



UNIVERSITI PUTRA MALAYSIA

**LOCALIZATION OF NEWCASTLE DISEASE VIRUS (NDV-AF2240) IN
4T1 XENOTRANSPLANT BREAST CANCER BALB/c MICE**

GHOLAMREZA MOTALLEB

FPSK(P) 2009 2



**LOCALIZATION OF NEWCASTLE DISEASE VIRUS (NDV-AF2240) IN 4T1
XENOTRANSPLANT BREAST CANCER BALB/c MICE**

By

GHOLAMREZA MOTALLEB

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfilment of Requirements for the Degree of Doctor of Philosophy**

September 2009



DEDICATION

With love and appreciation to:

*My mother (Kobra Norouzi Ghotb abadi), Father (Nematollah Motalleb), My wife
(Niloufar Nabi), My daughter (Mehrafarin), My son (Arian), My brother
(Mohamadreza) and my sisters*

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

LOCALIZATION OF NEWCASTLE DISEASE VIRUS (NDV-AF2240) IN 4T1 XENOTRANSPLANT BREAST CANCER BALB/c MICE

By

GHOLAMREZA MOTALLEB

September 2009

Chairman : Professor Dr. Fauziah bt. Othman, PhD

Faculty : Medicine and Health Sciences

In situ reverse transcriptase polymerase chain reaction (*in situ* RT-PCR), polyclonal chicken antibody and goat anti-chicken antibody conjugated with fluorescein isothiocyanate (FITC) using confocal laser scanning microscopy (CLSM) and negative staining transmission electron microscopy (NSTEM) were carried out to detect the NDV-AF2240 in tumor, liver, brain and lung during intratumoral injection in 4T1 xenotransplant breast tumor in female BALB/c mice. A total of 300 female BALB/c mice were divided randomly into 15 groups (5 non cancerous groups, 10 cancerous groups) consisting 20 mice per group. The normal control (NC), normal treated with 8, 16, 32 and 64HA units of NDV-AF2240 respectively named as N/NDV8, N/NDV16, N/NDV32 and N/NDV64. The mice in cancerous groups were initially inoculated sub-cutaneously with 4T1 cells; co-culture either with NDV-



AF2240 or/and tamoxifen. Cancerous groups were divided into cancer control (CC), cancer treated with only 5 µg/ml tamoxifen citrate (CT), cancer treated with 8, 16, 32 and 64HA units of NDV-AF2240 without tamoxifen respectively named C/NDV8, C/NDV16, C/NDV32, C/NDV64, cancer treated with 8, 16, 32 and 64HA units of NDV-AF2240 with tamoxifen respectively named as CT/NDV8, CT/NDV16, CT/NDV32 and CT/NDV64 daily for four weeks. The normal mice treated with 8, 16, 32 and 64 HA unit of NDV-AF2240 did not affect its lifespan. All of the cancerous and non cancerous mice survived well and completed the 4-weeks treatment. Only 4 groups of mice developed tumor that was CC, CT, CT/CNDV32 and CT/NDV64, however these groups survived until end of the 4 weeks of treatment. Significant difference ($p < 0.05$) in mean body weight was found between N/NDV16, N/NDV64 and NC. Whereas, for the cancerous groups, mean body weight of the mice in CC group were significantly different ($p < 0.05$) to compare with C/NDV8, C/NDV32, CT/NDV16, CT/NDV32 and CT/NDV64 groups. The mean tumour volume and mass of CT/NDV32 and CT/NDV64 were not significantly different ($p > 0.05$) to compare with each other and cancer control (CC), however, there was significant difference ($p < .05$) in the changes of tumour volume and mass over time. The CC and CT groups had a significantly ($p < 0.05$) higher lung weight compared with the other groups. The CC group had a significantly ($p < 0.05$) higher of liver weight compared with all groups. There was no significant ($p > 0.05$) different in the brain weight between CC and all cancerous groups. To localize HN gene expression of NDV-AFF2240 in tissues, *in situ* RT-PCR was applied on formalin fixed paraffin-embedded (FFPE) sections that were positive by negative staining

transmission electron microscopy. The HN gene expression was detected in all the breast tumor cells. However, it was found mainly in the blood vessels of the brain, liver and lung. The intensity of the HN gene expression in all the organs within the same group is significantly similar except the breast tumor tissue. There was no significant difference ($p>0.05$) in HN gene intensity between CT/NDV8 and CT/NDV16 groups, however, it was significantly different ($p<0.05$) compared to CT/NDV32 and CT/NDV64 groups. Virus dissemination seems to be determined by the infusion dose during intratumoral injection. β actin as internal control was expressed in breast cancer tissue, brain, lung and liver. *In situ* RT-PCR showed similar constant strong intensity of β actin gene expression in all mentioned tissues. Immunofluorescence and CLSM successfully detected the virus particles in tumor and all the organs of the cancerous groups during intratumoral injection. In tumor tissue the virus are found in the cells, whereas, in the lung, brain and liver are found mainly in the blood vessels. They are mainly found at the central vein (C.V.) and sinusoidal capillaries of the liver. This phenomenon was similar to results of *in situ* RT-PCR. Negative staining with transmission electron microscopy as a gold standard method was successfully used to detect the NDV-AF2240 at breast tumor, lung, liver and brain tissues during intratumoral injection in 4T1 xenotransplant breast cancer induced in mice. The results illustrated the presence of NDV-AF2240 in all organs of cancerous groups, but not in the normal groups treated with virus. The morphology of Newcastle disease virus was seen pleomorphic, spherical and ranging from 60-320 nm. The virion has an envelope and prominent surface projections. Occasionally, virions were seen to be rod in

shape. Besides observing the whole virus, nucleocapsids which is confined in the virion was frequently detected outside the virion and are also seen filamentous. The findings of this study showed that NDV-AF2240 suppressed the growth of breast cancer and it is disseminated in blood vessels of the brain, lung and liver, however, found in the cells of the breast cancer.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**PENGESANAN NEWCASTLE DISEASE VIRUS (NDV-AF2240) DI DALAM
PAYUDARA BARAH 4T1 XENOTRANSPLANT MENCIT BALB/c MICE**

Oleh

GHOLAMREZA MOTALLEB

September 2009

Pengerusi : Profesor Dr. Fauziah bt Othman, PhD

Fakulti : Medicine and Health Sciences

Kaedah *in situ* reverse transcriptase polymerase chain reaction (*in situ* RT-PCR), antibodi poliklonal ayam dan antibodi kambing anti-ayam FITC menggunakan mikroskop pengimbasan laser konfokal dan pewarnaan negatif menggunakan mikroskop elektron pancaran telah dijalankan untuk mengesan virus NDV-AF2240 di dalam barah payudara, hati, otak dan paru-paru semasa suntikan intratumoral dalam xenotransplant ketumbuhan payudara dalam mencit betina BALB/c. Sejumlah 300 mencit betina BALB/c dibahagi secara rambang ke dalam 15 kumpulan (5 kumpulan tiada barah, 10 kumpulan berbarah) yang mempunyai 20 mencit sekumpulan. Kumpulan mencit normal (NC), normal dirawat dengan titer virus 8, 16, 32 dan 64 HA unit NDV-AF2240 masing-masing dinamakan N/NDV 8, N/NDV 16, N/NDV 32 dan N/NDV 64. Mencit dalam kumpulan berbarah disuntik subkutaneus

dengan sel kanser payudara mencit 4T1;ko-kultur dengan NDV-AF2240 atau bersama tamoxifen. Kumpulan diaruh barah dibahagi kepada barah kawalan (CC), barah dirawat dengan 5µg/ml tamoxifen citrate (CT), barah dirawat dengan 8, 16, 32, 64 dan 64 HA unit NDV-AF2240 masing-masing ditambah dengan tamoxifen; CT/NDV8, CT/NDV16, CT/NDV32 dan CT/NDV64 setiap hari selama 4 minggu. Mencit normal yang dirawat dengan 8, 16, 32, 64 HA unit NDV-AF2240 tidak menjejaskan jangka hayat. Semua mencit dalam kumpulan diaruh barah dan tidak diaruh barah hidup dan menghabiskan rawatan 4 minggu tersebut. Hanya 4 kumpulan mencit tersebut ada ketumbuhan barah payudara iaitu CC, CT, CT/NDV32 dan CT/NDV64, walaubagaimanapun mencit dalam kumpulan ini hidup hingga ke hujung 4 minggu rawatan. Terdapat perbezaan signifikan ($p < 0.05$) dalam min berat badan antara N/NDV16, N/NDV64 dan NC. Antara kumpulan diaruh barah, min berat badan mencit kumpulan CC berbeza secara signifikan ($p < 0.05$) dibandingkan kepada kumpulan C/NDV8, C/NDV32, CT/NDV 16, CT/NDV 32, dan CT/NDV64. Min isipadu dan berat barah CT/NDV32 dan CT/NDV64 tiada perbezaan signifikan ($p > 0.05$) apabila dibandingkan sesama kumpulan ini dan juga kumpulan barah kawalan (CC), walaubagaimanapun, terdapat perbezaan signifikan ($p < 0.05$) dalam perubahan isipadu dan berat barah dari awal hingga hujung eksperimen. Kumpulan CC dan CT mempunyai berat paru-paru yang lebih tinggi secara signifikan ($p < 0.05$) dibandingkan kepada kumpulan lain. Kumpulan CC mempunyai berat hati yang tinggi secara signifikan ($p < 0.05$) dibandingkan kepada kumpulan lain. Tiada perubahan signifikan berat otak ($p > 0.05$) antara kumpulan CC dan kumpulan diaruh barah yang lain. *In situ* RT-PCR dijalankan untuk menentukan

pengekspresan gen HN NDV-AF2240 dalam seksyen tisu yang diawet formalin dan dibekukan dalam lilin. Gen HN diekspreskan dan ditemui dalam semua sel-sel barah payudara, walaubagaimanapun, ia ditemui khususnya dalam saluran darah otak, hati dan paru-paru. Keamatan pengekspresan gen HN dalam semua organ dalam kumpulan yang sama adalah serupa secara signifikan kecuali dalam tisu barah payudara. Tiada perbezaan signifikan ($p > 0.05$) dalam keamatan gen HN antara CT/NDV 8 dan CT/NDV 16, walaubagaimanapun, terdapat perbezaan signifikan ($p < 0.05$) apabila dibandingkan kepada CT/NDV 32 dan CT/NDV 64. Nampaknya penyebaran virus ditentukan oleh dos yang diberi semasa suntikan intratumoral. β actin sebagai kawalan dalam, diekspres dalam tisu barah payudara, otak, hati dan paru-paru. *In situ* RT-PCR menunjukkan keamatan tinggi ekspresi gen β actin yang serupa dalam semua tisu yang disebut sebelum ini. Immunofluoresence dan mikroskop pengimbasan laser konfokal telah berjaya mengesan partikel-partikel virus di dalam barah dan kesemua organ kumpulan diaruh barah semasa suntikan intratumoral. Dalam tisu barah, virus ditemui dalam sel-sel, tetapi dalam organ paru-paru, otak dan hati, virus banyak ditemui dalam saluran darah. Virus ini khususnya ditemui dalam vena sentral dan kapilari sinusoidal hati. Fenomena ini adalah sama dengan keputusan *in situ* RT-PCR. Perwarnaan negatif menggunakan mikroskop elektron pancaran sebagai kaedah standard emas berjaya digunakan untuk mengesan NDV-AF2240 dalam sampel tisu barah, paru-paru, hati dan otak semasa suntikan intratumoral. Keputusan menunjukkan kehadiran NDV-AF2240 di dalam semua organ dalam kumpulan diaruh barah, tetapi bukan dalam kumpulan normal dirawat dengan virus. Morfologi virus newcastle

disease ditemui dalam bentuk pleomorf, sfera dan berukuran dari 60-320 nm. Virionnya ada sampul dan permukaan unjuran yang ketara. Adakalanya, virion nampak dalam bentuk rod. Selain daripada memerhatikan virus secara keseluruhan, nukleokapsid yang biasanya di dalam virion, kerap ditemui di luar virion dan ditemui berfilamentos. Keputusan projek ini menunjukkan NDV-AF2240 disebarkan ke otak, paru-paru dan hati semasa suntikan intratumoral barah payudara 4T1 dalam mencit betina BALB/c.

ACKNOWLEDGEMENTS

Glory and praise to the Almighty Allah, the Omnipotent, Lord of all creation, Omnipresent, for his heavenly, luxurious blessings me and opening the windows of opportunity throughout my life, giving me the strength and health to achieve what I have done so far.

This research project program couldn't be carried out without the help and cooperation of my family who assist me during my PhD thesis. I would like to express my heartfelt gratitude and appreciation my supervisor, Professor Dr. Fauziah Bt. Othman for her ideas, kindly and valuable guidance and assistance throughout the period of my project. I appreciate the innumerable seconds she spent to show me the correct way to live and also doing my research.

I would like to take this opportunity to especially thank Professor Datin Paduka Dr. Aini Ideris, Deputy Vice Chancellor for Academic and International Affairs Division, UPM, for her advice, support, and comments. I wish to express my deepest thanks to my co supervisor Professor Dr. Asmah Rahmat for her kind assistance and guidance. Also very special acknowledgement is given to Associate Professor Dr. Rozita Rosli, Deputy Dean of Postgraduate and Research, Faculty of Medicine and Health Sciences of UPM. I wish to extend my thanks goes to President of Majlis Kanser Malaysia, Y. Bhg Dato' Mohd Farid Ariffin who opened new windows for cancer patients to know they are not alone in Malaysia. I also would like



to express my best wishes and regards to Dr. Cheah Yoke Kqueen for his pure helping throughout my project.

Ministry of Science, Research and Technology (MSRT) of Islamic Republic of Iran is greatly acknowledged.

Special thanks go to my friends and colleagues in the Microscopy Imaging and Nanoscience Unit, Institute of Biosciences in UPM (Mr. Ho, Mrs. Aini, Mr. Rafi, and Mrs. Ida), Laboratory of Biology of Faculty of Veterinary Medicine of UPM, and Institute of Medical Molecular Biotechnology (IMMB) of UiTM, Electron Microscopy unit of IBS in UPM, Laboratory of Immunotherapeutic and Vaccines (LIVES) of UPM.

Last but not least , I would like to express my warmest and deepest gratitude to my father (Nematollah Motalleb), my mother (Kobra Norouzi Ghotb Abadi), my dear wife (Niloufar Nabi) and beloved children (Mehrafarin and Arian) my brother (Mohamadreza), my sisters (Elham and Mojgan) for being patience, understanding, support and their belief in me during the course of this research. I love you all.



I certify that an Examination Committee has met on 10 September 2009 to conduct the final examination of Gholamreza Motalleb on his Doctor of Philosophy thesis entitled “Localization of Newcastle Disease Virus (NDV-AF2240) in 4T1 Xenotransplant Breast Cancer BALB/c Mice” in accordance with Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U. (A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Examination Committee were as follows:

SAIDI MOIN, PhD

Associate Professor,
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

PATIMAH ISMAIL, PhD

Professor,
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

MOHD HAIR BEJO, PhD

Professor,
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

MARY NG MAH LEE, Ph.D

Professor,
Faculty of Science
National University of Singapore
(External Examiner)

BUJANG BIN KIM HUAT, PhD

Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 15 October 2009



This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirements for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Fauziah Othman, PhD

Professor,
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Aini Ideris, PhD

Professor,
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Asmah Rahmat, PhD

Professor,
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

HASANAH MOHD. GHAZALI, PhD

Professor and Dean,
School of Graduate Studies
Universiti Putra Malaysia

Date : 16 November 2009



DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

GHOLAMREZA MOTALLEB

Date : 7 September 2009

TABLE OF CONTENTS

	Page
DEDICATION	ii
ABSTRACT	iii
ABSTRAK	vii
ACKNOWLEDGEMENTS	xi
APPROVAL	xiii
DECLARATION	xv
LIST OF TABLES	xix
LIST OF FIGURES	xxi
LIST OF ABBREVIATIONS	xxvi
CHAPTER	
1	1
INTRODUCTION	
1.1 Background of study	1
1.2 Significant of the study	2
1.3 Statement of the problems	3
1.4 Objectives of the Study	3
2	5
LITERATURE REVIEW	
2.1 Newcastle disease virus	5
2.1.1 HN gene of Newcastle disease virus	8
2.1.2 NDV- AF2240	11
2.2 Breast cancer	11
2.2.1 Breast cancer cell line	12
2.2.2 Animal model for breast cancer	13
2.2.3 Breast cancer and risk factors	16
2.2.3.1 Age	16
2.2.3.2 Family history	16
2.2.3.3 Previous benign breast disease	18
2.2.3.4 Radiation	18
2.2.3.5 Oral contraceptive	18
2.2.3.6 Hormone replacement therapy	18
2.2.3.7 Alcohol	19
2.2.3.8 Obesity	19
2.2.3.9 Induced abortion	20
2.2.3.10 Breast implants	20
2.2.3.11 Tobacco smoke	20

2.3	Breast cancer therapy	20
2.3.1	Locoregional treatment	20
2.3.2	Systemic treatment	21
2.3.2.1	Treatment options for minimal to average risk patients	22
2.3.2.2	Treatment options for high risk patients	23
2.3.2.3	Endocrine therapy	23
2.3.3	Chemotherapy: current standard	24
2.3.4	Metastatic disease	25
2.3.5	Hormone sensitive metastatic breast cancer	26
2.3.6	Her2 positive metastatic breast cancer	27
2.3.7	Treatment of skeletal metastases	28
2.3.8	Virotherapy	28
2.3.8.1	Ideal replication selective oncolytic virus attributes	33
2.3.8.2	Mechanisms of tumor selectivity	33
2.3.8.3	Use of inherently selective viruses	37
2.3.8.4	RNA viruses as virotherapy agents	38
2.3.8.4.1	Newcastle disease virus	39
2.3.9	Gene therapy	42
2.3.10	Viral dissemination during intra tumoral injection	44
2.4	Tamoxifen	47
2.5	Reverse transcription <i>in situ</i> polymerase chain reaction (RT <i>in situ</i> PCR)	49
2.6	Housekeeping gene	51
2.7	Immunohistochemistry	54
2.8	Negative staining and transmission electron microscopy	59
2.9	Confocal laser scanning microscopy	60
3	METHODOLOGY	62
3.1	Experimental design	62
3.2	Propagation of the virus	65
3.2.1	Collection of chicken eggs	65
3.2.2	Preparation of seed virus dilution	65
3.2.3	Inoculation the virus	66
3.2.4	Harvesting	66
3.2.5	Storing of allantoic fluid	66
3.2.6	Purification of the virus	67
3.2.7	Virus titration	67
3.2.7.1	Preparation of chicken RBC	67
3.2.7.2	Heamagglutination test	68
3.3	Cell culture	68
3.4	Breast cancer induction	69
3.5	Drug preparation	70
3.6	Sample Collection	70



3.7	Mean Survival Time (MST)	70
3.8	Reverse transcription <i>in situ</i> polymerase chain reaction	71
3.8.1	Tissue section preparation	71
3.8.2	Primer designing	71
3.8.3	Tissue processing	72
3.8.4	Proteolytic digestion and DNase treatment	73
3.8.5	One step RT <i>in situ</i> PCR assay	73
3.8.5.1	Controls of <i>in situ</i> RT-PCR	77
3.8.5.2	Scoring system for <i>in situ</i> RT-PCR	77
3.9	Immunofluorescence	78
3.10	Negative staining and transmission electron Microscopy	79
4	RESULTS	78
4.1	Profile of Experimental animals	80
4.1.1	Mean Survival Time (MST)	80
4.1.2	Body weight profile	81
4.1.3	Tumor volume profile	83
4.1.4	Tumor mass volume	87
4.1.5	Gross weight of lung, liver and brain of mice	88
4.2	<i>In situ</i> RT-PCR	92
4.3	Confocal laser scanning microscopy	111
4.4	Negative staining transmission electron Microscopy	125
5	GENERAL DISCUSSION	131
5.1	Body weight, gross and tumor weight profile of mice	132
5.2	<i>In situ</i> reverse transcription polymerase chain Reaction	138
5.3	Confocal laser scanning microscopy	142
5.4	Negative staining and transmission electron Microscopy	145
6	CONCLUSIONS AND RECOMMENDATIONS	149
	REFERENCES	153
	APPENDICES	173
	BIODATA OF STUDENT	185



LIST OF TABLES

1	Origins of a number of commonly used breast cancer cell lines	13
2	Example of replication selective viruses in clinical trials for cancer	32
3	Mechanisms of tumor specific viral replication	35
4	Pathotype and pathogenicity of NDV in chickens	40
5	House keeping genes and cellular function	54
6	Oligonucleotide primer sequences of HN and β -actin gene	72
7	Volume and concentration of reagents used in the final RT-PCR reaction	74
8	Thermal cycler condition for HN gene primers in gradient PCR	75
9	Thermal cycler condition for β -actin gene primers in gradient PCR	76
10	Intensity of <i>in situ</i> RT-PCR of HN and β actin gene expression in female BALB/c mice	108
11	<i>In situ</i> RT-PCR detection of HN gene expression of NDV-AF2240	109
12	Detection of virus particles using CLSM technique	112
13	Results of NSTEM in all groups of mice	126
14	Mean body weight of 4T1 breast cancer model of mice treated with NDV-AF2240	180



15	Mean tumor volume of 4T1 breast cancer model treated with NDV-AF2240	181
16	Gross weight profile of 4T1 breast cancer model treated with NDV-AF2240 and tamoxifen	182
17	The reagent involved and the tissue processing time used for the tissue processing by an automated tissue processor	183
18	MST and percentage of increase in lifespan in experimental groups treated with NDV-AF2240 strain	184



LIST OF FIGURES

1	Schematic representation of the virion structure of NDV	10
2	NDV genome organization and the viral transcripts	11
3	Schematic representation of tumor-selective viral replication and oncolysis	30
4	Schematic representation of mechanisms of tumor destruction with viral agents	36
5	Diagram of viral dissemination in a tumor mass	46
6	Strategies of oncolytic viruses for tumor specificity	47
7	Experimental design	62
8	The effect of NDV-AF2240 on body weight changes in 4T1 breast cancer model in BALB/c mice	82
9	The effect of NDV-AF2240 on mean tumour volume of 4T1 breast cancer induced in female BALB/c mice	83
10	4T1 cancer cell line	84
11	Female BALB/c mice with the tumor before sacrificing	85
12	The mice with tumor before and after sacrificing	86
13	The effect of NDV-AF2240 on mean tumour mass of 4T1 breast cancer induced in female BALB/c mice	87
14	The effect of NDV-AF2240 on mean lung weight of 4T1 breast cancer induced in female BALB/c mice	89

15	The effect of NDV-AF2240 on mean liver weight of 4T1 breast cancer induced in female BALB/c mice	90
16	The effect of NDV-AF2240 on mean brain weight of 4T1 breast cancer induced in female BALB/c mice	91
17	Representative light micrograph of <i>in situ</i> RT-PCR of β actin mRNA in breast tumour tissue of CT/NDV32 and CT/NDV64 groups	94
18	Representative light micrograph of <i>in situ</i> RT-PCR of HN gene in breast tumour tissue of CT/NDV32 and CT/NDV64 groups	95
19	Representative light micrograph of <i>in situ</i> RT-PCR of β actin mRNA in lung tissue of CT/NDV32 and CT/NDV64 groups	96
20	Representative light micrograph of <i>in situ</i> RT-PCR of HN gene in lung tissue of CT/NDV32 and CT/NDV64 groups	97
21	Representative light micrograph of <i>in situ</i> RT-PCR of β actin mRNA in liver tissue of CT/NDV32 and CT/NDV64 groups	98
22	Representative light micrograph of <i>in situ</i> RT-PCR of HN gene in liver tissue of CT/NDV32 and CT/NDV64 groups	99
23	Representative light micrograph of <i>in situ</i> RT-PCR of β actin mRNA in brain tissue of CT/NDV32 and CT/NDV64 groups	100
24	Representative light micrograph of <i>in situ</i> RT-PCR of HN gene in brain tissue of CT/NDV32 and CT/NDV64 groups	101
25	Representative light micrograph of <i>in situ</i> RT-PCR of HN gene in brain tissue of CT/NDV8	102

and CT/NDV16 groups

26	Representative light micrograph of <i>in situ</i> RT-PCR of β actin mRNA in lung tissue of CT/NDV8 and CT/NDV16 groups	103
27	Representative light micrograph of <i>in situ</i> RT-PCR of HN gene in lung tissue of CT/NDV8 and CT/NDV16 groups	104
28	Representative light micrograph of <i>in situ</i> RT-PCR of β actin mRNA in liver tissue of CT/NDV8 and CT/NDV16 groups	105
29	Representative light micrograph of <i>in situ</i> RT-PCR of HN gene in liver tissue of CT/NDV8 and CT/NDV16 groups	106
30	Mean intensity of HN and β actin genes	110
31	Confocal laser scanning micrographs of NDV-AF2240 in 5 μ m of FFPE of CT/NDV32 (B) and CT/NDV64 (D) in breast tumor tissue	113
32	Confocal laser scanning micrographs of NDV-AF2240 in 5 μ m of FFPE of CT/NDV32 (B) and CT/NDV64 (D) in liver tissue	114
33	Confocal laser scanning micrographs of NDV-AF2240 in 5 μ m of FFPE of CT/NDV32 (B) and CT/NDV64 (D) in brain tissue	115
34	Confocal laser scanning micrographs of NDV-AF2240 in 5 μ m of FFPE of CT/NDV32 (B) and CT/NDV64 (D) in lung tissue	116
35	Confocal laser scanning micrographs of NDV-AF2240 in 5 μ m of FFPE of CT/NDV8 (B) and CT/NDV16 (D) in lung tissue	117

36	Confocal laser scanning micrographs of NDV-AF2240 in 5µm of FFPE of CT/NDV8 (B) and CT/NDV16 (D) in brain tissue	118
37	Confocal laser scanning micrographs of NDV-AF2240 in 5µm of FFPE of CT/NDV8 (B) and CT/NDV16 (D) in liver tissue	119
38	Confocal laser scanning micrographs of NDV-AF2240 in 5µm of FFPE of C/NDV8 (B) and C/NDV16 (D) in lung tissue	120
39	Confocal laser scanning micrographs of NDV-AF2240 in 5µm of FFPE of C/NDV8 (B) and C/NDV16 (D) in liver tissue	121
40	Confocal laser scanning micrographs of NDV-AF2240 in 5µm of FFPE of C/NDV8 (B) and C/NDV16 (D) in brain tissue	122
41	Confocal laser scanning micrographs of NDV-AF2240 in 5µm of FFPE of C/NDV32 (B) and C/NDV64 (D) in liver tissue	123
42	Confocal laser scanning micrographs of NDV-AF2240 in 5µm of FFPE of C/NDV32 (B) and C/NDV64 (D) in brain tissue	124
43	Transmission electron micrograph of NDV-AF2240 isolated from tumor, lung, brain and liver at CT/NDV32 group	127
44	Transmission electron micrograph of NDV-AF2240 isolated from the lung of CT/NDV8 group by NSTEM	128
45	Transmission electron micrograph of NDV-AF2240 isolated from the liver of CT/NDV64 group by NSTEM	128
46	Transmission electron micrograph of rod shape NDV-AF2240	129

