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Serial Transplantation: A Method for Inducing Mammary Tumours in Sprague Dawley Rats

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ABSTRACT

Study of breast cancer biology has been limited due to scarcity of available human samples, cost and preservation of cell lines thus, necessitating the use of chemically-induced rodent models. This study defines a protocol demonstrating that serial transplants obtained from 7,12-DMBA-induced mammary tumours had ability to generate and propagate mammary tumour cells with shorter latency. It also compared the changes in morphology and gene expression in the primary tumour cells and serial transplants. Virgin 50-day old female Sprague-Dawley rats were administered 10 mg/kg DMBA sub-dermally. Primary tumours were harvested, half processed for histology while the remainder was processed as a cell suspension and transplanted into clean rats. Five percent (5%) of rats developed multiple mammary carcinoma after DMBA injection with a latency of 112 days. However, 35% and 56% developed mammary tumours in the first and second transplants with mean latency of 18 and 9 days respectively. This protocol induced significant palpable tumour response as well as reduced expression of ER and PR in the serial transplants as compared to the primary tumours while HER2 was undetectable in all tumours. Morphologically, tumours were carcinomas having different levels of differentiation: primary tumours showed moderate differentiation, first transplant showed poor differentiation and second transplant showed well-differentiated carcinomas. It can be concluded that serial transplants can continuously generate and propagate mammary tumour cells in shorter time periods while losing their ER and PR dependence. This could provide a useful tool for testing of therapeutic drugs for breast cancer particularly in developing economies.

Keywords: Serial transplantation, immunohistochemistry, estrogen receptors, progesterone receptors.

INTRODUCTION

Breast cancer is a major global health challenge as it is the second leading cause of cancer related deaths globally (i.e. 11.6%), and the leading cause of cancer-related deaths among females[1]. It is a heterogeneous and highly diverse disease with respect to its clinical presentation, morphology, expression of molecular markers, prognosis and treatment outcome[2]. In Nigeria, the incidence of breast cancer is on the rise, being the leading cause of cancer related morbidity and mortality among females[3], with hormone receptor positive cancers having a lower incidence[4] as compared to the triple negative breast cancers[5].

Reports have shown that some human cancers express either estrogen receptors (ER) or progesterone receptors (PR), thus making the expression of these receptors a routine diagnostic marker for breast cancer[6]. Based on these receptor expression, (ER, PR, and the oncogene ERBB2 receptors - HER2), breast cancer has been classified into three subtypes termed ER^+, $HER2^+$ ($ER^-/PR^-/HER2^+$) and triple negative ($ER^-/PR^-/HER2^-$)[7], which differ in progression, patterns of metastatic spread, clinical prognosis and response to therapy thus, having important implications on patient stratification, treatment planning and clinical management for breast cancer[8,9].

Chemically-induced mammary tumours in experimental animals have been shown to be closely related to those found in humans, thus results obtained from studies done using such experimental models could be extrapolated to humans. Furthermore, animal models for carcinogenesis provide an invaluable resource for the identification of tumour markers, development of therapeutic interventions, as well as allow for manipulation and study of the processes involved in carcinogenesis[10]. Studies have also shown that chemical carcinogens such as 7,12-dimethyl benz (a)anthracene, N-methyl-N-nitrosourea (NMU) among others can be used for the induction of mammary tumours experimentally in rats[11].

Tumour transplant entails the transfer of living cancer cells or solid tumours from a donor to another animal and this may either be a xenograft or an allograft based on whether the donor tumours and host belong to different or same species respectively[12]. In both cases, the transplants can be orthotropic, subcutaneous or intravascular in order to study the different stages of tumour progression[13]. Transplantation assay has gained significance in cancer research, being used to validate some animal models of cancer, test the malignancy of some human cancers, screen for potential therapeutics that may play a role in inhibiting tumour malignancy as well as identify putative cancer stem cells[14-18]. Besides, it has been used to test blood cancers in order to differentiate between a myelo-proliferative or lympho-proliferative disorder from a leukemia[16, 19]. Using this procedure, immunodeficient and nude mice are used as host for transplantation studies of chemotherapeutic agents in cancer research[20], but research using these models are expensive as the rat models are not readily accessible and besides, need to be kept under sterile conditions. There is therefore a need for the development of a simple, cheap and reproducible method for inducing mammary tumours in experimental animals in a resource limited setting where facilities for cell culture in cancer research are not readily available. In addition, studies have concentrated on the primary tumours derived from the use of these chemicals but not much is known on the use of cells isolated from serially transplanted 7,12-DMBA-induced tumours in Sprague Dawley rats.

This study was aimed at investigating the growth indicated by the ability to generate and propagate mammary tumours as well as the changes which occur in the phenotype and hormonal expression of these transplant tumours as they develop.

MATERIALS AND METHODS

Experimental animals: Fifty-nine female Sprague Dawley rats of about 7-8 weeks old were used for the study. They were housed in plastic cages with free access to food and water in an environment of approximately 12 hour light and dark cycle. The animals were acclimatized for two weeks before use. Ethical approval was obtained from Ahmadu Bello University Zaria Nigeria's Animal Care and Use Committee prior to the commencement of the study.

Tumour Induction in Donor Animal (Primary tumours): The primary mammary tumours were induced based on the method of Barros *et al*.[11] with modification and 25 Sprague Dawley rats were used. Animals were divided into two groups of 5 and 20 animals serving as control and experimental groups respectively. The rats in the treatment group were immunosuppressed with a single dose of dexamethasone at 20 mg/kg intraperitoneally and then administered a booster dose of 10 mg/kg of dexamethasone on day 8[21] alongside 10 mg/kg DMBA dissolved in sesame oil sub-dermally within the mammary pad. Animals were palpated for appearance of tumours weekly.

Tumour cell isolation from donor animal: Following tumour development, i.e. 30 days post tumour development, the tumour bearing animal was humanely sacrificed under ketamine anaesthesia (150 mg/kg) and the mammary tumour harvested and weighed and sectioned into smaller pieces which were placed in phosphate buffered saline (PBS) on ice. The pieces of mammary tumour in (PBS) were teased using the flat surface of a syringe plunger and a 70 µ Falcon cell strainer placed on a 50 ml Falcon tube. The cell suspension obtained in the Falcon tube was passed through 18, 22 and 26 gauge needles respectively so as to obtain a single cell suspension. This was then centrifuged at 3000 rpm for 10 min. The supernatant was decanted using a disposable pipette and the cells were re-suspended in PBS and centrifuged. The procedure was repeated thrice. The cells were re-suspended in 20 ml of PBS, stained with trypan blue to check for cell viability and counted using a haemacytometer. All harvested cells were used within one hour of preparation.

Tumour cell transplant in experimental animals: Twenty-five female Sprague Dawley rats were used for the first transplant. 5 rats served as control while the remaining twenty rats were inoculated with the tumour cells isolated from the primary DMBA-induced mammary tumour obtained above. The twenty experimental rats were immunosuppressed using 5 mg/kg dexamethasone injection twice daily for three days[22] after which they were innoculated. 0.1 mL of the cell suspension (i.e. 6 x 10^6 cells in 20 ml PBS) was injected subcutaneously into the mammary fat pad using a 1 ml syringe with a 26 gauge needle. For the tumour cell second transplant, the mammary tumour obtained from the first transplant was used. This was harvested 14 days post tumour development (i.e. 35 days post tumour transplant). Tumour cells were isolated and inoculated into rats as previously described. Nine rats were used for the study.

Tumour measurements: All animals were monitored for tumour development. The tumour mass was

measured horizontally and vertically using a digital Vernier calliper and the tumour volume was calculated using Carlsson's formula[23].

$$V = \frac{(ab)^2}{2}$$

Where V = volume of tumours
a = longest diameter of tumours and
b = shortest diameter of tumours

Morphological Studies: The experimental animals were weighed before transplant, weekly following transplantation and every three days post-tumour development. At the end the experiment (day 30 post tumour development), the experimental animals were humanely sacrificed following ketamine (150 mg/kg) anaesthesia and the mammary tumours, harvested and weighed.

Histopathological analysis: Tissues obtained from the mammary tumours were fixed in 10% neutral buffered formalin, sectioned and dehydrated via graded concentrations of ethanol (70%, 85%, 95% and 100% respectively), cleared in xylene and embedded in paraffin. Tissue sections of 4 µm thick were cut to water, stained with haematoxylin and eosin stains and mounted on distyrene plastisizer xylene (DPX, Sigma Aldrich, Germany). The slides were studied using light microscopy and photomicrographs were taken using a microscope with a camera (Leica ICC50E, Leica microsystems Wetzlar, Germany) attached to a computer.

Immunohistochemical analysis: Mammary tumours tissues were fixed in 10% neutral buffered formalin, dehydrated in graded concentrations of ethanol (70%, 85%, 95% and 100% respectively), cleared in xylene and embedded in paraffin. Tissue sections of 4 µm thick was cut to water and mounted on poly-L-lysine coated slides (Boster biolaboratories, Pleasanton, USA). The appropriate antigen epitope for ER, PR and HER2 (DDBiotech, Slovakia) were separately retrieved at pH 6.0 for 40 minutes at 95 ºC and the slides incubated in their primary antibody (i.e. ER antibody, PR antibody and HER2 antibody respectively) for 60 minutes at 1:100 dilution. These were washed and incubated in mouse + rabbit horse radish peroxidase (HRP) for 30 minutes and a mixture of 3, 3'-diaminobenzidine (DAB) and substrate for 7 minutes respectively. Slides were washed twice with PBS and counter stained with hematoxylin for 2 minutes. The slides were studied using light microscopy and photomicrographs were taken using a microscope with a camera (Leica ICC50E, Leica microsystems Wetzlar, Germany) attached to a computer.

Statistical analysis: The data obtained from the tumour occurrence and latency period was analyzed using IBM Statistical Product for Service Solutions (SPSS) Statistics 20 and the results expressed as the mean ± standard error of mean (SEM). Differences among the means was determined using one way analysis of variance (ANOVA) and a value of $P<0.05$ was considered statistically significant. Bonferroni's post hoc test was used to determine where the level of significance lay. Kaplan Meier was used to plot the survival curve.

RESULTS

Tumour occurrence and latency period: Results obtained from the study showed the presence of palpable tumours across experimental groups as seen in Fig. 1a-c. Tumour occurrence rate increased across transplant groups with 5%, 35% and 55.5% tumour occurrence in the primary, first transplant and second transplant groups respectively (Fig. 2a). Furthermore, tumour latency period varied across experimental groups with a longer latency period (112 days) observed in the primary tumours while 14-21 days and 7-10 days latency period was observed in the first and second serially transplanted groups respectively (Fig. 2b).

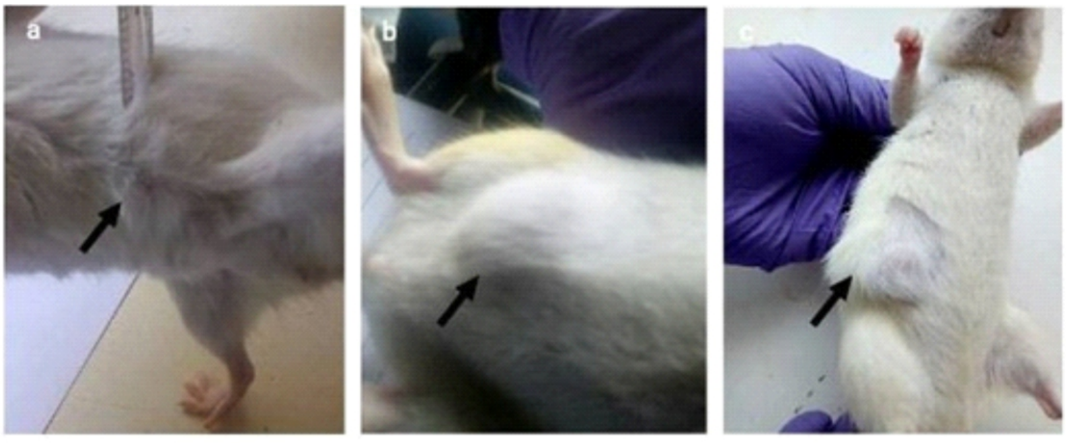

Figure 1: DMBA induced primary and serially transplanted mammary tumours in experimental animals showing multiple mammary tumours in the donor animal (a) and site specific tumours in the first and second serial transplants (b and c).

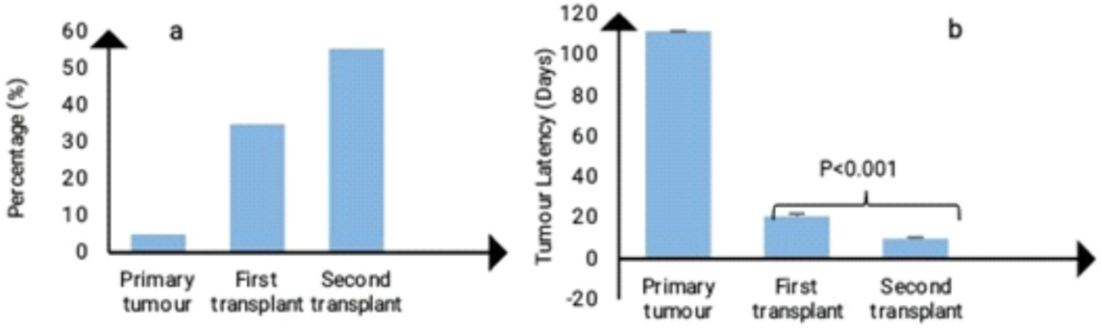

Figure 2: (a) Percentage tumours development in the primary donor animals, first and second serial transplants showing 5%, 35.5% and 55.5% tumours development in rats respectively. (b) Latency period for tumours development in the primary donor animals, first and second serial transplants showing a significantly reduced tumours latency period in the transplant groups.

Tumour growth rate and animal survival: Results revealed a slow but steady growth in tumours obtained from the first transplant with a change in only one tumour, which spiked high from day 15 post tumour development (Fig. 3a). On the other hand, the tumour growth pattern in the second transplant exhibited rapid increase in tumour volume with a spike on day 9 post tumour development (Fig. 3b). The highest mortality (60% of tumour bearing rats) in the second transplant occurred day 9 post tumour development. Results obtained from the study further revealed that animals from the first serial transplant survived up to 30 days post tumour appearance which is longer as compared to the second serial transplant where animals from the group survived 15 days post tumour appearance (Fig. 3c).

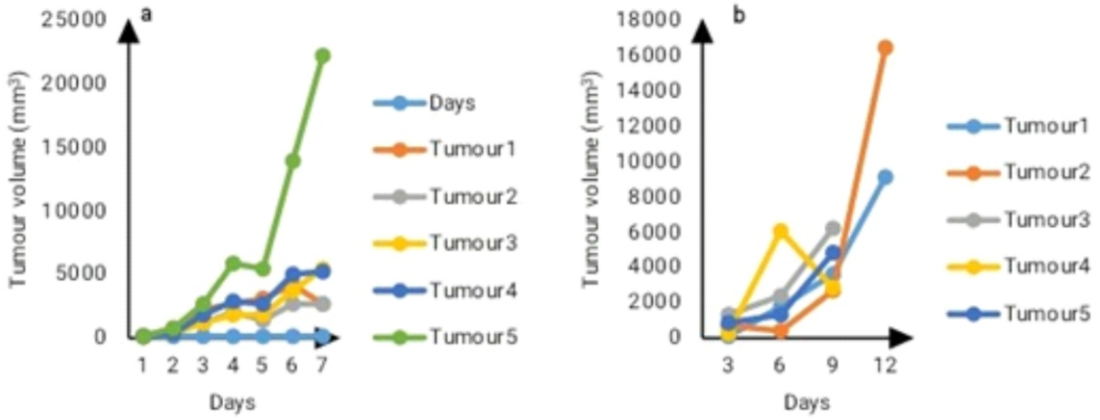

Figure 3: (a) Change in tumours volume of the primary tumours showing an initial slow tumours growth that became more aggressive as the tumours progressed. (b) Change in tumours volume of individual tumours that developed in the first serial transplant. Tumours was measured every three days post tumours development.

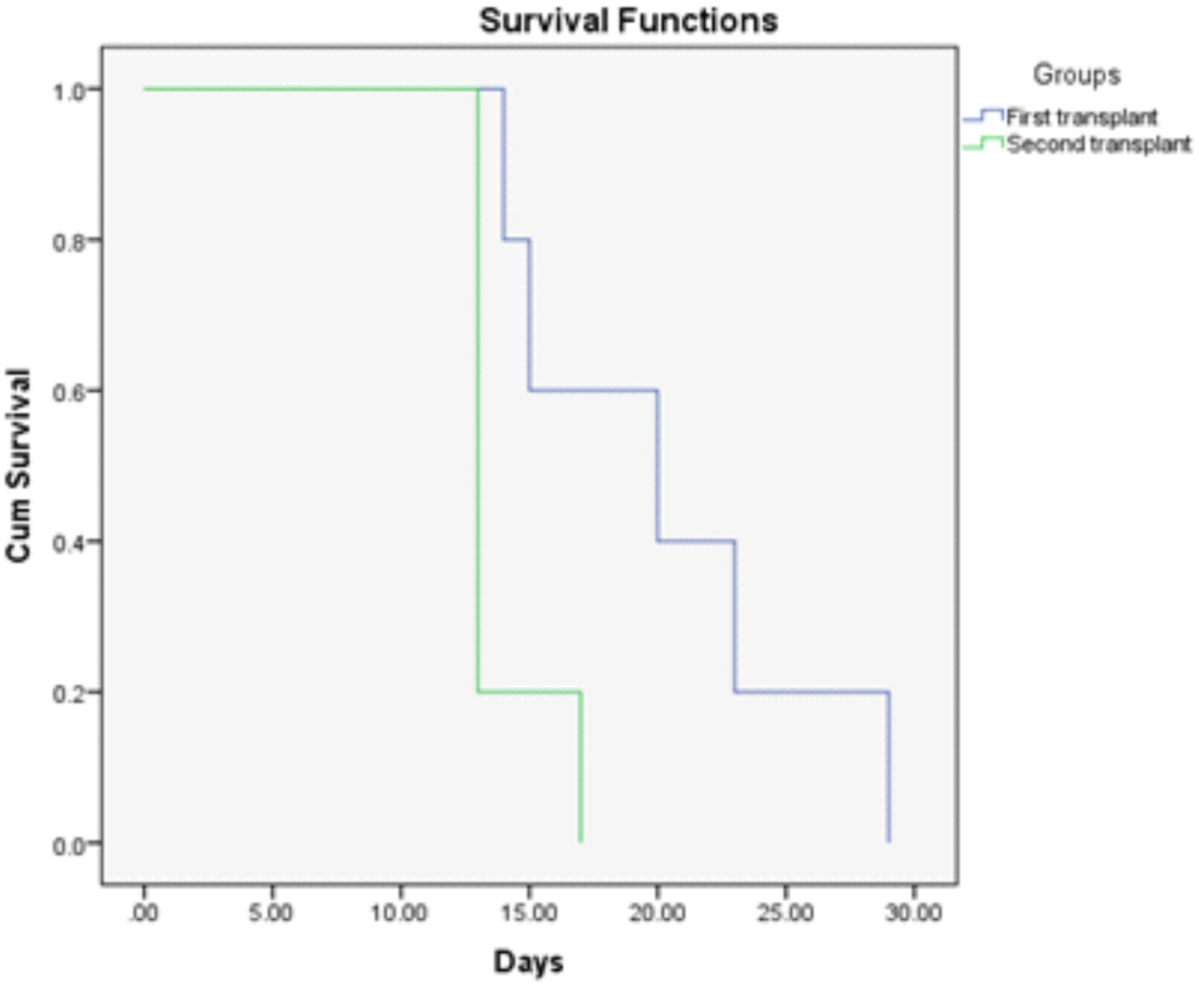

Figure 3c: Kaplan Meier plot showing survival analysis of animal that developed tumours in the first and second serial transplants with the rats in the first serial transplanted tumours surviving longer than those of the second transplant.

Histopathological studies: Mammary glands obtained from non-tumours bearing rats showed mammary glands having well-formed ducts and epithelial lining (Fig. 4a). The primary breast tumour showed a moderately differentiated breast carcinoma as seen in Fig. 4b. The tumours that developed from the first generation serial transplant revealed regions of geographical necrosis with infiltration of inflammatory cells, and single tumour cells indicative of a poorly differentiated breast carcinoma (Fig. 4c). On the other hand, histopathological analysis of tumours that developed from the second generation serial transplant revealed the presence of tumour cells characterized by pleomorphic vesicular nuclei to hyperchromatic nuclei with well-formed ducts indicative of a well differentiated ductal breast carcinoma (Fig. 4d). Tumour metastasis was not observed in experimental animals but circumscribed lesions that did not express ER, PR or HER2 were seen in the liver and lung of both first and second generation transplants (Fig. 4e and Fig.

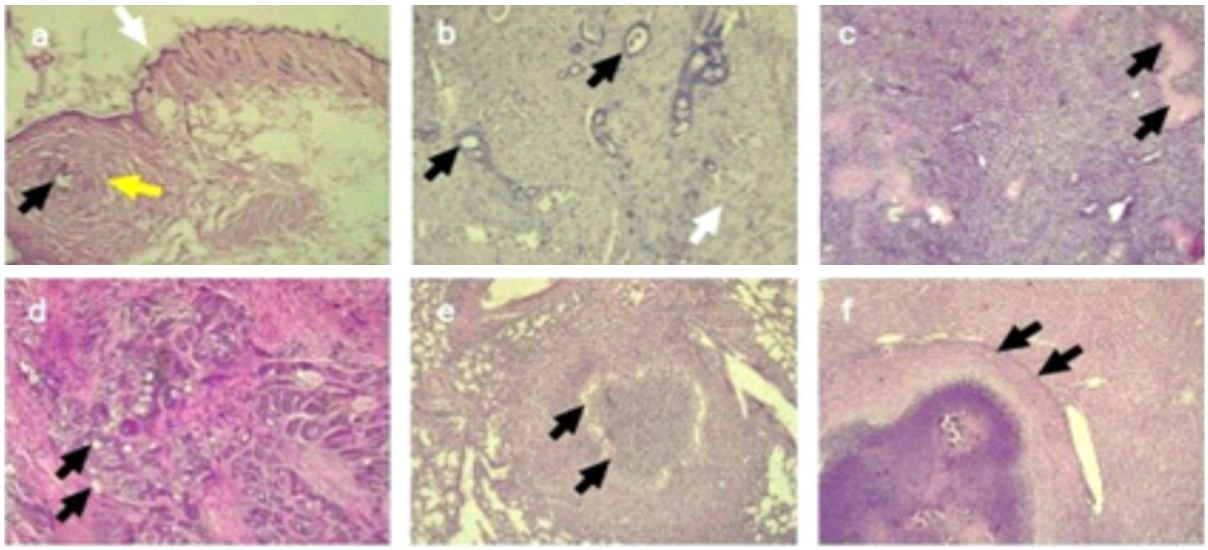

Figure 4: (a) Mammary gland of the control animal showing duct (black arrow) and stroma (yellow arrow), skin and adnexia structures (white arrow) of the mammary gland; (b) Tumours obtained from DMBA-induced primary mammary tumours in donor rat showing distorted tissue lobular architecture (white arrow) and few to moderate tubule formation (black arrows); (c) Tumours from first serial transplant showing areas of geographic necrosis embedded within the tumours; (d) Tumours from second serial transplant showing well differentiated mammary tumours having well-formed tubules (arrows); (e) Circumscribed lesions in the lungs of a rat from the second generation transplant. (f) Liver of a rat from the first generation transplant showing circumscribed lesion with necrotic area within it. H and E stain, x 40 magnification

Immunohistochemical expression of ER, PR and HER2 in serially transplanted DMBA-induced mammary tumours in Sprague Dawley rats: Results obtained from the study showed a moderate positivity for ER in the primary tumours (Fig. 5a) and weak positivity in the tumours from the first serial transplant (Fig. 5b) while being negative in the second serial transplant (Fig. 5c). PR was weakly expressed in the primary tumour (Fig. 5d) and negative in both first and second serial transplant groups while HER2 expression was negative in the primary tumour and tumours obtained from the first and second generation transplant groups.

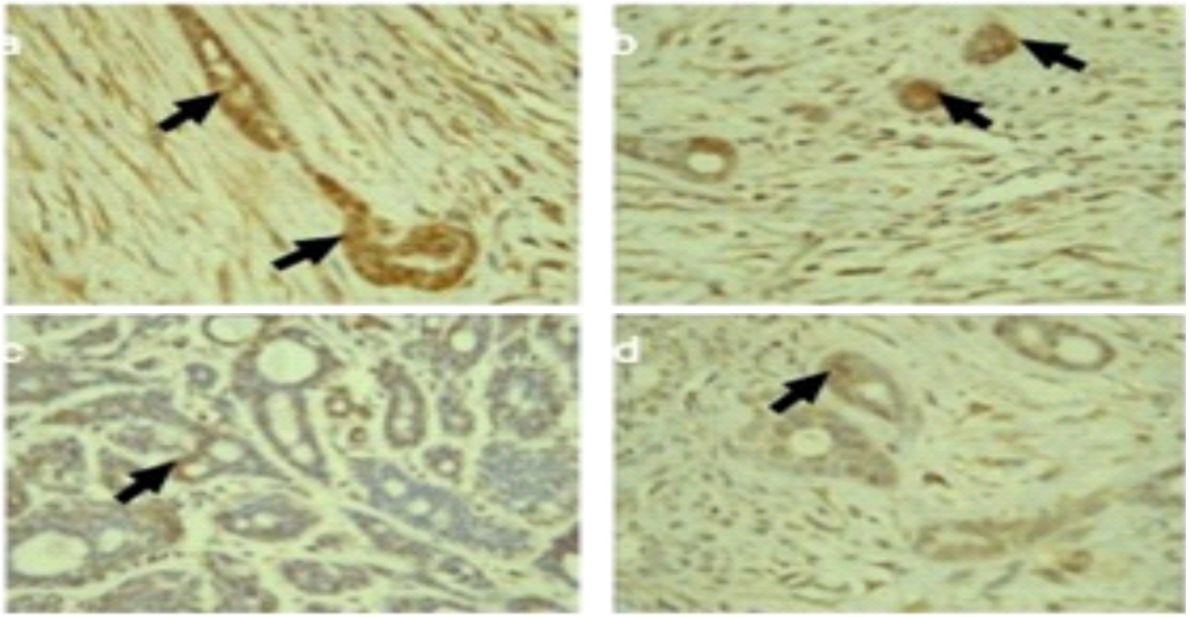

Figure 5: (a) Rat primary breast tumours with moderate positivity for ER; x 400 magnification. Arrows showing cells expressing ER (b) Rat mammary tumours from the first generation serial transplant showing a weak positivity for ER; x 400 magnification. Arrows showing cells expressing ER (c) Rat mammary tumours from the second generation serial transplant with weak positivity for ER; x 400 magnification. Arrows showing cells expressing ER (d) Rat mammary tumours from rat primary breast cancer with weak positivity for PR; x 400 magnification. Arrows showing cells expressing PR

DISCUSSION

Cancer is a systemic disease that initially presents a local manifestation that progresses via a series of processes such as rapid proliferation, evasion of apoptosis, neoangiogenesis, local invasion, metastasis etc.[24] In this present study, breast cancer models were developed by transplanting DMBA-induced mammary tumours in Sprague Dawley rats into the mammary fat pad of syngeneic animals. Mammary tumours developed following serial transplantation of tumour cells isolated from 7, 12- DMBA-induced tumours and these had a significantly shorter latency as compared to the primary tumours. The tumours developed between day 14 and 21 post inoculation in the first generation transplant while in the second generation transplant, the mammary tumours developed between day 7 and day 14 post inoculation suggesting a shorter latency period as compared to the first transplant. It has been reported that methods involving the use of chemical carcinogens for the induction of mammary tumours in experimental animals are expensive, time consuming and most times not reproducible[25], thus corroborating the 5% tumour development after 112 days that was observed in the study following administration of DMBA to Sprague Dawley rats to induce the primary tumours. The method of serial transplantation of tumour cells resulted in significant palpable tumour response having shorter latency thereby presenting a cheaper and easier method for tumour induction in experimental animals. On the other hand, 5 out of the 9 Sprague Dawley rats inoculated in the second transplant developed tumours. Kaliss[26] observed a similar tumour occurrence in a previous study where he transplanted tissue grafts into experimental animals to induce mammary tumours. He reported that primary transplants of tumours originating in an inbred mouse will not grow in 100% of host of the same inbred strain at first transplant but with continued transfers, graft will grow in all hosts, and at faster rates as observed in the second transplant in this study, so that transfers have to be made at shorter intervals to avoid losing the tumour line. Thereby, making the transplantation method of tumour cell generation of great significance in the study of breast cancer biology *in vivo*.

The reason for the shorter latency period that was observed in subsequent transfers are not fully understood but have been attributed to increased virulence or antigenic simplification of the cells[26]. Furthermore, Kreso and Dick[27] reported that the heterogeneous nature of the tumours and the presence of tumour initiating cells within it may be responsible for the tumours that developed following inoculation. Individual malignant cells within a tumour possess variation in growth, apoptosis, metabolism and other hallmarks of cancer[27]. Since transplantation assays are aimed at evaluating the potential of cancer cells to form tumours rather than their actual fate in the native tumour[28], the capacity of a tumour cell to initiate a new tumours was variable with not every tumour cell able to function as a tumour initiating cell. Thereby establishing that tumours are not a collation of homogenous cells with equal capacity for proliferation. Instead, they are a complex network where individual cells display a diverse set of characteristics and function together to support the growth and maintenance of the tumour as a whole[27].

Breast cancer is associated with a wide range of tumour growth rates[29-30] and for several decades, the rapid tumour growth rate was suspected to be associated with poor prognosis. The slow growing tumours were reported to have a lower incidence of local recurrence and distant metastasis than the more rapidly growing tumours[31]. Yoo *et al*.[32] reported that in vivo growth rate of tumours are associated with other worse prognostic factors and disease free survival but not an independent prognostic factor in breast cancer patients. Results obtained from the study showing rapidly growing tumours in the second transplant as compared to the first transplant may be responsible for their short survival time, as 60% of the tumour bearing rats of the second transplant died 15 days post tumour appearance as compared to the first transplant which had no mortality at day 15 post tumour appearance. The high growth rate observed in the second serial transplant may be as a result of excessive growth factor in the tumours as it has been reported that in several types of human cancers, autonomous growth has been attributed to the excessive synthesis of growth factors or growth factor receptors, or due to an excessive amplification of the signal generated by the growth factor receptor such as transforming growth factor alpha in breast cancer[31].

Following histological analysis, results obtained from the study revealed areas of tissue desmorplasia and well-formed tubules in over 70% of the primary tumour parenchyma indicating a moderately differentiated invasive ductal adenocarcinoma of the mammary gland which corroborates results reported by Huggins *et al.*[33] that revealed that adenocarcinomas were the type of cancers that develop following DMBA administration in Sprague Dawley rats. It has been reported that invasive ductal carcinoma of the breast is the most common cancer type diagnosed among Nigerian women and in most cases, tumours were grade II lesions, with tumour grades correlating with the 5-year survival of cancer patients[34]. The higher the tumour grade, the worse the prognosis[35]. The tumours that developed from the first and second serial transplants showed a change in tumours morphology from poorly differentiated to a well differentiated adenocarcinoma were similar to those seen by Bartsch *et al*.[36] who studied serial transplants of DMBA mammary tumours in Fischer rats up to the 12th passage. They observed that the tissue of the second passage consisted of a chaotic mixture of neoplastic cells, epithelial carcinomatous cells forming a few irregular small tubes or solid nests and predominantly elongated plum or spindle shaped atypical myoepithelial cells. Necrosis and infiltration of inflammatory cells observed in the tumours may be indicative of inflammatory responses.

Hormone receptors (ER and PR) and human epidermal

growth factor 2 receptor are considered as immunohistochemical markers of prognosis and predictors of response to therapy, and knowing their levels of expression is highly significant in cancer prognosis. This is because ER/PR positive tumours have a more favourable outcome as compared to ER/PR negative tumours[37]. Estrogen and progesterone are steroid hormones that play vital roles in sexual differentiation and fertility and act by binding to specific nuclear receptors which are commonly localized within the same cell[38]. Normal cycling mammary epithelial cells do not possess ER/PR[39], hence, ER and PR are considered as prognostic factors for mammary carcinomas. Simultaneous expression of both receptors in a tumours have been associated with a less aggressive type of mammary tumour as compared to those that express only one of these receptors[40]. Reports on ER/PR expression in chemically induced mammary tumours are conflicting as Kordon[41], reported that most genetically modified mouse breast cancer models as well as most spontaneous, chemically-induced or mouse mammary tumours virus-induced (MMTV) tumours do not express ER and PR. The few MMTV models that express ER and PR are usually pregnancy dependent. On the other hand, Shirai *et al*.[42] observed that DMBA-induced mammary tumours are hormone dependent expressing both ER and PR. These findings corroborates with results obtained from the present study where ER and PR expression were observed. It has been reported that the higher the content of ER and PR in a breast tumours, the higher the likelihood of response to hormonal therapy[43]. Furthermore, high levels of ER and PR have been associated with lobular and tubular breast cancers and ER expression is inversely associated with the size of the primary tumour[44]. Expression of ER and PR is not constant and changes with disease progression i.e. the number of cells expressing ER and/or PR decreases as disease progresses[45-46]. This may be the reason for the decreasing expression of ER and PR across the transplant groups - with expression levels higher in the primary tumours, ER low and PR negative in the first transplant, and ER low and PR negative in tumours from the second transplant. Furthermore, studies by Abba *et al*.[47] in mice revealed that long latency (254 days) tumours showed strong ER and PR positivity as compared to short latency (55 days) tumours which were ER^-/PR^-. The short tumour latency period observed in this study of 112 days (primary tumours), 14-21 days (first transplant tumours) and 7-14 days (second transplant tumours) may also be responsible for the weak to negative expression of ER and PR observed across the study groups.

CONCLUSION

Serial transplantation method for the induction of mammary tumours is a faster and economical method for inducing mammary tumours in Sprague Dawley rats, with the tumours obtained histologically identical to the primary tumour. The data represent significant similarities to human cancers and hence, this method may be used for laboratory-based breast cancer research in settings where cell culture facilities are limited.

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