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Newcastle Disease Virus as Virotherapy Agent Targeting p53 in Rat Fibrosarcoma Models

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Abstract

Repairing wild-type p53 or destroying of mutant p53 is one of the therapeutic targets in cancer. Newcastle disease virus (NDV) is a natural oncolytic virus that has potential as a virotherapy agent in cancer. This virus has been shown to induce cancer cell death. The aim of this study was to determine the expression of p53 in cytoplasm and nucleus of cancer cells and its correlation to the grade of cancer malignancy after NDV therapy in rat fibrosarcoma model. Rat fibrosarcoma model were divided into two groups, i.e., the control group (P0) and the treatment group (P1), each consist of 3 rats. The control group (P0) was injected with 0.5 mL phosphate buffered saline and treatment group (P1) was injected with 0.5 mL NDV Tabanan-1/ARP/2017 intratumorally once a day for four consecutive days. At the end of the study, 15 days post-treatment, all rats were euthanized and fibrosarcoma tissue was collected. Fibrosarcoma tissue was examined using immunohistochemistry to determine p53 expression and histopathological examination with hematoxylin-eosin staining to determine the grade of malignancy. The results of this study, the mutant p53 were more expressed in the control group (P0) than the treatment group (P1). It showed that NDV was significant ($P<0.05$) to the decrease of mutant p53 expression and positively correlated ($P<0.05$) to the cancer malignancy in rat fibrosarcoma model. In conclusion, NDV has potential as a virotherapy agent targeting mutant p53 in rat fibrosarcoma models.

KEYWORDS
Fibrosarcoma, Mutant p53, Newcastle disease virus, Virotherapy

INTRODUCTION

P53 has a very important role in maintaining the integrity of the genome in normal cells. P53 is a nuclear transcription factor with a tumor suppressor function to prevent the growth of abnormal cells. The role of p53 as a tumor suppressor is based on its ability to induce apoptosis, cell cycle arrest, and cell senescence (Mello and Attardi, 2017). The importance of this role, the p53 gene is widely referred to as the 'guardian of the genome'. In cancer, p53 is the gene most mutated so it loses its function as a tumor suppressor. The mutation causes abnormal cell proliferation and is commonly associated with the malignancy of cancer (Ganci *et al*., 2013). Therefore, efforts to restore p53 activity are the focus of cancer therapy in both laboratory studies and clinical trials (Cheok *et al*., 2011).

Virotherapy using oncolytic viruses has been developed as a promising cancer therapeutic agent. Oncolytic viruses can selectively infect and induce cancer cell death without damaging normal cells. Many studies have shown several types of viruses to have oncolytic activity, including the Newcastle disease virus (Lawler *et al*., 2017; Schwaiger *et al*., 2017; Yurchenko *et al*., 2018). Newcastle disease virus (NDV) or Avian orthoavulavirus 1 (AOV-1) is a naturally oncolytic virus of the genus Orthoavulavirus, family Paramyxoviridae. The oncolytic activity of NDV is derived from the type I IFN signaling pathway and type I IFN receptors less sensitive on cancer cells (Krishnamurthy *et al*., 2006; Zamarin and Palese, 2012). In mammals, this virus is non-pathogenic and does not cause severe disease or hematological and biochemical abnormalities, so it can be used as a cancer therapeutic agent (Buijs *et al*., 2014; Schwaiger *et al*., 2017; Sewoyo *et al*., 2022).

Indonesian NDV isolates have shown potential as virotherapy agents in cancer. NDV isolates from Indonesia have shown potential as virotherapy agents in cancer. The virulent NDV Gianyar-1/AK/2014 has been shown to inhibit the growth of fibrosarcoma in mice. Histopathological examination showed few anaplastic fibroblasts, low mitotic figure and no angiogenesis after virotherapy (Rakhmawati *et al*., 2022). Other studies have also shown the ability of NDV as a cancer therapeutic agent. Virotherapy with NDV Tabanan-1/ARP/2017 has been shown to reduce the number of blood vessels in fibrosarcoma tissue and cause a decrease in tumor volume (Sewoyo *et al*., 2021)

In recent years, there has been renewed interest in the process of autophagy in cancer cells. NDV has been known to have autophagy activity in cancer cells. Autophagy is a cellular homeostatic process that can degrade damaged macromolecular proteins or organelles (Ueno and Komatsu, 2017). This study was carried out to determine the expression of p53 in the cytoplasm and nucleus of cancer cells and its correlation to the grade of cancer malignancy after NDV therapy in rat fibrosarcoma models.

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MATERIALS AND METHODS

Ethical Approval

This study has been officially approved by the Ethical Commission for the use of experimental animals, the Faculty of Veterinary Medicine, Udayana University, with the approval number B/75/UN14.2.9/PT.01.04/2022. The procedures carried out in this study are under the provisions and requirements of the ethics committee.

Experimental Animal

The animal used in this study was 12 a male white rat (Rattus norvegicus) Sprague Dawley strain aged 3 months with a body weight of 100-150 grams. These rats were kept in the Laboratory of Veterinary Pathology, Faculty of Veterinary Medicine, Udayana University. The rats were adapted for one week and given food and drink ad libitum.

Induction of fibrosarcoma

Induction of fibrosarcoma in rats using benzo(a)pyrene (Sigma-Aldrich B-1760, St. Louis, MO, USA) dissolved in oleum olivarum (0.3% w/v). This solution was sterilized by heating using an autoclave at a temperature of 115°C for 15 min. The benzo(a) pyrene solution was administered in 0.1 mL by subcutaneous injection in the interscapular area. The injection is given ten times in stages with an interval of once every two days (Sewoyo *et al*., 2021).

Virus and viral propagation

The NDV Tabanan-1/ARP/2017 (Fusion gene accession number MH215997) is an Indonesian field isolate (Adi *et al*., 2019; Sewoyo *et al*., 2021). The virus was propagated in 9 to 11-days-old embryonated chicken eggs via the allantoic cavity and incubated at 37ºC for 48-72 h. The infective allantoic fluid was then collected and the titer of virus was determined by the hemagglutination assay (HA) as described by OIE (2021).

Treatment group

In this study, fibrosarcoma is seen macroscopically at the 93rd day after injection of benzo(a)pyrene. Six rats that had tumors with a diameter of 3-4 mm were divided into two groups. The control group (P0) was injected with 0.5 mL phosphate buffer saline (PBS) and the treatment group (P1) was injected with 0.5 mL of 29 HA titer of NDV Tabanan-1/ARP/2017. Both Groups P0 and P1 received four injection doses for four consecutive days as previously done (Yurchenko *et al*., 2018; Sewoyo *et al*., 2021).

Tissue collection

The rats were euthanized, followed by necropsy after 15 days post-treatment. Euthanasia was performed according to guidelines from the American Veterinary Medical Association (AVMA) using ketamine HCL 10% (Ket-A-100, Agrovet Market SA) and xylazine HCL 2% (Xyla, Interchemie) in five time the normal doses via the intraperitoneal route (Leary *et al*., 2020). Tumor tissue specimens were collected and fixed in 10% neutral buffered formalin (NBF) for 18-24 h, then processed into paraffin-embedded tissue block.

Histopathological Assessment

Three micrometer-thick sections were deparaffinized and rehydrated using xylene and ethanol, followed by routine Hematoxylin Eosin (HE) staining. Fibrosarcoma malignancy was assessed based on the scoring system of the French Federation of Cancer Centers Sarcoma Group (FNCLCC). Three prognostically relevant factors, the tumor cell differentiation, mitotic index and the amount of necrosis, are scored independently. Finally, those scores were summed up and the grade of the tumor was assessed. The examination was carried out under a bright-field microscope in 10 high-power fields (x400) (Augsburger *et al*., 2017).

Immunohistochemistry

Three micrometer-thick sections were deparaffinized and rehydrated using xylene and ethanol. For antigen retrieval, sections were immersed in citrate buffer (pH 6.0), then heated in a microwave oven at 95ºC for 20 min and cooled at room temperature. Endogenous peroxidase was blocked by immersion in 3% H2O2 in methanol for 20 minutes. Sections were then added Peroxide Blocking Reagent (BioLegend) for 5 min. Anti-p53 antibody (p53 polyclonal antibody, Bioss) diluted with Da Vinci Green Diluent (Biocare Medical) (dilution 1:200) was applied for 2 h at room temperature. Sections were incubated for 1 h at room temperature with goat anti-rabbit polymer-HRP (N-Histofine Simple Stain MAX PO MULTI, Nichirei Bioscience Inc.) and visualized with Diaminobenzidine (N-Histofine DAB-2V, Nichirei Bioscience Inc.). Sections were counterstained with hematoxylin staining, then rinsed with PBS, dehydrated, cleared and coverslipped. Positive cells are characterized by a brown color in the nucleus or cytoplasm. Positive cells were counted using the Allred score method. The examination was carried out under a bright-field microscope in 5 high-power fields (x400) (Qureshi and Pervez, 2010).

Statistical analysis

Score data of mutant p53 were analyzed with Mann-Whitney U test. The correlation between mutant p53 expression and fibrosarcoma malignancy was analyzed using the Spearman correlation test. Statistical analysis was performed using SPSS version 25 for windows, the $P<0.05$ value is considered statistically significant.

RESULTS

Scores of mutant p53 and fibrosarcoma malignancy are presented in Table 1. Immunohistochemical assessment, the P0 group showed high number of positive cells for mutant p53 with strong intensity compared to P1 group (Figure 1). Based on the Mann-Whitney U test, there was a significant difference ($P<0.05$) between the P0 group and the P1 group after 15 days post-NDV injection. Histopathological examination showed that the P0 group had a high-grade malignancy compared to the P1 group (Figure 2). In P0 group, 2 of 3 samples had fibrosarcoma with grade 3 (poorly differentiated). In the P1 group, 2 of 3 samples had fibrosarcoma with grade 1 (well differentiated). The result of the Spearman correlation test showed a positive correlation ($P<0.05$) between mutant p53 expression and fibrosarcoma malignancy.

DISCUSSION

P53 is the gene that is mostly mutated in cancer (Bailey *et*

Table 1. Scores of mutant p53 expression and fibrosarcoma malignancy

Group	Sample	Immunohistochemistry[a]		Histopathology[b]	
		Proportion	Intensity	Cell differentiation	Mitotic
P0 (Control)	1	4	3	2	3
	2	3	3	3	3
	3	3	3	3	3
P1 (NDV virotherapy)	1	1	1	2	1
	2	1	2	2	2
	3	1	1	2	1

[a]Immunohistochemical assessment for p53 using a proportion score (score 1: 1% positive cells, 2: 1-10% positive cells, 3: 10-33% positive cells, 4: 33-66% positive cells, 5: 66-100% positive cells) and intensity scores (score 1: weak, 2: moderate, 3: strong).
[b]Histopathological assessment was based on scores of cell differentiation (score 1: closely resembling normal tissue, 2: histological typing is certain, 3: embryonal or undifferentiated sarcoma) and mitotic score (score 1: 0-9 mitoses, 2:10-19 mitoses, 3 : >19 mitoses per 10 HPF).

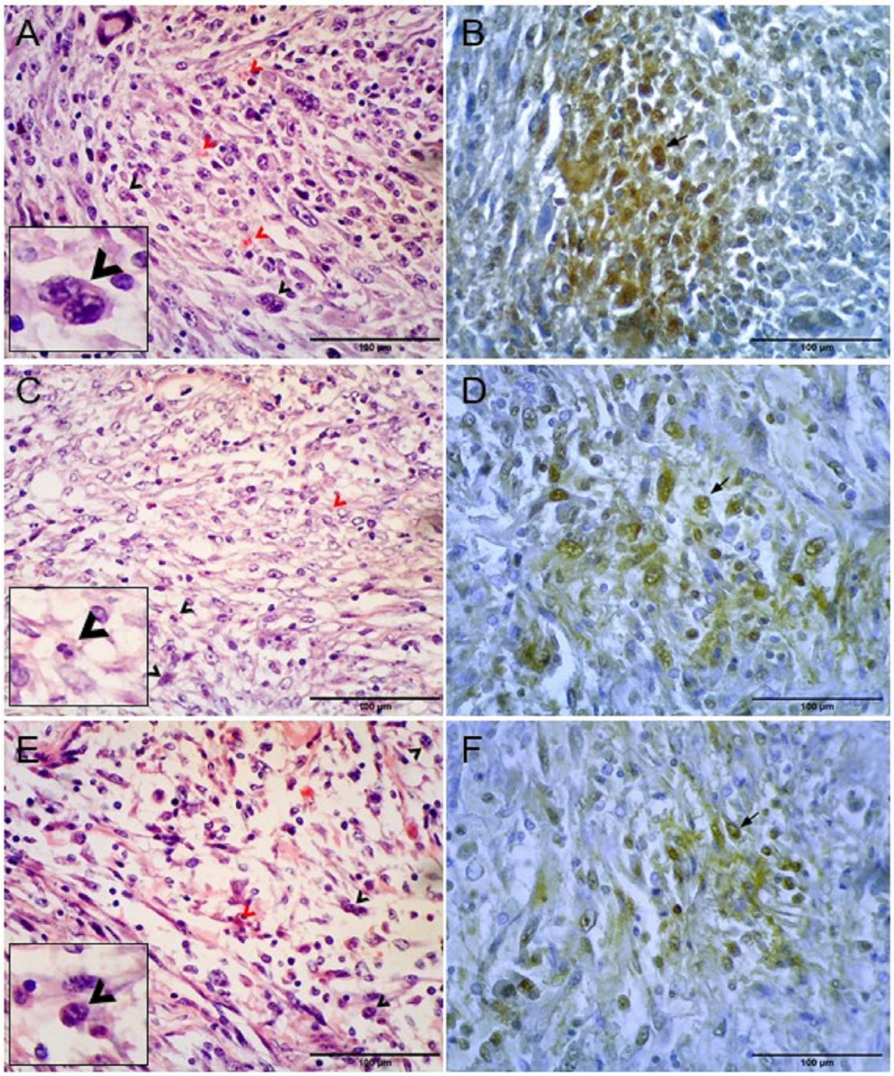

Fig. 1. Histopathological features of fibrosarcoma in P0 group (A, C, E). The neoplastic cells are not well demarcated with a proliferation of collagen fibers. A large number of mitotic are observed (black arrowhead). The neoplastic cells are prominent with a variable number of nuclei with limited cytoplasm and numerous blood vessels with red blood cells in the lumen found (red arrowhead). HE stain. x400. Representative images of immunohistochemical staining for p53 (B, D, F). x400. Brown color indicated immunopositive cell againt p53 antibody (arrow). The majority of fibrosarcoma cells are immunopositive for p53 with strong staining intensity.

al., 2018). Most of the mutations in p53 occur in the DNA-binding domain (DBD) and 73% of them are missense mutations that cause protein transcription errors (Bouaoun *et al*., 2016). In fibroblasts, exposure to benzo(a)pyrene can cause mutations in the DNA-binding domain (DBD) of p53, especially exons 5, 7, and 8 with the most common mutation occurring at codon 248 (Paget *et al*., 2012). The p53 gene with mutations in the DNA-binding domain (DBD) still has a tetramerization process because it has a functional tetramer domain (TD). Tetramerization will form a mutant p53 tetramer and allow binding to DNA in the cell nucleus, but not activate the transcription process (Gencel-Augusto and Lozano, 2020). A mutant p53 gene with a functional tetramer domain (TD) can partially inhibit normal p53 (wild-type p53) activity due to the formation of mixed tetramers (Natan *et al*., 2011).

Mutant p53 expression is associated with poor prognosis and potential for tumor recurrence (Ganci *et al*., 2013). This is because the mutant p53 gene has lost its normal function as a tumor suppressor and has acquired a new function (gain of function) that supports tumor development and progression. GOF activity can accelerate the proliferation, migration and metastasis of tumor

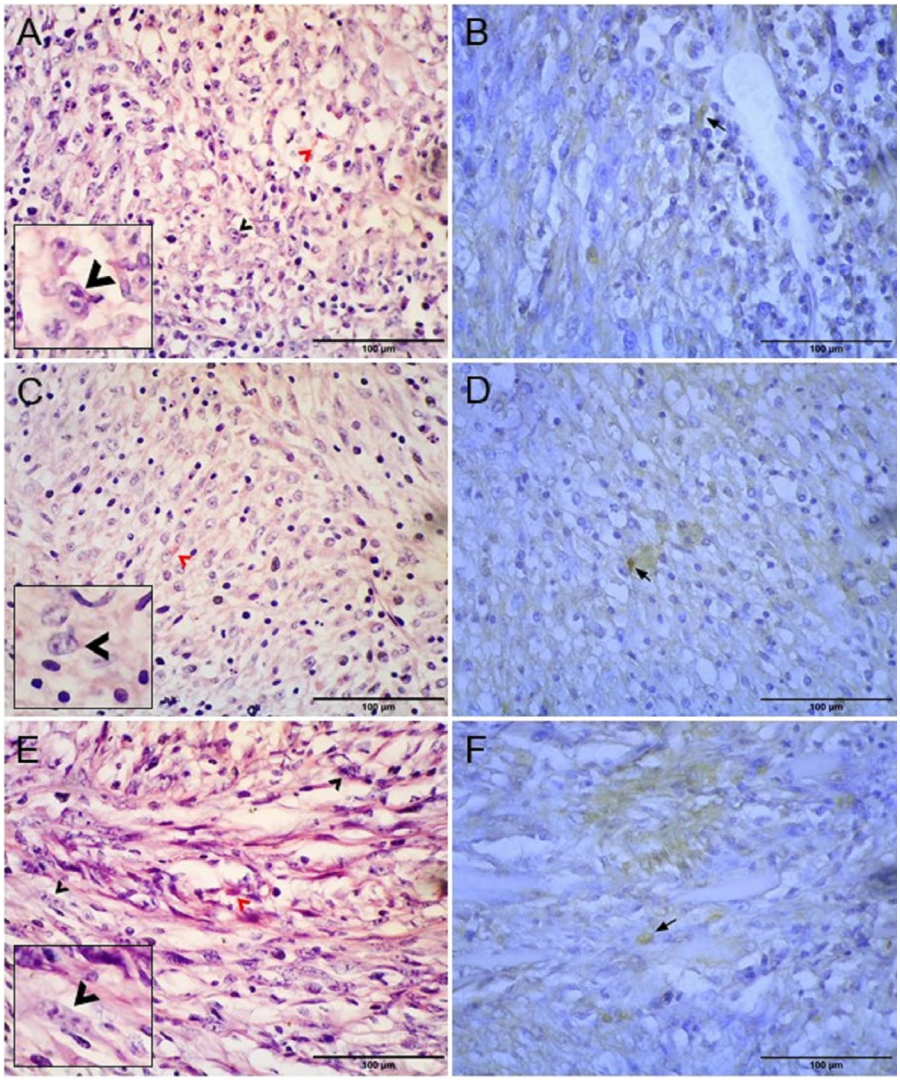

Fig. 2. Histopathological features of fibrosarcoma in P1 group (A, C, E). Less anaplastic fibroblast cells and a moderate number of mitotic are observed (black arrowhead). Only a few blood vessels with red blood cells in the lumen found (red arrowhead). HE stain. x400. Representative images of immunohistochemical staining for p53 (B, D, F). x400. Brown color indicated immunopositive cell againt p53 antibody (arrow). Very few fibrosarcoma cells are immunopositive for p53 with weak staining intensity.

cells through the endosome pathway leading to the activation of receptors and integrins. Overexpression of mutant p53 has been shown to increase the translocation of EGFR (Epidermal Growth Factor Receptor) and integrin α5β1 on the cell membrane surface. Consequently, many intracellular pathways associated with cell proliferation, including the PI3K/AKT or MAPK signaling pathways are activated by mutant p53 (Alvarado-Ortiz *et al*., 2021).

The p53 gene is one of the targets for the development of effective cancer therapies. The aim is to restore the function of wild-type p53 and degrade mutant p53 in cancer cells (Mantovani *et al*., 2019). In this study, it was shown that NDV significantly ($P<0.05$) could decrease mutant p53 expression in the rat fibrosarcoma model. Research on the oncolytic activity of NDV related to p53 expression has been reported in U251 glioma cells. NDV can induce U251 glioma cell death by triggering ferroptosis through increased expression of wild-type p53 (Kan *et al*., 2021). Another study also reported that therapy using rNDV-p53 can induce cell death by increasing the expression of wild-type p53 in HepG2 hepatoma cells and mice H22 hepatocellular carcinoma model (An *et al*., 2016). Decreased mutant p53 expression after virotherapy with NDV has not been previously reported in fibrosarcoma, so the results of this study are novel.

Decreased mutant p53 expression can cause GOF activity to be low in fibrosarcoma. Low GOF activity will reduce the proliferation, migration and metastasis of tumor cells, which will affect the malignancy of a tumor (Alvarado-Ortiz *et al*., 2021). Mutant p53 expression is often found in tumors with poor histopathological grade and high proliferative activity. In several types of tumors, mutant p53 expression correlates with tumor malignancy (Brunetti *et al*., 2021). The results of this study showed that there was a positive correlation ($P<0.05$) between mutant p53 expression and fibrosarcoma malignancy. Indirectly, virotherapy with NDV can reduce fibrosarcoma malignancy by decreasing mutant p53 expression. This shows that NDV has the potential as a virotherapy agent in fibrosarcoma.

Although NDV has been shown to decrease mutant p53 expression, but this study has not been able to explain the mechanism of NDV in targeting mutant p53. For further studies, it is necessary to study more deeply the mechanism of NDV by examining the expression of other genes or proteins. The cell mechanisms namely autophagy, is most likely the cause of the decreased expression of mutant p53. Autophagy is a cellular homeostatic process required to maintain normal physiology in cells (Cooper, 2018). Based on that mechanism, autophagy can degrade mutant p53 and restore its normal function in cancer cells (Shi *et al*., 2021). Several studies have proven that NDV can trigger autophagy activity in lung cancer, stomach adenocarcinoma and glioma (Ye *et al*., 2018; Bu *et al*., 2015; Meng *et al*., 2012). Therefore, it is possible that NDV can also induce autophagy in fibrosarcoma and degrade mutant p53.

CONCLUSION

It could be concluded that NDV has potential as a virotherapy agent targeting mutant p53 in rat fibrosarcoma models.

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CONFLICT OF INTEREST

The authors declare no conflict of interest with respect to the publication of this paper.

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