Thrombocytopenia and Non-Expression of Tn And TF Antigens Define Non-Metastasizing Breast Cancer in Serial Transplants of Dimethyl Benz(A)Anthracene Induced Mammary Tumours in Sprague Dawley Rats

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ABSTRACT

Functional mammary tumours were generated as serial transplants of dimethyl benz(a)anthracene (DMBA) in immunosuppressed Sprague Dawley rats (SD). These serial transplants were evaluated for their cellular behaviour and biomarkers. Breast cancer was induced by 10 mg/kg of 7, 12 dimethylbenzanthracene (DMBA) in 10 female SD rats. Following tumour development, the primary tumour was passaged into apparently healthy rats for first and second serial transplants. Cellular behaviour and biomarkers of transplants included growth of tumour, haematological indices, expression pattern of Tn and TF antigens and TNF-alpha. An elevation of TNF-α, non-induction of Tn antigen with thrombocytopenia were observed in the serial transplants derived from a malignant tumour induced by 7, 12, DMBA in female Sprague Dawley rats. Furthermore, neutrophil to lymphocyte and platelet to lymphocyte ratios were found to be significantly low in animals from the first serial transplant while being significantly high in animals of the second serial transplant group. Results indicate that DMBA-induced mammary tumours in Sprague Dawley rats undergo divergent cellular behaviour and antigenic expressions during serial transplantation.

Keywords: Serial Transplants; DMBA; Breast Cancer; Thrombocytopenia; Tn Antigen; TF Antigen; Sprague Dawley Rats

INTRODUCTION

Breast cancer is the major cancer type affecting women globally (Bray *et al*., 2018) and treatment options requires the use of clinical and molecular diagnostic and prognostic markers such as age, tumour grade, hormone receptor status, human estrogen receptor 2 (HER-2) status, number of

involved regional lymph nodes, tumour histology amongst others (Senkus *et al*., 2015; Orditura *et al*., 2016). inflammatory responses associated with cancer have been reported to play significant roles in the initiation and progression of many cancers (Orditura *et al*., 2016), as the resulting changes in blood parameters which is a reflection of systemic inflammation have been linked with poor outcome in cancer patients (Pierce *et al*., 2009; McMillan, 2013). In addition, weakened adaptive immune response during chronic inflammation promotes cancer growth, angiogenesis and cancer cell survival (Grivennikov *et al*., 2010; Elinav *et al*., 2013).

Full blood count is a routine test carried out by clinicians to compliment the working diagnosis of a number of ailments such as anaemia, infections, immune disorders and cancer (Akaani *et al*., 2013). Studies have shown that high lymphocyte density within the tumour stroma is associated with better clinical outcome as compared to low lymphocyte infiltration within the tumour stroma. On the other hand, high density neutrophil infiltration has also been associated with poor disease outcome (Szkandera *et al*., 2013; Teramukai *et al.,* 2009). This is so because the inflammatory cells such as leucocytes present within the tumour microenvironment are responsible for the inflammatory response and hence, supports proliferation, survival and metastasis of tumour cells by preventing angiogenesis and DNA damage (Szkandera *et al*., 2013). In addition, the role of neutrophils in cancer development and progression is multifactorial and hence, not fully understood as they have been reported to participate in different stages of the oncogenic process such as tumour initiation, growth, proliferation or metastatic spread (Swerczar *et al*., 2015; Coffelt *et al*., 2016). High neutrophil count is associated with poor prognosis (Okada et al., 2020).

Assessment of the neutrophil to lymphocyte ratio (NLR) is a simple and less-invasive method used to determine the systemic inflammation as NLR is a good indicator of systemic inflammatory response (Semiz *et al*., 2014). Elevated levels are linked with cancers (Szkandera *et al*., 2013; Seretis *et al*., 2013a), chronic stress (Erminio *et al*., 2009) and coronary heart disease (Fowler Fowler and Agha (2013). NLR is a marker of an impaired cell-mediated immunity that is associated with systemic inflammation (McMillan, 2009) and reflects a balance between pro-tumour inflammatory status and anti-tumour immune status. This balance have been suggested to be tilted in favour of the pro-tumour inflammatory status in cancer patients with elevated NLR, hence resulting in poor outcome which may be due to an enhanced tumour progression alongside suppression of the immune system and lymphopenia (Szkandera *et al*., 2013). Furthermore, elevated NLR level was found to be an independent predictor of poor outcome in several solid tumours such as breast cancers (Forget *et al*., 2014).

Studies by Zhang *et al*. (2017) revealed that a high platelet to lymphocyte ratio (PLR) is associated with poor disease outcome and clinicopathological features such as tumour stage, lymph node metastasis and distance metastasis, and thus, could be used as a prognostic factor for breast cancer.

The Thomsen Friedenreich (TF) and Thomsen Nouvelle (Tn) antigens are precursor molecules of the MN-blood group antigens. They are generated by sialic acid depletion and are found in tissues with cancer characteristics (Kolbl *et al*., 2016). In the normal tissues, TF and Tn antigens are coated by glycosyl structures, thus, forming glycoproteins which have been reported to account for the MN-blood group antigens, while in malignant tissues, these molecules are uncovered (Kolbl *et al*., 2016). TF and Tn antigens are associated with cell adhesion properties hence, playing a role in tumourigenesis. Tn antigen on the other hand, stimulates cell adhesion to the extracellular matrix, cell migration and invasiveness and is present in the lamellipodia of migrating cells (Gill *et al.*, 2013). An upregulation of TF and Tn antigens which is generated by changes in glycosl transferases is correlated with tumour progression, higher tumour, node, metastasis (TNM) staging and reduced survival respectively (Kolbl *et al*., 2016).

Animal models are significant tools in the study of cancer initiation and progression, and these models have been used to study the therapeutic effects of some chemicals and natural compounds on the developing tumour. For this to be achieved, tumours must to a large extent, be able to mimic the processes of tumour initiation and progression, the tumour microenvironment as well as the associated inflammatory processes etc. as seen in humans (Babino *et al*., 2000). Serially Transplanted tumours are tumour models that are obtained following inoculation of tumour cells that were isolated from a resultant spontaneous or chemically -induced tumour into syngeneic hosts (Ni *et al*., 2009).

Preoperative full blood counts, NLR, PLR, TNF-alpha, Tn and TF antigens as well as their association with cancer prognosis have been studied in both chemically-induced tumours and cancer patients but

there is no record in the medical literatures of their levels in serially transplanted chemically-induced mammary tumours in experimental models. This study was therefore carried out to determine the changes in some haematological indices, neutrophil to lymphocyte ratio and platelet to lymphocyte ratio, TNF-alpha, Tn and TF antigens in serially transplanted DMBA-induced breast tumours in female Sprague Dawley rats.

MATERIALS AND METHODS

Experimental Animals

Fifty-nine (59) female Sprague Dawley 7-8 weeks old, weighing 110 g to 140 g were used for the study. They were housed in plastic cages with free access to food (standard pellet diet, Grand Cereal Ltd, Jos Plateau State) and water in an environment of approximately 12-hour light and dark cycle. The cages were cleaned and beddings changed every two days. The animals were acclimatized for two weeks prior to use. Ethical approval was obtained from the Ahmadu Bello University, Zaria's Animal Care and Use Committee.

Breast Tumour Induction

Mammary tumours were induced according to the method of Barros *et al*. (2004) with modification. The primary breast tumour was induced with a single dose of 10 mg/kg DMBA dissolved in 1ml sesame oil administered sub-dermally within the mammary pad following dexamethasone immunosuppression at 20 mg/kg intraperitoneally and then administered a booster dose of 10 mg/kg on day 8 (Anafi *et al*., 2004). After tumour development, the mammary tumour cells were isolated and inoculated into clean rats. Breast tumours obtained from the first transplant were harvested, cells isolated and further inoculated into a second set of clean rats.

Haematological Analysis

This was carried out according to the method described by Kratz and Brugnara (2015). Blood samples were collected before transplant and after tumour development via tail bleeding into plain and EDTA vacutainers. At the end of the study, experimental animals were humanely sacrificed following ketamine anaesthesia (150 mg/kg). Blood samples were collected via cardiac puncture in EDTA vacutainers. Blood samples were analyzed for haematological indices (white blood cell count, (WBC), percentage neutrophils, percentage lymphocyte, packed cell volume (PCV), red blood cell count (RBC), mean corpuscular haemoglobin (MCH), mean cell volume (MCV), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW) and mean platelet volume (MPV) using a haematology auto analyser (Swelab Omega 2.0, China).

Immunological Analysis for TNF-α

The assay for TNF-α in the serum was done using enzyme-linked immunosorbent assay (ELISA) technique according to the method of (Ma *et al*., 2017) using rat TNF-α picokine ELISA kit according to the manufacturer's instructions

Immunohistochemical Analysis for Thomsen Friedenreich (TF) and Thomsen Nouvelle (Tn) Antigens

Immunohistochemical analyses for TF and Tn antigens were carried out according to the methods of Karacosta *et al.* (2018) and Babino *et al*. (2000) respectively. Mammary tumour 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 Tn and TF antigens) were separately retrieved at pH 6.0 for 40 minutes at 95 ºC. The slides were cooled to room temperature and then incubated in hydrogen peroxide (H_2O_2) for 10 mins after which they were washed twice in phosphate buffered saline (PBS). After washing, the slides were incubated in their primary antibody (i.e. Tn antibody and TF antibody respectively) for 60 minutes at 1:100 dilution. These were washed again twice in PBS and further incubated in mouse + rabbit horse radish peroxidase (HRP) for 30 minutes and washed twice with PBS. Finally, the slides were incubated in a mixture of 3, 3'-diaminobenzidine (DAB) and substrate for 7 minutes and then washed twice in PBS and counter stained with haematoxylin for 2 minutes and washed twice in PBS. 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 Analyses

The results obtained was analysed using IBM Statistical Product and Service Solutions (SPSS) Statistics 20 and the results expressed as mean ± standard error of mean (SEM). Differences among

the means was determined using one-way analysis of variance (ANOVA) while the student t-test was used to determine the difference in mean values before tumour transplant and after tumour development 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

Animal survival

Results obtained from the study showed that animals from the first serial transplant survived a longer period i.e. up to 30 days post-tumour development as compared to the second serial transplant where most animals died 15 days post-tumour development. Both set of transplants were characterized by exponential tumour growth (Figure 1).

Changes in Haematological Indices

***Changes in Haematological Indices before Transplant and after Tumour Development in the First Serial Transplant*:**

Results obtained from the first transplant revealed no significant difference in the levels of MPV, platelets, PCV, RBC and MCV before transplant and after tumour development. On the other hand, a significant decrease in the levels of neutrophils (p<0.001), MCH (p=0.002) and MCHC (p<0.001) as well as a significant increase in the WBC count (p<0.014) and lymphocyte count (p<0.001) were observed as seen in Table I.
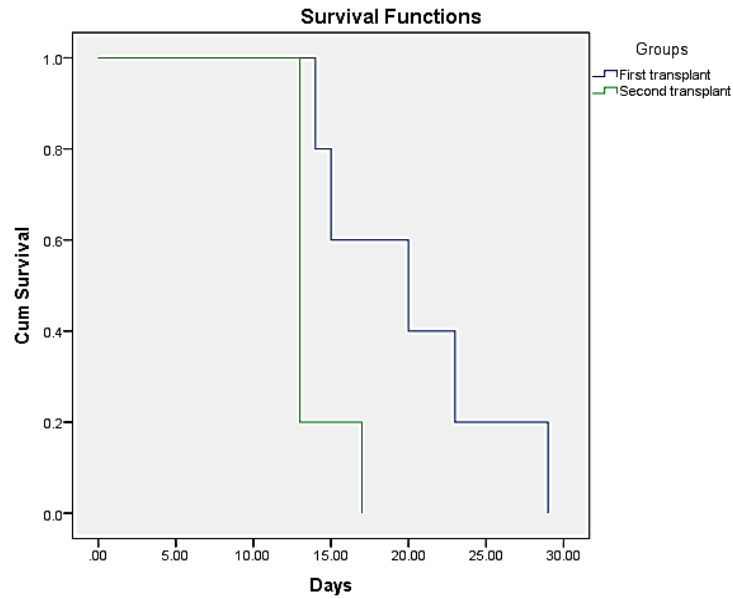
Changes in Haematological Indices before Transplant and after Tumour Development in the Second Serial Transplant

Results obtained from the second serial transplant showed no significant change in the levels of MCH, MCHC, RDW, MPV, platelets, PCV, RBC, WBC and MCV before transplant and after tumour development. On the other hand, a significant increase was observed in the level of neutrophils (p=0.028) alongside a significant decrease in lymphocyte count (p=0.020) as seen in Table II.

Changes in Haematological Indices across Transplant Groups

Haematological indices following tumour development across transplant groups as shown in Table III revealed that RBC, PCV and neutrophil levels were significantly lower in rats of the first transplant group as compared to the second transplant while MCV, MCH, MPV and RDW levels were significantly lower in the second transplant as compared to the first transplant. Furthermore, RBC, PCV, platelets and MPV levels were significantly lower, while RDW, MCV and MCH levels were significantly higher in rats from the first transplant as compared to those of the control group. Lymphocyte count was significantly lower in the second transplant as compared to the control while no significant change was observed in the levels of WBC and MCHC across the experimental groups.

Figure 1. 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

Table 1. Haematological indices before tumour transplant and after tumour development in animals from the first serial transplant

Haematological indices	Time		p-value
	Before transplant	After tumour development	
White blood cell (10^3/µL)	1.31±0.21	10.66±2.59	0.022[*]
Neutrophils (10^3/µL)	69.90±0.37	6.54±1.42	0.001[*]
Lymphocytes (10^3/µL)	15.70±1.44	85.52±2.99	0.001[*]
Red blood cell (10^{12}/L)	4.63±0.94	3.73±0.74	0.472
Packed cell volume (%)	30.38±1.89	28.12±4.15	0.639
Platelet (µl)	535.00±125.72	351.20±74.06	0.251
Mean platelet volume (fL)	8.04±0.66	6.66±0.15	0.103
Red blood cell distribution width (%)	74.30±9.72	112.82±17.94	0.107
Mean corpuscular volume (fL)	85.84±5.04	78.90±4.81	0.348
Mean corpuscular hemoglobin (pg)	72.40±7.93	32.48±4.29	0.004[*]
Mean corpuscular hemoglobin concentration (g/dl)	83.2±4.65	40.50±4.24	0.001[*]

[*]= significant at p<0.05

Table 2. Haematological indices before tumour transplant and after tumour development in animals from the second serial transplant

Haematological indices	Time		p-value
	Before transplant	**After tumour development**	
White blood cell (10^3/μL)	12.16±2.11	13.26±1.32	0.672
Neutrophils (10^3/μL)	8.26±2.08	8.73±3.91	0.028*
Lymphocytes (10^3/μL)	81.6±5.32	59.46±5.49	0.020*
Red blood cell (10^{12}/L)	5.09±0.66	6.15±0.38	0.211
Packed cell volume (%)	33.72±8.54	39.08±1.45	0.568
Platelet (μl)	524.00±37.35	471.80±96.57	0.635
Mean platelet volume (fL)	7.14±1.07	5.86±0.09	0.298
Red blood cell distribution width (%)	53.87±20.07	55.78±2.15	0.933
Mean corpuscular volume (fL)	67.40±7.94	64.80±1.76	0.766
Mean corpuscular hemoglobin (pg)	31.08±9.59	20.58±0.31	0.335
Mean corpuscular hemoglobin concentration (g/dl)	30.92±3.98	31.80±0.61	0.834

* = significant at $p<0.05$

Table 3. Haematological indices across transplant groups

Haematological Indices	Control	First transplant	Second transplant	p-value
White blood cell (10^3/μL)	7.24±2.68	13.94±2.25	11.58±0.92	0.112
Neutrophils (10^3/μL)	8.98±1.11	5.84±1.04	13.50±1.87[c]	0.007*
Lymphocytes (10^3/μL)	80.62±2.22	86.66±2.78	65.76±8.32[b]	0.001*
Red blood cell (10^{12}/L)	6.83±0.36	2.91±0.89	6.07±0.36[a,c]	0.001*
Packed cell volume (%)	38.82±1.27	22.80±4.99	37.56±2.85[a,c]	0.010*
Platelet (μl)	614.50±39.96	276.00±93.35	476.25±83.07[a]	0.038*
Mean platelet volume (fL)	5.63±0.17	6.58±0.15	5.60±0.91[a,c]	0.001*
Red blood cell distribution width (%)	38.68±1.37	115.63±22.88	49.63±1.57[a,c]	0.005*
Mean corpuscular volume (fL)	56.90±1.08	79.98±6.05	61.90±1.68[a,c]	0.004*
Mean corpuscular hemoglobin (pg)	19.80±0.31	33.77±5.28	20.75±0.41[a,c]	0.018*
Mean corpuscular hemoglobin concentration (g/dl)	34.88±0.39	41.48±3.99	33.48±0.34	0.080

a=significant between control and first transplant, b=significant between control and second transplant, c=significant between first transplant and second transplant, *=significant at $p<0.05$

Neutrophil / lymphocyte ratio (NLR)

Changes in NLR before tumour cell transplant and after tumour development:

Results obtained from the study showed a significant decrease (p<0.001) in NLR following tumour development in the first serial transplant as seen in Figure 2. On the other hand, a significant increase (p=0.014) was observed in NLR in animals from the second serial transplant before tumour cell inoculation and following tumour development (Figure 3).

Change in NLR across transplant groups:

The NLR was lower in the first transplant as compared to the control and second transplant following tumour development. This level was significantly higher (p=0.018) in the second serial transplant as compared to the first transplant (Figure 4) but difference was not significant between the second transplant group and control.

Platelet to lymphocyte ratio (PLR)

Changes in PLR before tumour cell transplant and after tumour development:

Results obtained from the study revealed a significant decrease (p<0.001) in the PLR in the first serial transplant following tumour development (Figure 5). On the other hand, a significant increase (p=0.009) in its level was observed in the second serial transplant following tumour development as seen in Figure 6.

PLR before and after tumour development across transplant groups

Results obtained from the study revealed a decrease in PLR in the transplant groups as compared to the control. Similarly, decrease in PLR was also observed in the first transplant as compared to the second transplant. These changes were all not to significant levels as seen in Figure 2.

Changes in TNF-α Levels across Treatment Groups

Results obtained from the study showed an increase in the levels of TNF-α in the first and second serial transplants as compared to the control. A significant increase in TNF-α levels was also observed in the second transplant as compared to the control as seen in Figure 4.

Immunohistochemical Expression of TF and Tn Antigens in Serially Transplanted DMBA-Induced Mammary Tumours

Result obtained from the study revealed that TF antigen was negative in both primary and serially transplanted tumours. However, Tn antigen was expressed in the DMBA-induced primary tumour while being negative in the serially transplanted DMBA-induced mammary tumours. The number of cells positive for Tn antigen were few compared to the entire tumour load and the intensity of staining varied between the tumour cells stained indicative of an invasive ductal carcinoma (Plate I).

Table 4. Neutrophil to lymphocyte **ratio and platelet to lymphocyte ratio**

| Haematological indices	First transplant			Second transplant		
	Before transplant	After tumour development	p-value	Before transplant	After tumour development	p-value
N/L ratio	4.80±0.30	0.08±0.01	0.001[*]	0.28±0.20	1.59±0.33	0.014[*]
P/L ratio	32.92±5.66	3.92±0.93	0.001[*]	6.28±0.79	39.36±8.70	0.009[*]

* = significant at p<0.05
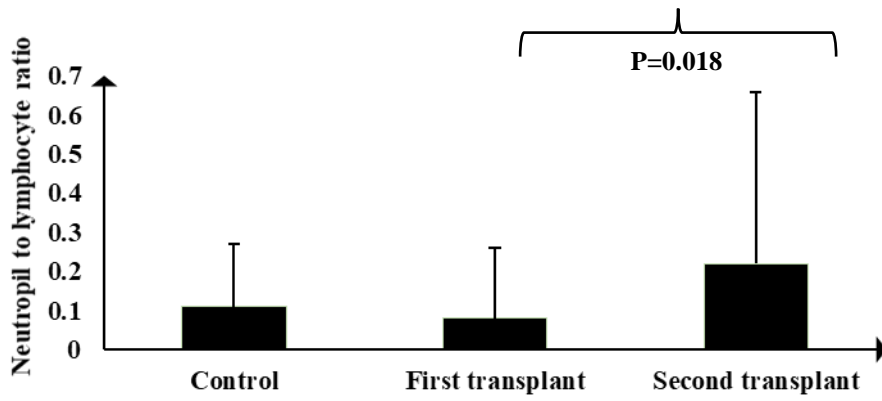
Figure 2. Changes in neutrophil to lymphocyte ratio post-tumour development in both first and second serial transplants.
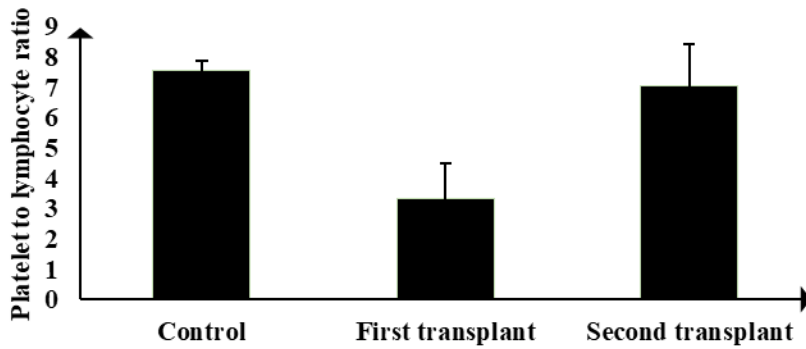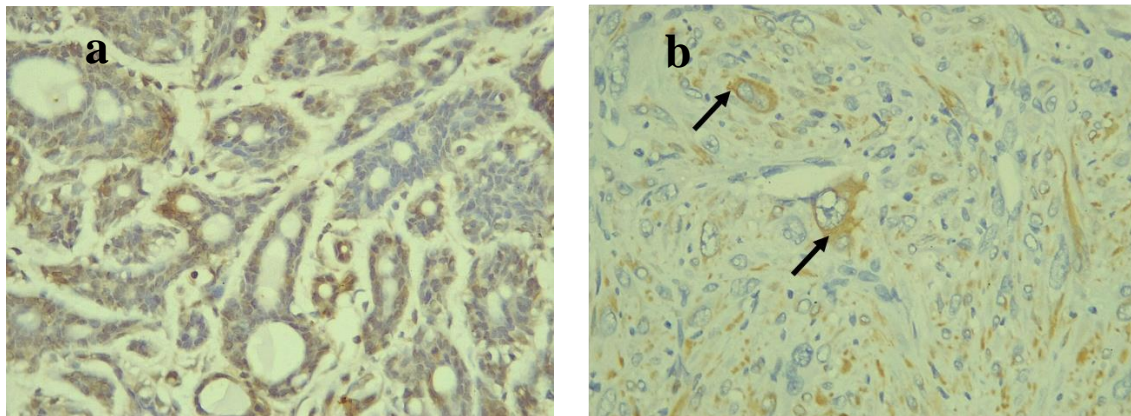

Figure 3. Changes in platelet to lymphocyte ratio post-tumour development in both first and second serial transplants
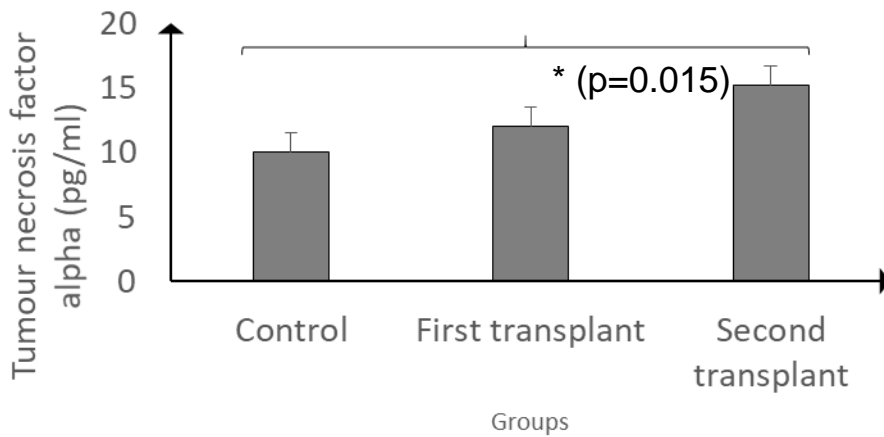
Figure 4: Changes in TNF alpha levels post-tumour development in the first and second serial transplants. *= significant between control and second transplant group

Plate I. Photomicrograph of Eosin Stained; (a) Control group showing negative Tn antigen expression (b) Tn antigen expression in the primary tumour showing areas positive for Tn.X 100 and X 400 magnifications respectively

DISCUSSION

Full blood count is a vital investigation requested from all cancer patients before surgery, use of chemotherapy or radiotherapy (Dezayee and Nimer, 2016) and these parameters have been associated with the outcome of cancers as changes in haematological parameters and markers of inflammatory response reflect disease progression and prognosis in several malignancies (Shrivastava *et al*., 2017).

Lymphocytes are crucial components of the adaptive immune system. It has been reported that infiltrating lymphocytes indicate the generation of an effective anti-tumour cellular immune response (Bobdey *et al*., 2017). Thus, a low peripheral lymphocyte level may indicate a poorer lymphocyte mediated immune response to tumour and hence, suggests a poorer outcome (Bobdey *et al*., 2017). Results obtained from the study revealing significantly high levels of circulating lymphocytes in the first transplant as compared to the second may be responsible for the longer period of survival of animals in the first transplant group as compared to the second transplant.

Levels of circulating lymphocytes prior to treatment goes a long way to determine disease outcome, as it has been reported that the higher its pretreatment values, the longer the survival time (Ray-Coquard *et al*., 2009) while a decrease in total lymphocytes in the blood is a significant immunologic change observed in advanced malignancy (Ahmad *et al*., 2012) resulting in an inadequate cell-mediated immunologic response toward the tumour (Jain *et*

al., 2016) which may have been responsible for the shorter survival time of the second transplant group.

Neutrophils constitute about 50-70% of all polymorphonuclear leucocytes in human circulation and act as the first line of defense against various infections (Sinov *et al*., 2015). In cancer, they are reported to play conflicting roles i.e. take on both anti-tumour and pro-tumour roles via their different subpopulations (Sinov *et al*., 2015). Tumour infiltrating neutrophils release different mediators that enhance tumour angiogenesis and intravasation of malignant cells (Huang *et al*., 2015). Besides, neutrophils are actively involved in both systemic and local inflammatory response via multiple mechanisms which include the release of proinflammatory factors, promotion of growth and metastasis via remodeling of the extra cellular matrix (Ciftci *et al.,* 2015), release of reactive oxygen species, nitric oxide, arginine and suppression of T cell response (De Larco *et al*., 2004). Previous study by Suzuki *et al*. (2011) revealed that neutrophils plays a role as a tumour promoting leucocyte through the transforming growth factor β-induced signaling pathway. Result obtained from the current study revealed low levels of neutrophils in the first serial transplant as compared to the second transplant though reasons for the low levels of neutrophils observed is not known. The significantly high levels of neutrophils observed in the second serial transplant as compared to the first transplant in the present study corroborates reports by Akingbami *et al*. (2013) who found that neoplasms of all types are associated with neutrophilia. Besides, higher neutrophil percentage may also indicate various conditions such as bacterial infection (Pfeiler

et al., 2017) or cytokine release by cancer cells which helps them to disseminate to other sites (Al-Arifi *et al.*, 2018).

Chronic diseases such as cancer have been associated with low levels of erythropoietin thus, leading to decrease in the production of RBCs with resulting anaemia (Poggiali *et al.*, 2014). Results obtained from the study showing significantly high (p<0.05) levels of RBC, PCV, MCV, MCH, MCHC and RDW in the first serial transplant as compared to the control and second serial transplants suggests the presence of anaemia in the second transplant group and this may also be responsible for the poor survival of rats in the second transplant group. Akingbami *et al.* (2013) reported that low PCV, RBC, MCV, MCH and MCHC are associated with anaemia from chronic disorders such as cancer. Furthermore, low Hb levels could cause activation of the metastatic genes leading to metastasis and hence resistance to chemotherapy, radiotherapy and ultimately, poor survival (Gilkes, 2016). The red cell distribution width (RDW), an independent risk factor of poor survival in cancer (Riedl *et al.*, 2014), and a marker of breast cancer activity as significantly high levels were observed in breast cancer as compared with breast fibroadenomas (Seretis *et al.*, 2013b). Its levels are elevated when there is an increase in red cell distribution or ineffective red cell production. It may also be a reflection of underlying issues such as nutritional deficiency (e.g. iron, vitamin B12 or folic acid), bone marrow depression or chronic inflammation which conditions are found to be more or less prevalent in cancers (Wang *et al.*, 2014). RDW is also used in parallel with the platelet distribution width in the assessment of breast cancer (Wang *et al.*, 2015a).

Inflammation is a significant event that occurs during the process of tumourigenesis (Seretis *et al.,* 2013a). It is recognized as the 7th hallmark of cancer and plays a role in tumour proliferation, angiogenesis, metastasis as well as resistance to hormonal and chemotherapy (Wu and Zhou, 2009). Peripheral blood tests taken at the time of diagnosis could reflect the inflammatory conditions present within the tumour (Chen *et al.*, 2015). Furthermore, inflammatory related markers such as absolute WBC count, C-reactive proteins, cytokines, PLR and NLR have been reported to be associated with specific outcomes in cancer patients (Templeton *et al.*, 2014). The NLR is reported to be a biomarker for systemic inflammatory response (Cingoz *et al.*, 2013) and can be easily and more conveniently examined as compared to other conventional markers (Chen *et*

al., 2015). In addition, when compared to other parameters, it is superior in predicting the long- or short-term mortality in breast cancer patients (Azab *et al.*, 2013). It is significantly associated with age, gender, tumour type and depth of invasion (Balta *et al.*, 2013). Individual parameters have been proven to be altered by physical, physiological and pathological factors but the NLR was found to be more stable even when under the influence of such factors (Ciftci *et al.*, 2015); hence, it was proposed that the NLR might represent both the inflammatory and immune pathways which though opposing in nature, exist together in cancer patients (Tomita *et al.*, 2011). Results obtained from the study indicating a significantly high NLR in the second transplant as compared to the control and first transplant, may be responsible for the poor survival observed in the second transplant group as elevated NLR have been reported to be strongly associated with poor survival in breast cancer patients, and hence, can be described as a predictive and prognostic factor for patients with breast cancer (Chen *et al.*, 2015). Similarly, the PLR is considered to be a marker for endogenous residual anti-cancer pre-inflammatory and pre-coagulative response that arises in malignancies (Wang *et al.*, 2015b); and together with NLR, they combine the evident pre-inflammatory and pre-coagulative status in cancer with the endogenous residual anticancer ability (Proctor *et al.*, 2011). Increase in NLR and PLR level is associated with increase in systemic inflammatory response associated with cancer, indicating an advanced stage in several types of malignancies (Raungkaewmanee *et al.*, 2012) which are often times associated with poor survival as observed in the present study.

Tn antigen is widely expressed in cancers of epithelial origin being expressed in more than 90% of breast cancers (Zhang *et al.*, 1997; Sorensen *et al.*, 2006). It has been characterized as one of the most specific human cancer associated structures where it serves as an important recognition element mediating several cell-to-cell interactions, being implicated in tumour cell organotropic metastasis (Freire and Osinaga, 2003). Teresawa *et al.* (1996) who studied Tn expression in carcinoma of the uterine cervix have reported that the expression of Tn antigen in a tissue reflects a specific change in the neoplastic progression from a carcinoma in situ to an invasive carcinoma. Results obtained from the present study showed Tn antigen expression in the primary tumour, with cell membranes stained positive and staining extending to the apical and basal part of the cell membrane. This corroborates results reported by Babino *et al.* (2000) who

observed that immunolabeling of Tn antigen in the mammary gland of N-Methyl-N-Nitroso Urea – induced (NMU-induced) mammary carcinomas were found mainly in the cell cytoplasm though staining was also observed on the cell membranes. Furthermore, they found out that Tn antigen staining was highest in carcinomas where most of the cells stained strongly or moderately with less than 10% of cells unstained (Babino *et al*., 2000), this was also similar to staining pattern observed by Osinaga *et al*. (1996). Konska *et al*. (2016) observed diminished staining intensity of tumour cells as the disease progressed. This may be responsible for its negative expression observed in the serially transplanted groups as the tumours obtained in the transplant groups had a short latency period and were well differentiated mammary tumours particularly the second serial transplant group. On the other hand, TF antigen expression was negative in the primary tumour as well as the serially transplanted groups. Reasons for the negative expression is not fully understood. Attempts by this researcher to review the literature on TF antigen in chemically-induced mammary tumours of rats were not so productive. This could be a pointer to this as a recommended area of research interest.

TNF-α, a proinflammatory cytokine has been implicated in the in the processes of apoptosis, inflammation and immunity (Zhou *et al*., 2014). Results obtained from the present study showing an increase in TNF-α levels across the tumour transplant groups as compared to the control may be responsible for the exponential growth observed in the tumours. This is consistent with reports by Waters *et al*. (2013) who noted that besides apoptosis, TNF-α plays a role in cancer cell proliferation and survival. TNF-α may influence tumour growth and progression via inducing the expression of pro-malignant chemokines, metalloproteinases, endothelial adhesion molecules and various angiogenic mediators, or through DNA damage as a result of reactive oxygen species intermediates whose overall effect contribute to tumourigenesis (Sirotkovic-Skerlev *et al*., 2006).

CONCLUSION

DMBA-induced mammary tumours in Sprague Dawley rats undergo divergent cellular behaviour and antigenic expressions during serial transplantation characterized by an elevation of TNF-α, non-induction of Tn antigen with thrombocytopenia.

RECOMMENDATION

Further studies should be done to evaluate the underlying mechanisms involved in the non-expression of TF antigens in chemically-induced mammary tumours of experimental animals.

CONFLICT OF INTEREST: Authors declare no conflict of interest.

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