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Indonesian Newcastle Disease Virus Field Isolate Reduces c-Jun Expression in Rat Mammary Cancer Models

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Abstract

c-Jun is often found to be overexpressed in various cancers, so this gene might be a target for cancer therapy. Newcastle disease virus (NDV) is recognized for its oncolytic properties and potential as a cancer virotherapy agent, with various mechanisms reported to trigger cancer cell death. This study aimed to assess the c-Jun expression in rat mammary cancer models. Rat mammary cancer models were categorized into two treatment groups: the control group (C) and the virotherapy group (V). Group C was administered with 0.5 cc of sterile PBS, while group V received 7 log 2 HAU per 0.5 cc of the Indonesian NDV field isolate Tabanan-1/ARP/2017 intratumorally. The treatment was carried out for four days in a row. Two weeks after treatment, all rats were humanely euthanized, and mammary cancer tissues were excised for further examination. Mammary cancer tissues were examined histopathologically and analyzed using immunohistochemistry to determine intranuclear c-Jun expression, quantified by the H-Score. The results demonstrated that NDV significantly reduced c-Jun expression. It can be inferred that NDV Tabanan-1/ARP/2017 holds potential as a mammary cancer therapy agent by reducing c-Jun expression. This finding is considered novel, as there have been no previous reports of decreased c-Jun expression following virotherapy with NDV.

Keywords

c-Jun, mammary cancer, Newcastle disease virus, virotherapy

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Introduction

C-Jun, encoded by the JUN gene, is a member of the activator protein-1 (AP-1) family (Vleugel *et al*., 2006). It plays a crucial role in various biologic activities, such as apoptosis, morphogenesis, proliferation, and survival of cells and tissues (Meng and Xia, 2011). Under normal conditions, the c-Jun expression is regulated by several mechanisms, including the ubiquitin-proteasome system (UPS), which triggers c-Jun degradation (Hochstrasser *et al*., 1995), and the Mitogen-Activated Protein Kinase (MAPK) pathway, which triggers c-Jun phosphorylation (Kayahara *et al*., 2005).

A study analyzing samples from human breast cancer patients revealed that c-Jun expression was found elevated in breast cancer tissue compared to benign lesions (Vleugel *et al*., 2006; Brennan *et al*., 2020; Binato *et al*., 2021). C-Jun promotes cancer progression through several mechanisms, including angiogenesis, enhanced cell proliferation, invasion, metastasis, and drug resistance (Brennan *et al*., 2020). Given its involvement in mammary cancer progression based on several reports, c-Jun might be an attractive target for cancer therapy.

Virotherapy is a treatment that uses viruses to combat cancer. This therapy employs viruses that have oncolytic activity that specifically infect, replicate, and destroy cancer cells without affecting normal cells (Lin *et al*., 2023). Among the various viruses with potential anti-cancer properties, Newcastle Disease Virus (NDV) is one of the most promising. NDV, or Avian Orthoavulavirus-1, causes Newcastle disease in birds and has the ability to selectively infect and destroy cancer cells in mammals. This selective infection is associated with aberrant interferon responses in cancer cells. NDV not only demonstrates direct oncolytic effects but also employs various other mechanisms to induce cancer cell death and then subsequently suppress tumor growth, such as immune cell stimulation, necrosis, apoptosis, and autophagy (Song *et al*., 2019; Schirrmacher, 2022).

Several studies have reported on the oncolytic activity of NDV from Indonesia. One such strain is the Tabanan-1/ARP/2017 isolate, classified as virulent and belonging to genotype VII (Adi *et al*., 2019). Previously, its oncolytic activity has been reported in rat fibrosarcoma models (Pradnyandika *et al*., 2023; Sewoyo *et al*., 2021;) and rat mammary carcinoma models (Sewoyo *et al*., 2024). To further understand the mechanism of NDV field isolate Tabanan-1/ARP/2017 in suppressing cancer cell growth, this study aimed to determine the intranuclear c-Jun expression after virotherapy with the Indonesian NDV field isolate Tabanan-1/ARP/2017 in rat mammary cancer models.

Materials and Method

Animals

This study used 12 rats (*Rattus norvegicus*) with these criteria: Sprague Dawley strain, female, never mated, aged between 30-55 days. The rats were housed in the Laboratory Immunology and Pathology, Veterinary Medicine Faculty of Udayana University. They had *ad libitum* access to clean drinking water and were given standard feed.

Mammary Cancer Induction

The rats were acclimatized for one week prior to mammary cancer induction. The rats were induced with mammary cancer using 7,12-dimethylbenz[α]anthracene (DMBA) at 80 µg/g BW (El-Makawy *et al*., 2022), modified by dissolving in 0.75 cc of corn oil and administered twice with a one-week interval.

The DMBA solution was injected directly into the mammary fat pad of the mammary gland. After eight weeks post induction with DMBA, several rats showed tumor mass macroscopically. Rats with tumors measuring 5-10 mm in diameter were selected for treatment. Histopathological examination confirmed that the rats had *ductal carcinoma in situ*.

Experimental Group

The rats were separated into two groups, i.e., a control group (C) and a virotherapy group (V). The C group was administered 0.5 cc of sterile phosphate-buffered saline (PBS), while the V group received 7 log 2 HAU per 0.5 cc of Indonesian NDV field isolate Tabanan-1/ARP/2017 (GenBank No. MH215997.1), administered intratumorally. Therapy was given for four days in a row (Rakhmawati *et al*., 2022; Sewoyo *et al*., 2024). The viral was prepared according to Joao *et al.* (2022).

Euthanasia

After two weeks post-treatment, the rats were euthanized following the AVMA guidelines using toxic doses of ketamine and xylazine (10 times the normal dose) intraperitoneally (Leary *et al*., 2020).

Tissue Processing and Immunohistochemistry

The block paraffin from histologic processing was cut into 5 micrometers thick, and subsequently processed for immunohistochemical staining. Immunohistochemical staining was performed using the universal immuno-enzyme polymer method with c-Jun Polyclonal Antibody bs-0670R (Bioss Antibodies) diluted 1:100 with DaVinci Green Diluent (Biocare Medical) coupled with anti-rabbit-mouse horseradish peroxidase (HRP) polymer conjugate (N-Histofine®) and diaminobenzidine (DAB) chromogen (N-Histofine®). The immunohistochemistry was carried out according to manufacturer instruction. Section was counterstained with hematoxylin.

Immunohistochemistry Assessment

C-Jun protein expression levels in the tissue were performed using the H-score calculation. The H-score is determined by combining staining intensity with the proportion of cells stained at each intensity level. Staining intensity is classified as: no staining (0), weak (1), moderate (2), or strong (3). The formula used is: (0 x percentage of unstained nuclei) + (1 x percentage of weakly stained nuclei) + (2 x percentage of moderately stained nuclei) + (3 x percentage of strongly stained nuclei).The final H-score value ranges from 0 to 300 (Wen *et al*., 2024). Assessment of staining intensity was assisted by ImageJ.

Statistical Analysis

H-score results of c-Jun were analyzed using the Mann-Whitney U test using IBM SPSS version 26.

Result and Discussions

H-score Tumor tissues were processed immunohistochemically to determine the c-Jun expression. Staining using the c-Jun antibody was considered immunopositive if the nucleus was stained brown.

In this study, immunopositive cells with varying intensity, ranging from weak to strong, were found in both the control and virotherapy groups (Figure 1). The results of the H-score calculation showed that the C group had an average score of 42.14 ± 7.96, while the V group had an average score of 7.96 ± 9.52 (Table 1).

Based on the Mann-Whitney U test, there was a significant difference in the c-Jun expression between the control and virotherapy groups ($p<0.05$).

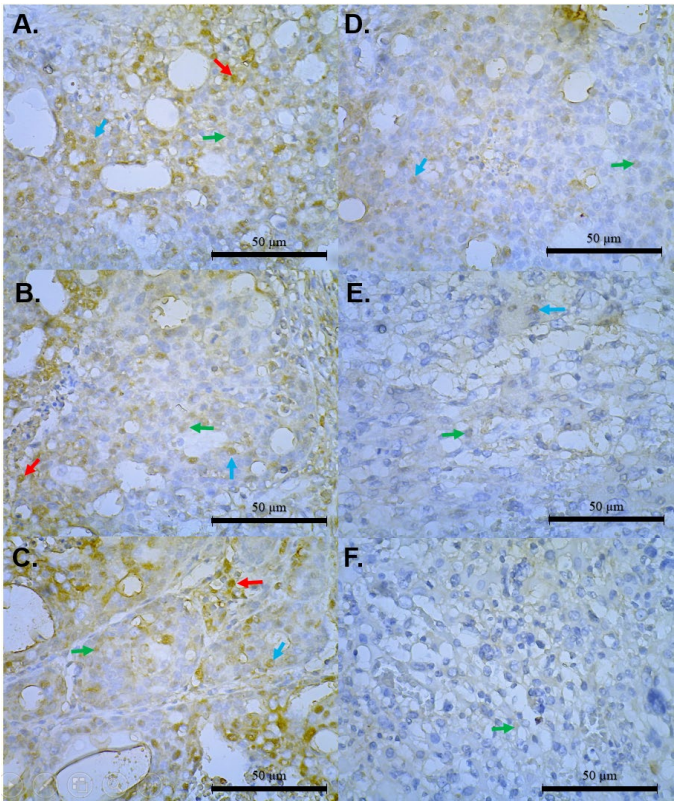

Figure 1. Immunohistochemical staining for c-Jun in control (A, B, C) and virotherapy (D, E, F) groups. The brown color indicates immunopositive cells stained with c-Jun antibodies in the cell nucleus. Description: Strong intensity (red arrow), medium intensity (blue arrow), and weak intensity (green arrow).

Table 1. c-Jun expression in each treatment

Group	Sample	H-score
C (Control)	1 (K1)	60.69
	2 (K2)	30.21
	3 K(3)	35.54
	Mean ± St. Dev.	42.14±16.27*
V (Virotherapy)	1 (T1)	18.91
	2 (T2)	1.55
	3 (T3)	3.44
	Mean ± St. Dev.	7.96±9.52*

Note: (*) $p<0,05$ was considered significant based on Mann-Whitney U test.

In this study, rat mammary cancer models were induced with DMBA. The control group showed significantly higher c-Jun expression than the virotherapy group based on the H-

Score calculation ($p<0.05$). c-Jun expression is known to be increased or overexpressed in several types of malignant tumors, such as colorectal cancer, fibrosarcoma, glioma, breast cancer, lung cancer, and urothelial carcinoma (Brennan *et al*., 2020). It also has been reported that high c-Jun expression is closely related to proliferation, angiogenesis, and lower survival rate. In MCF-7 cell lines, c-Jun overexpression can trigger invasive characteristics and contribute to bone metastasis (Han *et al*., 2023). Additionally, c-Jun overexpression has been reported to cause breast cancer resistance to tamoxifen treatment (He *et al*., 2017).

Elevated expression of c-Jun is linked with disruption of the mitogen-activated protein kinase (MAPK) signaling pathway in cancer (Brennan *et al*., 2020). This pathway becomes dysregulated due to mutations in the RAS gene, causing persistent activation that leads to unchecked cell proliferation and resistance to apoptosis (Bahar *et al*., 2023). Increased ERK/MAPK activity can elevate c-Jun transcription and protein stability, therefore contributes to c-Jun overexpression in cancer cells. The c-Jun protein is also protected from degradation when bound to basic leucine-zipper proteins (Brennan *et al*., 2020). Mutation in components such as RAF or MEK also reported contributing to the increase of ERK/MAPK activity (Guo *et al*., 2020).

Several possible mechanisms related to the decrease in c-Jun expression after virotherapy with NDV include the virus's ability to trigger cancer cell death through various mechanisms. As mentioned before, NDV has been reported to trigger direct oncolysis in cancer cells, apoptotic pathways to enhance the oncolysis effect, and cause autophagy, immunogenic death and necrosis (Song *et al*., 2019; Schirrmacher, 2022). The direct oncolysis effect can kill cancer cells, suggesting that cells containing c-Jun are destroyed by this virus. NDV can also stimulate an immune response that helps destroy cancer cells. Activation of the immune system can stimulate the release of cytokines and other immune factors that may suppress the MAPK pathway, thereby reducing c-Jun phosphorylation. Several studies have reported that NDV can induce autophagy (Bu *et al*., 2015; Ye *et al*., 2018), a defensive reaction to cellular stress against viral infection. The autophagy process is known to degrade damaged or excessive proteins, potentially reducing excessive c-Jun expression. It is also possible that this virus targets the ERK/MAPK pathway directly.

Previous studies by Pradnyandika *et al*. (2023) using the same isolate as this study demonstrated the ability of the virus to reduce the expression of mutant p53 (mutp53) in rat fibrosarcoma models. The p53 acts as a tumor suppressor. However, when a mutation occurs, this gene loses its tumor-suppressor function and gains a new function as a tumor promoter (Alvarado-Ortiz *et al*., 2020). The mutp53 protein has a synergistic effect with oncogenic RAS in regulating signaling components such as ERK/MAPK, PI3K, and NFκB (Solomon *et al*., 2011). The decrease in mutp53 is also thought to contribute to the reduction in ERK/MAPK activity, thereby reducing c-Jun phosphorylation. Further research is needed to elucidate the detailed mechanism of NDV Tabanan-1/ARP/2017 in reducing c-Jun expression.

A study by Sewoyo *et al*. (2024) reported that NDV Tabanan-1/ARP/2017 therapy can reduce angiogenesis activity in rat mammary carcinoma models. Immunohistochemical analysis revealed that the protein involved in angiogenesis, VEGF, significantly decreased,

accompanied by a lower proliferation rate as indicated by reduced Ki67 expression (Sewoyo, 2024). The decrease in c-Jun expression is thought to contribute to the reduction in angiogenesis activity. In this present study, intratumoral injection of NDV Tabanan-1/ARP/2017 can significantly reduce c-Jun expression. This is supported by a study by Vleugel *et al.* (2006), which stated that c-Jun expression is closely related to proliferation and angiogenesis in invasive breast cancer. Intranuclear expression levels of c-Jun are positively correlated with factors contributing to angiogenesis, such as VEGF in breast cancer patients (Vleugel *et al*., 2006). Shao *et al*. (2021) reported that targeting c-Jun in A549 cancer cells can enhance anti-angiogenic activity both in vitro and in vivo via the exosome/miRNA-494-3p/PTEN signaling pathway. Both studies consistently suggest that targeting c-Jun/AP-1 could offer a novel strategy for inhibiting tumor angiogenesis.

Based on several reports, decreased c-Jun expression has several benefits for breast cancer therapy. *In vitro* studies have shown that c-Jun inhibition is an effective therapy for luminal breast cancer with bone metastasis (Han *et al*., 2023). In breast cancer cells that were positive against ER, deletion of endogenous c-Jun was reported to increase sensitivity to tamoxifen therapy (He *et al*., 2017). Decreased c-Jun expression after NDV virotherapy has not been reported before, making this finding novel. Therefore, it is imperative to further elucidate the mechanism by which this virus decreases c-Jun expression.

Conclusion

The Indonesian Newcastle disease virus Tabanan-1/ARP/2017 reduces c-Jun expression and holds promise as a virotherapy agent in rat mammary cancer models.

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Author's Contribution

FGSM: Writing – Original Draft, Investigation, Methodology, Data Curation. PSS: Writing – Original Draft, Investigation, Methodology, Formal Analysis, Software, Project Administration. INM: Conceptualization, Validation, Resources, Writing – Review & Editing, Supervision. AAAAM, IBOW: Conceptualization, Validation, Writing – Review & Editing, Visualization, Supervision. IBKS, IKB: Validation, Writing – Review & Editing.

Conflict of Interest

The authors declare no conflict of interest regarding the publication of this article.

Aproval of Ethical Commission

This study obtained ethical approval from the Ethics and Animal Use Committee, Veterinary Medicine Faculty of Udayana University (Approval Number: B/UN14.2.9/PT.01.04/2024).

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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