Newcastle disease virus Tabanan-1/ARP/2017 suppresses Ki67 and VEGF expression in rat mammary carcinoma models

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ABSTRACT

Targeting proteins involved in angiogenesis represents a promising strategy in cancer therapy, with vascular endothelial growth factor (VEGF) emerging as a notable candidate. The oncolytic Newcastle disease virus (NDV) has shown potential as a virotherapy agent for cancer. This study aims to investigate the oncolytic potency and mechanism of Indonesian NDV in rat mammary carcinoma models, by examining the expression of Ki67 and VEGF protein. Rats were divided into two groups: control (K^0) and virotherapy (K^1). The K^0 groups received intratumoral administrations of 500 µL phosphate-buffered saline, while K1 received 2^7 HAU per 500 µL of NDV Tabanan-1/ARP/2017 once per day for four consecutive days. On the 15th day following the initiation of therapy, all the rats were euthanized, and tumor tissue was collected. The tissue was then processed histologically and stained with anti-Ki67 and anti-VEGF. Histopathologically, the virotherapy group exhibited fewer blood vessels than the control group. NDV therapy significantly reduced both the Ki67 and VEGF expression. In conclusion, NDV Tabanan-1/ARP/2017 demonstrates potential as a virotherapy agent in rat mammary carcinoma models.

Key words: Ki67; mammary carcinoma; Newcastle disease virus; oncolytic virotherapy; VEGF

Introduction

Vascular endothelial growth factor (VEGF) tends to be overexpressed in the majority of solid tumors, including mammary carcinoma, and its overexpression is related to tumor aggressiveness, reduced therapy efficacy (AYOUB et al., 2022), and poor prognosis (QUI et al., 2008). Elevated VEGF expression is often induced by tumor cells experiencing hypoxia, a condition that arises from poorly vascularized areas within the tumor mass. Given its central role in the mechanisms underlying tumor growth and metastasis, VEGF has emerged as a promising therapeutic target. In experimental animal models, VEGF inhibition has demonstrated efficacy in inhibiting tumor growth (NIU and CHEN, 2010).

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Oncolytic viruses (OVs) have been garnering increasing attention from researchers due to their perceived direct oncolytic effects and their ability to stimulate cancer-specific immune responses. Leveraging advancements in genetic engineering technology, OVs have evolved into a versatile platform for the development of novel antitumor strategies, either as stand-alone therapies or in conjunction with other treatment modalities (LIN et al., 2023). Numerous studies have documented the oncolytic properties of various viruses, with the Newcastle disease virus (NDV) standing out as a particularly promising candidate (BUIJS et al., 2014; SCHIRRMACHER, 2017; KALYANANSUNDRAM et al., 2018; SEWOYO et al., 2021).

The Newcastle disease virus (NDV) or Avian Orthoavulavirus-1 (AOAV-1) is an enveloped, non-segmented, single-stranded RNA virus with negative polarity, primarily known for causing lethal Newcastle disease in poultry (AMARASIGHE et al., 2019). Despite the conventional negative perception associated with viruses causing disease in poultry, NDV presents an alternative facet wherein it holds promise as a viable cancer therapy in mammals. Its ability to replicate within cancer cells is intricately linked to abnormalities in the interferon (IFN) response (SCHIRRMACHER, 2017). Notably, NDV has been observed to replicate at a slower rate in normal cells (KALYANANSUNDRAM et al., 2018), and assessments in rat models and non-human primates have demonstrated no alterations in hematology and blood biochemistry (BUIJS et al., 2014; SEWOYO et al., 2022).

The Indonesian NDV have demonstrated promising potential as agents for cancer virotherapy. Specifically, NDV Tabanan-1/ARP/2017 exhibited the capability to inhibit the growth of fibrosarcoma and mammary carcinoma in rat models (SEWOYO et al., 2021; SEWOYO et al., 2024a). This tumor suppression was attributed to a reduction in the number of blood vessels. However, additional mechanisms by which this virus suppresses the growth of mammary carcinoma have yet to be reported.

To investigate the underlying mechanisms of the NDV Tabanan-1/ARP/2017 further, the present study aims to evaluate the expression of Ki67 and VEGF in rat mammary carcinoma models.

Materials and methods

Animals. This study used 15 female virgin white rats of the Sprague-Dawley strain, aged 55 days, induced with 7,12-dimethylbenz[α]anthracene (DMBA) at a dosage of 80 mg/kg BW in 0.75 mL corn oil to establish mammary carcinoma (SEWOYO et al., 2024b). The experimental animals were housed in the Pathobiology Laboratory, Faculty of Veterinary Medicine, Udayana University, Indonesia. They had access to tap water *ad libitum* and were maintained in accordance with the ethical standards for the humane treatment of animals.

Experimental group. Out of the total of 15 rats induced with DMBA, six rats developed mammary tumors with approximately equal diameters. These rats were divided into two groups: control (K_0) and virotherapy (K_1). The K_0 group received intratumoral administration of 500 µL sterile phosphate-buffered saline (PBS, pH 7.4), while K_1 group received Indonesian NDV Tabanan-1/ARP/2017 (GenBank No. MH215997.1) at a dosage of 2^7 hemagglutination units (HAU) per 500 µL, as per the protocols outlined by SEWOYO et al. (2024a). The therapy regimen was administered once daily four times on consecutive days, and on the 15th day following the initial therapy, the experiment concluded, leading to the euthanasia of the animals.

Tissue collection and paraffin-embedded tissue blocks preparation. Euthanasia of all the rats was carried out using toxic doses of ketamine and xylazine, which were five times the normal dose, i.e., 200 mg/kg BW and 40 mg/kg BW, respectively, administered intraperitoneally (LEARY et al., 2020). The tumor tissue was excised and cut into 1×1×1 cm dimensions, then deposited into sample pots containing 10% neutral buffered formaldehyde (NBF). Following this, the tissue samples were processed into paraffin blocks.

Histology and immunohistochemistry. The paraffin blocks containing the tissue were sectioned at a thickness of 5 µm. For the histological slides, the paraffin-embedded tissue blocks were processed for routine histological staining with Harris hematoxylin and eosin (H&E). The immunohistochemical staining procedure was carried

out using the universal immuno-enzyme polymer method, with anti-mouse Ki67 monoclonal antibody (Invitrogen, Waltham, Massachusetts, USA) and anti-mouse VEGF monoclonal antibody (NB100-664, Novus Biologicals, Colorado, USA) coupled with conjugate anti-rabbit-mouse horseradish peroxidase (HRP) polymer (N-Histofine® Simple Stain MAX PO Multi, Nichirei Bioscience Inc., Tokyo, Japan) and diaminobenzidine/DAB chromogen (N-Histofine® DAB-2V, Nichirei Bioscience Inc., Tokyo, Japan). Antigen retrieval was performed by submerging the slides in citrate buffer (pH 6.0) and heating them in a microwave at 95°C for 30 min, followed by cooling to room temperature. Endogenous peroxidase was blocked by immersing the slides in H_2O_2 in methanol for 20 min at room temperature, followed by PBS rinsing. Anti-Ki67 (1:50) and anti-VEGF (1:100) antibodies were diluted with antibody diluent (DaVinci Green Diluent, Biocare Medical, USA), then introduced to the slides and incubated overnight at 4°C. The slides were counterstained using Mayer hematoxylin (Merck KGaA, Darmstadt, Germany).

Ki67 assessment. The proliferation index (PI) was determined by quantifying the number of Ki67 immunopositive cells per a minimum of 500 neoplastic cells (SOLIMAN and YUSSIF, 2016).

VEGF expression scoring. The cytoplasmic staining intensity of invasive tumor cells was evaluated semi-quantitatively following the method outlined by MAAE et al. (2011), which involves assessing staining intensity scores and staining area (Table 1). The two scores are accumulated, and if the cumulative score falls within the range of 0-2, it is classified as negative (-); a score of 3-4 is regarded as positive (+), while a score of 5-6 indicates strong positivity (++).

Table 1. VEGF expression assessment scoring

Score	Staining intensity	Staining area
0	-	<10%
1	Weak	10-25%
2	Moderate	25-50%
3	Strong	>50%

Reference: MAAE et al. (2011)

For both Ki67 and VEGF assessments, tumor sections with the highest cellular and vascular density were selected. The staining extent was quantified using ImageJ (National Institute of Health, Bethesda, Maryland, USA). Initially, the image underwent color deconvolution to separate the hematoxylin counterstain color from DAB. Subsequently, the threshold was adjusted to isolate the brown immunopositive area, and the area percentage was determined using the area fraction function.

Statistical analysis. The PI of slides stained with anti-Ki67 was subjected to parametric t-test, while the VEGF score for slides stained with anti-VEGF was analyzed using the non-parametric Mann-Whitney test. Statistical analyses were conducted using SPSS version 26 software for Windows. The values of $P<0.05$ were considered significant.

Results

Histopathology. Histopathological examination revealed a combination of cribriform carcinoma and comedo carcinoma in all rats induced with DMBA. The number of blood vessels in the virotherapy group was fewer than in the control group (Fig. 1). Additionally, the virotherapy group also exhibited infiltration of mononuclear inflammatory cells with multifocal necrotic tumor tissue.

Immunohistochemistry. Staining with anti-Ki67 is considered immunopositive when the cell nucleus exhibits a brown coloration. In this study, numerous brownish cell nuclei were observed, indicating Ki67 immunopositivity (Fig. 2). Both the control (K_0) and virotherapy (K_1) groups exhibited immunopositive cells. The results of the PI calculation revealed that the K_0 group had a mean PI of 8.67±2.08, whereas the K_1 group had a mean PI of 4.33±1.52 (Table 2). According to the t-test, a significant difference in PI ($P<0.05$; $P=0.014$) was observed between the two groups.

Immunohistochemical staining with anti-VEGF antibodies is considered immunopositive when the cytoplasm exhibits brown staining. In this study, the K_0 group displayed a stronger intensity of brown coloration and a larger staining area compared to the K_1 group. The K_1 group exhibited

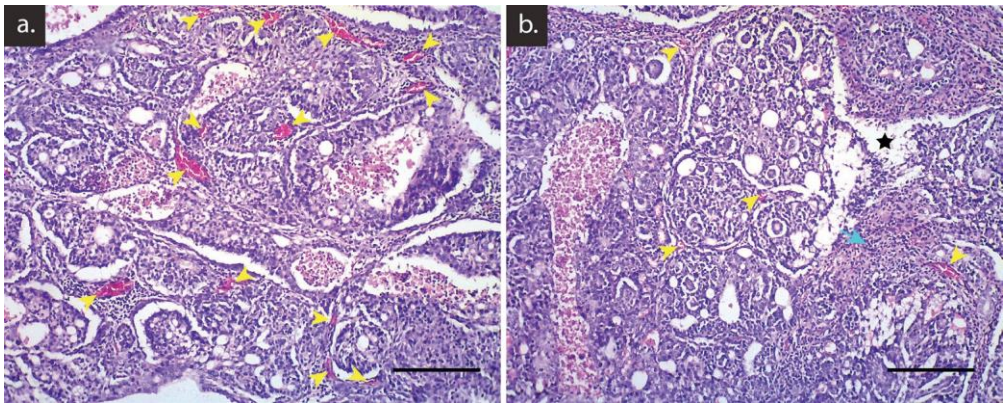

Fig. 1. Histopathology of mammary carcinoma in each treatment group

The control group (a) exhibited several more blood vessels engorged with red blood cells (arrowhead) than the virotherapy group (b) virotherapy group was also severely infiltrated with mononuclear cells (arrow) with area of necrosis (star). H&E Staining (100×; Bar 100 μm)

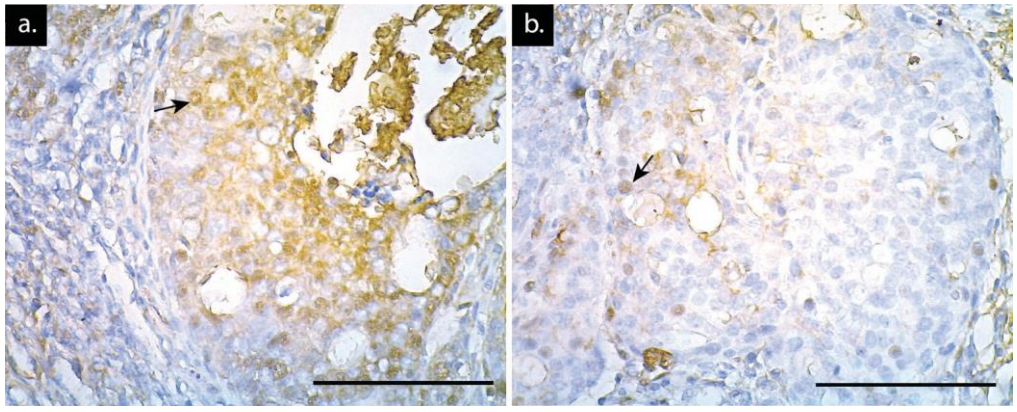

Fig. 2. Ki67 expression in mammary tumor tissue of the control group (a) and virotherapy group (b)

The control group exhibited a higher number of immunopositive cells (indicated by arrow) compared to the virotherapy group. (400×; Bar: 50 μm)

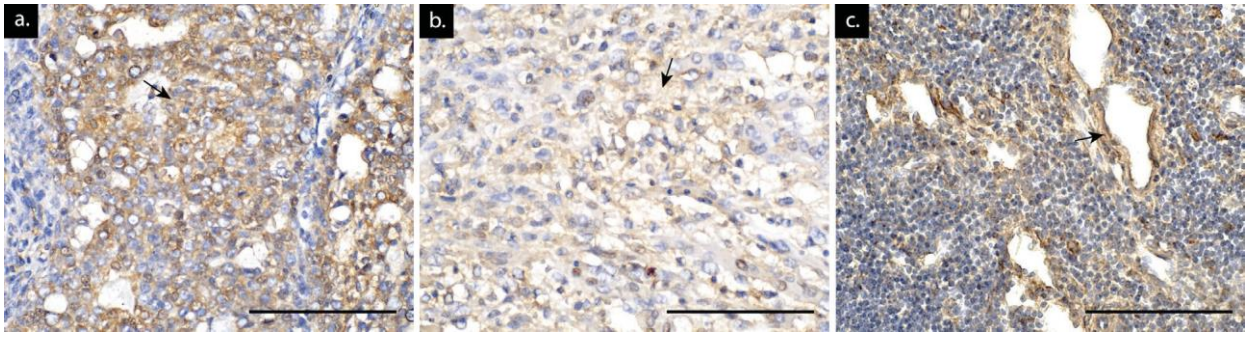

Fig. 3. The VEGF expression in mammary tumor tissue from the control group (a) and the virotherapy group (b,c)

The control group (a) exhibited stronger staining intensity and a larger staining area compared to (b) the virotherapy group (arrow). (c) In the virotherapy group, lymphocytic cell infiltration in necrotic tumor tissue was observed, which was not stained by VEGF. Additionally, VEGF was slightly expressed on the blood vessel endothelium, as indicated by the arrow. (400×; Bar: 50 μm)

Table 2. Ki67 and VEGF protein expression in each treatment

Treatment	Sample	VEGF		Ki67
		Staining area	Staining intensity	Proliferation index
K_0 (Control)	1	3	3	11
	2	3	2	7
	3	3	3	8
	Mean	3 ± 0	2.66 ± 0.56	8.67 ± 2.08
K_1 (Virotherapy)	1	2	2	6
	2	2	2	4
	3	1	1	3
	Mean	1.67 ± 0.57	1.67 ± 0.57	4.33 ± 1.52

*The proliferation index is calculated on the basis of the number of immunopositive cells per 500 neoplastic cells. The VEGF score is assessed using the extent of staining (score 0: <10%, score 1: 10-25%, score 2: 25-50%, score 3: >50%) and intensity (score 1: weak, score 2: moderate, score 3: strong).

varying intensities of brown staining, ranging from low to medium, with generally smaller staining area (Table 2). In the control group, VEGF was expressed in the tumor cytoplasm (Fig. 3a), whereas in the virotherapy group, VEGF was observed to be expressed in the tumor cytoplasm (Fig. 3b) and also slightly expressed in the vascular endothelium. Additionally, the necrotic tumor tissue in the virotherapy group, which was heavily infiltrated with lymphocytic cells, did not express VEGF (Fig. 3c). According to the Mann-Whitney test, a significant difference in VEGF protein expression ($P<0.05$; $P=0.034$) was observed between the two groups after 15 days of treatment.

Discussion

In the clinical settings, AL-HARRIS et al. (2008) reported that VEGF overexpression was also positively correlated with Ki67, tumor grade, stage, lymph node metastasis, and breast cancer recurrence. Evidence of reduced angiogenesis activity was apparent in the rats treated with NDV Tabanan-1/ARP/2017, as indicated by lower blood vessel counts correlated with lower VEGF expression levels. Previous preliminary reports utilizing NDV Tabanan-1/ARP/2017 in rat mammary carcinoma also yielded similar results (SEWOYO et al., 2024a), aligning with the findings presented in this study. Decreased VEGF expression is also accompanied by a decrease in the proliferation rate of cancer cells, as indicated by decreased expression of the Ki67 protein. Thus, NDV Tabanan-1/ARP/2017 appeared to reduce mammary carcinoma vascularization, possibly through suppression of VEGF. The reduction in protein expression may be attributed to the oncolytic capacity of the NDV, which effectively destroys tumor cells. VEGF protein is typically expressed in tumor cells (KIERAN et al., 2012); hence, the viral-induced destruction of tumor cells likely contributes to the diminished expression of this protein. Additionally, tumor cell destruction may occur through the activation of host immune cells involved in the oncolytic process. Evidence of this includes the infiltration of mononuclear inflammatory cells into the tumor tissue in the virotherapy group in the present study, as well as the presence of necrosis.

PRADNYANDIKA et al. (2023), using the same virus isolate as in the current research, demonstrated that intratumoral administration of the virus in rat fibrosarcoma models led to a decrease in mutant p53 (mutp53) expression. Overexpression of mutp53 in cancer cells supports tumor progression due to its gain-of-function (GOF) effects (ZHANG et al., 2020). Mutp53 can activate protein kinase C (PKC), which in turn increases VEGF expression (KIESER et al., 1994). In breast cancer cells, mutp53 can binds with long non-cod-

ing RNA MALAT1, enhancing its chromatin association and stimulating VEGF expression (PRUSZKO et al., 2017). There is a strong correlation between mutp53 and VEGF expression, as well as tumor aggressiveness, and in humans, it has been reported to be linked with poor prognosis (ZHANG et al., 2020). In contrast, wild-type p53 is known to suppress VEGF expression and inhibit angiogenesis (PAVLAKIS and STIEWE, 2020). It is hypothesized that the virus reduces mutp53 expression through the autophagy, consequently reducing GOF activity, which may decrease VEGF expression. The mutp53 is either degraded or repaired, thereby regaining its tumor suppressor function.

Numerous studies highlight the efficacy of NDV isolates in suppressing carcinoma growth through anti-angiogenic mechanisms. For instance, research by AL-SHAMMARI et al. (2022), utilizing the NDV strain AMHA1 from Iraq, demonstrated its effectiveness in suppressing mammary adenocarcinoma growth in murine models *in vivo* by reducing blood vessel formation. *In vitro* experiments revealed that the NDV AMHA1 virus inhibited angiogenesis in AMN3 mammary adenocarcinoma cells by downregulating the expression of angiopoietin-1, angiopoietin-2, and epidermal growth factor (EGF). Another study by SUI et al. (2017) reported that NDV-D90 suppresses growth of gastric cancer by reducing cancer-related vascularization, possibly through suppression of VEGF-A and Matrix Metallopeptidase 2 (MMP2). Other protein expressions, such as CD31+, were also significantly reduced. Similarly, YURCHENKO et al. (2021) reported that the wild-type NDV Altai/Pigeon/770/2011 virus from Russia exhibited an antiangiogenic effect in a Krebs-2 carcinoma mice model by inhibiting the expression of vascular endothelial growth factor receptor (VEGFR). Notably, VEGFR is mainly expressed by endothelial cells lining blood vessels (APTE et al., 2019). This suggests that, besides replicating in tumor cells, the NDV likely replicates in blood vessel endothelium, thereby contributing to the reduction of angiogenesis activity.

The results of this study are quite promising, indicating the potential for further research to explore how NDV Tabanan-1/ARP/2017 suppresses VEGF expression, thereby reducing tumor angiogenesis. We also suggest to examining the other proteins that are involved in angiogenesis, such as CD31+ and VEGFR, in future research. The results of this research are novel, as studies on the oncolytic activity of NDV isolates from Indonesia remain scarce. NDV Tabanan-1/ARP/2017 demonstrated the ability to decrease the expression of Ki67 and VEGF in rat mammary carcinoma models. A notable limitation of the present study is replication, as the induction of tumors using chemical carcinogens often results in variability in tumor size and type. This inherent challenge arises due to differences in the biological response of individual animals to the carcinogen. To address this limitation, it is recommended to conduct studies involving a larger number of animals induced with chemical carcinogens, to increase replication and improve the statistical power of the findings. Alternatively, employing other tumor models with more consistent tumor characteristics could provide a more reliable basis for comparison and enhance the reproducibility of the study results.

Conclusions

It can be inferred that this virus isolate holds promise as a potential cancer virotherapy agent in rat mammary carcinoma models.

Ethics approval

This research has obtained approval from the Experimental Animals Ethics and Use Committee Faculty of Veterinary Medicine, Udayana University, Indonesia (certificate number: B/135/UN14.2.9/PT.01.04/2023). All actions and procedures conducted in this research strongly adhered to the guidelines and regulations of the committee.

Declaration of competing interest

No potential conflicting interest was reported by the authors.

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SAŽETAK

Ciljano djelovanje na proteine uključene u angiogenezu obećavajuća je strategija u liječenju karcinoma, pri čemu se kao dobar kandidat pojavljuje vaskularni endotelni čimbenik rasta (VEGF). Onkolitički virus newcastleske bolesti (NDV) pokazao se kao potencijalan lijek u virusnoj terapiji karcinoma. Cilj je rada bio istražiti onkolitički potencijal i mehanizme NDV-a iz Indonezije u modelima štakora s karcinomom mliječne žlijezde, analizirajući ekspresiju proteina Ki67 i VEGF. Štakori su podijeljeni u dvije skupine: kontrolnu skupinu (K^0) i skupinu u kojoj je provedena virusna terapija (K^1). Štakori u skupini K^0 primili su intratumorski 500 µL fosfatno puferirane fiziološke otopine, dok su štakori u skupini K^1 primili 2^7 HAU na 500 µL NDV-a Tabanan-1/ARP/2017 jedanput dnevno tijekom četiri uzastopna dana. Petnaesti dan od početka terapije svi su štakori eutanazirani te je prikupljeno tumorsko tkivo. Tkivo je histološki obrađeno i obojeno upotrebom antitijela anti-Ki67 i anti-VEGF. Histopatološki, u štakora kojima je dana virusna terapija razvio se manji broj krvnih žila nego u kontrolnoj skupini. NDV terapija znakovito je smanjila ekspresiju i Ki67 i VEGF-a. Zaključuje se da NDV Tabanan-1/ARP/2017 ima potencijal kao virusno terapijsko sredstvo u modelima štakora s karcinomom mliječne žlijezde.

Ključne riječi: Ki67; karcinom mliječne žlijezde; virus newcastleske bolesti; onkolitička virusna terapija; VEGF