Original Article

Dissemination of Newcastle Disease Virus (NDV-AF2240) in Liver during Intratumoral Injection of Xenotransplant Breast Cancer in BALB/c Mice

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Abstract

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Objective: Newcastle disease virus (NDV) or avian paramyxovirus type1 possesses several unique properties that make it an excellent anticancer agent. The hemagglutinin neuraminidase (HN) protein of NDV plays an important role in viral infection. The purpose of the present study is to investigate the dissemination of Newcastle disease virus (NDV) AF2240 strain in the liver during intratumoral injection in 4T1 breast cancer in female BALB/c mice.

Materials and Methods: A total of 200 female BALB/c mice were divided randomly into 10 cancerous groups consisting of 20 mice per group. The mice were initially induced with 104 4T1 cells, NDV-AF2240 and tamoxifen co-culture. Cancerous groups were divided into: cancer control (CC), cancer treated with 0.5 µg/ml tamoxifen citrate (CT), cancer treated with 8, 16, 32 and 64HA units of NDV-AF2240 (respectively named C/NDV8, C/NDV16, C/NDV32, C/NDV64), cancer treated with 8, 16, 32 and 64HA units of NDV-AF2240 and tamoxifen (respectively as CT/NDV8, CT/NDV16, CT/NDV32 and CT/NDV64 daily for four weeks). *In situ* reverse transcription polymerase chain reaction (*In situ* RT-PCR), negative staining electron microscopy (NSEM), polyclonal chicken antibody and goat anti-chicken antibody conjugated with fluorescein isothiocynate (FITC) using confocal laser scanning microscopy (CLSM) were used to detect the virus in the tumor and liver.

Results: *In situ* RT-PCR, NSEM and CLSM successfully detected NDV-AF2240 in tumor cells and liver.

Conclusion: The findings showed NDV-AF2240 disseminated into liver during intratumoral injection.

Keywords: Breast Cancer, Newcastle Disease Virus, Reverse Transcriptase Polymerase Chain Reaction, Confocal Laser Scanning Microscopy, Negative Staining

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Introduction

An estimated 182,460 new cases of invasive breast cancer are expected to occur among women in the US during 2008; about 1,990 new cases are expected in men (1). In Malaysia, out of 100 women diagnosed with cancer, 30 are breast cancer patients. A Malaysian woman has a 1 in 19 chance of getting breast cancer in her lifetime (2). The idea of using viruses in the treatment of cancer is not new (3). Observations made in the early 1920s indicated that viruses replicated in and lysed murine and other experimental tumors. Amongst the earliest reports on regression of human tumors is the case of cervical carcinoma that

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regressed after inoculation of the patient with an attenuated rabies vaccine (3). In addition, there are reports of remissions of Burkitt's and Hodgkin's lymphomas following natural infections with measles virus (4, 5). Intratumoral injection is a routine method for local viral therapy in tumor tissues and reduces systemic toxicity. Virus dissemination has been reported to reduce gene expression of the virus in the tumor and leads to the accumulation of viruses and transgene products in normal tissues (6). Newcastle disease virus (NDV), a synonym for avian paramyxovirus type 1, is a nonsegmented, single-stranded, negative-sense, enveloped RNA virus (7) composed of approximately 15, 200 nucleotides (8) which is classified as the only member of the genus *Avulavirus* belonging to the family *Paramyxoviridae* within the order *Mononegavirales* (9). NDV is considered a potential oncolytic agent in the treatment of cancer because it can selectively kill tumor cells (10). NDV isolates are categorized as velogenic (highly virulent), mesogenic (intermediate), or lentogenic (nonvirulent); depending on the severity of the disease they cause (11). The Malaysian heat stable, viscerotropic NDV (AF2240) isolated in the 1960s has antineoplastic properties and is currently being tested as an anticancer agent *in vivo* and *in vitro* in Malaysia (12). In the present study, dissemination of NDV-AF2240 during intratumoral injection in 4T1 breast cancer BALB/c mice in liver was investigated by *in situ* reverse transcription polymerase chain reaction (*In situ* RT-PCR), negative staining electron microscopy (NSEM) and confocal laser scanning microscopy (CLSM).

Materials and Methods

Cell maintenance

Mouse mammary tumor cells (4T1) were purchased from American type culture collection (ATCC) (Cat. no. CRL2539). All culture work was performed under strict aseptic conditions. The cells were cultured in 10% RPMI-1640 (R1383, Sigma, St Louis, MO, USA) cell culture media supplemented with 10% fetal calf serum (FCS) (N4637, Sigma Aldrich, St. Louis, MO, USA) and 1% penicillin/streptomycin (P0781, Sigma Aldrich, St. Louis, MO, USA) in a humidified incubator supplied with 5% CO_2 at 37°C. The cells were counted using a hemocytometer (Hawksley, England) according to Freshney (13).

Virus Propagation

The stock of NDV-AF2240 was originally obtained from the faculty of Veterinary Medicine of University Putra Malaysia (Biology laboratory), Serdang. The virus was grown in the allantoic cavity of 8 to 9 day-old chicken embryonated eggs (Linggi Poultry Farm, Negeri Sembilan, Malaysia) according to the procedures of Blaskovic and Styk (14). After 3-4 days of incubation at 37°C, the eggs were chilled overnight at 4°C. The allantoic fluids were clarified at 6000g for 10 minutes at 4°C using a refrigerated centrifuge (Beckman Coulter, Optima XL-100K). The supernatant were centrifuged at 20000 rpm (Beckman, USA, T21 rotor) for 3 hours at 4°C. The supernatant was discarded and the pellet was resuspended and dissolved in 1ml NTE buffer (0.5 M NaCl, 10 mM Tris–HCl and 5 mM EDTA, pH 8.0). Sucrose gradients at concentrations of 30%, 40%, 50% and 60% were prepared in ultra-clear tubes and kept in the refrigerator overnight. A few drops of the virus in NTE buffer were added into the sucrose gradient. The tubes were then centrifuged at 38000 rpm (Beckman, USA, SW41 Ti rotor) for 4 hours at 4°C. The band of the virus was collected and transferred into polyalomer tubes (Beckman Coulter, Inc., USA) and then centrifuged at 20000 rpm for 2 hours at 4°C. The pellet was dissolved using NTE buffer, filtered with a 0.4 mm filter (Sartorius Stedim Biotech. S.A., France) and kept at -80°C. The titer of the virus was determined by hemagglutination (HA) test (15) using a V-bottom 96-well plate (Eppendorf, Germany).

Mouse maintenance

Three to four week-old female BALB/c mice were purchased from the Institute of Medical Research (IMR) at Kuala Lumpur in Malaysia. All animal experiments were approved by the faculty of Medicine and Health Sciences of University Putra Malaysia Animal Care and Use Committee (ACUC No.UPM/FPSK/USH/00108). The mice were housed, five to a cage, fed *ad libitum*, and observed daily. The cages were kept in a climate controlled animal suite and cleaned weekly.

Drug preparation

Tamoxifen citrate [(Z)- 1- (p-dimethylaminoethoxyphenyl) -1, 2-diphenyl-1-butene] was purchased from Sigma Aldrich (St. Louis, MO, USA). The tamoxifen citrate powder was dissolved in a phosphate buffered saline (PBS) (Sigma Aldrich, St Louis, MO, USA) solution to a concentration of 1000 µg/ml as a stock solution and stored at −20°C. Each mouse was injected subcutaneously with 0.5 µg of tamoxifen citrate in 0.1ml of PBS. Stocks of purified NDV-AF2240 were quantified

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as described above. Mice in different groups received subcutaneous injections of 8, 16, 32 and 64 HA units of the virus prepared from the stocks.

Tumor development study

Tumor development in this study was carried out according to the modified method of Xanthopoulos (16). Two hundred, six week-old female BALB/c mice were divided randomly into 10 cancerous groups consisting of 20 mice per group. Cancerous groups were: cancer control (CC), cancer treated with 0.5 µg/ml tamoxifen citrate (CT), cancer treated with 8, 16, 32 and 64HA units of NDV-AF2240 respectively as C/NDV8, C/NDV16, C/NDV32, C/NDV64, and cancer treated with 8, 16, 32 and 64HA units of NDV-AF2240 and tamoxifen respectively named as CT/NDV8, CT/NDV16, CT/NDV32 and CT/NDV64 daily for four weeks. The mice were injected subcutaneously with 104 4T1 cells suspended in 0.1ml of PBS at the left breast region. The individual or combination regimens of each drug dissolved or suspended in 0.1ml PBS were injected subcutaneously inside the breast region daily. The tumor was detected by palpation around the induction area. The tumor size was measured weekly with a digital microcaliper (Mitutoyo, Japan) and the volume was calculated according to Basler and Shapiro (17) as shown below: ($\pi \times$ length $\times$ width $\times$ height) / 6. The sample collection was done weekly after the treatment started, for 4 weeks. At each sampling, 5 mice from each group were humanely sacrificed with diethyl ether and tumor volume and weight were recorded.

In situ reverse transcriptase polymerase chain reaction

Formalin-fixed paraffin-embedded (FFPE) tumor and liver tissues were used for *in situ* RT-PCR. Briefly, the tissues were washed in PBS, fixed in 10 % neutral buffered formalin for at least 24 hours and were processed in an ascending series of alcohol solutions for 22 hours in an automatic tissue processor (Leica, ASP300). The tissues were then embedded into blocks with paraffin by an embedder machine (Leica EG1160, Germany) and cooled at a low temperature. The tissues were trimmed and cut by microtome (Leica RM 2135, Germany) at 4-5 µm and pasted on poly-L-lysine-coated slides (Menzel Glaser, Germany) for *in situ* RT PCR and CLSM. A modified one step *in situ* RT-PCR approach was performed using the techniques of Nuovo (18). The slides were incubated for 2 hours at 70°C and subjected for paraffin wax removal with xylene (I, II) and decreasing concentrations of ethanol and treated with 100 µl proteinase K (9 µg/ml) (Bioron GmbH, Germany) for 10-20 minutes at 37°C. PBS and DEPC treated water were used to wash off the proteinase K and its digestion was inactivated by incubating the slide for 1 minute at 95°C on a heat block (Leica HI 1220). After that, the slides were rinsed firstly in PBS and subsequently in diethyl pyrocarbonate (DEPC) treated water and then air dried. The slides were incubated with 50 µl RNase-free DNase I (20 U) overnight at 37°C in a humid chamber. Then the slides were washed for 1 minute with DEPC water and 100% ethanol and air dried. *in situ* synthesis and amplification of cDNA were performed in one step using the QIAGEN® OneStep RT-PCR Kit (QIAGEN Hamburg GmbH, Germany) by a Mastercycler® gradient PCR machine (Eppendorf, Germany). All reagents were prepared with RNase-free water and glassware was made RNase-free by double autoclaving at 120°C for 20 minutes. A master mix containing RNAse-free water, 5X QIAGEN OneStep RT-PCR buffer, 400 µM dNTP mix, 0.6 µM forward and reverse primers, QIAGEN OneStep RT-PCR Enzyme Mix, 40 U/ml RNase inhibitor (Promega, USA), Digoxigenin-11-2´-deoxy-uridine-5´-triphosphate (Roche Diagnostics Ltd., Basel, Switzerland) and 2 % bovine serum albumin (BSA) (Sigma, USA) was prepared. The thermal cycling conditions for HN gene were: reverse transcription: 1 cycle of 50°C for 30 minutes; initial PCR activation: 1 cycle of 95°C for 15 minutes followed by 34 cycles of 45 seconds each at 94°C (DNA denaturation), 45 seconds at 58°C (primer annealing) and 1 minute at 72°C (primer extension). The HN gene of NDV-AF2240 and house keeping gene (β-actin, as internal control) were used to study protein gene expression. The gene sequences of HN and β-actin were obtained from the genomic DNA sequence database, GenBank (National Center for Biotechnology information, USA). The forward-5´GTGGGCCGCTCTAGCCACCAA3´ and reverse-5´TCTTTGATGTCACGCACGATTTC3´ primers of β-actin were obtained according to Ramos-Paya´n (19). The primers of the HN gene of NDV-AF2240 were designed by Primer Premier Software according to the sequence of EMBL/GenBank/DDBJ databases: X79092 (20). The criteria of 20 ± 2 base pairs in length with a 40-60% GC content and Tm of 55°C ± 5°C were used to select the most appropriate primers using the program. The forward-5´CCGCATCACCAATAGCA GAC3´(nucleotide position 97-115) and reverse-5´TTAGCAACGCCAACGGAGAC3´ (nucleotide position 395-414) primers of HN gene were used for the amplification of amplicons. All four sets of

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primer sequences were sent to Germany (Operon Biotechnologies GmbH) to be chemically synthesized. The thermal cycling conditions for β-actin gene were: 1 cycle of 94°C for 2 minutes followed by 34 cycles of 94°C for 45 seconds, 53°C for 40 seconds, 68°C for 1 minute followed by 1 cycle of 68°C for 4 minutes. After RT-PCR process, the *in situ* frame was gently removed and the slides were flooded with PBS for 5 minutes and then it was properly washed twice in PBS. Before the detection of PCR products, endogenous peroxidase in the tissue was blocked by incubation in 6% hydrogen peroxide in absolute methanol for 30 minutes in a dark humid chamber, followed by rinsing with PBS in Tween 20 for 2 minutes. The slides were treated with 2% BSA for 15 minutes in a humid chamber. Then, the sections were flooded with PBS in Tween 20 for 2 minutes and then washed for 10 minutes with PBS in Tween 20. The digoxigenin-labeled PCR products were detected by incubation with 60µl anti-digoxigenin-POD (150 U/ml) (Roche Diagnostics Ltd., Basel, Switzerland) diluted 1:80 in 100 mM Tris-HCl, 150 mM NaCl, pH 7.5 for 30 minutes at room temperature in a humid chamber. The slides were washed in PBS in Tween 20 and then in deionized water. The sections were developed for 10-20 minutes at room temperature with 100µl DAB chromogen (Millipore, USA). Then the slides were kept in dark and monitored at interyals until color development (brown color) in a dark humid chamber. The slides were flooded with PBS in Tween 20 and were rinsed in distilled water. The slides were counterstained with hematoxylin for 4 minutes, dehydrated in ascending concentrations of ethanol [75%, 95%, 100% (I) and 100% (II)] followed by xylene I and II with 5 minutes of each. Finally, the slides were viewed under light microscope and image analysis was carried out.

Confocal laser scanning microscopy

Polyclonal chicken antibody and goat anti-chicken antibody conjugated with fluorescein isothiocynate (FITC) (Jackson ImmunoResearch Laboratories, Inc., USA) were used to detect NDV-AF2240 by CLSM in the tumor and liver according to the modified method of Oldoni et al. (21) and Gestel et al. (22). Samples for immunohistochemistry were deparaffinized as described above and dehydrated in 100%, 95%, 85%, 80% and 75% alcohol. The slides were washed with PBS and placed at room temperature for 30 minutes. Non- specific protein binding sites were blocked by incubation with blocking buffer (0.05% goat serum; Jackson ImmunoResearch Laboratories, Inc., USA), 0.001% BSA and PBS) for 1 hour at room temperature and subsequently incubated with polyclonal chicken antibody (diluted 1:50 in PBS containing 0.5% (w/v) BSA and 0.05% (v/v) Tween 20). Antibody incubations were performed in a humidity chamber at 37°C for 1 hour. After washing three to five times with PBS containing 0.05% (v/v) Tween 20, the slides were incubated with goat anti-chicken antibody FITC conjugated diluted 1:50 to a final concentration of 1µg/ml in PBS containing 0.5% (w/v) BSA and 0.05% (v/v) Tween 20. The washing procedure was repeated, and the slides were sealed with Fluoro Guard (Bio Rad, USA). Slides were examined using an inverted spectral Confocal Laser Scanning Microscope (Leica TCS SP5, Leica GmbH, Wetzlar, Germany). The samples were illuminated with a 488 nm Ar Laser; emitted photons (530 nm).

Negative staining electron microscopy

The negative staining used in this study was according to Horne and Whittaker (23) and Laliberte et al. (24). Dissected tissues (tumor and liver) were crushed in a glass Elvehjem tissue grinder (Jencon, USA). The homogenate tissues were clarified at 6000g for 10 minutes at 4ºC. The supernatant was collected and then centrifuged at 20000 rpm using rotor T21 (Beckman) for 3 hours at 4ºC. The supernatant was discarded and the pellet was dissolved in NTE buffer at pH 8 in the tube. Sucrose gradients with 30%, 40%, 50% and 60% sucrose were prepared in an ultra clear tube (Beckman Coulter, Inc., USA) using a peristaltic pump and kept at room temperature overnight. The sucrose gradient with the virus was centrifuged at 38000 rpm using rotor SW41 for 4 hours at 4ºC. The purified virus band was marked and the rest of the sucrose was discarded. The purified virus collected in the polyalomer tube was centrifuged again at 38000 rpm using rotor SW41 for 2 hours at 4ºC. The pellet was then dissolved with 500 µl NTE buffer.

One drop was mixed with 1.5% aqueous potassium phosphotungstate previously adjusted to pH 7.4 (PTA) and put onto grids and viewed by electron microscope (Philips HMG 400).

Statistical analysis

Data was expressed as Mean ± Standard deviation. Analysis of variance (ANOVA) was used to compare the means. A level of $p<0.05$ was considered as statistically significant.

Results

in situ RT-PCR, CLSM and NSEM were carried out to detect NDV-AF2240 in the tumor and liver during intratumoral injection in 4T1 breast cancer in female BALB/c mice. Indirect single im-

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munofluorescence staining was performed. Fig 1 shows the virus particles at breast tumor and liver tissues of the CT/NDV32 and CT/NDV64 groups. Arrowhead illustrates the virus particles.

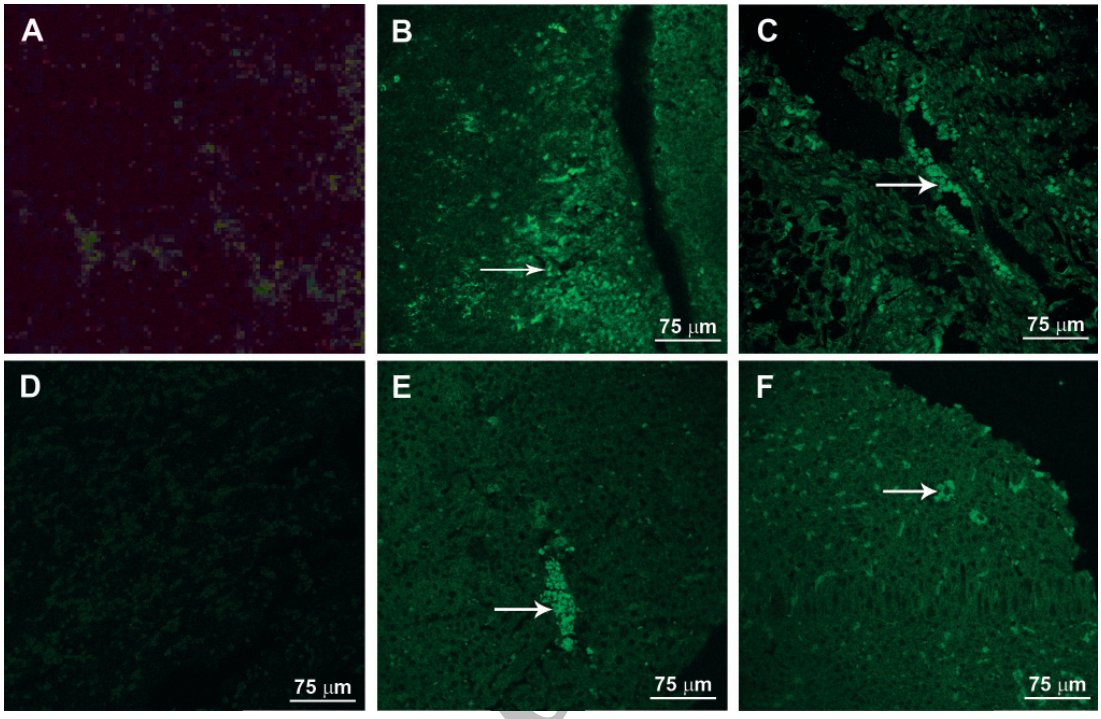

Fig 1: Confocal laser scanning micrographs of NDV-AF2240 in 5µm of FFPE of CT/NDV32 (B & E) and CT/NDV64 (C & F) in breast tumor tissue (A, B & C) and liver (D, E & F). PBS was used as secondary antibody and no signal was observed in the negative control (A & D). Magnification: x200. The results were replicated 3 times.

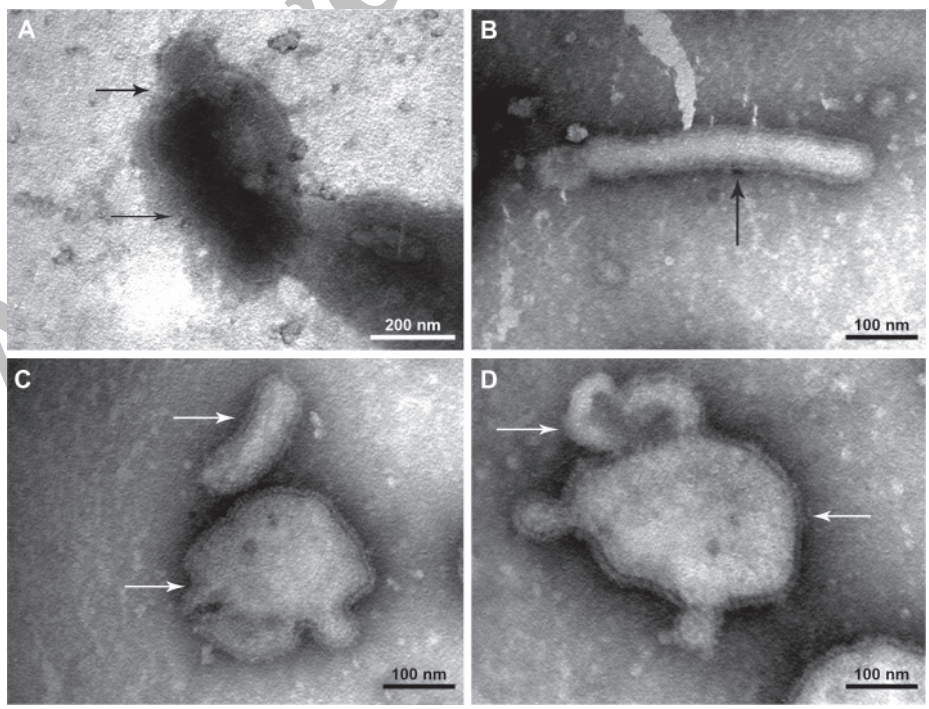

Fig 2: Negative staining electron micrograph of NDV-AF2240 in CT/NDV32 (A & C) and CT/NDV64 (B & D) in breast tumor (A & B) and liver (C & D).
A. bar = 200 nm, magnification = x100000; B. bar = 100 nm, magnification = x165000
C. bar = 100 nm, magnification = x165000; D. bar = 100 nm, magnification = x215000

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The combination of negative staining with 1.5% PTA (pH 7.4) and observation at 80-120 kV by electron microscopy was successfully used to detect the virus at the tumor and liver tissue of the CT/NDV32 and CT/NDV64 groups. Fig 2 shows NSEM of NDV-AF2240 at the mentioned tissues.

To localize HN gene expression of NDV-AFF2240 in breast tumor and liver tissues, *in situ* RT-PCR was applied on FFPE sections. Fig 3 illustrates the results of the *in situ* RT-PCR amplifications from breast tumor and liver tissues of the CT/NDV32 and CT/NDV64 groups. Experience with immunohistochemistry on tissue has shown that sections on poly-L-lysine coated microscope slides were less prone to detachment during processing (25), so these were chosen for the *in situ* RT-PCR experiments. The specificity of the signal with RT *in situ* PCR was demonstrated by its loss with the omission of the RT step (negative control). In the negative control experiments no amplification was observed. The results confirmed that the PCR product was consistent with that of the NDV-AF2240 HN gene. Fig 3 (G and H) shows that, at the liver, the virus particles were seen at the central veins. Figs 4 and 5 show the effect of 32 and 64 HAU of NDV-Af2240 and tamoxifen on tumor volumes and mass during 4 weeks. There was no significant effect of virus and tamoxifen to compare with the cancer control.

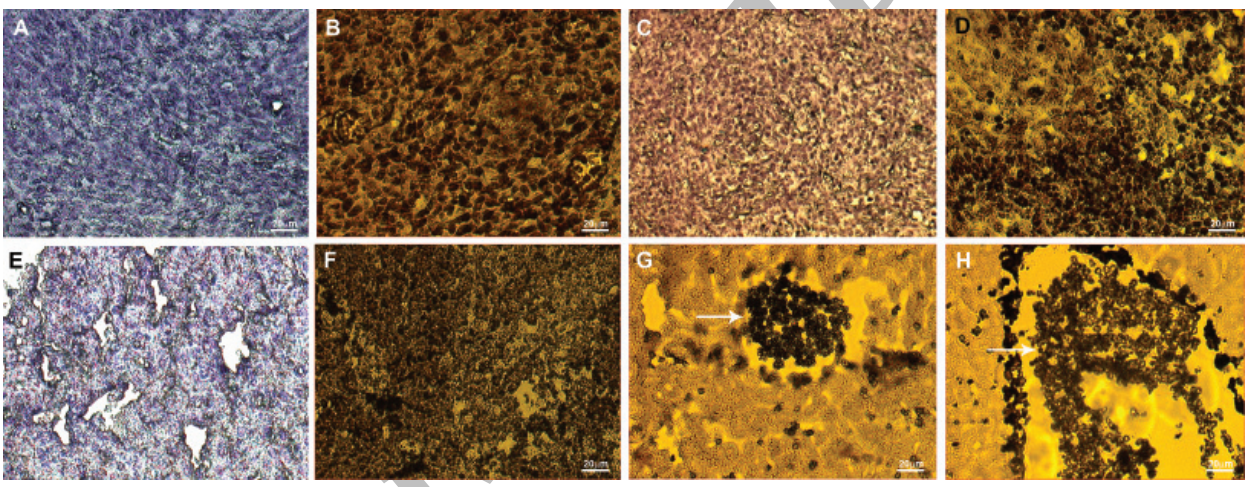

Fig 3: A. The negative control of in situ RT-PCR with omission of primer showed no positive signal at the tumor tissue (magnification x200); B. In situ RT-PCR detection of β actin mRNA in breast tumor tissue. A strong brown staining was observed in positive cells (magnification x200); C & D. In situ RT-PCR detection of HN gene expression of NDV-AF2240 in breast tumor tissue of CT/NDV32 and CT/NDV64 groups. A strong brown staining was observed in positive cells (magnification x200). E. The negative control of in situ RT-PCR with omission of primer showed no positive signal at the liver tissue (magnification x200). F. In situ RT-PCR detection of β actin mRNA in liver tissue. A strong brown staining was observed in positive cells (magnification x200); G & H. In situ RT-PCR detection of HN gene expression of NDV-AF2240 in central vein of liver tissue of CT/NDV32 and CT/NDV64 groups. A strong brown staining was observed in positive cells (magnification x200).

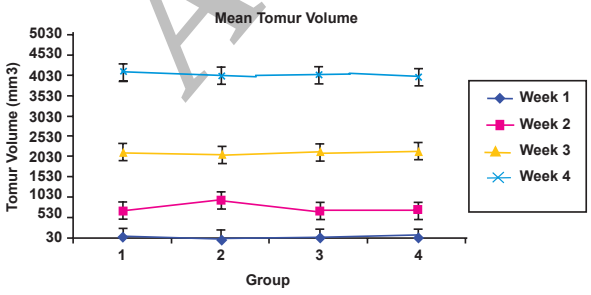

Fig 4: The effect of NDV-AF2240 on mean tumor volume of 4T1 breast cancer induced in female BALB/c mice. 1) Cancer control; 2) Cancer treated with 64 HAU of NDV-AF2240 and tamoxifen; 3) Cancer treated with 32 HAU of NDV-AF2240 and tamoxifen; 4) Cancer treated with tamoxifen.

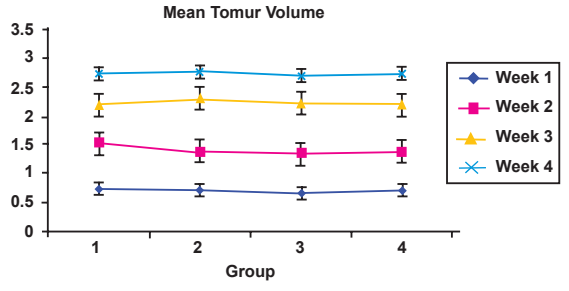

Fig 5: The effect of NDV-AF2240 on mean tumor mass of 4T1 breast cancer induced in female BALB/c mice. 1) Cancer control; 2) Cancer treated with 64 HAU of NDV-AF2240 and tamoxifen; 3) Cancer treated with 32 HAU of NDV-AF2240 and tamoxifen; 4) Cancer treated with tamoxifen.

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The mean tumor volume and tumor mass of 4T1 breast cancer induced in female BALB/c mice treated with 32 and 64 HAU NDV-AF2240 and tamoxifen co-cultured daily during 4-weeks is shown in Figs 4 & 5. The mean tumor volume and tumor mass were not significantly different ($p > 0.05$) to compare with the cancer control (CC). However, among these four groups (CC, CT/NDV32, CT/NDV64 and CT) there was a significant difference ($p<0.05$) in the changes of tumor volume and mass over time.

Discussion

Intratumoral infusion is currently the most common method for viral gene delivery in cancer treatment because it can circumvent transvascular barriers and enhance interstitial transport in tumors. In this study, dissemination of NDV-AF2240 in the liver during intratumoral injection of breast cancer was investigated using 3 molecular techniques: *in situ* RT-PCR, CLSM and NSEM. Fig 6 shows a diagram of viral dissemination in a tumor mass. The results of our study indicate that NDV-AF2240 disseminated into the liver during intratumoral injection in BALB/c mice. Our results are similar to Wang et al. (26). They found up to 90% of adenoviral vectors in the liver 10 minutes after intratumoral infusion in a mouse study. Most viruses spread from an initial focus of infection to a target tissue via the blood stream (27). CLSM and *in situ* RT-PCR showed the virus particles in the central vein of the liver during intratumoral injection (Fig 1E & F, Fig 3G & H). The virus was isolated from the tumor and liver after intratumoral injection to confirm our results by NSEM as a gold method to distinguish the virus. Fig 2 shows ultra structure of NDV-AF2240 virions in tumor and liver by negative staining. Examination revealed the intact virion with fine surface projections (arrow) representing viral glycoprotein spikes. The surfaces of NDV particles contain two important functional glycoproteins: fusion (F) and HN proteins (28). The HN protein of NDV is a multifunctional protein. It possesses both receptor recognition and neuraminidase (NA) activities associated with the virus. It recognizes sialic acid-containing receptors on cell surfaces; it promotes the fusion activity of the F protein, thereby allowing the virus to penetrate the cell surface; and it acts as an NA by removing the sialic acid from progeny virus particles to prevent self-agglutination of the progeny virus (29). Thus, the HN protein plays an important role in viral infection. *in situ* RT-PCR to FFPE sections are successfully applied in order to detect HN gene expression in the tumor and liver during intratumoral injection. The use of *in situ* RT-PCR to examine gene expression in disease tissues has certain advantages over more established hybridization, PCR amplification or antibody based techniques (30). As with immunohistochemistry, detection of gene expression is at the level of individual cells, whereas polyclonal antibody production by immunization may take a long time (4 months or longer) and require extensive optimization, it is relatively easy to characterize and optimize oligonucleotide primers which have considerably less chemical complexity and, therefore, inherently more predictable properties. Moreover, while cross-reactivity is a frequent problem when selecting antibodies for protein detection, it is a simple matter to select PCR primers that are specific to a single member of a gene family, or even a particular splice variant of that gene (30). This present finding is the first report in Malaysia as a preclinical study of dissemination of NDV-AF2240 during intratumoral injection in the liver before starting the clinical trial phase on humans using NDV as a vaccine.

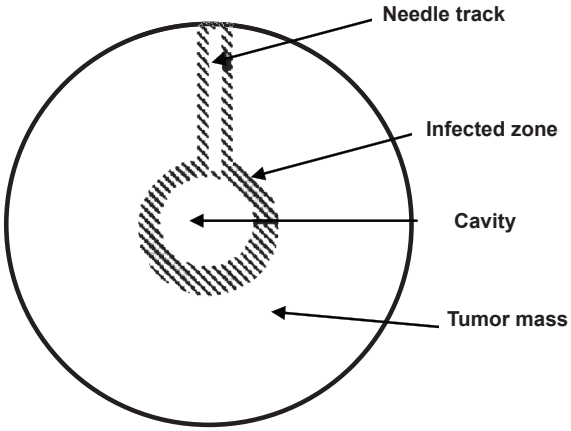

Fig 6: Diagram of viral dissemination in a tumor mass. The wall of a needle track and the cavity are formed by a few layers of infected tumor cells (infection zone) (31).

Conclusion

The findings of this study showed NDV-AF2240 disseminated in the liver during intratumoral injection in 4T1 breast cancer in BALB/c mice. The virus particles were mostly detected in the central vein of the liver. *in situ* RT-PCR, NSEM and CLSM were successful in detecting the virus.

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