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Profile of Histopathological Type and Molecular Subtypes of Mammary Cancer of DMBA-induced Rat and its Relevancy to Human Breast Cancer

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Abstract

BACKGROUND: Animal models with mammary cancer that closely mimic human breast cancer for treatment development purposes are still required. Induction of 7,12-dimethylbenzanthracene (DMBA) to rats shows the histopathological features and mammary cancer characterization similar to humans. Examinations of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and Ki67 expressions are crucial in deciding the treatment and prognosis of breast cancer.

AIM: This research aimed to view histopathology images of mammary glands and expressions of ER, PR, Ki67, and HER2 of DMBA-induced rats.

METHODS: After 1-week adaptation, 11 5-weeks-old female rats were induced with 20 mg/kg body weight (BW) of DMBA 2 times a week for 5 weeks. On week 29, nodules taken from the mammary gland were examined for hematoxylin-eosin staining and immunohistochemistry with p63, ER, PR, HER2, and Ki67 antibodies. The grading score used the Nottingham Grading System and molecular classifications based on St. Gallen 2013.

RESULTS: Six rats had nodules, but the histopathologic features of one nodule showed normal mammary gland without cancer. The histopathological type of mammary cancer was cribriform carcinoma, comedo carcinoma, lipid-rich carcinoma, adenocarcinoma squamous, and adenomyepithelioma. Histopathological grading showed 60% of grade 3 and 40% of grade 2. P63 expression showed 60% positive and 40% negative. The frequency of ER, PR, HER2, and Ki67 of five nodules showed positivity: 40%, 60%, 60%, and 60%, respectively. Molecular subtypes of Luminal A, B, HER2, and triple-negative were 0%, 60%, 20%, and 20%, respectively.

CONCLUSION: Histopathological features and molecular subtype of mammary cancer on rats induced with 20 mg/kg BW of DMBA showed similarity to human breast cancer.

Introduction

Breast cancer is the most common cancer case in women around the globe including in Indonesia. The new cases of breast cancer keep increasing both in Indonesia and globally [1]. Even though the mortality level in developed countries has tended to decline, mortality in developing countries keeps increasing [2]. Research and development to overcome the limitations in both prevention and treatment of breast cancer are still direly needed. Animal experiments are always conducted before clinical trials in humans. Research on humans needs a complicated ethical procedure for approval from institutional review boards before beginning the study. To continue this sensitive research in the interim, the use of animal models in which mammary cancer characteristics closely mimic human breast cancer is still required [3].

There are various types of animal models for cancer. They are cancer line cell transplantation or cancer tissue transplantation, animal study with genetic intervention or transgenic subjects, and environmental intervention or chemical material exposure which involves increasing the risks for the occurrence of cancer. Compared to others, the experimental animal type with chemical substance tends to look like the mechanism of human carcinogenesis from the initiation phase, continued with promotion and progression. Therefore, the model of cancer with chemical exposure can be used for evaluation and research that is related to etiology, prevention, diagnostic, and treatment for all cancer stages [4]. One of the chemicals carcinogenic that is commonly used is 7,12-dimethylbenzanthracene (DMBA) [4].

DMBA is a Polycyclic Aromatic Hydrocarbon: a carcinogen that depends on the ovarium hormone. The DMBA rat model was selected since DMBA can

induce preneoplastic lesions in the form of intraductal proliferation and mammary intraepithelial neoplasia that are similar to ductal carcinoma of humans *in situ* [4].

The molecular subtype of breast cancer is currently needed to classify treatment determination and breast cancer prognosis. These molecular subtypes of breast cancer can be classified into Luminal A, B, human epidermal growth factor receptor 2 (HER2), and triple-negative. The classification is based on the expressions of estrogen receptor (ER), progesterone receptor (PR), HER2, and Ki67 [5]. By this classification, patients with positive hormone or positive HER2 can be treated with anti-hormone or anti HER2 which consequently has a better prognosis [6]. The existence of a profile of molecular subtype in DMBA-induced rats that are similar to the molecular type of human breast cancer is significant for research development in breast cancer treatment. Research of the classification of molecular subtypes in DMBA-induced mammary cancer rats is still limited. The most-reported frequency of mammary cancer in DMBA-induced rats is positive estrogen (luminal A): 76.29%, which is similar to the type that is mostly happening to women with breast cancer [7], [8]. However, the past research has not reported the existence of HER2 and Ki67 expressions. This research aimed to reveal how the image of mammary cancer molecular subtype is in rats after being induced with DMBA and the relationship with grading and histopathological type of breast cancer.

Methods

Research subjects

This research was a randomized post-test only using 11 female Sprague Dawley rats gained from the Integrated Research and Testing Laboratory of Universitas Gadjah Mada (UGM). According to Charan and Biswas in 2013 [9], this amount met the minimal size for animal studies in which the total of rats subtracted by total groups (1 group) = around 10–20. Rats aged 4 weeks had body weight (BW) around 40–70 g. After 1-week adaptation, they were induced with 20 mg/kg BW of DMBA (@Sigma-Aldrich) dissolved in corn oil twice a week for 5 weeks. Rats were fed a standard AIN93 diet and drank ad libitum until they were 33 weeks old. They were kept under controlled conditions including temperature state, humidity, sanity, and breeding room according to guidelines for animal welfare. This study was approved by the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health and Nursing, UGM, Yogyakarta, Indonesia.

Tissue processing

Besides taking the breast nodule, macroscopically, each organ of the rats was inspected and palpated to see the possibility of finding any nodules. Mammary nodules were soaked in 10% phosphate-buffered saline (PBS) formalin for 2 × 24 h before tissue processing. It was done using alcohol, xylene, and liquid paraffin before being made into paraffin embedded samples. Paraffin-embedded blocks of rat mammary cancer were cut (4 μm thick) before hematoxylin-eosin staining and immunohistochemistry.

Immunohistochemistry

Slides from paraffin-embedded tissue sections were deparaffinated and rehydrated using xylol 3 times each for 5 min, then using ethanol 100%, 95%, and 70%, and aquadest for 5 min each. Hydrogen peroxidase block was dripped into the slide and incubated for 10 min, and then it was washed twice in PBS with Tween® detergent (PBST). Heat-induced antigen (epitope) retrieval was conducted using heat 95°C for 20 min in tris EDTA Ph 8. After it cooled down, the slide was moved into a humidity chamber and washed 3 times in PBST. Immunoblock (@Biotna Biotech Cat.No. TAHC02D) was then applied and was incubated for 10–30 min at room temperature to block non-specific background staining. After that, it was washed 2 times in PBST. Primary antibodies: ER1 (Abclonal Cat No. A3198), PR (Fine test Cat No. FNab09774), HER2 (Abclonal Cat No. A2071), Ki67 (Fine test Cat No. FNab09788), and p63 (Fine test) were applied and incubated for 1 h in a room temperature with each dilution 1:200. Thereafter, the slide was re-washed with PBST for 5 min (3 times). Rabbit probe horseradish peroxidase was incubated for 30 min at room temperature and then washed 3 times using PBST. DAB chromogen 1:20 was applied for 2 min and then washed 4 times in PBST. Hematoxyline was applied for 2 min. Then, finally, it was washed with aquadest.

Histopathological type

The slide was observed using a magnification microscope 10 × 40 (Olympus CX21). Histopathology type was determined based on Goldschmidt *et al*. [10], Rudmann *et al*. [11], Russo [8], and Nascimento and Otoni [12]. Evaluation of histopathological grading of the mammary tumor was based on the scoring method from the Nottingham Grading System by evaluating tubular formation, mitotic rate, and nuclear pleomorphic. The total of the three scores were classified as grade I (well-differentiated) with 3–5 Score, grade II (moderately differentiated) with 6–7 Score, and grade III (poorly differentiated) with 8–9 Score [13]. The observation was conducted with objective lens magnification 40× with 10 fields of view. Evaluation included tubular feature >75% with Score 1, 10–75% was scored 2 and <10% (Score 3). Pleomorphic nucleus evaluation was a uniform relative cell (Score 1), size and nuclear form that

was slightly varied (Score 2), and highly pleomorphism with vesicular chromatin, and prominent nucleoli (Score 3). Meanwhile, mitosis activity was scored for its mitosis amount with 0–9 (Score 1), 10–19 (Score 2), and >20 (Score 3).

Molecular subtype classification

ER, PR, and Ki67 expressions were counted from 1000 cells in the area with the highest number of brown cell nuclei (hot spot) with a magnification microscope 400×. Sample was positive for ER and PR if the brown in the cell nucleus ≥1% cell; positive Ki67 if the brown nucleus cell ≥14% [14]. The intensity of the brown color was not differentiated (Figure 1). HER 2 was positive if the intact cell membrane was strongly stained with brown and was observed for more than 30% [15]. P63 expression was considered positive marked by dark brown color, observed at peri ductal myoepithelial cell nucleus. P63 expression was assessed into three categories [16]: Positive diffusion if ≥10% of positive cells and myoepithelial cells also showed continued expression; positive focal if myoepithelial cells showed disjointed expression; and negative expression if no positive cells (Figure 2). The molecular subtype was classified based on St. Gallen 2013. It is classified as Luminal A if ER/PR (+) and Ki67/HER2 (-); Luminal B type if ER/PR (+) and Ki67/HER2 (+); HER2 type if PR/ER (-) and HER2 (+). Triple-negative was determined when the expressions of ER, PR, and HER2 were negative [14].

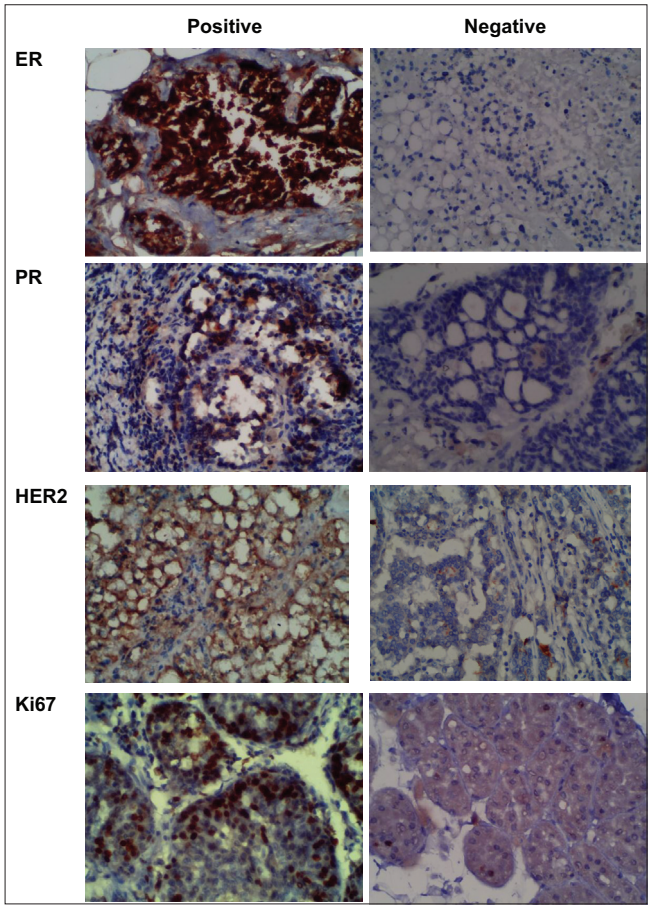

Figure 1: Expression of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 and Ki67 (magnification 400×)

Results

Five rats died before any nodules appeared. Six rats with nodule formation underwent histology examination and one rat nodule found showed normal mammary gland features. Five others showed malignant neoplasm features because of increased proliferation, mitotic figure, nuclear pleomorphic, and decrease in tubular feature. Based on the examination of all organs, there was one rat with a nodule in the lung, but no metastasis was found in four other rats. The histological type found was mostly carcinoma ductal: Type cribriform, type comedo, and others were lipid-rich carcinoma, adenocarcinoma squamous, and adenomyepithelioma (Figure 3). The most common histopathological grading (60%) showed was poor differentiation level (Grade 3), 40% of Grade 2, and none were found with grade 1 (Table 1).

P63 expression showed that two cancer tissues experienced invasion due to negative p63 expression. Two other cancer tissues mostly showed no infiltration, but some places experienced infiltration toward muscle and connective tissues. There was one nodule that showed scattered p63 expression. There were two nodules from five nodules (40%) with ER expression. PR expression was 60%, HER2 expression was 60%, and Ki67 expression was 60%. Based on the St. Gallen classification, the largest number of occurrences was Luminal B (60%), followed by HER2 (20%), triple-negative (20%), and there was no Luminal A-type.

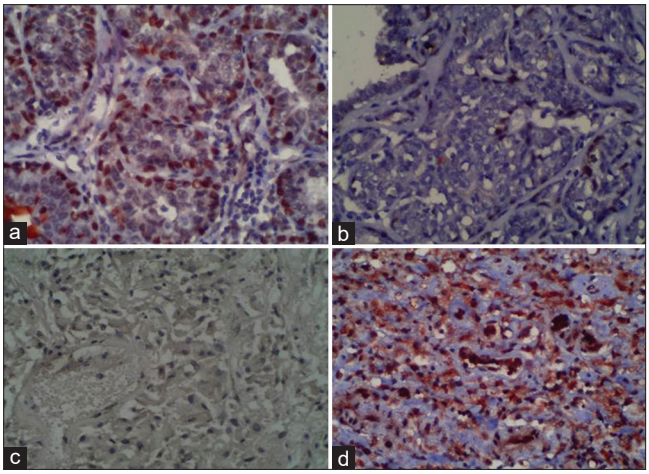

Figure 2: P63 expression of breast nodule after given 20 mg/kg body weight of 7,12-dimethylbenzanthracene 2 times a week for 5 weeks: (a) Diffuse expression; (b) focal expression; (c) negative expression; (d) scatter expression

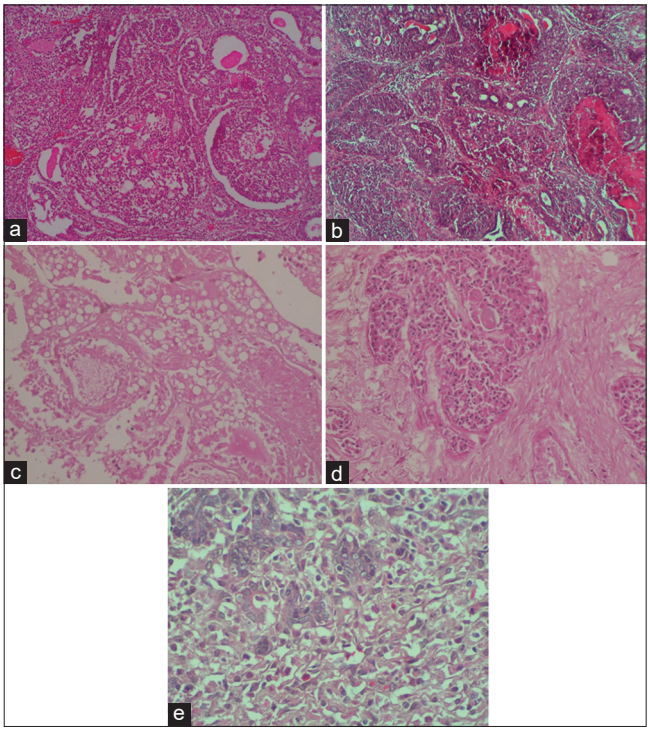

Figure 3: Histopathological image of breast nodule after being given 20 mg/kg body weight of 7,12-dimethylbenzanthracene twice a week for 5 weeks (a) comedo carcinoma; (b) cribriform carcinoma; (c) lipid-rich carcinoma; (d) adenocarcinoma squamous; e) adenomyoepithelioma (H and E staining; magnification 100×)

Discussion

The clinical state of subjects

The experimental animals were cared for maximally according to animal welfare guidelines such as room temperature (23 ± 2°C) 12 h dark and light adaptation, and air humidity 70–80%. Animals lived in a cage made of waterproof, robust, and easily cleaned materials. The cage has sufficient space and was always kept clean. The researchers provided standard animal feed and access to drink every day with suitable pellets (AIN93M) ad libitum. However, several test animals became sick and ended up dying. The disease was be suspected by a combination of *Mycoplasma pulmonalis* and *Streptobacillus moniliformis* with the possibility of virus involvement. Unfortunately, those possibilities could not be proved yet since the bacteria culture examination was not conducted. The assumption came from the fact that those sick rats showed inactive rat symptoms: Blood coming out from eyes, porphyrin secretion or known as chromodacryorrhea, hair falling out or rough hair, and loss of appetite [17]. This disease attacks the respiratory tract and is called the chronic respiratory disease (CRD). Besides those symptoms, other symptoms were often unspecific such as sneezing and coughing. The researchers randomly found a grey lesion in the lung. Some rats also experienced additional symptoms such as slanting and spinning walks. The symptoms were assumed to be caused by the involvement of *Pasteurella* sp. [18]. In standard laboratories, rat infection disease is still often found. A survey on rat bacteria in laboratories in North America, Europe, Australia, New Zealand, and Singapore showed that the most common causative bacteria are parvoviruses, rat rhinovirus, and *Helicobacter* spp. Meanwhile, *Pasteurella* is in the next place with around 4.81% cases in America and Europe and 17.9% in Australia [19], [20]. In addition, CRD disease with *M. pulmonalis* and *S. moniliformis* as the causes generally happen to experimental animals in tropical areas as found in this research [18], [21]. DMBA administration causes rats to easily get sick because DMBA causes organ toxicity such as liver damage [22]. In addition, this induction also increased oxidative stress, and number of granulocytes, but decreased the number of erythrocytes [23]. It increased inflammation [24] and decreased immunity [25]. In a previous report, administration of 12 mg single dose of DMBA to Sprague Dawley rats caused death in 30% of subjects [26].

Table 1: Histopathology, grading, expressions of ER, PR, HER2, Ki67, and p63 after induced with 20 mg/kg BW of DMBA

Histopathology type	Rat 1 Comedo carcinoma	Rat 2 Cribriform carcinoma	Rat 3 Lipid rich carcinoma	Rat 4 Adenocarcinoma squamous	Rat 5 Adeno myoepithelioma
Grading	3	2	2	3	3
Tubular	2	2	3	3	3
Pleomorphic	3	2	1	2	2
Mitosis	3	3	3	3	3
Metastasis	no	no	no	yes	no
p63	diffusion, focal	diffusion, focal	negative	negative	scatter
ER	(+)	(+)	(-)	(-)	(-)
PR	(+)	(+)	(-)	(+)	(-)
HER2	(-)	(+)	(+)	(+)	(-)
Ki67	(+)	(+)	(-)	(+)	(-)
Molecular subtype	Luminal B	Luminal B	HER2	Luminal B	Triple negatives

DMBA: 7,12-dimethylbenzanthracene, ER: Estrogen receptor, PR: Progesterone receptor, HER2: Human epidermal growth factor receptor 2.

Histopathological type of DMBA-induced mammary cancer

Histology types of induced breast cancer using DMBA are generally papillary carcinoma and cribriform carcinoma [8], [27]. In this research, the most common types found were cribriform and comedocarcinoma, but papillary carcinoma was not seen in this research. Instead, it found lipid-rich carcinoma and adenomyoepithelioma; a rarely found type of cancer. However, according to Russo *et al*. [28], this might be because, in both benign and malignant breast tumors, fat droplets could appear, so it showed a cytoplasm image with the fat vacuole. The research by Wahyuniari *et al*. [29] found one case in DMBA-induced Sprague Dawley rat with this type. There are several case reports on both human and rat breast cancer with lipid-rich carcinoma images, but there are only a few reports related to the prognosis of this case. In the 17 lipid-rich carcinoma cases in Jinjling Hospital China, there was connectivity between grade and involvement of lymph node with prognosis [30]. As in those 17 cases, this research has a similar histopathological image: ER, PR, and negative p63, but positive HER2.

Adenomyoepithelioma is a rare case in terms of both human and rat breast cancer. However, there was a report about this in several imaging cases in DMBA-induced mice [31], induced DMBA and knockout TRP53 mice [32], and induced DMBA with a high-fat diet [33]. Histopathology of breast adenomyoepithelioma showed a multinodular mass with the proliferation of epithelium and myoepithelium cells. Epithelium cells usually form the gland room. It lined up high with a little of eosinophilic cytoplasm surrounded by a myoepithelium cell with clear cytoplasm. Myoepithelium cells may be common and may have forms like a spindle, clear or polygonal. Myoepithelium appeared as a small or scattered group with epithelioid morphology, intranuclear vacuole, or intracytoplasmatic. There was mild nucleus atypia. The myoepithelial cell was confirmed by positive p63 expression (appeared brown color in immunohistochemistry examination). The prognosis of this case depends on whether it is benign or malignant [34], [35]. In this research, adenomyoepithelioma tended to be malignant, because a rare tubular feature was found (Score 3), mild variation of pleomorphic nucleus image (Score 2), and high rate of mitosis (Score 3).

Adenocarcinoma squamous is also a rare type of human breast cancer [36]. Histopathology showed metaplasia squamous cells and experienced partial or no keratinization. This type is reported in DMBA-induced cancer rats [11], [37]. This cancer is often malignant and has a bad prognosis [36]. This research also found the existence of necrosis, inflammation, rare tubular image, variation of nucleus shape and size, increased mitosis, and infiltration in the connective tissue around it.

The present study found comedocarcinoma and cribriform carcinoma. In rat 1, microscopically, histopathologic presents center necrotic in neoplastic cell aggregates. The tissue consists of closely packed cells arranged in solid foci and nests supported by a fibrovascular stroma. In rat 2, the cribriform-type images showed islands of uniform tumor cells forming a sievelike arrangement. Both cancer tissues showed positive p63 expression, intact basalis membrane, and continuous p63 expression. However, other parts showed negative p63 expression, and in H and E staining seemed to invade muscle. The prognosis of breast cancer type depends on the expressed molecular type [38]. In this research, both are luminal B types with positive ER, PR, and Ki67 expressions.

In this research, grade 3 histopathological features ensued the most frequent (60%) after induction of 20 mg/kg BW DMBA 10 times. The previous research used a 5 mg dose of DMBA induction and 65 mg/kg BW single dose which showed that the most frequent image was grade 1, then grade 2, and the fewest was grade 3 [37], [39]. In general, in human breast cancer, the most frequent is grade 2 [40], [41]. Histopathological grading showed cell differentiation level and it had a relation with a bad prognosis of human breast cancer [42]. In this research, rat number 4 showed grade 3, had negative p63 expression, infiltration in surrounding tissues, and metastasis nodule was seen in lung tissue. In this study, DMBA induction got a less aggressive mammary cancer, because only one metastasis to the lung was found. Meanwhile, in Korea and Indonesia, patients with breast cancer commonly experience metastasis which is around 45.8% and 64.06%, respectively [43], [44].

P63 examination is used as one of the markers of invasion in breast gland cancer. Continuous positive p63 expression means that the myoepithelial cells were intact and still surround the gland, meaning that the development is still *in situ* and did not penetrate the basalis membrane. The p63 expression in this study was negative in two rats (40%). The previous research showed no difference in p63 expression between rats and humans. There was a significant difference in p63 expression between hyperplasia and invasive ductal carcinoma. In invasive ductal carcinoma, the expression of p63 was negative but positive in hyperplasia [45].

A molecular subtype of DMBA-induced mammary cancer

The frequency of ER and PR expressions in this research was 40% and 60%. In the previous study, DMBA induction showed a frequency of ER expression of 39.89% and PR expression of 21.56% [27]. In other researches, they showed 65.8% and 34.2% [46]. Indeed, DMBA induction can increase ER expression. There are not many reports presenting molecular subtypes based on the consensus classification of St. Gallen 2013 for DMBA-induced rats. Meanwhile, this classification of human breast cancer becomes a treatment guide. In this research, molecular subtype found after the administration of 20 mg/kg BW of DMBA as much as 10 times showed 60% of Luminal type B as the most frequent subtype, followed by HER2 and triple-negative (20%) and no type of Luminal A found. The previous study showed Luminal A (76.2%) and Luminal B (23.8%). However, this classification had not included the Ki67 expression yet (Russo, 2015b). In human breast cancer, generally, the highest frequency is Luminal A which is around 20–30%, then Luminal B (20–30%), HER2 (12–20%), and triple-negative (15–20%) [47].

HER2 expression in this study was about 60%, meanwhile research on rats with 10 mg/100 g BW dose of DMBA was 29.4% [48]. The frequency of HER2 expression on human breast cancer varies of which 22% in Switzerland [49], 34.4% in Iran [50], and 48.5% in Lampung, Indonesia [51]. The research with rat HER2 (+) model showed an increased frequency of HER2 expression with DMBA induction [52]. Positive HER2 on human breast cancer was associated with higher histopathological grading, high proliferative index, and poor prognosis, except for the patients who were given antiHER2 [12].

The best prognosis on human breast cancer is Luminal A subtype followed by Luminal B, HER2, and triple-negative [53]. This research found nodules with low Ki67, but the grading showed a bad differentiation level. In general, high expression of Ki67 relates to higher grading and poor prognosis [54]. However, other reports in Iran and Afghanistan showed there is no relevance between Ki67 and grade [55], [56].

In this research, 60% of nodules showed Ki67 expression. The previous research on induced rats with 10 mg/100gr BW of Ki67 expression was 35.5% [48]. The expression of Ki67 on human breast cancer varies. The research in Egypt showed 62.8% of Ki67 expression [15]. Ki67 is a marker of cell proliferation and cycle. Cell cycle occurred as the result of the c-myc activity. DMBA induction increases aryl hydrocarbon receptor, c-myc, and cyclin D1 expressions [57]. This research found one nodule with a triple-negative molecular subtype, but it had low Ki67 expression. In addition, a previous study in triple-negative breast cancer patients had low or negative Ki67 expression [58]. Triple-negative cancer patients with high Ki67 expression showed a good prognosis [59]. However, patients with triple-negative breast cancer have high Ki67 expression and progress with poor prognosis generally [60].

Conclusions and Recommendations

The histopathology features and ER, PR, HER2, and Ki67 expressions on induced rats with 20 mg/kg BW as much as 10 times showed similarity with human breast cancer with different frequencies, but were less aggressive. The limitation of this research is the small number of nodules found. Furthermore, even though the number of samples in this research met the minimal size for animal research, but since there was a death case, it is recommended to increase the number of rats for DMBA induction until the maximum limit meets the requirement of animal research for anticipation of the occurrence of death.

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