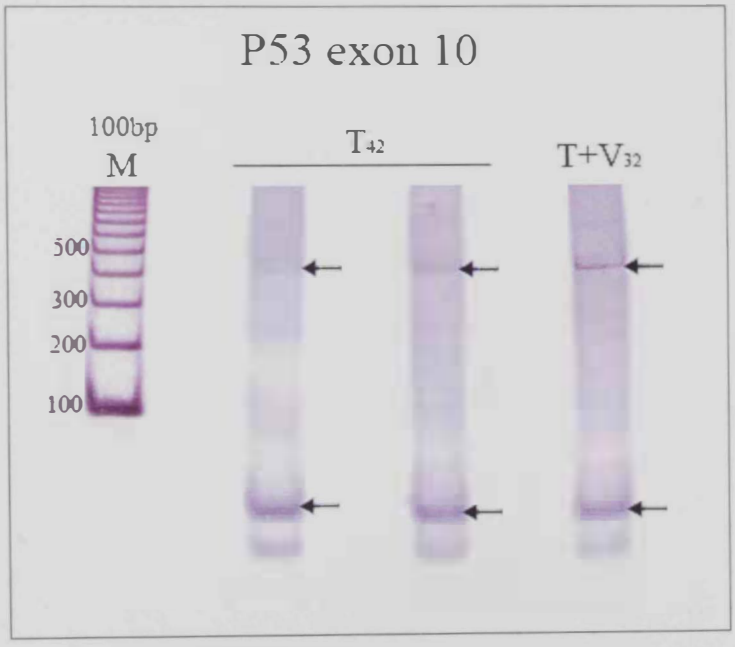

Fig. 27: SSCP analysis of the PCR products of p53 gene exon 10 of tumors obtained from DMBA-treated (T) and DMBA-plus-alkaloids-treated rats (T+V). Molecular marker (100bp) indicates the size of resulted fragments.

The PCR products of exon 11 of BRCA1 gene revealed more than one band on the gel (Fig. 28, lane undigested C). Therefore, we used RFLP method to detect the polymorphism.

Four different enzymes were used to find the appropriate cutter for BRCA1 exon11 PCR product, they are: XhoI, PSTI, BamHI, and EcoRI. BamHI was found to be the appropriate cutter for BRCA1 fragment. PCR product of BRCA1 exon 11 was at 1600 bp, which was the biggest size obtained using specific primer sequence mentioned in the material and methods section. In figure 28, the digested products showed no remarkable difference between control and tumor samples. Also, no difference was observed between tumors from rats treated with or without *Vinca* alkaloids extract.

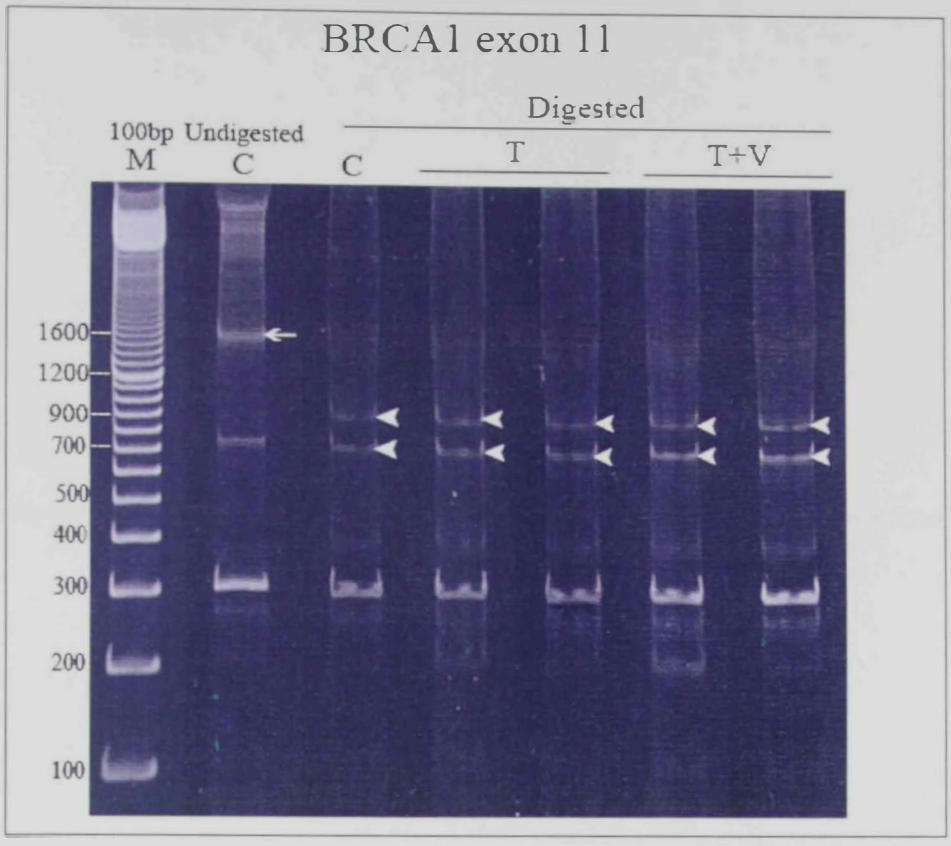

Fig. 28: PCR-RFLP analysis of BRCA1 exon 11 in tumors obtained from DMBA-treated (T) and DMBA-plus-alkaloid-treated (T+V) rats. Undigested band at 1600 bp in lane C (from the left) represents BRCA1 target product which was digested with BamHI and produced two bands at 900 bp and 700 bp (lanes digested C, T and T+V). All samples produced same pattern compared to digested control.

CHAPTER IV

DISCUSSION

CHAPTER IV
DISCUSSION

In the present study, some of the morphological and molecular changes that occur during breast cancer development have been characterized in female Wistar rats treated with a single dose (80 mg/kg body wt) of DMBA. Some of these DMBA-treated rats received crude extract of *Vinca* alkaloids to test whether it has an effect on the incidence of breast cancer and the early changes that occur during its progression. This study was carried out with special emphasis on PCNA, as a cell cycle protein, and p53 and BRCA1 as tumor suppressor genes.

IV.1. The sequence of events that occur during development of pre-neoplastic lesions in DMBA-treated rats

A series of microscopic changes have been observed in the mammary glands of DMBA-treated rats. These changes are taken to be representative of the multistep process that occurs before development of breast cancer.

In the past, Beckmann et al. (1997) described the first two stages of breast cancer development in humans as hyperplasia and carcinoma *in situ*. When Singh et al. (2000) induced breast cancer in Sprague Dawley rats with 1-methyl-1-nitrosourea, hyperplasia was also the first histopathological change observed. Even though these hyperplastic ducts of mammary glands are poorly studied, their presence has become a valuable indicator of malignant progression in both humans and rats (Lee et al., 2006; Weroha et al., 2006).

In the DMBA-treated rats examined in this study, some mammary glands developed hyperplasia in association with other pre-neoplastic lesions (42.4% of all

mammary glands examined in DMBA-treated rats). In a few mammary glands (2.4%) carcinoma *in situ* was also observed.

The majority of DMBA-treated mammary glands (93.4%) examined in the present study developed another form of a histopathological change (Table 3). These glands exhibited moderately dilated terminal ducts characterized by numerous dead cells extruded into their lumens (Fig. 5). This morphological change was observed either alone (46% of all DMBA-treated mammary glands examined) or in association with other pre-neoplastic lesions (47.4%). It is generally believed that when a cell dies, it releases some factors that stimulate neighboring post-mitotic cells to re-enter the cell cycle. It was reported earlier that in lymphoid and vascular epithelial tissues, apoptosis precedes and leads to hyperplasia (Volkmann et al., 1996; Izumi et al., 2003). Thus, the stage of cell death described has been considered to occur earlier than hyperplasia, and taken to be the first stage in the multistep process of breast cancer development in the DMBA-treated Wister rats.

Dysplasia of the mammary glands is the third pre-neoplastic lesion detected in the DMBA-treated rats. Dysplasia appeared either alone (1.2%) or, more commonly (18.2%) in association with other pre-neoplastic lesions (Table 3). Similar features of this lesion were also described by Xie et al. (1999) when breast cancer was induced in rats with a combination of 17β-oestradiol and testosterone.

It has been reported earlier that pre-neoplastic changes of the mammary gland includes intraductal noninfiltrating carcinoma, or carcinoma *in situ* (Komitowski et al., 1982). In the present study, 9.5% of the rats treated with DMBA developed intraductal noninfiltrating carcinoma *in situ*. When all the DMBA-treated mammary are considered, it is estimated that carcinoma *in situ* occurs in only 2.4% of them. It occurs in association with other pre-neoplastic lesions (Table 3). It has already been shown that DMBA

treatment alters the normal process of mammary gland differentiation of terminal ducts to alveoli and lobules, producing instead the sequence of terminal ducts → intraductal proliferation → carcinoma *in situ* → invasive carcinoma (Russo et al., 1982). Extensive studies on the unique features of *in situ* carcinoma reveal its significance in predicting the aggressiveness of breast cancer (Lennington et al., 1994; Rehman et al., 2000).

IV.2. Tumors developed in the mammary glands of DMBA-treated Wister rats are mostly malignant.

In this study, the incidence of mammary tumors which was induced in Wistar rat using DMBA is 29%. In contrast, Bojkova et al. (2000) found that administration of 20 mg of DMBA in Wistar:Han rats resulted in about 8% tumor incidence. Repeated DMBA administration (3 doses of 10 mg/rat) increased the incidence to 85%. When the more sensitive Sprague Dawley rats were administered with the same single dose of DMBA, they showed higher incidence of tumor development, 46 and 67%, which reached up to 100% with multiple doses (Kubatka et al., 2002).

These differences in rat susceptibility could, possibly, be due to the age when the carcinogen was administered. The incidence reached the highest rate between postnatal days 40-46, when cells of the terminal ducts have maximal proliferative activity. The physiological changes through seasons may also play a substantial role in determining the susceptibility to carcinogenesis (Kubatka et al., 2002). All rats used in the present study received DMBA at almost the same age and during the same season.

The vast majority of tumors observed in DMBA-treated rats of the present study are carcinomas (Table 2). This is consistent with previous studies using DMBA or 1-methyl-1-nitrosourea as a carcinogenic agent (Russo and Russo, 1996; Liska et al., 2000; Costa et al., 2002).

The most highly malignant tumors in the rat have some common features with the infiltrating ductal carcinoma of the human (Russo et al., 1990c). In this study, two different forms of carcinoma were observed: cribriform and papillary; the former has been observed more commonly. However, Russo and Russo (2000) reported that invasive papillary carcinoma is the most typical and frequent DMBA-induced tumor.

Two other forms of tumors are also observed in the present study and classified as "benign". They include: 1) lactating adenoma which was previously identified and well characterized (Russo and Russo, 2000; Costa et al., 2002), and 2) squamous cell papilloma which is identified in the present study in DMBA-treated rats for the first time. Based on the features described in the results section, it is hypothesized that the origin of squamous cell papilloma is probably the lactiferous or large ducts which are close to the nipple.

While benign tumors of the mammary glands that occur in the rats closely resemble those of humans, they are observed more frequently in humans than in rats treated with 1-methy-l-nitrosourea (Russo et al., 1990b; Singh et al., 2000). However, Liska et al. (2000) were not able to detect any benign tumors by using similar carcinogen in rats. In the present study, benign tumors are observed less frequently than malignant lesions. It is postulated that the less differentiated terminal ducts develop invasive cancer lesions when attacked by DMBA, while benign lesions such as adenomas arise from the more differentiated big ducts (Russo et al., 1990b). If this is the case, then the reason of developing more carcinomas than benign lesions could simply be due to the structural organization of the tree-like branching duct system of the mammary gland in which the small terminal ducts are much more numerous than the big ones.

IV.3. An initial decrease followed by a gradual increase in cell proliferation features breast cancer development in DMBA-treated rats.

PCNA has been described as one important predictive and prognostic factor which is used widely as a marker for cell proliferation in the clinical assessment of various tumors including breast cancer (Wu et al., 2003). In the present study, the PCNA-labeling has also been used to correlate the rate of cell proliferation with each of the various pre-neoplastic and neoplastic changes observed in DMBA-treated rats.

The nuclear expression of PCNA in the mammary glands of control rats shows that cell proliferation is mainly found in the small terminal ducts. This finding confirms previously published data (Qiu et al., 2005). The occasional presence of PCNA-labeled myoepithelial cells indicates that these differentiated cells maintain some capacity for mitosis (Fig. 15A).

In DMBA-treated rats, progression toward breast cancer is found to be associated with an increase in the number of PCNA-labeled cells starting at the stage of hyperplasia (Table 5). This observation correlates with those of Funakoshi et al. (2000) who studied canine mammary gland tumors. However, unexpectedly, there was a reduction in PCNA labeling during the initial stage of "cell death". This might be related to the deregulation phenomena of neoplastic progression (Hoshino, 1992).

In an attempt to evaluate the correlation between breast cancer progression and the intensity of PCNA labeling, quantitative measurements were carried out using Scion image program. It is generally noticed that the intensity of staining gradually increases with progression toward breast cancer. Similarly, Mo et al. (2004) reported that the expression of PCNA was significantly related to progression of nasopharyngeal tumor in humans. However, Surowiak et al. (2005) used another proliferation marker, Ki-67, and

found it useful in correlating its labeling intensity with the grading of ductal breast cancer.

IV.4. Alteration of p53 during development of breast cancer in rats

P53 is a tumor suppressor gene which has an apparent role in the development breast cancer in humans, mice, and rats (Hollstein et al., 1991; Olivier et al., 2002; Zurer et al., 2004). In the present study, the expression of p53 protein was studied in all lesions obtained from DMBA-treated rats using mouse monoclonal antibody, PAb-240 clone. This antibody detects both wild-type and mutant forms of p53 (Gannon et al., 1990). The results reveal that p53 is differentially expressed during breast cancer progression. It is down-regulated in pre-neoplastic lesions, but up-regulated in malignant tumors. Lee et al. (2004) reported the significant correlation between p53 mutations and p53 over-expression. Also it was reported that p53 protein expression in benign tumors is much less compared to that in malignant lesions. By using the same clone of antibody, upregulation of the mutant form of p53 is observed in the advanced stages of human breast cancer (Andersen et al., 1993). These results are consistent with the data obtained in the present study.

The correlation between p53 accumulation and the increase in tumor cell proliferation has been also demonstrated in the present study. Therefore, as previously reported, it seems that p53 negatively regulates cell division and its mutation leads to aberrant regulation of cell proliferation (Kesari et al., 1997).

An increasing number of studies are using molecular methods to analyze P53 mutations in cancer tissues. Mutations in p53 gene are very common in human cancers; they occur in 20-40% of breast cancer cases (Kalemi et al., 2004). In comparison to

breast cancer, 50% of adult cancers such as colon, stomach, liver and others are also characterized by p53 mutations (Jerry et al., 2000).

Most of the P53 mutations reported in different types of tumors are clustered within exons 4–8, which encode a highly conserved region, containing the DNA binding domain of the protein (Jerry et al., 2000). In the present study, four of the exons of p53 gene (exon 5, 6-7, 8-9 and 10) were studied for possible mutations that may occur during breast cancer development. It was possible to detect P53 polymorphism in some rats with hyperplastic mammary glands using SSCP technique (Fig. 23). The present results correlate with those of Keohavong et al. (2004) who reported P53 mutations in individuals with pre-invasive mammary gland lesions: atypical ductal hyperplasia and ductal carcinoma *in situ*. In mice, Jerry et al. (1993) also reported over-expression of p53 and its mutation in hyperplastic mammary glands.

It has been estimated that 33.3% of the tumors analyzed in the present study show polymorphism in P53 exon 5. In the DMBA-treated rats, base substitutions at A or G in the sense strand of the cDNA accounted for 95% of all the point mutations of P53 gene (Manjanatha et al., 1996). The predominance of purine (A or G) mutations is consistent with the fact that DMBA-adduct formation preferentially occurs on dA and dG (Manjanatha et al., 1996), leading to depurination (Chakravarti et al., 1995).

IV.5. Blockage of BRCA1 translocation during development of breast cancer in DMBA-treated rats

In the present study, BRCA1 localization using immunohistochemistry revealed nuclear expression in luminal epithelial duct cells. Progressive histopathological changes toward breast cancer are associated with alteration in nuclear localization of BRCA1 until it becomes cytoplasmic in malignant tumors. Similar cytoplasmic localization of BRCA1

in breast carcinoma was detected in studies conducted in humans using the same clone of the antibody (Chen et al., 1996; Schofield et al., 2000). By using different antibodies specific for BRCA1, similar results were obtained. Feng et al. (2004) reported increased amount of cytoplasmic expression of BRCA1 in breast cancer cells after exposure to ionizing radiation, suggesting a role played by p53 in the alteration of this translocation process. It has been proposed that elimination of BRCA1 from the nucleus may be required for the cell to stop dividing in response to DNA damage to avoid genomic instability (Feng et al., 2004).

In order to examine whether BRCA1 gene has been altered, RFLP technique was used. In the present study, no mutation has been detected in exon 11 of BRCA1 gene of all rats examined. Therefore, based on these results and those available in the literature (Lee et al., 1999), it seems that alteration of BRCA1 localization may be due to abnormality of molecules involved in the transport rather than mutation of the BRCA1 gene itself. Feng et al. (2004) reported that DNA damage cause breast cancer progression that is associated with the production of an inactive form of BRCA1. The latter can not be targeted to the nucleus and, therefore, remains in the cytoplasm of cancer cells. Thus, impairment of the nuclear localization signal and/or the nuclear export signal of BRCA1 protein are possible explanations for the abnormal cytoplasmic localization of BRCA1 during breast cancer progression.

IV.6. Inhibition of breast cancer progression in rats by *Vinca* alkaloids

The DMBA-induced cancer model has been used extensively to test the preventive and curative potential of a variety of chemical compounds (Himes, 1991; Rajeshkumar et al., 2005; Kotsopoulos et al., 2005; Lai and Singh, 2006). It has been shown that a single purified or semi-synthetic *Vinca* alkaloid, such as vinorelbine, has an

inhibitory effect on breast cancer progression. Sui and Fan (2005) found that *Vinca* alkaloids (vincristine and vinblastine) alter some DNA-binding proteins, which in turn inhibited cell cycle progression. Other studies reported that *Vinca* alkaloids (vincristine and vinorelbine) may induce bcl-2 phosphorylation, and therefore inhibiting its antiapoptotic activity (Pathan et al., 2001, Wang et al., 1999). Recently, Martinez-Campa et al. (2006) attributed the effect of *Vinca* alkaloids (vincristine, vinblastine, and vinorelbine) to decreased expression of estrogen receptor alpha protein and its mRNA levels in the human breast cancer cells.

In addition to the effects of a single alkaloid on the progression of breast cancer, we hypothesize that a combination of more than one alkaloid may have a synergistic effect and become more effective in the inhibition of breast cancer cell growth.

Grover et al., 2002 reported that oral administration of 200 mg/kg body wt/day of crude extract of the areal parts of *Vinca rosea* plant for several weeks has no toxic effect on the rats. In the present study, the same dose, 200 mg/kg body wt, but of a crude *Vinca* alkaloids extracted from the areal parts of *Vinca rosea* has been introduced into rats during the initiation phase of breast carcinogenesis (7 days post DMBA administration). An inhibitory effect on the tumor development has been observed. Only 15% of the rats developed tumors, i.e. 50% less than what developed in DMBA-treated rats. Tumors appeared after alkaloid treatment were not only few in number, but were also very small in size (2.5 and 0.14 gm).

It seems that the early treatment during the initiation phase of breast cancer development is crucial. Rao et al. (1985) reported that an extract prepared from the leaves of *Piper betle* blocks the progression of the neoplastic progression in DMBA-treated rats. In contrast, treatment of the rats with the same extract at a later phase after development of tumors didn't show any significant inhibition in tumor growth. Similar

results were also observed by Russo et al. (1990a). They found that early administration of chorionic gonadotropin for 21 days into DMBA-treated rats decreased the incidence of mammary tumors. These results were attributed to the ability of chorionic gonadotropin to induce p53-dependent programmed cell death in tumor cells (Srivastava et al., 1997). However, Rao (1981) found that exposure of virgin female rats to asparagus-root-extract-diet before the administration of DMBA was associated with a reduction in the incidence of mammary tumors. This effect was due to enhanced differentiation of terminal ducts which, therefore, became less susceptible to carcinogenesis. In the present study, an attempt has been made to provide some explanation for the inhibitory effects of crude *Vinca* alkaloids on the incidence of breast cancer by examining some genes involved in cell cycle regulation.

While immunohistochemistry and RFLP analysis revealed no effect for *Vinca* alkaloids on the protein product and DNA sequence of BRCA1 gene, protein products of both PCNA and p53 genes appeared to be directly or indirectly affected by these crude alkaloids.

IV.6.1. Treatment with *Vinca* alkaloids down-regulates PCNA expression in DMBA-treated rats

It is well known that antibodies specific for PCNA can be used as a useful cell proliferation marker to assess the effectiveness of cancer therapeutic agents (Denoyelle et al., 2003; Thangapazham et al., 2006). Aziz et al. (2005) reported that PCNA expression correlates positively with tumor size and histological differentiation of the mammary gland. Also, in colon cancer, Zhou et al. (2000) found that the decrease in the size of tumors induced by 1-methyl-1-nitrosourea correlate with the decrease in the amount of PCNA labeled cells. In the present study, administration of *Vinca* alkaloids to DMBA-

treated rats induced an effective inhibitory action on PCNA protein expression in the mammary glands. Counts of PCNA-labeled cells in each of the pre-neoplastic and neoplastic mammary gland lesions developed after DMBA-plus-alkaloids treatment are significantly lower than those in rats treated only with DMBA. In addition, the labeling intensity of PCNA in the nuclei showed significant decrease in some tumors developed in the alkaloids-treated rats. Therefore, the decrease in both the PCNA labeling indices and its amount, as measured by immunohistochemistry, may explain the small tumor size observed in some alkaloids-treated rats.

The inhibitory effect of *Vinca* alkaloids on PCNA expression has been explained by Blajeski et al. (2002), who stated that microtubule-disrupting agents, which thought to arrest cells in mitosis initiate a cascade of events involving upregulation of p21 leading to inhibition of Cdc2-cyclin B complex and PCNA, preventing interaction of the latter with other components of the DNA polymerase complex.

In spite of the changes observed at the level of PCNA protein, no polymorphism has been detected in exon 1 of PCNA gene using PCR-SSCP analysis for both DMBA-treated and DMBA-plus-alkaloids treated rats. In 1998, Bechtel et al. also found that alteration of PCNA during cancer development was not associated with genetic mutation. However, the contribution of altered PCNA in the accumulation of genetic mutations and genomic instability was not excluded.

IV.6.2. *Vinca* alkaloids down-regulates expression of mutant p53 protein in DMBA-treated rats

Alteration of p53 expression and its mutation are common events that occur during cancer development. In the present study, the reduction in the incidence and size of the DMBA-induced tumors in the mammary glands of *Vinca* alkaloids-treated rats is

associated with a reduction in p53 protein expression in two of the three tumors detected. The amount of p53 protein detected by Western blot analysis becomes more or less similar to that of control mammary glands (Fig. 19). Therefore, the reduction in PCNA-labeling in *Vinca* alkaloids-treated rats is associated with down-regulation of p53 expression. These results are in agreement with those of Korkolopoulou et al. (1994) who found similar correlation between p53 expression and PCNA labeling indices. One may hypothesize that treatment with the crude extract of *Vinca* alkaloids inhibits the accumulation of mutant p53 protein in the cells during progression to breast cancer. This assumption could be supported by Schwartz and Shklar (1996), who reported that carotenoids and retinoids prevent DMBA-induced malignant transformation *in vitro* by inhibiting expression of the mutant form of p53 and enhancing the tumor suppression function of p53.

When Vayssade et al. (2002) examined the effect of the *Vinca* alkaloid, vincristine, on breast cancer cell lines, up-regulation of p53-inducible genes was observed along with some increase in p53 mRNA and protein levels. Therefore, wild-type p53 facilitates the response to anti-microtubule agents which increase the expression of p53-associated proteins, such as p21, 14-3-3σ, GADD45, and bax. These proteins are known to be involved in cell cycle arrest, apoptosis or DNA repair (Vayssade et al., 2002).

SUMMARY & CONCLUSION

In this study, a series of histopathological changes were induced in the mammary glands of Wister rats by administering a single oral dose of DMBA. These changes appeared to be representative of the various pre-neoplastic and neoplastic events that occur during breast cancer development. Alteration of cellular dynamics leading to enhanced cell death was the first pre-neoplastic event observed. This was followed by hyperplastic and dysplastic changes leading to carcinoma *in situ*. Malignant lesions appeared in two forms cribriform and papillary carcinomas and were more common than benign tumors: lactating adenoma and squamous cell papilloma.

These morphological changes were associated with alteration in the expression of PCNA, p53, and BRCA1 proteins. An initial down-regulation followed by up-regulation in the expression of both PCNA and p53 follows the sequence of the morphological changes. This was associated with polymorphism of exon 5 of the p53 gene. While no change in the level of BRCA1 protein was detected, its translocation from the cytoplasm to nucleus was blocked during breast cancer progression.

By using a crude extract of *Vinca* alkaloids, it was possible to reduce the number of neoplastic lesions developed in DMBA-treated rats. These few lesions were small in size and were characterized by inhibition of PCNA expression and reduction in the amount of p53 mutant form.

It is very likely that the observations reported in the present study are applicable to humans because: 1) the overall organization of the epithelial duct cells of rat mammary glands are comparable to those of the human, 2) DMBA is a well known environmental factor that may contribute to the etiology of breast cancer in humans, 3) most of the histopathological changes observed in the mammary glands of DMBA-treated rats are similar to those reported in humans and 4) the three genes examined in this study are

highly conserved among mammalian species. Therefore, one of the important challenges now is to know whether this very early stage of pre-hyperplastic cell death occurs in humans. Then to use gene profiling technology to pinpoint some genes that could be useful as early markers for screening women susceptible to develop breast cancer. Finally, this genetic approach could also help pharmaceutical companies to design therapeutic and/or preventive agents against breast cancer.

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سرطانية موضعية. هذه التغيرات تطورت أخيرا لأنواع خبيثة من السرطان لها القدرة على غزو الخلايا المجاورة. هذا الإضافة إلى تكون بعض الأورام الحميدة عند مجموعة من الفئران المحقونة بمادة DMBA.

من خلال دراسة تكون بروتين PCNA في مراحل سرطان الثدي المختلفة، لاحظنا نقصان مبدئي في تكون هذا البروتين في مرحلة "موت الخلايا"، تلتها زيادة مطردة في المراحل المتقدمة من هذا المرض. كما وجدنا من خلال فحصنا لتكون بروتين BRCA1 في المرحل المختلفة لسرطان الثدي- والذي يقع في أنوية الخلايا المبطنة للقنوات الطرفية في غدة الثدي لفئران المجموعة الضابطة- يتغير موقعه من النواة إلى السيتوبلازم تدرجيا حتى يصبح تماما في السيتوبلازم مميزا للمراحل المتقدمة وخاصة في الأورام الخبيثة.

أظهرت دراسة بروتين p53 بتقنية Western blotting نقصان مبدئي في تكون هذا البروتين في المراحل المبكرة لسرطان الثدي، مع زيادة مطردة في تكوين النوع المتحول(الطافر) في المراحل الأكثر تقدما. هذا وقد وجدنا أن هذه التغيرات لازمها وجود طفرات في جينة P53. من جهة أخرى، لم يوجد أي طفرات في كل من جينة PCNA و BRCA1.

أظهرت بعض الدراسات الحديثة في مجال علاج مرض السرطان أن استخدام خليط مركب من مجموعة من القلوانيّات يعمل بشكل تآزري في تثبيط نمو الخلايا السرطانية. بالاعتماد على هذه النتائج، قمنا بدراسة تأثير مستخلص القلوانيّات الخام من نبتة "الوينكا" على مراحل تطور سرطان الثدي باستخدام نموذج الفئران المحقون بمادة DMBA. بعد أسبوع من حقن الفئران بمادة DMBA، تم معالجة الفئران يوميا عن طريق الفم بالمستخلص الخام لنبتة "الوينكا (200 مجم / كجم من وزن الجسم) لمدة 21 يوم. في نهاية فترة المتابعة، لاحظنا تناقص في نسبة تكون الأورام في غدة الثدي بشكل عام بنسبة 50%. مقارنة بالأورام المتكونة في المجموعة الأولى (المحقونة بمادة DMBA فقط)، وجدنا أن الأورام مع العلاج أقل في الحجم، كما أظهرت تكوين كميات أقل من بروتين PCNA والشكل المتحول لبروتين p53.

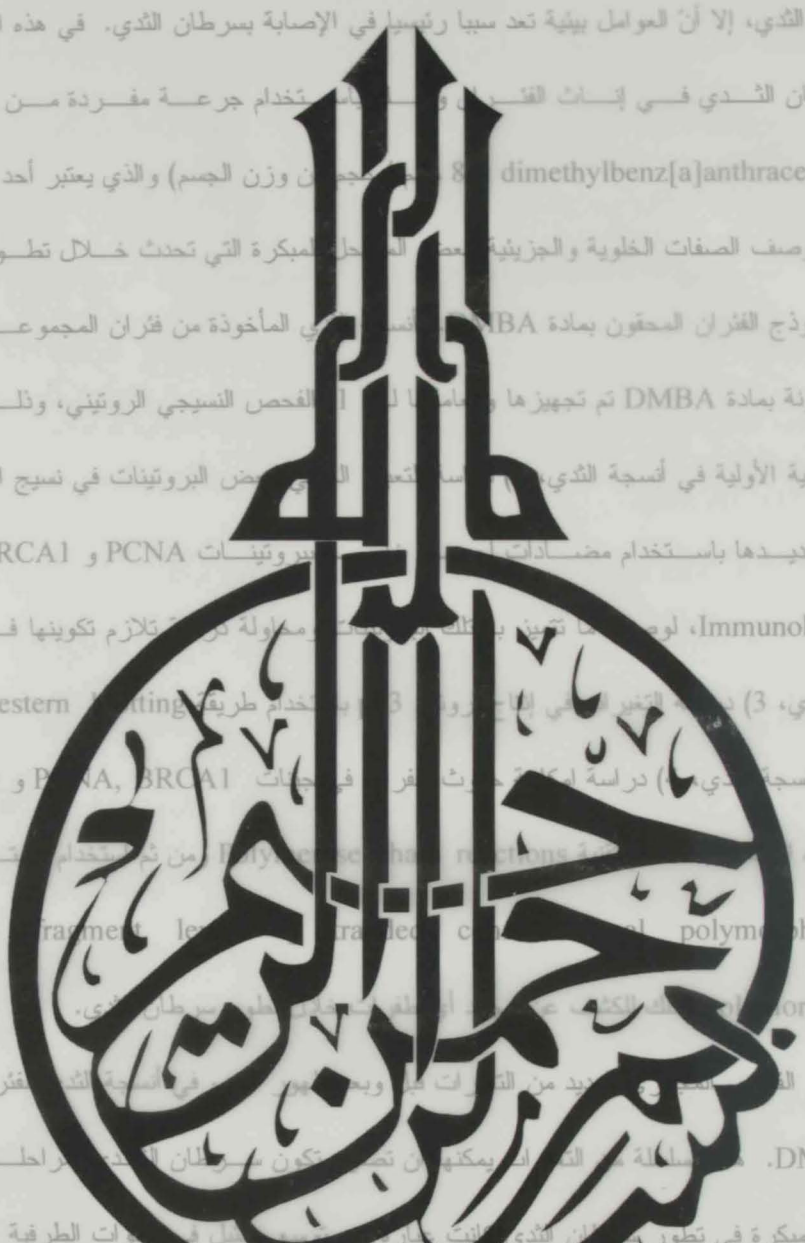
وقد خلصت الدراسة إلى أن التعرف على الصفات المميزة للمراحل المبكرة من سرطان الثدي يزيد من فهمنا وإدراكنا لمنشأ وتولد هذا المرض، كما أنه قد يفيدنا في تحسين الوسائل الوقائية. هذا وقد يساعدنا التعريف بهذه الصفات من جهة أخرى على الكشف المبكر لهذا المرض.

الملخص العربي

يعتبر سرطان الثدي من أكثر أنواع السرطانات شيوعا بين النساء، كما أنه سبب في العديد من وفيات السرطانات بين النساء في دولة الإمارات ودول العالم. بالرغم من تعدد العوامل المساهمة في ارتفاع حدوث الإصابة بسرطان الثدي، إلا أنّ العوامل بيئية تعد سببا رئيسيا في الإصابة بسرطان الثدي. في هذه الدراسة، تم احداث سرطان الثدي في إناث الفئران وذلك باستخدام جرعة مفردة من 7,12- dimethylbenz[a]anthracene (DMBA) (80 مجم / كجم من وزن الجسم) والذي يعتبر أحد المواد البيئية المسرطنة وذلك لوصف الصفات الخلوية والجزيئية لبعض المراحل المبكرة التي تحدث خلال تطور سرطان الثدي باستخدام نموذج الفئران المحقون بمادة DMBA. أنسجة الثدي المأخوذة من فئران المجموعة الضابطة والمجموعة المحقونة بمادة DMBA تم تجهيزها ومعاملتها لـ: 1) الفحص النسيجي الروتيني، وذلك لتعريف التغيرات الباثولوجية الأولية في أنسجة الثدي، 2) دراسة التعبير الجيني لبعض البروتينات في نسيج الثدي وذلك عن طريق تحديدها باستخدام مضادات أجسام خاصة ببروتينات PCNA و BRCA1 بطريقة Immunohistochemistry، لوصف ما تتميز به تلك البروتينات ومحاولة دراسة تلازم تكوينها في مراحل تطور سرطان الثدي، 3) دراسة التغيرات في إنتاج بروتين p53 باستخدام طريقة Western blotting قبل وبعد ظهور الورم في أنسجة الثدي، 4) دراسة إمكانية حدوث طفرات في جينات PCNA, BRCA1 و P53 وذلك بمضاعفة نسخ هذه الجينات باستخدام تقنية Polymerase chain reactions ومن ثم استخدام تقنيتي single stranded conformational polymorphism (SSCP) و restriction fragment length polymorphism (RFLP) وذلك للكشف عن وجود أي طفرات خلال تطور سرطان الثدي.

لقد أظهر الفحص المجهري العديد من التغيرات قبل وبعد ظهور الورم في أنسجة الثدي للفئران التي تم حقنها بمادة DMBA. هذه السلسلة من التغيرات يمكنها أن تصور تكون سرطان الثدي بمراحله المتعددة. فإحدى التغيرات المبكرة في تطور سرطان الثدي كانت عبارة عن توسع ضئيل في القنوات الطرفية الموجودة في غدة الثدي، مع تراكم مجموعة من الخلايا الميتة في تجويف هذه القنوات. فبالاعتماد على هذه المميزات تم الإشارة لهذه المرحلة "بمرحلة موت الخلايا". تلت هذه المرحلة عدة مراحل من الزيادة في إنتاج نسيج الثدي عن الحد الطبيعي، كما أظهر البعض الآخر خلل في إنتاج هذا النسيج، والعديد من أنسجة الثدي بدأ بتكوين خلايا

الملخص العربي



يعتبر سرطان الثدي من أكثر أنواع السرطانات شيوعا بين النساء، كما أنه تسبب في العديد من وفيات السرطانات بين النساء في دولة الإمارات ودول العالم. بالرغم من تعدد العوامل المساهمة في ارتفاع حدوث الإصابة بسرطان الثدي، إلا أنّ العوامل بيئية تعد سببا رئيسيا في الإصابة بسرطان الثدي. في هذه الدراسة، تم إحداث سرطان الثدي في إناث الفئران وباستخدام جرعة مفردة من -12 ,7 dimethylbenz[a]anthracene (DMBA) 8 ملجم/كجم من وزن الجسم) والذي يعتبر أحد المواد البيئية المسرطنة وذلك لوصف الصفات الخلوية والجزيئية لبعض المراحل المبكرة التي تحدث خلال تطور سرطان الثدي باستخدام نموذج الفئران المحقون بمادة DMBA. تم تشريح الغدد الثديية المأخوذة من فئران المجموعة الضابطة والمجموعة المحقونة بمادة DMBA تم تجهيزها وتقييمها للفحص النسيجي الروتيني، وذلك لتعريف التغيرات الباثولوجية الأولية في أنسجة الثدي وكذلك تعبير بعض البروتينات في نسيج الثدي وذلك عن طريق تحديدها باستخدام مضادات البروتينات PCNA و BRCA1 بطريقة Immunohistochemistry، لوصف ما تعبر عنه تلك البروتينات ومحاولة تحديد تلازم تكوينها في مراحل تطور سرطان الثدي، 3) دراسة التغير الجيني الناجم عن الـ DMBA باستخدام طريقة Western blotting قبل وبعد ظهور الورم في أنسجة الثدي و4) دراسة إمكانية حدوث تغيرات في جينات PCNA, BRCA1 و P53 وذلك بمضاعفة نسخ هذه الجينات من ثم استخدام تقنية Polymerase chain reactions التي single restriction fragment length polymorphism (SSCP) و restriction fragment length polymorphism (RFLP).

لقد أظهر الفحص النسيجي العديد من التغيرات قبل وبعد ظهور الورم في أنسجة الثدي لفئران التي تم حقنها بمادة DMBA. هذه الدراسة قد تشير إلى إمكانية أن يكون سرطان الثدي في مراحله المتعددة. فإحدى التغيرات المبكرة في تطور سرطان الثدي كانت ملاحظة تغيرات في الفتحات الطرفية الموجودة في غدة الثدي، مع تراكم مجموعة من الخلايا الميتة. فبالاعتماد على هذه المميزات تم الإشارة لهذه المرحلة "بمرحلة موت الخلايا". تلت هذه المرحلة عدة مراحل من الزيادة في إنتاج نسيج الثدي عن الحد الطبيعي، كما أظهر البعض الآخر خلل في إنتاج هذا النسيج، والعديد من أنسجة الثدي بدأ بتكوين خلايا

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عمادة الدراسات العليا
برنامج ماجستير علوم البيئة

دراسة الجينات المثبطة للأورام أثناء تطور سرطان الثدي في الفئران

رسالة مقدمة من الطالبة

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إلى

جامعة الإمارات العربية المتحدة
استكمالا لمتطلبات الحصول على درجة الماجستير في علوم البيئة

2006