Abstract. Breast cancer is the most common type of cancer and the leading cause of cancer-related deaths among women in the United Arab Emirates and worldwide. Although many factors contribute to the high incidence of breast cancer, a considerable number of cases are related to environmental factors. In the present 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 of the present study was to characterize some of the 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) immunohistochemical probing using anti-BRCA1 antibody to characterize and correlate the localization of this cell cycle protein during progression to cancer, ii) western blotting to analyze the alteration of p53 protein expression in preneoplastic and neoplastic lesions of the mammary glands, and iii) polymerase chain reactions using primers specific for 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 preneoplastic and neoplastic lesions providing a sequence representing the multistep process of breast cancer formation in DMBA-treated rats.

Probing for BRCA1 protein revealed 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 preneoplastic lesions, BRCA1 was localized in both the cytoplasm and nuclei of the epithelial duct cells. In all malignant lesions, BRCA1 was mostly found in the cytoplasm. Western blotting revealed initial downregulation in the expression of p53 protein during breast cancer development. However, with progression towards malignancy, upregulation of p53 was observed. These changes were associated with polymorphism in p53 gene, which was detected in exon 5 using SSCP assay. However, using RFLP and BamHI to digest the PCR products of exon 11 of BRCA1 gene revealed no detectable polymorphisms. In conclusion, molecular characterization of the early changes that occur during development of breast cancer provides some clues for better understanding of its pathogenesis.

Introduction

Breast cancer has been increasing in incidence and remains the most frequent and the deadliest cancer in women worldwide (1). Many factors contribute to the development of breast cancer such as age, life style and genetic factors (2). However, in many cases there is no obvious predisposing factor, supporting the view that a variety of environmental carcinogens may play a major role in the initiation of breast cancer (3). Polycyclic aromatic hydrocarbons, such as 7,12-dimethylbenz[a]anthracene (DMBA), are genotoxic environmental pollutants which may play a major role in the initiation of breast cancer (3). Polycyclic aromatic hydrocarbons, such as 7,12-dimethylbenz[a]anthracene (DMBA), are genotoxic environmental pollutants which may play a major role in the initiation of breast cancer (3).
genes (7). Identification of these alterations can thus improve our understanding of carcinogenesis and help in the development of new diagnostic tools and therapeutic modalities.

Tumor suppressor genes, such as P53 and BRCA, are frequently involved in the development of breast cancer (8). While the wild-type p53 protein suppresses cell growth, its mutated form acts as an oncogene. Mutations in the P53 gene usually result in stabilization and accumulation of its translated protein which is a frequent feature of many malignant tumors (9). It has been estimated that up to 58% of breast cancer patients have mutated P53 gene with accumulation of altered p53 protein as detected by immunological assays (10). Mutant forms of p53 protein lose the ability to bind DNA and cause abnormal cell growth (11).

BRCA1 protein interacts with many nuclear proteins, such as Rad51 and BRCA2 (12), and consequently plays several critical functions in the cell (13). BRCA1 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 a transcriptional activator when linked to DNA binding domain. Moreover, BRCA1 plays a role in the regulation of cell cycle checkpoints and centrotermes (13).

Individuals carrying mutations in the BRCA1 gene have an increased risk of developing breast and ovarian tumors (14). Mutations in BRCA1 alone account for ~45% of families with high incidence of breast cancer and up to 80% of families with both breast and ovarian cancer (15,16). It has been shown that BRCA1 knockout mice are hypersensitive to γ-irradiation which induces chromosomal aberrations (17). Therefore, loss of transcriptional activation by BRCA1 is an important factor in oncogenesis (18). BRCA1 is also involved in the development of sporadic breast cancer by loss of heterozygosity, downregulation of mRNA expression, and methylation of the promoter region (19).

In the present study, preneoplastic and neoplastic mammary gland tissues of DMBA-treated rats were processed for protein expression and mutation analyses of P53 and BRCA1 genes. The data provide new information on this commonly used animal model of breast cancer.

Materials and methods

Animals and study design. Female virgin Wistar rats (43-50 days old) were supplied by the animal facility of the College of Medicine and Health Sciences, UAE University. The protocol described below was approved by the Animal Research Ethics Committee of the College of Medicine and Health Sciences, UAE University. All rats were kept in standard conditions with 12:12 light-dark regimen and ad libitum access to food and water.

Rats were divided into two groups. The first group included 21 rats and used for breast cancer induction using a single gavage of DMBA solution containing 80 mg/kg body weight (5). The second group included 9 rats which received only vehicle (corn oil) to serve as age-matched control. Thus, each 2 or 3 DMBA-induced rats had a control littermate.

Mammary glands of all rats were gently palpated every other day to detect development of any abnormal mass. After 15, 25, 30, 35 or 40 weeks of DMBA or corn oil gavage, rats in each group were sacrificed by an overdose of anesthetic. For each rat, the mammary glands of one side were dissected along with their covering skin and immediately fixed for 12-24 h in Bouin's solution and processed for immunohistochemistry. The opposite group of mammary glands were immediately dissected and stored at -80°C for protein and DNA analysis. In case that a mammary gland had a mass or tumor, it was weighed and divided for immunohistochemistry and protein/DNA analyses.

Immunohistochemical studies. Bouin's fixed tissues were dehydrated in graded ethanol, cleared in xylene, and finally infiltrated and embedded in paraffin. Tissue blocks were cut at 5 µm thickness. Tissues were deparaffinized, rehydrated, and washed in phosphate-buffered saline (PBS). Endogenous peroxidase activity was inhibited by incubating the tissue sections in methanol containing 1% hydrogen peroxide for 30 min. The slides were placed horizontally in a humid chamber. To ensure equal conditions for all tissue sections, slides were drained off, area around sections were wiped dry, and circled with a thin film using PAP-pen (DakoCytomation, Glostrup, Denmark). Non-specific binding was blocked by incubating sections in PBS containing 1% bovine serum albumin for 45 min. Then, sections were incubated overnight with rabbit polyclonal anti-BRCA1 antibody, clone I-20 (Santa Cruz Biotechnology Inc., Dallas, TX, USA) at 4°C. This antibody is specific for the C-terminal region between codons 1823-1842. The catalyzed signal amplification CSA kit (DakoCytomation) was used according to the manufacturer's instructions. Tissue sections were counterstained with Harris hematoxylin.

For quantification, two different approaches were used. First, semi-quantitatively using the 100x objective of the light microscope, the overall amount of cells with labeled nuclei and those with labeled cytoplasm were estimated and scored as low (+), medium (++), high (+++), and very high (++++) expression. The score of low characterized the moderate staining of widely scattered cells. Medium was defined by focal moderate staining in less than half of the cells. High was indicated by focal moderate staining of more than half of the cells. Very high was characterized by dark staining of more than half of the cells. Second, quantitatively, light micrographs prepared using the 100x objective lens were examined to determine the percentage of labeled cells in glandular profiles and localization of the BRCA1 protein (nuclear or cytoplasmic). Only cells with visible nuclei in the micrographs were considered. The means of labeled cells were compared in control and DMBA-treated rats using the ANOVA with post hoc Tukey test and GraphPad Prism Software (GraphPad Inc., San Diego, CA, USA).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis. Frozen mammary gland tissues of control and DMBA-treated rats were homogenized under liquid nitrogen temperature and then lysed with 1 ml buffer containing: 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10% sucrose, 10 mM 1,4-dithio-DL-threitol, 0.1% 3-[3-cholamidopropyl]dimethylammonio]1-propanesulfonate, 150 mM NaCl, and protein inhibitors: 100 mM phenylmethylsulfonyl fluoride, 0.1%
leupeptin and aprotinin. Tissue lysates were centrifuged at 14,000 rpm for 30 min at 4°C. Protein concentrations in the supernatants were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, 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 sulfate, 2-mercaptoethanol, and bromophenol blue. Samples were boiled for 5 min and electrophoresed on 8% polyacrylamide gel at 80 volts for 2 h. To check the expected size of the protein, Bio-Rad prestained protein marker was used.

After electrophoresis, proteins in the gels were transferred onto nitrocellulose membrane. Non-specific binding was blocked by PBS containing 5% non-fat dry milk and 0.1% Tween-20 for 1 h. Following two washes in PBS-Tween, blots were incubated overnight with anti-p53 antibody (clone PAb240; dilution 1:1,000; DakoCytomation) at 4°C. Blots were washed with PBS-Tween, and then incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Ig) G (Cell Signaling Technology, Danvers, MA, USA) at dilution 1:1,000 for 2 h at room temperature. Blots were then washed with PBS-Tween and immunoreactive proteins in the blot were detected using enhanced chemiluminescence reagents (Pierce Biotechnology, Rockford, IL, USA) on X-ray film (Fuji Medical, Japan). To confirm equal loading of proteins, the same blot was immunoprobed with rabbit polyclonal anti-actin antibody (dilution 1:1,000; clone AC40; Sigma, St. Louis, MI, USA). Films were scanned using BioDocAnalyze system (Biometra, Göttingen, Germany) to estimate the intensity of the bands. The percent change in protein band intensity as compared to control samples was determined.

DNA extraction. Genomic DNA was obtained from mammary glands of control and DMBA-treated rats using DNA extraction kit (Qiagen, Toronto, Canada). Tissues were quickly washed in cold PBS, weighed, and then homogenized in a mortar at liquid nitrogen temperature. The fine powder was mixed with cold PBS (100 µl PBS per 20 mg tissue powder). Homogenized 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 at 260 nm using spectrophotometer (WPA; Cambridge, UK).

Polymerase chain reaction. PCR was performed using PuReTaq Ready-to-Go PCR beads (Amersham Pharmacia Biotech, Uppsala, Sweden). The procedure was carried out according to the manufacturer's instructions. Primers of P53 gene were obtained from Pharmacia (Piscataway, NJ, USA). BRCA1 primers were obtained from Operon Biotechnologies (Sweden). Sequences of all primers used are listed in Table I.

The PCR amplifications of P53 exons were performed using the Techne Genius PCR thermal cycler (Burlington, NJ, USA). Each sample was initially denatured at 95°C for 5 min, and then subjected to 40 cycles, each included denaturation at 95°C for 1 min, annealing for 1 min, and then extension at 72°C for 1 min. A final 5 min extension step was included. PCR products (5 µl) were loaded into 1.5% agarose gel and run at 100 volts for 1 h at room temperature. To determine the expected size of PCR products, 100 base pair ladder marker (Amersham Biosciences Corp., Piscataway, NJ, USA) was used. The bands were visualized by staining the gel with ethidium bromide (10 mg/ml) and exposing it to the UV transilluminator (Life Technologies, Carlsbad, CA, USA). PCR amplification of BRCA1 exon 11 was performed with genomic DNA, 2.5 µl of each primer at 10 pmol/µl, and DEPC water was added to a total volume of 25 µl. The same conditions of P53 exon amplification were applied, except that the annealing temperature of BRCA1 exon 11 was adjusted to 48°C.

Single-strand conformation polymorphism (SSCP). The p53 PCR-product (5 µl) was denatured by adding 5 µl loading buffer containing formamide, xylene cyanol and bromophenol blue and incubated at 96°C for 10 min. The mixture was immediately chilled on ice. Then, 10 µl of the denatured PCR products were loaded onto a 10% non-denaturating polyacrylamide gel and run at 60 volts for 4 h at 4°C, using Bio-Rad vertical electrophoresis. The silver staining kit (Amersham Pharmacia Biotech) was used to stain DNA bands in the polyacrylamide gels. The procedure was carried out according to
the manufacturer's instructions. The stained polyacrylamide gels were dried using cellophane membrane (Promega, Fitchburg, WI, USA) and scanned using HP Officejet scanner and HP Image Zone software.

**Restriction fragment length polymorphism (RFLP).** BamHI was used to cut the PCR product of BRCA1. The enzymatic reaction included 3 µl of 10X enzyme buffer E (Promega), 0.3 µl 1% bovine serum albumin (Promega), 30 U BamHI enzyme (Promega) and 10 µl BRCA1 PCR product. The reaction mixture was brought up to 30 µl with DEPC-treated water. All samples were incubated overnight at 37˚C in block heater (Stuart Scientific, Staffordshire, UK). The digested products were loaded in 10% polyacrylamide gel for 2 h at 110 V. Gels were stained in the running TBE buffer containing ethidium bromide. Bands were finally visualized using the UV transilluminator and photographed.

**Results**

Gross observation of the mammary glands of DMBA-treated rats revealed tumor formation in the cervical, thoracic or abdominoinguinal regions (6). Fig. 1 shows an example of these DMBA-treated rats with a tumor in the cervical mammary gland, but all other mammary glands appeared small in size as those of the control. However, microscopic examination of all the mammary glands of DMBA-treated rats sacrificed after 15, 25, 30, 35 and 40 weeks revealed the development of a wide range of pathological changes. None of the mammary glands appeared microscopically normal as in the control. The changes included increased cell death, hyperplasia, dysplasia, adenoma, carcinoma *in situ* and invasive carcinoma (Fig. 2).

Figure 1. A rat anesthetized after 15 weeks of a single dose of DMBA. The lower arrows and green circles show mammary glands with normal size and shape. Note the tumor developed in the mammary gland of the cervical region (upper arrow and red circle).

These preneoplastic and neoplastic lesions were similar to those previously identified and characterized (6). In addition, mammary gland tumors were developed in ~29% of the treated

Figure 2. Light micrographs of rat mammary gland tissue sections stained with hematoxylin and eosin representing the features of control tissue as compared to DMBA-induced preneoplastic (hyperplasia and dysplasia) and neoplastic (*in situ* and invasive carcinomas) lesions.
rats. Approximately 14% of all mammary glands examined developed benign tumors and 19% were invasive or malignant.

**Immunohistochemical localization of BRCA1.** Control rats showed that BRCA1 protein was present mainly in the nuclei of some luminal epithelial duct cells (Fig. 3A). Faint cytoplasmic stain was also observed in cells of some small ducts, which was more apparent in the large ducts. Early microscopic lesions (hyperplasia and dysplasia) developed in DMBA-treated rats showed both nuclear and cytoplasmic localization of BRCA1 (Fig. 3B). This nucleo-cytoplasmic pattern of BRCA1 expression was also noted in some neoplastic lesions classified as 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 localized in the cytoplasm (Fig. 3C). Scoring of the immunolocalization of BRCA1 in rat mammary glands with different histopathological conditions are presented in Table II. It shows a change in the pattern of the expression of BRCA1 from more nuclear in control tissues to more cytoplasmic in DMBA-treated tissues. In addition, counts in tissue sections obtained from different mammary glands of control and DMBA-treated rats revealed that the percentages of cells with BRCA1 labeled nuclei gradually dropped from 69 to 54 to 12 in control, preneoplastic and neoplastic tissues, respectively. The differences between these percentages of nuclear labeling were statistically significant (Fig. 4). This change in BRCA1 nuclear labeling was associated with a significant increase in the percentages of cells with BRCA1 labeled cytoplasm from 9 to 58 to 91 in control, preneoplastic and neoplastic tissues, respectively (Fig. 4).

**Expression of p53 protein.** Homogenized tissue samples of the mammary glands obtained from control and DMBA-treated rats were analyzed by western blotting to study the expression of p53 protein during breast cancer progression. Representative results of 3 different experiments are shown in Fig. 5. Comparing to control tissue (Fig. 5; lane 1), initial
downregulation in some hyperplastic and benign lesions was observed (Fig. 5; lanes 2 and 5, respectively). These were from mammary glands of rats treated with DMBA and sacrificed after 35 weeks. In these rats, some mammary glands progressed toward malignancy, and therefore, the amount of p53 was increased. This was either the accumulated wild-type form or the mutant form detected using anti-p53 antibody clone PAb240 (Fig. 5, lanes 3, 4 and 6-8). Measurement of band densities confirmed these expression patterns of p53 (Fig. 5). Calculations of percent change in band intensity, as compared to control, showed an increase in tissues with hyperplasia by 33 and 96% (except for a 23% decrease in one case) and an increase in all neoplastic cases by 5, 15, 18 and 27%.

SSCP analysis of P53 gene in rat mammary glands. PCR products of exon 5 revealed 270 bp amplicon for all samples when compared to the standard DNA molecular marker. SSCP analysis of this 270 bp product of control mammary glands showed two bands as indicated in Fig. 6. Out of 9 tumors, two of them (22%) exhibited band shift (3 extra bands) with electrophoresis in addition to the two bands appeared in control sample. These two tumors were developed in rats treated with DMBA and sacrificed after 25 and 32 weeks. In addition, some hyperplastic mammary glands showed band shifting similar to that of tumor samples as shown in Fig. 6. PCR amplification of exon 6-7 produced 300 bp band for all samples in agarose gel. SSCP analysis displayed 3 bands representing the normal pattern of P53 exon 6-7 (Fig. 6). Analysis of DMBA-treated samples revealed bands similar to those of control with no shift detected in the polyacrylamide gels (Fig. 6). 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. The SSCP analysis of P53 exon 8-9 showed two bands similar to the pattern of exon 5. SSCP analysis of the 410 bp fragment of exon 10 of P53 gene showed two main bands in the control and DMBA-treated mammary gland (Fig. 6).
mutant forms of p53 (24). Using SDS-PAGE and western blot analysis, the p53 protein is found to be differentially expressed during mammary gland carcinogenesis. It tends to be down-regulated in preneoplastic lesions, but becomes upregulated in malignant tumors. These findings correlate with previous studies in human breast cancer (25). A significant correlation between p53 mutations and p53 overexpression was reported in both canines and humans (26,27). In addition, p53 protein expression in benign tumors is much less compared to that in malignant lesions (26).

Mutations in p53 gene are very common in human cancers; they occur in 20-40% of breast cancer cases (28). In addition, 50% of cancers of colon, stomach, and liver are characterized by p53 mutations (29). Most of these P53 mutations are clustered within exons 4-8, which encode a highly conserved region, containing the DNA binding domain of the protein (29,30). In the present study, exons 5, 6-7, 8-9 and 10 of p53 gene were studied for possible mutations that may occur during breast cancer development. Using SSCP, it was possible to detect P53 polymorphism in some rats with hyperplastic mammary glands (Fig. 6). In humans, previous studies reported P53 mutations in individuals with preinvasive mammary gland lesions: Atypical ductal hyperplasia and ductal carcinoma in situ (31,32). In mice, overexpression of p53 and its mutation in hyperplastic mammary glands was also reported (33).

It has been estimated that 33.3% of the mammary gland tumors developed in the present study acquire 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. The predominance of purine (A or G) mutations is consistent with the fact that DMBA-adduct formation preferentially occurs on dA and dG (34,35), leading to depurination (36).

There may be a concern that some models of breast cancer may not reflect the disease in humans. This is not the case for the DMBA model. It has been demonstrated that the different forms of preneoplastic and neoplastic histopathological changes of DMBA animal model are very similar to those in humans (6). Therefore, the in vivo model of DMBA-induced breast cancer is useful for understanding of disease progression and early detection in humans. In addition, the observations that BRCA1 and P53 genes are altered in DMBA breast cancer model are not surprising. They were also described in human breast cancer tissues (8,10,19). However, the present study has probed some questions regarding p53 and BRCA1 proteins. In humans, it is known that hyperplastic mammmary gland lesions precede tumor formation. However, the associated biochemical changes are not well characterized, at least for mammary gland lesions due to DMBA. The present study has demonstrated that alteration of BRCA1 subcellular localization and upregulation and mutations of P53 expression are common changes. Therefore, due to the similarities of the multistep process of breast cancer development in humans and rats, the limitations of DMBA model are minimal.

In conclusion, the present study demonstrates that the morphological changes in mammary gland induced by a single gavage of DMBA are associated with alterations in the expression of p53 and BRCA1 proteins. An initial downregulation followed by upregulation in the expression of p53 follows the sequence of the morphological changes. This is associated with polymorphism of exon 5 of P53 gene which is detected

Discussion

The present study demonstrates that DMBA-induced preneoplastic and neoplastic lesions develop a block in the nuclear translocation of BRCA1 protein and upregulation and polymorphisms of P53 gene.

Various studies previously indicated that BRCA1 acts as a nuclear-cytoplasmic shuttle protein, and its transport is altered by DNA damage (20,21). Thus, it seems that breast cancer progression is associated with the production of an inactive form of BRCA1 which cannot be targeted to the nucleus and therefore, remains in the cytoplasm of cancer cells. This could be due to impairment of nuclear localization signals and/or the nuclear export signal of BRCA1 protein (20). The data presented in the present study indicate that alterations in the localization of BRCA1 protein occur in preneoplastic lesions of the DMBA-rat model of mammary gland carcinogenesis.

P53 is a tumor-suppressor gene which has an apparent role in the development of breast cancer in humans and rodents (22,23). In the present study, the expression of p53 protein was studied in mammary glands of DMBA-treated rats using an antibody that detects both wild-type and mutant forms of p53 (24). Using SDS-PAGE and western blot
as early as during hyperplasia. While no change in the level of BRCA1 protein was detected, its translocation from the cytoplasm to nucleus is blocked during breast cancer progression. Therefore, it seems that P53 mutations in exon 5 and blocking the nuclear translocation of BRCA1 are important events for mammary gland carcinogenesis in DMBA-treated rats.

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