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RESEARCH ARTICLE

DMBA- Induced Breast Cancer: A Hormonal Camouflage

Anita K. Bakrania, Bhavesh C. Variya and Snehal S. Patel*

Department of Pharmacology, Institute of Pharmacy, Nirma University, Ahmedabad, Gujarat, 382 481, India

Abstract: ***Background*:** Breast cancer is one of the high prevalence cancers worldwide affecting women based on various factors. Hormonal changes are amongst the factors involved in the development of breast cancer. The basic hormones involved include estrogen and progesterone with human epidermal receptor-2 (HER2) which could be either over expressed or not at all expressed. Therefore, studies were conducted on the DMBA induced breast cancer model.

***Methods*:** As per literature review, there has been complete befuddlement on the hormonal crisis of the DMBA induced model hence we performed the immunohistochemistry studies of estrogen, progesterone and human epidermal receptor-2 in order to prove the presence or absence of the major hormones involved in progression of breast cancer. Preceding morphological tumor detection, it was further confirmed with hematoxylin-eosin stained sections.

***Results*:** These data initially detected the probable presence of mucin in the hematoxylin-eosin stain hence we performed specific mucin stain using Meyer's mucincarmine concluding mucinous carcinoma. Thereafter, we obtained negative estrogen and HER2, with positive progesterone in the immunohistochemistry studies.

***Conclusion*:** Conclusively, DMBA induced breast cancer is a suitable model for mucinous carcinoma in estrogen and human epidermal receptor-2 negative and progesterone positive breast cancer.

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INTRODUCTION

7,12-Dimethylbenz[a]anthracene (DMBA), a polycyclic aromatic hydrocarbon as well as a synthetic carcinogen, has been vastly used to study various potential factors involved in progression of breast cancer and is neither naturally present in the human environment nor produced endogenously in humans. Its mechanism is however not completely understood till date, but several studies have shown its interaction with cellular DNA leading to oncogene activation [1, 2].

*Address correspondence to this author at the Department of Pharmacology, Institute of Pharmacy, Nirma University, Ahmedabad, Gujarat, 382 481, India; Tel: +9107927600686;
E-mail: snehal.patel@nirmauni.ac.in

Recalling the history of the carcinogen DMBA, it was first reported by Silberman and Shklar that DMBA possessed a carcinogenic effect. They however studied this using the hamster's buccal pouch in combination with croton oil [3]. Looking into the effect of DMBA in mammary cancer, it was initiated by Rooks in his book entitled Steroidal Activity in Experimental Animals and Man in 1964. He studied the carcinogenic potential of DMBA in inducing breast cancer in rats and mice. Following this era, several animal models preceded using DMBA as a carcinogen for the induction of breast cancer in various animals [4].

Recently in 2014, novelty was observed whereby the airpouch technique was used to induce DMBA into the mammary fat pad. The pro-

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tocol made use of sterile air (1-2ml), carefully injected subcutaneously beneath the mammary fat pad in order to form an air pouch which was allowed to remain for 24 hours. Later, DMBA was injected into the air pouch for induction of mammary cancer. They also stated that this model is generally hormone dependent adenocarcinoma however, more evidence on hormone levels is still to be elucidated [5].

In 2002, the first and last study was performed for the expression of ER, PR and HER-2 in DMBA induced rat mammary cancer (supported by supplementary data). Surprisingly, the research concluded the presence of all the three mentioned hormones which was significantly lower in the groups treated with soy. Also, the presence of mucin in DMBA induced mammary cancer hasn't been researched upon in conjunction to hormones. Hence, we performed our research on the same hormones *i.e.* estrogen, progesterone and HER-2 for determination of the type of cancer animal model performed (Table **1**, Table **2**) [6].

MATERIALS AND METHODS

Materials

7,12-dimethyIbenz[a]anthracene (DMBA) was purchased from Sigma Aldrich Pvt. Ltd., India, Estrogen (abcam, ab32063), Progesterone (Abcam, ab2765) and HER2 (Abcam, ab106575) antibodies, DAB quanto substrate and chromogen, hydrogen peroxide block, HERP polymer were purchased from thermoscientific Pvt. Ltd. Xylene, alcohol, tris buffer were of analytical grade.

Experimental Animals

All experiments and protocols described in the present study were approved by the Institutional Animal Ethics Committee (IAEC) of Institute of Pharmacy, Nirma University. Five week old 16 female SD rats were procured and housed in the animal house of the Institute under controlled conditions of temperature 23±2 °C, relative humidity 55±5%, and 12 hr. light and dark cycle.

Experimental Protocol

5 week old female SD rats were randomized into 2 groups of 8 animals each namely; normal control group and disease control group. 25mg/kg DMBA was dissolved in a mixture of olive oil and saline in ratio (3:1) and was administered subcutaneously in the right side mammary gland. Simultaneously, the time of tumor incidence was recorded along with the tumor size. After 3 months, the tumors were isolated and stored in 10% neutral formalin solution and subjected to haematoxylin eosin staining, mucin staining and immunohistochemistry studies for estrogen, progesterone hormones and HER2 receptor.

Immunohistochemistry Studies for ER, PR and HER2

Initially, paraffin blocks were cut into 5µm sections on slides for immunohistochemistry staining. The slides were baked under pressure as a process of dehydration, removal of paraffin layer as well as receptor activation followed by incubation with xylene, alcohol and water. This serves as a hydration process. The slides for ER and PR staining were then heated in tris buffer pH 9 as a process of removal of formalin bonds for nuclear staining while slides for HER-2 stain were heated in tris buffer pH 6 for cytoplasmic membrane staining. The slides were then placed in a dark chamber and flooded with tris buffer pH 7.4. This was followed by addition of hydrogen peroxide block proceeded by a wash. It serves as consumption of peroxidase enzyme by conversion of hydrogen peroxide to water and oxygen free radical in order to prevent peroxidase from interfering further in the reaction process. Goat serum protein was added without wash and incubated for 10 minutes. The primary antibody for the respective hormone with dilution 1:400 was added and incubated in dark for 1 hour. After a wash with tris buffer, secondary antibody of the respective hormone was added and incubated in a dark chamber. After a wash with tris buffer, HRP conjugated antibody was added to the slides. DAB quanto substrate is added followed by 1 drop of DAB chromogen and incubated in dark. Finally a wash was given with distilled water and hematoxylin counter staining performed. The slide is then mounted and observed for immunohistochemical staining using Olympus microscope.

Determination of Mucin in the Mammary Gland Sections

Also, due to a specific observance in the microscopy, mucin staining was performed utilising

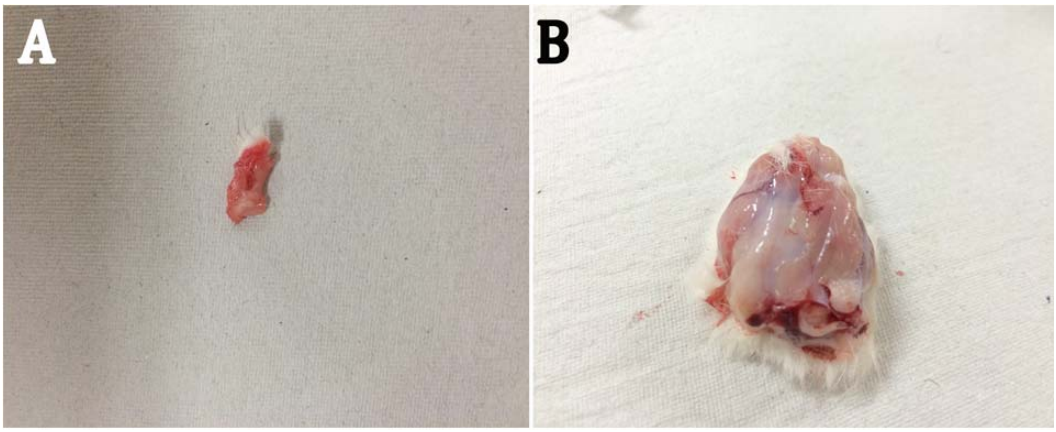

Fig. (1). Tumor size.

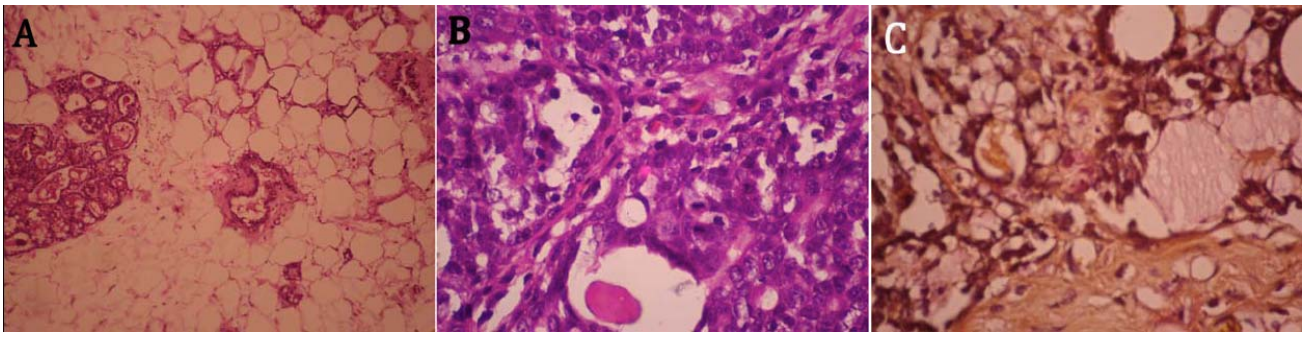

Fig. (2). H/E staining and mucin staining.

the same formalin embedded paraffin blocks. Mucin staining was done using Meyer's mucin-carmine staining after which the slides were observed for mucinous carcinoma under Olympus microscope.

Statistical analysis results are represented as mean ± S.E.M. Statistical analysis was performed using Graph pad prism 5 statistical software. Statistical differences between the means of various groups were evaluated using one way analysis of variance (ANOVA) followed by turkey's test. Data were considered statistically significant at $P<0.05$.

RESULTS

Tumor Incidence and Size

The tumor incidence time was observed daily for any observable characteristics. A minimal tumor swelling was observed on day 21 after DMBA intoxication. The tumor then gradually increased in size reaching an optimal size by 3 months (Fig. **1**).

Haematoxylin Eosin Staining for Tumor Cell Detection

In the Haematoxylin eosin staining it was observed that normal ductular and alveolar structure of the mammary gland was maintained in normal control with no signs of inflammation, tissue damage or hyperplasia. HE staining in the diseased sections showed marked hyperplastic cells as well as well demarcated tubular carcinoma. The tubular and alveolar epithelium showed diffused hyperplasia along with minimal rim of skeleton muscle (Fig. **2A**, **2B**).

Mucin Staining for Mucinous Carcinoma

Mucin was observed in the tumor cells as a pink froth attached to the cell membrane. Mucin was only observed in tumor cells while the normal cells when stained with Meyer's mucincarmine and counter stained with HE did not produce any mucinous stain. Hence, the model can be predicted to be a useful model of mucinous carcinoma (Fig. **2C**).

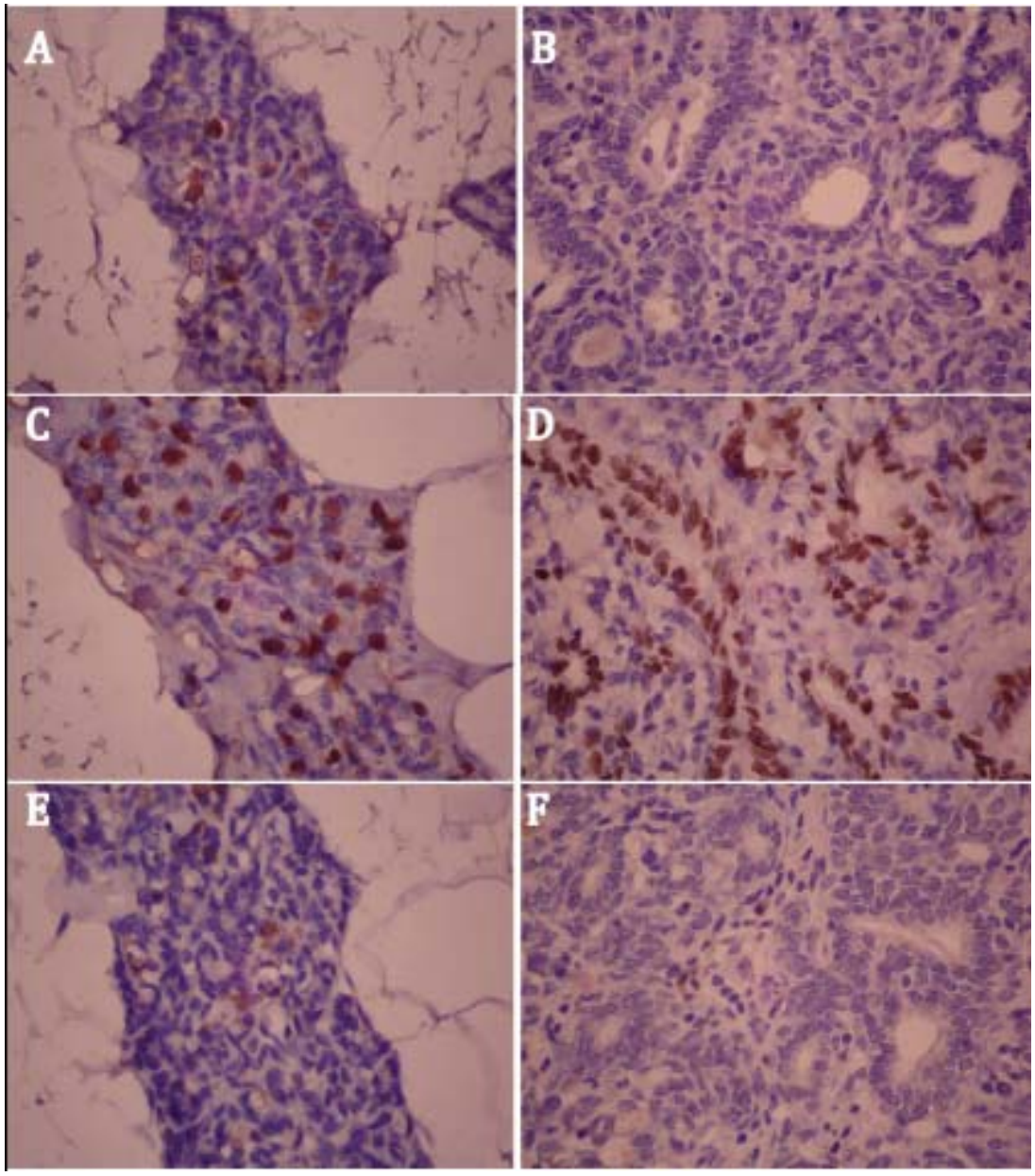

Fig. (3). Immunohistochemistry of ER, PR and HER-2.

Immunohistochemistry for ER, PR and HER-2

The tumor samples showed negative ER and HER-2 stains while PR staining was found to be positive. The immunoreaction was determined by the integrated optical density measurement. This indicates the model as double negative for ER and HER-2. The normal breast tissue showed positive nuclear staining for estrogen and progesterone hormone and positive cytoplasmic membrane staining for HER-2 receptor. The intensity of PR stain in normal and tumor cells was not altered. This indicates that DMBA has effect on hormone levels for the induction of mammary cancer in the rat model with alteration in ER and HER-2 levels but without any significant alteration in specifically PR levels (Fig. **3**, Fig. **4**).

DISCUSSION

DMBA is commonly used as a potential carcinogen for the induction of breast cancer in experimental animals. Basically, DMBA up-regulates cytosolic aryl hydrocarbon receptor (AhR). This translocates into the nucleus leading to a complex formation with the aryl hydrocarbon receptor nuclear translocator (ARNT) which further binds to specific AhR genes inducing

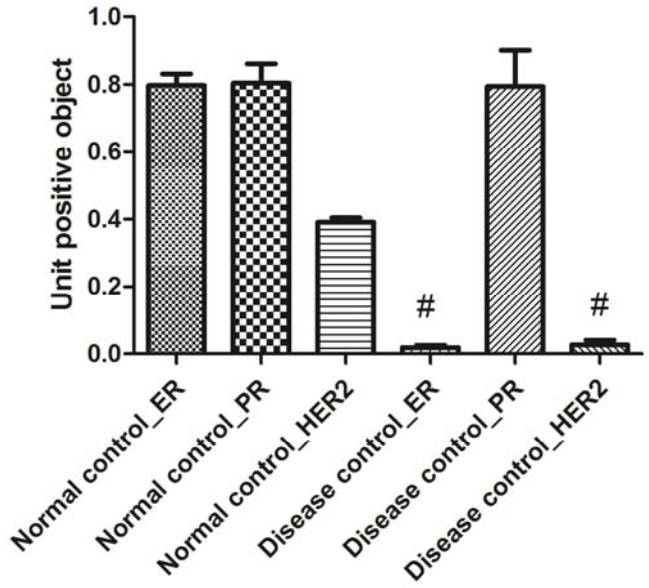

Fig. (4). Quantification of immunohistochemistry studies for ER, PR and HER2.

CYP1 gene transcription. Mechanism of DMBA, although not well understood, is found to covalently bind to DNA leading to lesion formation which could initiate tumorigenesis in the mammary gland. DMBA has found to be capable of inducing 8-OHdG (8-hydroxy-2' –deoxyguanosine) in the mammary gland [2]. 8-OHdG generation has found to be an important event in tumorigenesis. It leads to G to T trans-versions, the most frequent hot spot mutations in human tumor genes. In mammary cancer, this G to T conversion has been observed to cause p53 tumor suppressor gene mutational spectrum. It has been reported that 8-OHdG levels are not signi-ficantly correlated to the estrogen or progesterone hormone status in breast cancer [7].

The carcinogenic molecule DMBA is found to be activated by cytochrome P-450 enzymes and epoxide hydrolase to form ultimate carcinogen 3,4-dihydrodiol-1,2-epoxide. Later, looking into the detoxification of the corresponding carcinogen, it is mediated through various phase II enzymes such as glutathione-S-transferase [8]. Both CYP1A1 and CYP1B1 are major cytochrome isoforms required for the activation of DMBA based on various studies. They exhibit stereo-specific DMBA metabolism; CYP1A1 producing anti-diolepoxides and CYP1B1 producing the syn-isomer [9].

DMBA has been found to be organ and site specific procarcinogen, requiring metabolic activation for chromosomal damage by binding with adenine residues of DNA leading to mutagenesis as well as tumorigenesis. Also, it has found to have interaction with estrogen hormone partially mimicking both positive and negative feedback actions of estradiol in ovariectomized rats [10].

After a duration of 3 months, a prominent tumor was observed in each of the diseased animals which was absent in the normal control animals. In order to confirm presence of tumor induction in the diseased animals, the tumor was incised and subjected to haematoxylin eosin staining. Results obtained show presence of ductular carcinoma as well as well differentiated tumor cells in diseased animals while the normal animals showed no deformities in cellular architecture.

Our findings have shown the DMBA induced breast cancer model to be mucinous carcinoma. Mucinous carcinoma is basically a specific type of breast cancer which is characterised by extracellular mucin. Studies have suggested that mucinous carcinoma is usually negative for HER-2/neu overexpression and further suggesting that HER-2 is rarely involved in tumorogenesis of mucinous breast cancer [11]. The novel cell line UHKBR-01, has shown positive mucin which mimics with the results we have obtained. We have obtained high amounts of mucin in the tumor histopathology samples indicative of mucinous carcinoma. Mucin levels have been reported to be indicative of tumor aggressiveness in human breast tumor [12]. Hence, this study is in line with previous results obtained pertaining to mucinous carcinoma induced by DMBA.

There has been severe confusion into the hormone status of DMBA induced breast cancer and therefore various hypotheses have been reported and studied for the same. Similarly, we also studied the hormonal basis for this model. According to a cell line UHKBR-01, established from DMBA induced rat mammary tumor, it was reported to be positive for estrogen as well as progesterone hormone. Also, the same study indicated tumor regression following hypophy-sectomy, ovariectomy or testosterone regimens while moderate doses of estrogen or progesterone stimulated the tumor growth [12].

The role of estrogen in DMBA induced carcinoma was studied by Uchidaa *et al.* and they evaluated the role of Medroxyprogesterone acetate (MPA) in mammary as well as endometrial cancer. They concluded that mammary carcinoma can be inhibited by decreased estrogen levels by synthesis of estradiol or the precursor. However, as per their study estrogen was found to be present in the DMBA induced mammary cancer [13]. A study by Bishayeea *et al*. also stated the estrogen-dependent nature of DMBA induced mammary cancer model in relevance to human breast cancer with specificity to origin: ductal epithelial cells [14]. Similarly, another study showed that estrogens significantly enhance the mammary tumors induced by DMBA. However, they mentioned that the detailed elucidation for the nature of estrogen involvement is still to be elucidated. They also concluded that there exists a correlation between estrogens and DMBA for the development of the breast cancer [15]. Research has also shown the correlation of prolactin and estrogen in tumorigenesis whereby DMBA induced breast cancer is a hormone dependent model regulated by estrogens. After several confusions on whether estrogen is positive or negative in DMBA induced mammary cancer in SD rats, our results have shown reproducible negative estrogen levels during the immunohistochemistry protocol performed. No nuclear staining was observed during immunohistochemistry staining for estrogen levels. Various studies have opposed this result manifesting the presence of estrogen in the same model. McDougal *et al.* showed that a high percentage of initial DMBA intoxicated mammary tumors were estrogen positive responding to endocrine therapy [16].

The role of progesterone has a great impact in mammary cancer [17]. According to Chatterton *et al.* progesterone has been studied as an essential requirement for the induction of breast cancer. Their research is based on a conclusion that in the absence of progesterone, epithelial cells are highly resistant to DMBA [17]. Earlier studies have shown that progesterone is an essential element for the differentiation of alveolar structures and enhances proliferation in mammary gland. Also, it has been stated that early ductal branching occurs as a result of progesterone activity. Previous studies have proved that in the absence of progesterone, lesions had found to be absent in mammary glands and it is evident that also hormone-independent tumors require PR for tumor initiation as well as proliferation. In our study, we observed the presence of progesterone after immunohistochemistry with PR antibody. The animal model described is hence found to be positive for progesterone which complies with the previous studies indicating its role in tumor growth.

The human epidermal growth factors (HER) also known as ERBB receptors, are signal transduction proteins further classified as HER-1, HER-2, HER-3 and HER-4. Specifically, HER-2 (ERBB-2, NEU, or HER-2/neu) acts as a dimer with other HER receptor proteins. Upon activation, the tyrosine kinase domain activates downstream signaling molecules. HER2 activates multiple cellular signaling pathways, including the PI3K and MAPK cascades further leading to apoptosis. It has been studied that significant levels of HER2 are beneficial in order to prevent the occurrence of breast cancer. However, the upregulation or downregulation of HER2 imbalances the body physiology; leading to the activation of various pathways, and further causing initiation of tumor. Previous studies have reported the presence of HER-2 in DMBA induced model [6]. However, adding to the controversy, it has also been concluded by studies that; HER-2 expression has been found to be low to non-existent in mucinous, medullary, and tubular carcinomas; breast sarcomas and Phyllodes tumors; as well as hereditary breast cancer associated with BRCA1/2 mutations [18]. Hence, more proof required for the combination of mucinous carcinoma and HER-2 existence. Our findings indicate negative HER-2 expression in the tumor sections. This is in line with previous findings of negative HER-2 in conjunction to mucinous carcinoma.

Mucin, as previously stated, is an indicative of aggressive tumor in human breast cancer. Studies have shown that creatinine kinase B, myosin heavy polypeptide 11 and A28-RGS14p, a p53 target gene and regulator of G protein signaling, were expressed at higher levels in mucinous cancer. Our results have found positive mucin in the disease control animals which implements that DMBA induced mammary cancer is an aggressive type of breast cancer which has a rare prognosis. Also, we have proved the conjunction of HER2 with mucin which never co-exist as per the litera-

ture. Hence, this could indicate that HER2 negative breast cancer model could also be a model for mucinous carcinoma.

We have thoroughly assessed all the DMBA induced breast cancer models till date (supplementary data) for any justification on the hormone levels. However, we have reviewed that research work on the hormone status, either partially or fully, have been performed only five times by different investigators (supplementary data). These research work performed are partially contradictory to our results in terms of estrogen and HER-2 levels. Various studies have concluded presence of progesterone which complies with our results obtained. Although articles have mentioned presence of HER-2 and mucin in the same model, research has shown that they cannot occur at the same time which justifies our results of presence of mucin and absence of HER-2. Negative estrogen levels in the DMBA intoxicated mammary cancer model may be as a result of dose of DMBA and age of animals at cancer induction. However, systemic research upon various doses of DMBA as well as a range of cancer induction at different age groups is required to monitor the exact changes in hormonal status.

CONCLUSION

The research carried out eventually concludes the presence of mucin in the DMBA induced mammary cancer. This confirms the model suitable for mucinous carcinoma. Further, the hormonal involvement in the breast cancer model concludes double negative breast cancer with positive progesterone receptors. Conclusively, the animal model of DMBA induced breast cancer can be a suitable model for screening drugs acting on mucinous carcinoma with positive progesterone and negative estrogen and HER-2.

CONFLICTS OF INTEREST

The authors state that there are no conflicts of interest pertaining to this manuscript.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers Web site along with the published article.

REFERENCES

[1] Chatterjee M, Janarthan M, Manivannan R, Rana A, Chatterjee M. Combinatorial effect of fish oil (Maxepa) and 1alpha,25-dihydroxyvitamin D(3) in the chemoprevention of DMBA-induced mammary carcinogenesis in rats. Chem Biol Interact. 2010; 188: 102-10.

[2] El-Bayoumy K, Chae Y-H, Rosa JG, Williams LK, Desai D, Amin S, *et al.* The effects of 1-nitropyrene, 2-amino-1-methyl-6-phenylimidazo[4,5–b]pyridine and 7,12-dimethylbenz[a]anthracene on 8-hydroxy-2′-deoxyguanosine levels in the rat mammary gland and modulation by dietary 1,4-phenylenebis(methylene) selenocyanate. Cancer Lett. 2000; 151: 7-13.

[3] Silberman S, Shklar G. The Effect of a Carcinogen (Dmba) Applied to the Hamster's Buccal Pouch in Combination with Croton Oil. Oral surgery, oral medicine, and oral pathology. 1963; 16: 1344-55.

[4] Dorfman RI. Steroidal Activity in Experimental Animals and Man: Academic Press; 2013.

[5] Arivazhagan L, Sorimuthu Pillai S. Tangeretin, a citrus pentamethoxyflavone, exerts cytostatic effect *via* p53/p21 up-regulation and suppresses metastasis in 7,12-dimethylbenz(alpha)anthracene-induced rat mammary carcinoma. The Journal of nutritional biochemistry. 2014; 25: 1140-53.

[6] Gallo D, Ferrandina G, Giacomelli S, Fruscella E, Zannoni G, Morazzoni P, *et al.* Dietary soy modulation of biochemical parameters in DMBA-induced mammary tumors. Cancer Lett. 2002; 186: 43-8.

[7] Charles M-J, Schell MJ, Willman E, Gross H, Lin Y, Sonnenberg S, *et al.* Organochlorines and 8-Hydroxy-2′-Deoxyguanosine (8-OHdG) in Cancerous and Noncancerous Breast Tissue: Do the Data Support the Hypothesis That Oxidative DNA Damage Caused by Organochlorines Affects Breast Cancer? Arch Environ Contam Toxicol. 2001; 41: 386-95.

[8] Jung KJ, Wallig MA, Singletary KW. Purple grape juice inhibits 7,12-dimethylbenz[a]anthracene (DMBA)-induced rat mammary tumorigenesis and *in*

vivo DMBA-DNA adduct formation. Cancer Lett. 2006; 233: 279-88.

[9] Lin Y, Yao Y, Liu S, Wang L, Moorthy B, Xiong D, *et al.* Role of mammary epithelial and stromal P450 enzymes in the clearance and metabolic activation of 7,12-dimethylbenz(a)anthracene in mice. Toxicology letters. 2012; 212: 97-105.

[10] Baskaran N, Manoharan S, Balakrishnan S, Pugalendhi P. Chemopreventive potential of ferulic acid in 7,12-dimethylbenz[a]anthracene-induced mammary carcinogenesis in Sprague-Dawley rats. European journal of pharmacology. 2010; 637: 22-9.

[11] Hsu Y-H, Shaw C-K. Expression of p53, DCC, and HER-2/neu in mucinous carcinoma of the breast. Kaohsiung J Med Sci. 2005; 21: 197-202.

[12] Chow LW, Cheung MN, Loo WT, Guan XY. A rat cell line derived from DMBA-induced mammary carcinoma. Life sciences. 2003; 73: 27-40.

[13] Uchida M, Tsuboi H, Yamaji T, Murata N, Kohno T, Sugino E, *et al.* Inhibition by 9alpha-fluoromedoroxyprogesterone acetate (FMPA) against mammary carcinoma induced by dimethylbenz[a]anthracene in rats and angiogenesis in the rabbit cornea - comparison with medroxyprogesterone acetate (MPA). Cancer Lett. 2000; 154: 63-9.

[14] Bishayee A, Mandal A. Trianthema portulacastrum Linn. exerts chemoprevention of 7,12-dimethylbenz(a)anthracene-induced mammary tumorigenesis in rats. Mutation research. 2014; 768: 107-18.

[15] Keshava N, Mandava U, Kirma N, Tekmal RR. Acceleration of mammary neoplasia in aromatase transgenic mice by 7,12-dimethylbenz[a]anthracene. Cancer Lett. 2001; 167: 125-33.

[16] McDougal A, Wilson C, Safe S. Inhibition of 7,12-dimethylbenz[a]anthracene-induced rat mammary tumor growth by aryl hydrocarbon receptor agonists. Cancer Lett. 1997; 120: 53-63.

[17] Chatterton RT, Jr., Lydon JP, Mehta RG, Mateo ET, Pletz A, Jordan VC. Role of the progesterone receptor (PR) in susceptibility of mouse mammary gland to 7,12-dimethylbenz[a]anthracene-induced hormone-independent preneoplastic lesions *in vitro*. Cancer Lett. 2002; 188: 47-52.

[18] Ross JS, Slodkowska EA, Symmans WF, Pusztai L, Ravdin PM, Hortobagyi GN. The HER-2 receptor and breast cancer: ten years of targeted anti–HER-2 therapy and personalized medicine. Oncologis. 2009; 14: 320-68.