RESEARCH ARTICLE

pISSN: 0126-074X | eISSN: 2338-6223
https://doi.org/10.15395/mkb.v54n1.2479
Majalah Kedokteran Bandung. 2022;54(1):37–43

Majalah Kedokteran Bandung (MKB)

Received: July 13, 2021
Accepted: January 7, 2022
Available online: March 31, 2022

DMBA-induced Estrogen Receptors α, β, and AKT Modulation in Animal Model with Breast Cancer

Aziiz Mardanarian Rosdianto, [1,2,3,10] Ahmad Kurniawan,[4] Julia Windi Gunadi,[5,6] Isa Mahendra,[4] Iwan Setiawan,[2,3,10] Hanna Goenawan,[2,3,10] Nova Sylviana,[2,3,10] Yuni Susanti Pratiwi,[2,3,10] Mas Rizky A. A. Syamsunarno,[3,7] Roro Wahyudianingsih,[8] Unang Supratman,[3] Ronny Lesmana,[2,3,9,10]

[1]Veterinary Medicine Study Program, Universitas Padjadjaran, Indonesia
[2]Physiology Division, Department of Biomedical Sciences, Faculty of Medicine Universitas Padjadjaran, Indonesia
[3]Division of Biological Activity, Central Laboratory, Universitas Padjadjaran, Indonesia
[4]Center for Applied Nuclear Science and Technology, The National Nuclear Energy Agency Republic of Indonesia, Bandung, Indonesia
[5]Department of Physiology, Faculty of Medicine, Maranatha Christian University, Bandung, Indonesia, [6]Maranatha Biomedical Research Laboratory, Faculty of Medicine, Maranatha Christian University, Bandung, Indonesia
[7]Biochemistry and Molecular Biology, Department of Biomedical Sciences, Faculty of Medicine Universitas Padjadjaran, Indonesia
[8]Department of Pathology Anatomy, Faculty of Medicine, Maranatha Christian University, Bandung, Indonesia,
[9]Center of Excellence in Higher Education for Pharmaceutical Care Innovation, Universitas Padjadjaran, Indonesia,
[10]Laboratory of Sciences, Universitas Padjadjaran, Bandung, Indonesia

Abstract

The high incidence of breast cancer cases in the world requires the use of applicative methods. The 7,12-dimethylbenz(a)anthracene (DMBA) induced breast cancer animal model is a widely used chemical-induced animal models for research on breast cancer. However, the molecular mechanism related to DMBA induction remains unclear. Good understanding on DMBA-induced animal models is crucial for studies related to future breast cancer treatments as animal models will provide a deeper understanding of anticancer medication, specifically those aimed for treating breast cancer. The aim of this study was to develop an DMBA-induced animal model for breast cancer. This study used female Wistar rats injected subcutaneously with DMBA as a carcinogen-induced agent (20 mg/kg) to induce tumor. Rat tumors were then evaluated and breast appearance was observed weekly, starting from day 28th after DMBA injection. Breast cancer tissue was then sampled and stored at -80°C until it was used for western blot and histological study. This study indicated that DMBA induced cancer in female Wistar rat's breasts, and cytoplastic cells and lung metastatic was identified macroscopically and histopathologically. The metabolic sign was observed in the lung and breast sections. Interestingly, the DMBA induction in this study does not only induce organ cancers but also induces estrogen receptors and stimulates signaling of estrogen receptors α (ERα), ERβ, and Akt.

Keywords: Akt, Animal model, breast cancer, DMBA-induced, estrogen receptors

Introduction

Cancer is uncontrolled cell growth and can metastasize to different parts of the body depending on the individual condition.[1] The prevalence of cancer cases in 2000-2020 increased to almost 20 million people. By 2040, it is estimated that the number of cases will continue to increase by reaching 50% proportions.[2] Breast cancer is the fifth leading cause of death in women.[3,4] The uncontrollably changing and dividing of breast tissue cells leads to a mass or a lump and further gives rise to breast cancer. This cancer usually starts from the lobules (milk glands) or the ducts connecting the lobules and the nipple.[5] The incidence of breast cancer in Indonesia continues to increase every year.[6] The data from Indonesian Basic Health Research (*Riskesdas*) showed an increase in the prevalence of cancer and tumors from 1.4 per 1,000 in 2013 to 1.79 per 1,000 in 2018.[6] Currently, the incidence of cancer in Indonesia ranks 8th and 23rd in Southeast Asia and Asia.[3] Breast cancer

Corresponding Author:
Ronny Lesmana,
Physiology Division, Department of Biomedical Sciences,
Faculty of Medicine, Universitas Padjadjaran, Indonesia
Email: ronny@unpad.ac.id

pathogenesis and progressivity depend on many factors such as genetic and environment. As for genetic factors, a recent study has identified different kinds of genes as proliferation and nuclear factors for breast cancer. These genes have an important role in establishing breast cancer characteristics, patient's response to anticancer therapy, and their survival rate.[3,4] The potential of breast cancer to spread (metastatic properties) might determine the bad prognosis of the patient and lead cause of death in a patient. Limited exploration of the dimethylbenz(a) anthracene (DMBA)-induced model and the primary mechanism of cancer metastasis was an important basis for understanding the triggering mechanism of breast cancer in animal models with representative clinical observations.[3,4]

There is no perfect breast cancer model given the high level of heterogeneity. The modeling of combinative breast cancer in murine models is also widely used to understand prevention and therapy methods. However, a single model of cancer induction has not explained much about the pathway's mechanism for its success and for curing cancer in humans. So that further exploration will be useful.[7]

This study aimed to create an animal model of breast cancer induced by the carcinogenic chemical DMBA and explore the expression signaling pathways of ER-alpha and beta together with Akt to trigger the development of rat breast cancer. Finally, our study will examine the animal model justification and provide a direction for future research in creating a breast cancer animal model.

Methods

The experimental research on animals was conducted in September 2020 at the laboratory animal facility of The National Nuclear Energy Agency (BATAN) Republic of Indonesia, Bandung. Female Wistar rats weighing 150–160 g and aged between 6–7 weeks old were obtained from PT. Biofarma, Tbk., Bandung, Indonesia. Animals were acclimatized for one week after procurement and feed using standard rodent pellet diet and water *ad libitum* during the experimental period. The laboratory environment was maintaining 12:12 h light/dark cycle with an ambient temperature of 22 ± 3°C and humidity at 50±10%. Experimental procedures were approved by Ethical Committee for The Care and Use of Laboratory Animal (KEPPHP) BATAN (Ethical number: 003/KEPPHP-BATAN/V/2017).

The study was conducted by inducing cancer using chemicals and routine post-induction observations. 7,12-Dimethylbenz[a]anthracene (DMBA) was purchased from Sigma Aldrich (CAS Number:57-97-6, St. Louis, MO, USA), and corn oil was purchased from the common market. The doses of 20 mg/kg of DMBA were diluted with 0.5 mL of corn oil and saline using a sterile tube on single-dose injection. An injection of DMBA was administered subcutaneously into left breast rats.[7] Breast tumors of rats were evaluated and observed every week frequently starting from days 28th after DMBA injection for assessing tumor growth.

The study also used western blotting technical analysis methods. Western blot protocol was modified from Lesmana et al.[9] and Rosdianto et al[10] The whole breast tissue cancer was weighted and dissected, homogenized in lysis buffer (containing protease inhibitors, 10 mM Tris-HCl (pH 7.8), 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA). Following centrifugation, lysates were denatured (at 96 °C for 5 minutes). Lysates (administered 15 µg/lane) were homogenized, parted by SDS-PAGE, and made over to a nitrocellulose Western blotting membrane (GE Healthcare). The membranes after protein transfer were incubated for 60 minutes (22-25°C). The membrane was soaked overnight with Tris-buffered saline buffer with 0.1% Tween 20 as blocking solution at 4 °C in 2%**.** Western blotting was complete using a mouse monoclonal antibody of estrogen receptor alpha (#MA5-14501, diluted 1:1,000), estrogen receptor beta (#PA1-311, diluted 1:1,000), AKT (#9272, diluted 1:10000), and B-actin (Thermo scientific AM4300, diluted 1:1,000). The secondary antibody using anti-mouse (LI-COR, IRDye® 800CW Goat anti-Mouse IgG). The band was enhanced by chemiluminescence reagent (GE Healthcare, Chicago) and imaged (LI-COR Odyssey CLX, Cambridge, United Kingdom). The intensities are measured by LI-COR software.

Rat's mammary tumors are analyzed through histopathological methods. The rats were euthanized at week 18th after all the breast tumors were palpated. The breast cancer and lung tissue were isolated and fixed using 10% neutral buffered formaldehyde (Sigma Aldrich, St. Louis, MO, USA) for 24 h. Following protocols, by Eltania et al[10] The tissues were then dehydrated in ascending series of alcohol, kept in a 1:1 mixture of lab-grade alcohol, and subsequently incubated in lab-grade benzene for 1 hour. At last, tissue pieces were embedded

in paraffin wax, cut that paraffin blocks into 5 µm, applied on object glasses, color-wash with hematoxylin and eosin (H&E), and viewed under a light microscope with the same magnification and angle of view.

All experiment data were analyzed using Microsoft Office Excel 2019. Descriptive analysis data is presented based on the mean and SD. Non-parametric data were analyzed by Kruskal-Wallis with a CI of 95%.

Results

This animal model study indicates the success of breast cancer-induced in female Wistar rats by DMBA. The induction results were shown by macroscopic images of the rat breast (Figure 1). The lumps of the breast cancer were palpable and indicated tumor growth. A minor surgical procedure was performed to extract tumor mass under anesthesia conditions. The tumors in the DMBA-induced rats ranged in size from 0.1 to 3.0 cm in diameter and were unidentified in the neck, belly, or armpit. The tumor appears as a pale solid mass (Figure 1).

The mammary of DMBA-induced rats displayed proliferative cells with heterogeneous and uneven patterns under the microscope. Additionally, proliferating tumor cells damaged the basement membrane and fibrous connective tissue. Histopathology of breast cancer tissue showed grade 3 (poorly differentiated, high grade) and stage IV (metastatic cancer) values compared with normal rat breast tissue cells (Figure 2.). The results of histopathological analysis on rat breast cancer showed a lot of tumor cell formation. This result is visible when we compare it with normal rats (Figure 2b. and 2a.). Based on observations, breast cancer that occurs has metastases in the lungs of rats and showed the presence of many small metastases (Figure 2d. and 2c.). The tumor showed ductal and cribriform patterns 50% ductal differentiation characterized by mitotic features, nuclear pleomorphism, and tubule and gland formation in the carcinogen-induced group, which increased to 2 folds the normal rats (Figure 2e). The western-blot results indicate that DMBA induces cancer through the signaling of ERα, ERβ, and AKT (Figure 3).

Discussion

Rats are widely used animals for animal models included breast cancer. Rats have been chosen as an animal model compared to other animals in experimental terms to study mammary carcinogens because the mammary glands of rats have neoplasms that are very similar to humans.[11] However, using mammals for breast cancer animal models requires more detailed information, high costs, and long experiment periods.[7]

Currently, murine models are most widely

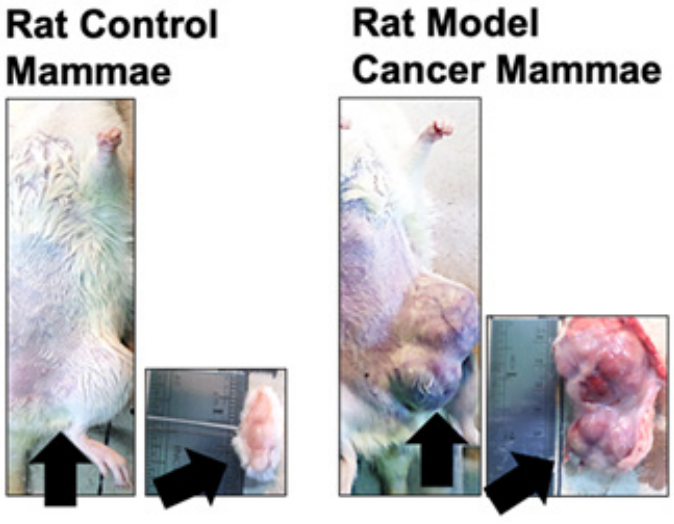

Figure 1 Macroscopic Breast of Female Wistar Rats by DMBA-induced

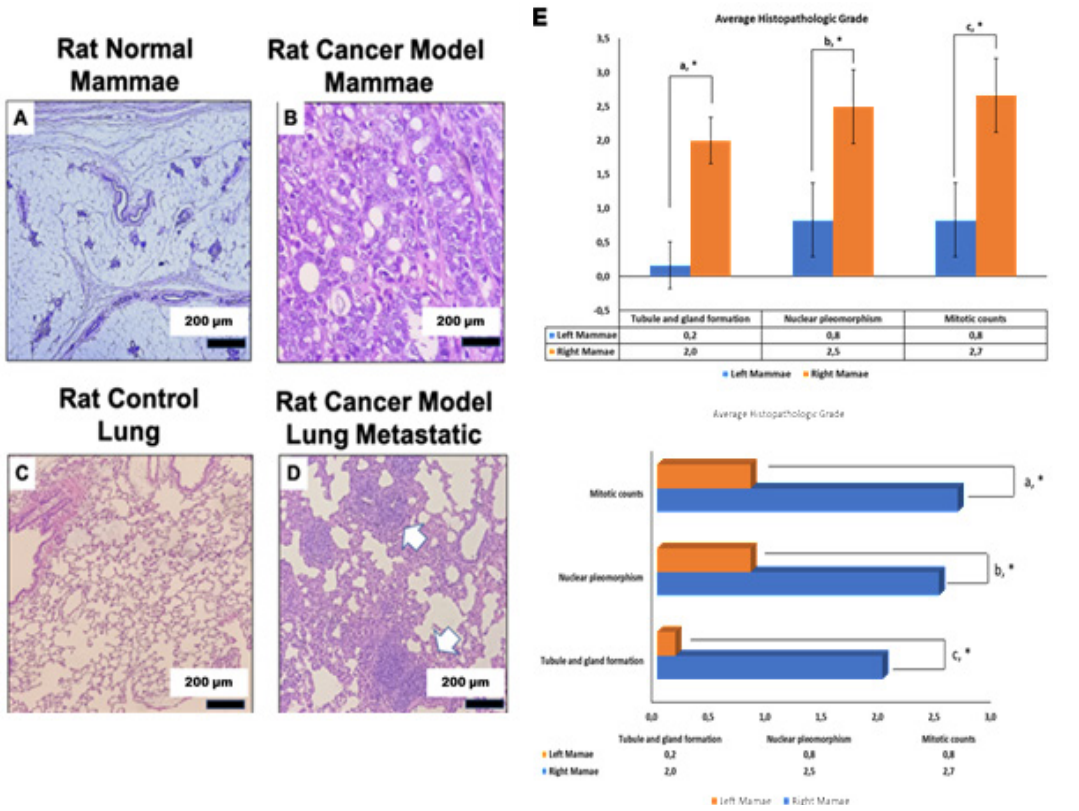

Figure 2 Histopathological Results were Shown in 40x Magnification

(a) normal and (b) carcinogen-induced breast cancer in rats. Metastases were seen in the lung compared with (c) control and (d) lung metastatic mouse cancer models, and a white arrow showed tumor cell invasion in a vessel of the lung. (e) The mean histopathology-score of breast cancer in female Wistar rats is based on mitosis, nuclear pleomorphism, and tubule and gland formation values.

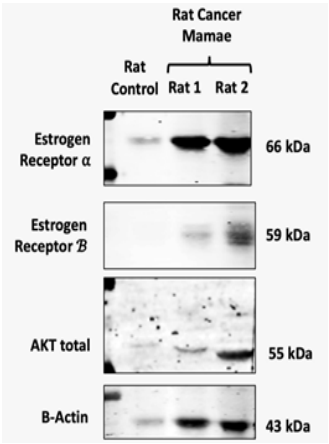

Figure 2 The protein level of ERα (66 kDa), Erβ (59 kDa), B-actin (43 kDa), and Akt (55 kDa) on Wistar rats' Breast-Cancer Tissues was performed by Western Blot Analysis

There were differences in gene expression between DMBA-induced rats compared to controls.

used as breast cancer animal models because they are smaller, cheaper, reproduced faster, have similarities to humans, have a greater variety of inbred strains, and have high gene-editing technology.[13] Nevertheless, using mice for breast cancer models has some disadvantages; for example, metastasis of breast cancer in humans usually occurs in the lung, lymph node, liver, bone, and brain, but in mice models, it only metastasizes to the lung.[14]

Various chemicals that have been used as an inducer of breast cancer in rats are DMBA, methyl nitrosourea (MNU), 3-methylcholanthrene (MCA), 2-acetylamino-fluorene (2-AAF), benzopyrene, ethyl nitrosourea, and or butyl nitrosourea.[14,15,16] Among those, DMBA and MNU are the most commonly used breast cancer inducer. DMBA and MNU model cancer have hormone-dependent characteristics.[14,15] In general, cases of human breast cancer and DMBA induction showed a similar tumor model, which occurred in the mammary duct area.[16,17] DMBA

is high carcinogenic properties. DMBA was dependent on the injection method. Skin contact with DMBA can develop papilloma. DMBA can also cause leukemia when injected directly into the vein.[12] Mammary cancer induction by DMBA can be done by oral administration and subcutaneous injection and is known effective in inducing breast cancer. DMBA is recognized to be prototypical of polycyclic aromatic hydrocarbons (PAH). The tumorigenesis process involves the aryl hydrocarbon receptor (AhR), which depends on the regulation of Cytochrome P450 enzymes that metabolize DMBA into mutagenic epoxide intermediates that are ready to form DNA adducts.[20] DMBA induced tumor (Figure 1), also histopathological results showed marked cancer in breast tissue (Figure 2b) accompanied by cancer metastases in lung tissue (Figure. 2d). This result becomes one of the main reasons for the utilization of DMBA for the induction of breast cancer in animals.

The utility of DMBA-induced mammary carcinogenesis models in human breast cancer studies has been widely used.[18,19]. The results showed the successful induction of rat breast cancer. Observationally, the tumor lumps indicated its growth in the rat breast. Microscopically, DMBA-induced rats were classified at grade 3 and stage IV for breast cancer form. In other results, we found metastatic conditions as evidenced by the histopathological picture of rat lung tissue. Tissue histology changes must be linear by a significant level of estrogen. This restrictive study was limited to exploring signaling pathways of estrogen receptor and AKT by using western-blotting methods. DMBA-induced breast tumors in female Wistar rats have shown significant breast carcinogenesis results. The use of Wistar rats in this study referred to previous research, which proved that this strain was DMBA sensitive.[21] On Sun et al[22] study intended to explore the mechanism of these tumors and determine the immune-expressions of reproduction hormone receptors (ER α and β).Moreover, DMBA-induced control group rats may exhibit the up-regulation of PI3K, AKT, and mTOR.[23] Similar to the results of Clarke *et al.,* which was later clarified in this study, that the Akt pathway then plays an important role in stimulating the expression of ERα and ERβ, which are directly involved in regulating tumor proliferation, growth, and motility (Figure 3).

The investigation results in breast cancer animal models could be used as a biological basis for understanding characteristics of breast cancer in humans to provide a better approach when determining novel therapy. The estimation of safety, efficacy, and bioavailability of new drugs applied for breast cancer could be obtained from preclinical studies using animal models.[24] Additionally, animal models also provided knowledge about cancer progression, sensitivity, and resistance mechanism to therapies.[25]

Our study has proven that DMBA induces rat breast cancer through estrogen receptor and AKT signaling pathways. Further research is important to evaluate the induction results based on different administration methods with similar doses and other molecular targets such as NF-kappa B and ROS. It is expected to obtain murine models that are stable and easy to develop, especially in testing novel and reliable anticancer candidates.

In this study, we can conclude that the administration of DMBA as the single carcinogenesis agent to induce breast cancer in rats can increase the expression of ERα, ERβ, and AKT signaling, which is involved in breast cancer progression. The results obtained parallel with a macroscopic and histological appearance, so the rat models of breast cancer become a relevant option for studying anticancer candidates.

Acknowledgment

The authors acknowledge (a) The Center for Applied Nuclear Science and Technology, The National Nuclear Energy Agency, Republic of Indonesia, for financial support, (b) Central Laboratory, Universitas Padjadjaran, (c) Grant scheme Penelitian Dasar Unggulan Perguruan Tinggi/PDUPT (document number: 1207/UN6.3.1/PT.00/2021) from Directorate General of Higher Education, Ministry of Education and Culture, Republic of Indonesia.

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