UNIVERSITI PUTRA MALAYSIA

MOLECULAR CHARACTERISATION OF VERY VIRULENT INFECTIOUS BURSAL DISEASE VIRUS AND THE EFFECTS OF ITS VIRAL PROTEINS ON CANCER CELL LINES

KENNY VOON GAH LEONG

FPV 2005 15

MOLECULAR CHARACTERISATION OF VERY VIRULENT INFECTIOUS BURSAL DISEASE VIRUS AND THE EFFECTS OF ITS VIRAL PROTEINS ON CANCER CELL LINES

KENNY VOON GAH LEONG

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

January 2005

Dedicated with love and gratitude to:

My parents, Joseph and Lisa and wife, Mandy

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

MOLECULAR CHARACTERISATION OF VERY VIRULENT INFECTIOUS BURSAL DISEASE VIRUS AND CYTOTOXIC EFFECTS OF ITS VIRAL PROTEINS ON CANCER CELL LINES

By

KENNY VOON GAH LEONG

January 2005

Chairman: Associate Professor Dr. Mohd Hair-Bejo, PhD

Faculty: Veterinary Medicine

Infectious bursal disease virus (IBDV) is an immunosuppressive virus causing bursa lesions and atrophy. There are several strains of IBDVs, namely classical, variant, attenuated and very virulent strains. Viral proteins, VP2 and VP5, from attenuated strain had been shown to induce apoptosis in chicken embryo fibroblast (CEF) and African green monkey cell line (BSC40). The objective of this study is to characterise local isolate IBDV namely, UPM01/10 and to determine the apoptotic effect of VP2 and VP5 proteins in neoplastic and normal cell lines. UPM01/10 was successfully characterised as vvIBDV, based on the molecular methods. The VP2 sequence of UPM01/10 isolate had amino acid substitutions at Ala[222], [Ile]256, [Ile]294 and [Ser]299, similar to other reported vvIBDV. This isolate did not have Lys[249] and Ser[254] amino acid residues which had been reported to be present in variant strains. The deduced amino acids of VP2 showed that the two hydrophilic regions and the serine-rich heptapeptide region were conserved in UPM01/10. Similar finding was reported for other

vvIBDVs such as UK661, HK46 and OKYM. The VP2 nucleotides sequence of UPM01/10 could be cut by restriction enzymes *Taq*I, *Acc*I, *Sty*I, *Spe*I but not by *Sac*I. UPM01/10 showed highest homologous similarity in nucleotides and amino acids to the reported Malaysian vvIBDVs. Phylogenetic analysis based on the nucleotides sequence of the VP2 and VP5 revealed that UPM01/10 isolate was clustered with other very virulent strains but distanced with classical, variant and attenuated strains. Serial samples of Chang, HepG2 and MCF-7 cells transfected with VP2 or VP5 at the interval of 6, 12, 18 and 24 hours were examined, respectively, in order to identify the onset of apoptosis. VP2 and VP5 of UPM01/10 were successfully expressed in Chang, HepG2 and MCF-7 cells. VP2 or VP5 alone was capable of triggering apoptotic response in Chang, HepG2 and MCF-7 cells. The onset of apoptosis started at 6 hours and preceded to necrosis at 24 hours. Both Chang and HepG2 cells were significantly susceptible to either VP5 or VP2 as compared to MCF-7 cells. VP5 induced significantly prominent apoptosis in HepG2 as compared to Chang cells at 6 hours, followed by maintaining similar level of apoptosis in both cells from 12 to 24 hours. However, VP2 induced higher level of cell death significantly in Chang cells as compared to HepG2 cells with exception at 6 hours. VP5 may be possible to be taken into consideration as candidate for liver cancer therapy as compared to VP2.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan Ijazah Master Sains

PENCIRIAN MOLEKULAR VIRUS PENYAKIT BURSA BERJANGKIT DAN KESAN SITOTOKSIK AKIBAT UNGKAPAN PROTIN VIRUS TERHADAP SEL –SEL BARAH

Oleh

KENNY VOON GAH LEONG

Januari 2005

Pengerusi: Profesor Madya Mohd Hair-Bejo, PhD

Fakulti: Perubatan Veterinar

Virus penyakit bursa berjangkit (IBDV) merupakan virus yang melumpuhkan sistem imun ayam dengan mengakibatkan lesi and pengecilan bursa. Terdapat beberapa baka jenis IBDV iaitu, klasikal, variant, nayhaktif dan amat virulen. Protin virus, VP2 dan VP5 daripada baka nyahaktif virus didapati mengakibatkan apoptosis dalam sel fibroblast embyo ayam (CEF) dan sel moyet hijau Africa (BSC40). Penyelidikan ini bertujuan untuk mengenalpasti cirri-ciri IBDV tempatan yang dinamakan UPM01/10 dan mengkaji kesan protin virus, VP2 dan VP5 terhadap sel normal dan sel kanser. UPM01/10, telah dikenalpasti sebagai serotip 1 virus penyakit bursa berjangkit yang amat virulen (vvIBDV) berdasarkan pencirian cara molekul. Jujukan asid amino VP2 daripada UPM01/10 mempunyai penggantian asid amino pada kedudukan Ala[222], Ile[256], Ile[294] dan Ser[299] seperti yang dilaporkan pada vvIBDV yang lain. Isolat ini tidak mempunyai asid amino Lys[249] dan Ser[254] seperti yang telah dilaporkan dalam baka variant. Jujukan asid amino VP2 menunjukkan bahawa kedua-dua kawasan hidrofilik

dan kawasan heptapeptid yang kaya dengan serine adalah terpelihara dalam UPM01/10. Penemuan yang sama telah dilaporkan pada vvIBDV yang lain seperti UK661, HK46 dan OKYM. Jujukan nukleotid UPM01/10 boleh dipotong oleh enzim pembatas *Taq*I, *Acc*I, *Sty*I, *Spe*I tetapi tidak boleh dipotong oleh *Sac*I. UPM01/10 menunjukan homologi yang paling tinggi dalam jujukan nukleotid dan asid amino terhadap vvIBDV dari Malaysia. Analisis filogenesis berdasarkan jujukan nukleotid VP2 dan VP5 menunjukkan baka ini bersekumpulan dengan vvIBDV yang lain dan berjauhan dengan baka klasikal, variant dan nyahaktif. Siri sampel daripada sel Chang, HepG2 dan MCF-7 yang telah ditransfek dengan VP2 dan VP5 pada setiap selang masa 6, 12, 18 dan 24 jam telah diperiksa untuk mengenalpasti masa bermula proses apoptosis. VP2 dan VP5 daripada UPM01/10 berjaya diungkap dalam sel Chang, HepG2 dan MCF-7. VP2 atau VP5, dengan sendiri, dapat mengakibatkan apoptosis dalam sel Chang, HepG2 dan MCF-7. Masa permulaan apoptosis adalah pada 6 jam, kemudian disusuli oleh nekrosi pada 24 jam. Kedua-dua sel Chang dan HepG2 adalah dengan nyata mudah dimatikan oleh VP2 dan VP5 berbanding dengan sel MCF-7. VP5 menyebabkan lebih banyak kematian sel dengan nyata dalam sel HepG2 berbanding dengan sel Chang pada masa permulaan 6 jam, kemudian diikuti dengan kematian sel yang sama banyak dalam sel HepG2 dan Chang. Namun, VP2 mengakibatkan lebih banyak kematian sel dengan nyata sekali dalam sel Chang berbanding dengan sel HepG2, kecuali pada waktu 6 jam. VP5 mungkin dapat dipertimbangkan untuk digunakan sebagai rawatan dalam kanser hati jika dibandingkan dengan VP2.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to Associate Professor Dr. Mohd Hair Bejo for providing invaluable advice and untiring assistance in this M.Sc. research. His constant guidance, support and encouragement were the motivation that enabled me to accomplish my work.

I would like to express my sincere thanks and appreciation to Professor Dr. Aini Ideris, a member of the supervisory committee for her constructive suggestion, proper guidance and encouragement throughout my study period. My sincere thanks and appreciation are also extended to Assoc. Prof. Dr. Abdul Rahman Omar, for his constant support and mostly for sharing knowledge on molecular techniques and guidance.

My special thank go to Professor Dr. Egbert Mundt, from Germany, for his generosity in providing the monoclonal antibodies toward VP2 and VP5 infectious bursal disease virus. I would also like to thank Tan Do Yew, Karuna Sharma, Wan Ken Fei and Khor Sok Fang for providing their technical assistance and support.

I wish to express my deepest gratitude to the Ministry of Science, Technology and Innovation for giving me the National Science Fellowship so that I could further my study in Master of Science.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AA	Arbor Acre
AIV	Avian influenza virus
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
atIBDV	Attenuated infectious bursal disease virus
Bcl-2	B cell lymphoma-2
BGM	Baby grivet monkey
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSC40	African green monkey kidney cell line
CAV	Chicken anemia virus
CMV	Cytomegalovirus
cDNA	Complementary DNA
CEF	Chicken embryo fibroblast
CEK	Chicken embryo kidney
CHSE	Chinook salmon embryo
CPE	Cytopathic effect
CsCl	Caesium chloride
cvIBDV	Classical strains
ddH_2O	Double-distilled water
dNTP	Deoxynucleotide triphosphate
DEPC	Diethyl pyrocarbonate
dH_2O	Distilled water
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
DTT	Dithiotheitol
dUTP	Deoxyurasil triphosphate
EDTA	Ethylenediaminetetraacetic
ELISA	Enzyme-linked immunosorbent assay
F	Fusion protein
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
HN	Hemagglutinin neuraminidase
IBD	Infectious bursal disease
IBDV	Infectious bursal disease virus
IFN	Interferon
Ig	Immunoglobulin
IPNV	Infectious pancreatic necrotic virus
L	Large protein
LB	Luria Bertani
M	Envelope matrix protein
MA	Rhesus monkey kidney
MDV	Marek's disease virus
MEM	Minimum essential medium
$MgCl_2$	Magnesium chloride
mRNA	Messenger ribonucleic acid

NCBI	National Centre for Biotechnology Information
NCRs	Non-coding regions
NDV	Newcastle disease virus
NP	Nucleocapsid protein
ORF	Open reading frame
P	Phosphoprotein
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI	propiduim iodide
PS	phosphatidylserine
R9	Chicken B-lymphocytes cell line
RFLP	Restriction fragment length polymorphism
RK	Rabbit kidney
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase- polymerase chain reaction
SD	Standard deviation
SPF	Specific-pathogen-free
SPSS	Statistical program for social science
TAE	Tris-acetate-ethylenediaminetetraacetic
TdT	Terminal deoxynucleotidyl transferase
TNF	Tumor necrosis factor
TUNEL	Terminal deoxynucleotidyl transferase biotin-dUTP nick labelling
Tris-HCl	Tris-hydrochloride
vaIBDV	Variant infectious bursal disease virus
vvIBDV	Very virulent infectious bursal disease virus

CHAPTER 1

INTRODUCTION

Infectious bursal disease virus (IBDV) is the aetiological agent of infectious bursal disease (IBD) or Gumboro disease that causes significant loss to the poultry industries resulting high mortality, impaired growth, excessive carcass condemnation and profound immunosuppression which lead to increase susceptibility to other pathogens (Lukert and Saif, 1997; van den Berg, 2000). IBDV has been infecting poultry farms since 1957 and until now, IBD is still hard to control due to the availability of different strains of IBDV.

IBDV is divided into two serotypes, namely serotype 1 and 2. Serotype 1 IBDV was isolated from chicken whereas serotype 2 IBDV was isolated from turkey (Becht *et al.*, 1988). Only the serotype 1 IBDV is pathogenic to chickens (Becht *et al.*, 1988). Serotype 1 IBDV can further sub-divide into different strains based on their virulence and antigenic variation. Classical IBDV (cvIBDV) varies in virulence, consisting of mild to intermediate strain, causing bursa damage and up to 30% mortality (Lasher and Shane, 1994). Variant IBDV (vaIBDV) can break the immunity that is induced by conventional classical vaccine to cause bursa atrophy without significant inflammation (Heine *et al.,* 1991). In contrast to vaIBDV, very virulent IBDV (vvIBDV) retains similar antigenicity to cvIBDV but cause more virulence, up to 100% mortality to specific-pathogen-free (SPF)

chicken (van den Berg *et al.,* 1991). Attenuated IBDV (atIBDV), which is mostly used as vaccine, is derived from cvIBDV. However, atIBDV may still produce mild lesions in bursa, despite being attenuated (Yao and Vakharia, 2001).

Even though different strains have been identified; physical attributes of IBDV are similar among strains. IBDV is a double-stranded ribonucleic acid (dsRNA) virus under the genus of *Avibirnavirus* in the family of *Birnaviridae.* IBDV posesses a bi-segmented genome (designated as segment A and segment B) which is well protected within a non-enveloped single shelled icosahedral capsid of approximately 60nm in diameter (Dobos *et al.,* 1979). The virus particles have sedimentation rate of 460S in sucrose gradients (Dobos *et al.,* 1979). The buoyant density of IBDV virus in caesium chloride (CsCl) gradient is in the range of 1.31 to 1.34g/ml, depending on whether the virus particles are full (type I tubules with 60nm diameter) or empty (type II tubules with 24 to 26nm in diameter) (Fahey *et al.,* 1985).

Segment B (2827 base pair (bp)) consists of a single open reading frame (ORF) that encodes VP1 (90kDa), a RNA-dependent RNA polymerase (Spies *et al.*, 1987). Segment A (3261bp) has a large ORF of 3036bp in length and a small ORF of 435bp, in which the small ORF partially overlaps at the 5' end of the large ORF (Kibenge *et al.*, 1990). The large ORF encodes the VP2-VP4-VP3 polyprotein (110-kDa) which is cleaved by auto-proteolysis into individual viral proteins (VP2, VP3 and VP4) (Azad *et al.,* 1985; Hudson *et al.*, 1986). The

small ORF encodes a small cystine-rich 17kDa protein (VP5) (Mundt *et al.*, 1995). Of all the four viral proteins, VP2 and VP3 are the major viral structural proteins, whereas VP4 is a minor protein involved in the processing of the precursor polyprotein. The VP2 protein is exposed on the surface of the virion and contains strain-specific epitopes that neutralizes specific monoclonal antibody (Eterradossi *et al.*, 1998). Sequence analysis reveals that the hypervariable region in VP2 displayed the greatest amount of amino acid residues variation between the classical and variant strains and, hence, these changes are responsible for the antigenic variations among IBDV serotype 1 isolates (Kibenge *et al.*, 1990; Vakharia *et al.*, 1994). The VP3 is considered to be group-specific antigen and is located on the inner surface of the virion, interacting with viral RNA (Becht *et al.*, 1988). On the other hand, VP5 is a nonstructural protein of IBDV that induces apoptosis and may involve in viral pathogenesis (Yao and Vakharia, 2001).

Fernandez-Arias *et al.* (1997) demonstrated that VP2 induce apoptosis in BSC40, which derived from African Green Monkey epithelial cells but not in chicken embryo fibroblast (CEF) cells. The inability of VP2 to induce apoptosis in CEF can be related to the cell tropism of Soroa strain (atIBDV) (Fernandez-Arias *et al.*, 1997). The original Soroa that was isolated from bursa does not replicate well in CEF unless the virus is adapted by serial passage in CEF. As a result, the virus is attenuated and its pathogenicity is reduced simultaneously. However, Yao and Vakharia (2001) have successfully induced apoptosis in CEF

and R9 (chicken B-lymphocytes) cells under the expression of VP2. This VP2 is derived from D78, a vaccine strain (atIBDV) from Intervet Inc., Millsboro, Delaware. The inconsistency of VP2 to induce apoptosis in CEF may be related to the origin of parental strains. However, VP2 from both isolates were attenuated and possesses the ability to induce apoptosis. Nevertheless, little was known about the role of VP2 from vvIBDV in inducing apoptosis in normal cell lines such as CEF, Vero and other human cell lines.

Besides VP2, VP5 (from atIBDV) has been reported to induce apoptosis in R9 and CEF cell line (Yao and Vakharia, 2001). This nonstructural protein plays an important role in viral pathogenesis, as it would induce apoptosis and, thereby, allowing the spread of viral progeny to the neighbouring cells. VP5 may induce apoptosis in human transformed cells. As a nonstructural protein, apoptin (Noteborn *et al.,* 1994) that was derived from chicken anemia virus (CAV) has been shown to induce apoptosis in lymphoblastoid T cells and human transformed cells (Danen-van Oorschot *et al.,* 1997). Thus, both VP2 and VP5 may have potential for cancer therapy if apoptosis is induced in human neoplastic cells only.

In Malaysia, IBD outbreak was first reported in 1991 (Hair-Bejo, 1992). Until now, only vvIBDV is reported in the country (Chong *et al.,* 2001, Phong *et al.,* 2003). Since the virulence and pathogenicity is determined by VP2 and VP5,

both proteins isolated from vvIBDV may induce different levels of apoptosis in human transformed cell lines.

In order to identify vvIBDV from field isolates, molecular methods such as reverse transcriptase-polymerase chain reaction (RT-PCR), restriction fragment length polymorphism (RFLP) and sequence analysis are used. Molecular analysis of IBDV sequences is used to identify the presence of molecular markers, such as restriction enzyme sites and amino acid residues, which are unique in certain IBDV strains. For example, the amino acids at position 279 and 284 in VP2 have been associated to virus virulence (Yamaguchi *et al.*, 1996). The molecular methods provide rapid identification and confirmation of the presence of different strains of IBDV. In comparison to the in vivo methods, molecular methods do not involve the use of experimental animals and thus is more humane and less laborious.

The objectives of the study are:

1) to isolate and characterise IBDV isolate UPM01/10.

2) to determine the effects of vvIBDV viral proteins, VP2 and VP5, in normal (Chang) and neoplastic (HepG2 and MCF-7) cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Infectious Bursal Disease Virus

Infectious bursal disease virus (IBDV) is classified into two serotypes; serotype 1 and serotype 2 (Jackwood *et al.*, 1982, Lukert *et al.*, 1979). Two isolates namely, OH and MO are recognized as serotype 2. Both isolates infected turkeys. IBDV serotype 1 is further categorized into different strains. The classical IBDV (cvIBDV) was first recognized as serotype 1 (Saif *et al.*, 1987). Classical IBDV, which varied in virulence, caused bursal damage and mortality up to 30% (Lukert and Saif, 1997). Variant IBDV (vaIBDV), which was first isolated by Rosenberger and Cloud (1986), is able to infect vaccinated chicks with maternity antibody known to be protective against classical IBDV. Chickens infected by vaIBDV rapidly developed bursa atrophy without inflammation phase and mortality could be less than 5% (van den Berg, 2000). Variant IBDV is antigenically and pathotypically distinct from classical IBDV (Becht *et al.*, 1988 and Rosales *et al.,* 1989). New very virulence IBDV (vvIBDV) emerged and caused mortality rates exceeding 25% in broiler, 60% in layer flocks and 100% in specific pathogen free (SPF) chickens (van den Berg *et al.*, 1991). Very virulent IBDV is related to classical IBDV antigenically, but is differed in pathogenicity. Attenuated IBDV (atIBDV) is usually derived from attenuation of