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Newcastle disease virus Tabanan-1/ARP/2017 inhibits growth of rat mammary carcinoma models

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ABSTRACT

The lack of effective therapeutic modalities for mammary cancer is attributed to side effects and therapy resistance, necessitating the exploration of alternative treatment options. Newcastle Disease Virus (NDV) exhibits oncolytic activity, making it a promising candidate for cancer therapy. This study aims to assess the effectiveness of the virulent NDV Tabanan-1/ARP/2017 on the growth of mammary carcinoma. The study involved 15 white female Sprague-Dawley rats induced with mammary carcinoma. After the tumors had developed, the rats were divided into two treatment groups, i.e., treatment 0 (P0) and treatment 1 (P1), which received 500 µL of phosphate-buffered saline and 128 HAU/500 µL of NDV Tabanan-1/ARP/2017, respectively. The rats were euthanized on day 15 post-virotherapy. Rats were necropsied, the tumor was excised to measure its weight, percentage of tumor inhibition, and subsequently routinely processed for histopathological preparations. The tumor weights in each treatment group were 3.70±0.72 and 2.34±0.64 grams, respectively, with a tumor inhibition percentage of 36.62%. The angiogenesis, hemorrhage, and mitotic activity of P1 were lower than those of P0, while inflammatory cell infiltration and areas of necrosis appeared more prominent in the group treated with the NDV. In conclusion, the NDV Tabanan-1/ARP/2017 shows potential as a virotherapy agent for rat mammary carcinoma models.

Introduction

The World Health Organization (WHO, 2022) asserts that cancer ranks as the leading cause of mortality in the global human population. Specifically, breast cancer constitutes a frequently diagnosed malignancy in women (Borecka *et al*., 2020). Statistics from the year 2020 indicate a disconcerting fatality rate, with approximately 600,000 female fatalities attributed to breast cancer, while an estimated 2.2 million new cases were diagnosed during the same period (Sammarco *et al*., 2020). Beyond humans, breast cancer, also called mammary cancer, prevails as the most prevalent neoplastic disease among female canines (Sewoyo *et al*., 2023). Notably, mammary cancer accounts for nearly half of all documented canine cancer cases, constituting a substantial proportion (Moe, 2010). In a comprehensive investigation by Salas *et al*. (2015), the incidence of mammary tumors in dogs was delineated, approximating 16.8%. Further categorization revealed benign tumors with a prevalence of 47.7%, juxtaposed with malignant counterparts at 47.5%, underscoring the heterogeneity of canine mammary neoplasms.

Advancements in cancer research and treatment have remarkably enhanced diagnostic precision and therapeutic options, including surgery, chemotherapy, and radiotherapy. These conventional therapy remains the standard for treating mammary cancer (Foulkes *et al*., 2010). However, it is crucial to recognize that these treatments have some risks and side effects. While surgery effectively eliminates tumor masses, its limitations, such as an increased risk of metastasis, warrant careful consideration (Tohme *et al*., 2017). Similarly, chemotherapy, while effective in eliminating metastatic cells, poses long-term negative impacts by affecting normal cells and disrupting physiological systems (Octavia *et al*., 2012).

In addition to the risks and side effects associated with conventional therapy, the emergence of therapy resistance poses a serious problem in cancer treatment. Resistance can occur either from the onset of treatment or develop during the therapy process (Schirrmacher *et al*., 2019). Consequently, researchers are actively exploring alternative approaches to address these challenges. One particularly promising alternative therapy candidate is virotherapy, which involves utilizing viruses as cancer therapy that selectively replicates within cancer cells while sparing normal cells from harm. The inception of virotherapy traces back to a fortuitous occurrence in 1950 when a patient with metastatic melanoma exhibited tumor regression following the administration of rabies vaccination. This serendipitous event spurred subsequent investigations into diverse viral agents for their potential utility in cancer therapy (Kelly and Russel, 2007).

To date, a multitude of viruses demonstrating oncolytic activity have been identified, including the Newcastle Disease Virus (NDV) (Fountzilas *et al*., 2017). While NDV is the causative agent of the lethal Newcastle disease in poultry, it exhibits potential as a therapeutic agent for cancer in mammals (Schirrmacher, 2017). NDV manifests an accelerated replication rate within mammalian cancer cells, surpassing that in normal cells by up to 10,000-fold, a phenomenon attributed to aberrant interferon (IFN) responses in cancerous cells (Kalyanasundram *et al*., 2018). Importantly, NDV does not induce significant physiological alterations, particularly in the hematological profile (Sewoyo *et al*., 2022) and histological of the lungs, spleen, and gut-associated lymphoid tissue (Adi *et al*., 2023) in rats. A study by Buijs *et al*. (2014) also further confirmed that intravenous administration of NDV in non-human primates is safe and there are no abnormalities in hematologic or biochemistry parameters.

Research on NDV isolates has been conducted in various countries, including Indonesia. Numerous studies have delved into the oncolytic capabilities of NDV isolates originating from Indonesia. The NDV Tabanan-1/ARP/2017 isolate, obtained from laying hens in Tabanan, Bali, in 2017, has

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been identified for its ability to inhibit the growth of fibrosarcoma in rat models (Adi *et al*., 2019a; Sewoyo *et al*., 2021). However, a comprehensive understanding of the oncolytic activity of this particular isolate, encompassing its underlying mechanisms and impact on other cancer cell types, necessitates further investigation. In this study, we employed the virulent NDV Tabanan-1/ARP/2017 isolate to extend our inquiry into its oncolytic potential, particularly within the context of a mammary carcinoma model in rats. This endeavor aims to elucidate the nuanced aspects of its oncolytic properties, contributing to a broader comprehension of its therapeutic efficacy in mammary carcinoma.

Materials and methods

Ethical approval

This research has received ethical approval and permission from the Ethics and Use of Experimental Animals Committee at the Faculty of Veterinary Medicine, Udayana University. All procedures conducted within this research strictly follow the guidelines and regulations established by the ethics committee.

Experimental animal

This study used 15 virgin female white Sprague Dawley rats (*Rattus norvegicus*) aged 55 days weighing 90-110 g. The animals that were used in this study were obtained from the Department of Pharmacology, Faculty of Medicine and Health Sciences, Udayana University. All rats were housed in the Department of Pathobiology, Veterinary Pathology Laboratory, Faculty of Veterinary Medicine, Udayana University, where they were maintained on a 12-h light and dark cycle under humane and ethical principles. The rats had access to clean tap water *ad libitum* and were provided with standard pellets that met their dietary needs throughout the study period. The rats were provided with standard pellets (Rat Bio®, PT. Citra Ina Feedmill, Indonesia), containing 60% carbohydrate, 20% protein, 4% fat, 4% crude fiber, 12% calcium, and 0.7% phosphorus.

Mammary carcinoma models establishment

Prior to the experiment, the rats underwent a one-week acclimatization. Mammary carcinoma was established using 7,12-dimethylbenz[a] anthracene (DMBA) (Tokyo Chemical Industry, Tokyo, Japan). The injection was carried out with a single dose of 25 mg per rat, following the protocol of Rajendran *et al*. (2019) with slight modification. DMBA was dissolved in 0.75 mL analytical-grade corn oil (Sigma Aldrich, St. Louis, MO, USA) and administered subcutaneously into the rat mammary fat pad in the right abdominal mammary glands. During the induction process, rats were anesthetized using ketamine (Keta-A-100®, Agrovet SA, Lima, Peru) and xylazine (Xyla®, Interchemie, Metaalweg, Holland) at doses of 40 mg/kg BW and 8 mg/kg BW respectively. The injection site was then shaved with a clipper, depilatory cream was applied for complete hair removal, and the area was prepared aseptically with 70% alcohol. Rats were palpated once every two days after induction with DMBA. The tumor appeared in nine rats (60%) with a diameter of 0.5-1 cm (Fig. 1b). Eight rats were randomly selected and divided into two group treatments, i.e., the control (P0) and treatment group (P1).

Virus, viral propagation and purification

The virulent NDV Tabanan-1/ARP/2017 isolate (GenBank accession number: MH215997.1) was used in this study. This virus was inoculated into 10-day-old embryonated chicken eggs (ECEs) via allantoic cavity and incubated at 37°C (Joao *et al*., 2022). Diffuse hemorrhage was observed on the chicken embryo skin 48 hours post-inoculation of the NDV (Fig. 1a). The infective allantoic fluid-containing NDV is collected using a sterile syringe, purified from debris by centrifugation at 1500×g for 10 min at 4°C, then filtered with 0.4 µm syringe filter and kept in microtubes at -80°C for further use (Adi *et al*., 2019b). The virus titer is measured using the hemagglutination (HA) test as described by the World Organization for Animal Health/WOAH (2021).

Treatment group

The control group (P0) consisted of rats that received 500 µL phosphate-buffered saline (PBS) intratumorally. The virotherapy group (P1) comprised rats that received intratumoral virotherapy using NDV Tabanan-1/ARP/2017 isolate at a dose of 128 HAU/500 µL (Rakhmawati *et al*., 2022). This treatment was given four times consecutively (Sewoyo *et al*., 2021; Pradnyandika *et al*., 2023).

Euthanasia procedure

At day 15 post-virotherapy, the experiment was completed, and the animals were euthanized. The euthanasia procedure adhered to guidelines from the American Veterinary Medical Association (AVMA), involved the administration of a toxic dose of ketamine and xylazine, i.e., 250 mg/kg BW and 25 mg/kg BW, respectively, via intraperitoneal route (Leary *et al*., 2020).

Measurement of tumor weight and growth Inhibition

After the rats were euthanized, the tumors were excised and weighed. The percentage of tumor inhibition was measured based on the formula of Ortega-Rivera *et al*. (2023): [(Mean tumor weight of control group – Mean tumor weight of treatment)/Mean tumor weight of control group)] x100.

Tissue collection

Following macroscopic examination, these specimens were then fixed in 10% neutral buffered formaldehyde for 24 hours. Subsequently, they were processed and embedded into paraffin-embedded tissue blocks then further processed for routine histological preparation and stained with Hematoxylin-Eosin (HE).

Histopathologic assessment

The tumor preparations that have been made are then evaluated histopathologically to determine the type of tumor and description of several parameters such as angiogenesis, inflammatory cell infiltration, areas of necrosis, and mitosis. These parameters were assessed qualitatively in five fields of view, then the results were presented in the following form: (-) None, (+) low, (++) moderate, and (+++) high.

Statistical analysis

The tumor weight obtained was analyzed using the student t-test, while the histopathological parameters were analyzed using Mann-Whitney. Statistical analysis using SPSS version 26 for Windows, the $p<0.05$ and $p<0.01$ values are considered significant.

Results

Following the euthanasia of experimental animals, necropsies were conducted, and tumor tissues were excised for subsequent analysis. The excised tumor tissues were then weighed, revealing an average weight of 3.70±0.72 g for P0 rats and 2.34±0.64 g for P1 rats (Fig. 1c). Based on the calculation of tumor inhibition rate, administration of NDV Tabanan-1/ARP/2017 inhibited the mass of the tumor significantly by a 36.62%

(p<0,05; p-value = 0.031).

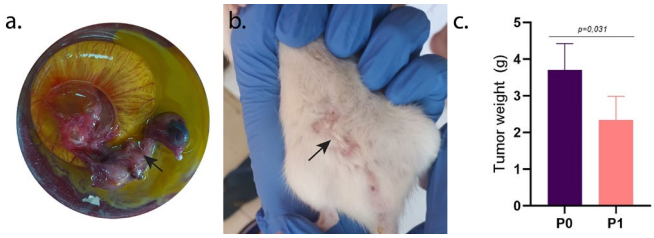

Fig. 1. (a) Cultivation of NDV Tabanan-1/ARP/2017 in ECEs. A general hemorrhage (arrow) was present in the chicken embryo skin (b) Tumor mass before treatment. The tumor exhibits a slightly dense consistency with a small area of alopecia (arrow) (c) Comparative graph showing the weights of tumor masses.

Eight rats DMBA-induced mammary tumors, were divided into two treatment groups and treatment was carried out. On the 15th day post-first therapy, rats were euthanized, and tumor tissues were collected for subsequent histopathological preparations.

Histopathologically, tumor tissue at P0 is characterized by high angiogenesis, low lymphocytic inflammatory cell infiltration, multiple areas of mild necrosis, and moderate to high mitotic rates. Conversely, in P1 angiogenesis levels range from low to moderate, with high infiltration of lymphocytic inflammatory cells with numerous areas exhibiting necrosis (Fig. 2). Additionally, a moderate level of mitosis was observed in P1 tumor tissues. According to the Mann-Whitney test, there were significant differences in the levels of angiogenesis (p<0.05; p value = 0.013), hemorrhage (p<0.05; p value=0.011), lymphocytic cell infiltration (p<0.01 ; p value=0.008), necrosis (p<0.05; p value= 0.013), and mitosis (p<0.05; p value=0.040).

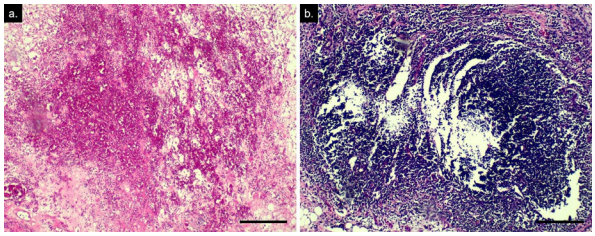

Fig. 2. Photomicrograph of mammary carcinoma in each treatment group. In the control group, (a) high vascularization areas with hemorrhage and limited inflammatory cell infiltration are evident. On the other hand, (b) the treatment group displays extensive necrosis and prominent lymphocytic cell infiltration with minimal vascularization. HE staining. Bar 100 µm.

Discussion

Virotherapy stands out as an innovative and promising approach to cancer therapy. Among the viruses considered for such therapy, NDV, known to cause Newcastle disease in poultry, emerges as a noteworthy candidate. Its distinct advantages include the ability to lyse various types of cancer cells and its non-pathogenic nature in mammals (Najmudin *et al*., 2023; Pathak *et al*., 2023). Several studies focusing on oncolytic activity have been conducted on NDV isolates across different regions of the world.

In this study, NDV Tabanan-1/ARP/2017 isolate was used which was isolated from laying hens in Tabanan, Bali, Indonesia in 2017. Notably, this virus belongs to genotype VII and is classified as virulent (Adi *et al*., 2019a). Macroscopically, a significant reduction in tumor weight was observed in P1 compared to P0. Based on the calculation of the percentage of tumor inhibition, administration of the NDV Tabanan-1/ARP/2017 was able to reduce tumor mass by 36.62%. This decrease in tumor mass can be attributed to NDV's capability to replicate within cancer cells. NDV demonstrates non-pathogenicity in normal mammalian cells and possesses natural lytic abilities against tumor cells. The virulent NDV Tabanan-1/ARP/2017 previously demonstrated its ability to suppress the growth of fibrosarcoma in rat models (Sewoyo *et al*., 2021). Further investigation showed that the virus decreased Ki67, VEGF, TNF-α (Adi *et al*., 2023), and p53 expression (Pradnyandika *et al*., 2023). The oncolytic potential of NDV is contingent upon its virulence, with a more virulent strain exhibiting stronger oncolytic capabilities (Buijs *et al*., 2014). The selective replication of NDV in cancer cells is caused by aberrant interferon (IFN) responses (Schirrmacher *et al*., 2017; Kalyanasundram *et al*., 2018).

Multiple mechanisms underpin NDV's efficacy in lysing tumor cells, including direct oncolytic effects stemming from viral replication within tumor cells, indirect effects through the stimulation of the host's innate and adaptive immune cells, initiation of the apoptotic cascade, and inhibition of the expression of proteins involved in the angiogenesis (Sewoyo *et al*., 2021; Yurchenko *et al*., 2021; Al-Shammari, 2022; Rakhmawati *et al*., 2022).

Consistent with the outcomes of the current study, other *in vivo* investigations employing the NDV to treat mammary cancer models, as demonstrated by Al-Shammari *et al*. (2022) and Ortega-Rivera *et al*. (2023), yielded analogous results. Al-Shammari *et al*. (2022), utilizing the NDV AMHA1 from Iraq, reported the inhibition of mammary tumor growth in adenocarcinoma mouse models. While Ortega-Rivera *et al*. (2023) utilized the recombinant NDV-P05, observing a substantial inhibition of mammary tumor growth in breast cancer murine models with a tumor inhibition percentage of 31%. These parallel findings across diverse studies underscore the potential efficacy of NDV-based therapies in the treatment of mammary cancer.

Microscopic observation revealed a high vascularization in the control group. Additionally, several regions exhibited hemorrhage, that may arise from the increased permeability and proteolytic activity associated with tumor vascularization (Tables 1 and 2). The irregular growth of blood vessels further contributes to the propensity for endothelial walls to rupture (Hodivala-Dilke *et al*., 2003). The formation of blood vessels in cancer differs significantly from the wound healing process, exhibiting distinctive morphological characteristics. In contrast to the stable distribution and hierarchical sequence of arteries, capillaries, and veins observed in normal blood vessels, angiogenic vessels in tumors display notable deviations. These vessels appear dilated and highly tortuous, and there is a lack of uniformity in vascular thickness and blood vessel diameter (Tong *et al*., 2004). In contrast, rats treated with the NDV exhibited a tendency toward low to moderate vascularization, leading to a notable reduction of hemorrhagic areas.

Table 1. Microscopic lesions in control group tumor tissue (P0).

Rat No.	1	2	3	4
Angiogenesis	+++	+++	+++	+++
Hemorrhage	++	+++	++	++
Lymphocytic infiltration	+	+	+	+
Necrosis area	+	+	+	+
Mitosis	++	++	+++	++

Note: (-) None; (+) Low; (++) Moderate; (+++) High

Table 2. Microscopic lesions in treatment group tumor tissue (P1).

Rat No.	1	2	3	4
Angiogenesis	+	+	++	++
Hemorrhage	-	-	+	+
Lymphocytic infiltration	+++	+++	+++	+++
Necrosis area	++	++	+++	+++
Mitosis	+	+	+	++

Note: (-) None; (+) Low; (++) Moderate; (+++) High

The proliferation of new blood vessels plays a pivotal role in the growth and development of cancer cells, as tumors are unlikely to exceed a size of 1-2 mm^3 without this vascular growth (Katayama *et al*., 2019). Furthermore, the growth of new blood vessels is implicated in the formation of metastases (Chen *et al*., 2017). The reduction in angiogenesis activity induced by NDV undoubtedly results in diminished oxygen and nutrient supply to cancer cells, concurrently reducing the potential for metastatic spread. In the NDV-treated group, extensive areas of necrosis and prominent lymphocytic inflammatory cell infiltration were observed. These infiltrations are likely attributed to both direct viral replication within tumor cells and damage to blood vessels, along with the inhibition of the angiogenesis cascade. The induced inflammation may also arise from the stimulation caused by NDV. In tumor cells, NDV infection induces an upregulation of class I major histocompatibility complex (MHC) molecules. These molecules play a pivotal role in presenting viral and tumor antigens to immune cells. Consequently, infected tumor cells are identified and eradicated primarily by natural killer (NK) cells and cytotoxic CD8+ T lymphocytes. Thus, it can be inferred that the activation of

the immune system by the NDV contributes significantly to its oncolytic activity (Tayeb *et al*., 2015).

A hallmark of malignancy is the compromise of the body's immune system by cancer cells. This compromise causes the body to mistakenly perceive these cells as normal, preventing it from mounting an effective defense against them (Hanahan, 2022). Replication of NDV within tumor cells can stimulate immune cells, fostering their engagement in the destruction of cancer cells. This mechanism underscores the potential of NDV to exploit the body's immune response to combat malignancy. The reduction in angiogenesis activity is intricately linked to the decreased expression of proteins involved in the angiogenesis cascade. Al-Shammari *et al*. (2022) study demonstrated that the Iraqi AMHA1 virus effectively suppressed the expression of angiopoietin-1, angiopoietin-2, and EGF *in vitro* within mammary cancer cell lines. Additionally, Yurchenko *et al*. (2021) reported that NDV/Altai/Pigeon/2011, wild-type NDV originating from Russia, successfully decreased the expression of VEGFR in a Krebs-2 carcinoma mouse model. It is plausible that the NDV Tabanan-1/ARP/2017 virus similarly hampers the expression of proteins associated with angiogenesis in mammary carcinoma. As mentioned before, a previous study showed that this isolate can decrease VEGF expression in rat fibrosarcoma models (Adi *et al*., 2023). Therefore, further research needs to be carried out on mammary carcinoma.

Conclusion

The findings suggest that Indonesian NDV Tabanan-1/ARP/2017 holds promise as a potential virotherapy agent for mammary carcinoma. However, further research is imperative to elucidate the underlying mechanisms by which this NDV isolate suppresses the growth of mammary carcinoma.

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Conflict of interest

The author declares that there are no competing interests exist.

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