北海道大学グローバルCOEプログラム

Global COE Program
Progress Report FY 2011
Presentation

2011年度 PD･RA研究成果発表会

Programme & Abstract

■場所：北大 獣医学研究科　講堂
■日時：　2012年3月7日(水)10:00～16：10

Venue: Conference Hall
Graduate School of Veterinary Medicine, Hokkaido University
Time & Date: 10:00～16:10, March 7 (wed), 2012

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10:00-10:05 Opening speech:

■Chairperson■ Chandika D. Gamage

10:05-10:30

1. **Knowledge, attitudes and practice on leptospirosis among community people in Sri Lanka**

   Yoshi Obayashi

2. **Evaluation of rabies elimination program in Sri Lanka**

   Kanae Shiokawa

■Chairperson■ Yoshi Obayashi

10:30-11:00

3. **A comparison of recombinant antigen base ELISA assays to diagnose carrier infection of leptospirosis in laboratory rats**

   Chandika D. Gamage

4. **Risk assessment apporach for control of foot-and-mouth disease outbreak**

   Norikazu Isoda

■Chairperson■ Jung-Ho Youn

11:00-11:30

5. **Epidemiological study of infectious diseases in wild birds in Hokkaido and molecular characterization of expressed sequence tags for *Dermanyssus gallinae* for the development of a novel control method of the mite**

   Masayoshi Isezaki

6. **The closest-neighbor trimming method: A resampling algorithm for nucleotide sequence datasets**

   Kouki Yonezawa

■Chairperson■ Kouki Yonezawa

11:30-11:55

7**. Genomic analysis of *Leptospira* species isolated in Japan using the next-generation sequencer**

Jung-Ho Youn

8. **Identification of a new polyomavirus from Vervet monkeys in Zambia**

Hiroki Yamaguchi

11:55-13:00 ==== Lunch ====

■Chairperson■ Masayoshi Isezaki

13:00-13:25

9. **Studies on the transmission route and pathogeneses of Seoul type hantavirus infection among urban rats populatin in Vietnam**

Shumpei P. Yasuda

10. **A novel cell line derived from the kidney of gray red-backed vole (*Myodes rufocanus bedfordiae*) for the isolation and the propagation of hantaviruses**

Takahiro Sanada

■Chairperson■ Naoki Yamamoto

13:25-13:45

11.**Experimental visceral leishmaniasis in bone marrow chimeric *aly/aly* and *aly*/+ mice**

Saruda Tiwananthagorn

12.**The role of Hokkaido sika deer in the transmission of zoonoses**

Kyunglee Lee

■Chairperson■ Yusuke Sakai

13:45-14:25

13.**Host cell factors involved in the synthesis and expression of Borna disease virus glycoprotein**

Yayoi Otsuka

14. **A decreased expression of pre-synaptic markers in neurons in differentiated neurospheres infected with BSE-derived prion strain**

Yukiko Sassa

■Chairperson■ Shumpei P. Yasuda

14:25-14:45

15. **Serological evidence of ebola virus infection in Indonesian orangutans**

Eri Nakayama

16. **1) Searching for a receptor for filovirus**
2) Comparison of the immunomodulatory effects of VP35 and VP24 between Zaire and Reston Ebola viruses

Makoto Kuroda

14:45-15:00 **Coffee Break**

■Chairperson■ Yayoi Otsuka

15:00-15:20

17. **Phosphatidylserine micelle as an immune-enhancing peptide carrier specifically taken by antigen presenting cells**

Toru Ichihashi

18. **The role of mouse oligoadenylate synthetase-1 family**

Enas Hamed Mahmoud Elkhateeb

■Chairperson■ Norikazu Isoda

15:20-15:45

19. **Expression level of the polymerase mRNAs of A/Hong Kong/483/97 (H5N1) was extensively higher than that of a mutant virus with PB2 of a single amino acid substitution at 627 Lys to Glu in mouse cells**

Naoki Yamamoto

20. **Host factors responsible for the pathogenesis of highly pathogenic avian influenza virus in chickens**

Saya Kuribayashi

■Chairperson■ Yukiko Sassa

15:45-16:10

21. **Roles of ubiquitin and heat shock protein 70(Hsp70) in the development of rabies virus (RBV) inclusion**

Yusuke Sakai

22. **Characterization of protooncogene TCL1b as an Akt kinase co-activator**

Manabu Hashimoto

16:10- Closing Speech

Knowledge, attitudes and practice on leptospirosis among community people in Sri Lanka

Yoshi Obayashi
Department of Global Health and Epidemiology
Hokkaido University Graduate School of Medicine

Leptospirosis is mainly distributed in tropical and subtropical areas. In Sri Lanka, the last outbreak of leptospirosis was reported in 2008 with an incident rate of 37.5 per 100, 000 population. Newly released data confirm that leptospirosis is expected to cause an outbreak again in Sri Lanka in 2010-2011. In prevention and controlling of leptospirosis, public awareness of the disease plays a major role. Assessing the actual situations about the awareness of leptospirosis among local people in the country is important to develop effective strategies and policies for prevention and control of the disease.

Thus, in this study, knowledge, attitudes and practices (KAP) towards leptospirosis was assessed in pilot study among general public in Sri Lanka. Other than assessing the knowledge of the people, this research focused on how to implement their awareness towards control of leptospirosis and to identify important issues for implementing better countermeasures for control of the disease.

A cross sectional survey was conducted from December 2011 to January 2012, among community people above 15 years of age in certain selected areas in Kegalle district. Participants were asked to participate in a 15-20 minutes face to face interview. Nine-hundred-thirteen people agreed to participate in the survey. The questionnaire consisted of one's demographics information, daily life style as well as KAP on leptospirosis. For ethical consideration, this procedure was pursued after informed consent from the participants.

In this presentation, preliminary results of analysis are shown and discussed. Respondents were female-dominant (64%). Among all, 98% knew leptospirosis as "rat fever" in their local language and they recognized the rat as the major reservoir animal. On the contrary, less than one-third of them knew that buffalos and dogs are also reservoir animals. The study suggests that further awareness-rasing programs towards control of the disease should be implemented for general public.

Evaluation of rabies elimination program in Sri Lanka

Kanae Shiokawa
Department of Global Health and Epidemiology,
Hokkaido University Graduate School of Medicine

Rabies is one of the neglected tropical diseases. The number of reported rabies cases is still prevalent in many developing countries, especially Asian and African nations, although both significant amounts of pre- and post- exposure vaccines (prophylaxis, PEP) have been provided every year. However, the high cost of PEP is a burden for many developing countries.

In Sri Lanka, rabies has existed since the mid 18th century. Rabies Ordinance, launched by British Colonial Administration in 1893, has been the legal instruments for rabies control activities in Sri Lanka. The country had elected to firstly implement the National Rabies Control Programme in 1975 and has further strengthened its activities since 1990 mainly including dog vaccination and elimination promotions. Dog culling operation was replaced with animal birth control program with chemical and surgical sterilization in 2007.

Despite of such legal frameworks and national programs, 41 human rabies cases and 658 animal rabies cases were still reported to the Epidemiology Unit, Ministry of Health, Sri Lanka in 2011 and 2010, respectively, throughout the country except no human case in 11 districts. According to the Ministry of Sri Lanka, all human deaths were the results of bites by dogs and approximately US$ 3 million are spent each year on treating these cases. Majority of the bitten cases are taking unnecessary PEP due to unavailability of animal vaccination certificates.

In the country, monitoring mechanism for rabies antibody titer in sera for dogs after vaccination programs is not in place, which remains a major obstacle to their evaluation. Therefore, it warrants the conduct of a molecular epidemiological study to evaluate the anti rabies vaccination programs as well as periodical review and assessment of community involvement and peoples' knowledge, attitude and practices for rabies, which is necessary to develop and implement the effective prevention and control programs in the country in the near future.

We have collected about 300 dog sera together with some epidemiological information in 3 cities (Colombo, Gampaha, and Kalutara) of the Western Province in collaboration with the Ministry (Public Health Veterinary Service) and Department of Veterinary Pathobiology, University of Peradeniya.

Some details of our study will be presented in the meeting.

A comparison of recombinant antigen base ELISA assays to diagnose carrier infection of leptospirosis in laboratory rats

Chandika D. Gamage
Department of Global Health and Epidemiology,
Hokkaido University Graduate School of Medicine

Introduction
Leptospirosis is a globally significant serious zoonotic disease with veterinary and public health impacts. Laboratory diagnosis of the disease is based on either demonstration of antigen or antibodies in diseased humans or animals. However serology is the most suitable for the rapid testing of a large number of samples in resource limited settings. Thus, in this study we have evaluated the diagnostic utility of four recombinant antigens in enzyme-linked immunosorbent assays (ELISAs) for serodiagnosis of carrier infection of leptospirosis.

Materials and methods
WKAH/hkm 6-week old female 24 rats were inoculated with *Leptospira interrogans* serovar Manilae and 6 rats were included as controls. Blood samples were collected on post infection days of 3, 6, 9, 12, 14, 21, 30, 45 and 60. Serum samples were analyzed using microscopic agglutination test (MAT) and ELISA based on recombinant antigens. Antigens were made by PCR amplifying of whole sequence of LipL32 and three segments of the LigA gene. Those sequence segments were designated as K, C, and V, and subcloned in to pET-43.1a. The recombinant plasmid was transformed in to *E.coli* BL21 and both fusion (NUS) proteins expression was induced by ITPG. MAT conducted using 4-5 days old *Leptospira interrogans* serovar Manilae culture. Samples collected between PID 9 - 60 were considered as immune phase samples. Each antigen's cutoff point optical density (OD) value for ELISA was determined by considering the control group's OD values.

Results
Out of 18 infected rats 15 had anti-leptospiral antibody titers of 400 or greater in MAT. Cutoff point OD values of ELSA assays were determined as 1.03 for LipL32; 0.12 for both LigA-K and LigA-V and 0.09 for LigA-C. ELISA using recombinant antigens showed 100% specificity. Sensitivities of these tests were 100% for both LipL32 and LigA-K antigens. On the other hand, the sensitivities of assays comprised LigA-C and LigA-V were 16.67% and 0%, respectively. A statistically significant correlation was revealed between MAT titers and the OD values of ELISA-LipL32 and ELISA-LigA-K assays (Correlation coefficient 0.59 and 0.63 respectively, $P< 0.01$).

Conclusion
The results indicate that recombinant antigens of LipL32 and LigA are useful for the laboratory diagnosis of carrier infection of leptospirosis in rats.

Risk assessment apporach for control of foot-and-mouth disease outbreak

Norikazu Isoda
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(Background)

The outbreak of foot-and-mouth disease (FMD) occurred in 2010 in Japan. It took approximately three months to control the whole outbreak using vaccination as well as "Stamping-out" and movement restriction policies. Total losses of this outbreak were much higher than for the last FMD outbreak in Japan in 2000. Several reasons why the control activities were not effective enough to control the outbreak quickly have been proposed.

(Objectives)

In order to achieve more quick and efficient control measure implementations, we proposed two research projects: 1) To develop a generic fault tree framework for the control of an FMD outbreak as a basis to systematically improve and refine control activities and general preparedness. 2) To develop a disease transmission simulation model able to simulate the loss of FMD outbreaks in Japan prospectively and compare the outputs of the model with different disease control scenarios.

(Methods)

A fault tree model is being developed which will map all activities and events where things can go wrong during an accident. Lack of rapid and complete disease control was disignated as the top event of the present fault tree which is a critical situation that represents system failure. Starting with the top event, the tree is expanded through several branches with logistic gates until the required level of detail is reached. In a second step, the specific fault tree for the 2001 outbreak in the UK was developed based on control weakness discussed in peer reviewed papers.

An existing disease transmission model was adapted to assess the effectiveness of control measures against FMD outbreak in Hokkaido. The parametes with a part of Hokkaido data were introduced and the molde was simulated in different "Stamping-out" policies and vaccination scinarios. The outputs of the disease model were compared with the number of culled animals, the number of infected premises, and the days to be controlled.

(Results)

We developed to a generic fault tree for FMD outbreak control. Specific fault tree for the 2001 outbreak in the UK indicated that there were 12 events responsible for failure of rapid control measure implementation.

The results of disease model indicated that the losses of FMD outbreak were decreased by earlier detection of index infected herd, though significant differences were not confirmed between control scenario and the scenario of earlier detection of index infected herd. However, vaccination size did not affect on the losses of outbreak in the disease model.

Epidemiological study of infectious diseases in wild birds in Hokkaido and molecular characterization of expressed sequence tags for *Dermanyssus gallinae* for the development of a novel control method of the mite

Masayoshi Isezaki
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Wild birds such as wild waterfowls play important roles as the infection sources in the epidemiology of many infectious diseases including the zoonosis. We previously established detection methods of JEV and WNV from feather tips using RT-PCR and nested RT-PCR. Therefore, we performed molecular epidemiological survey using the feather tips collected from the wild birds in Hokkaido. Total 100 samples were analyzed, but no positive birds of JEV and WNV in the survey of 2011 in the Hokkaido area. In addition, we also carried out epidemiological surveillance of other viruses (Influenza virus, Newcastle disease virus and Marek's disease virus) using the cloacal swabs or feather tips from the wild birds, and isolated Influenza viruses and Newcastle disease viruses but not Marek's disease virus.

The red mite, *Dermanyssus gallinae* is regarded as the most serious and economically important ectoparasite for the poultry industry all over the world. In addition, the red mite has been suggested as a potential vector of several pathogens. Recently, acaricide resistance is a major problem for the control of the mite in poultry farms. However, genetic information of this mite was scarcely available for candidates of vaccine antigens and for the development of novel acaricide. Hence, in this study, analysis of expressed sequence tags (ESTs) has been performed.

A plasmid cDNA library was constructed from the red mite collected from a poultry farm. A total of 2,466 cDNA clones were randomly picked and 1,147 cDNA clones except for shorter inserts were sequenced. These sequences were compared to those accumulated in NCBI databases, 373 sequences were identified as those known of functions. Some of these clones showed high similarity to drug-metabolizing enzymes (e. g., GST, cytochromeP450, microsomal epoxide hydrolase) and vaccine candidates (e. g., peroxiredoxins, type II allergen). Then, we focused on homologues of peroxiredoxins (Prx4 and Prx2) and type II allergen, which are suggested as possible vaccine candidates for other ticks, and their functions were analyzed by using recombinant proteins. Currently, functional analyses of other cDNA clones are in progress to identify new vaccine candidates and target molecules for the clarification of acaricide-resistant mechanism.

The closest-neighbor trimming method: A resampling algorithm for nucleotide sequence datasets

Kouki Yonezawa
Division of Bioinformatics,
Hokkaido University Research Center for Zoonosis Control

Understanding the transmission of an infectious disease requires a number of nucleotide sequences of its causative agent. Remarkable efforts are made at international and national levels to collect sequence information of important pathogens. As a result, a large number of pathogen-related sequences have been accumulated in the public databases. There exist more than 170,000 nucleotide sequences of influenza viruses in NCBI Influenza Virus Resources and more than 410,000 of human immunodeficiency viruses in HIV sequence database.

The rapid growth in the number of nucleotide sequences poses two critical problems. One problem is enormous increase in computational cost. Sequence data analyses—including multiple sequence alignment, phylogenetic analysis, and homology search of nucleotide sequences—involve time-consuming computation.

Another problem is sampling bias in public databases, which occurs when the sequences were not sampled randomly. One factor is the difference in the surveillance activity among countries. Developed countries having high surveillance activities would submit more sequences than other countries. Another factor is the advance in sequencing technology in the last two decades. The database contains more sequences from recent strains than those from old strains. Therefore it is important to develop methods to reduce sampling bias.

In this presentation we propose a novel algorithm --- called the closest-neighbor trimming method --- that resamples a given number of sequences from the large nucleotide sequence dataset. The method first constructs a phylogenetic tree with the whole sequence dataset. Then it repeats the following procedure until the number of remaining sequences reaches the given number: (1) pinch the pair of neighbors with the shortest distance among all pairs of neighbors and (2) trim the one of the neighbors with the longer edge length from their common parent. We compare the performance of our algorithm with three resampling algorithms.

Genomic analysis of *Leptospira* species isolated in Japan using the next-generation sequencer

Jung-Ho Youn
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Research Center for Zoonoses Control, Hokkaido University

Introduction
Leptospirosis is the most widespread zoonotic disease worldwide with a mortality rate of more than 10% in developing countries. There are more than 230 serovars among pathogenic leptospiras where 50% belonging to the species *interrogans* and *borgpetersenii*. However, until now, the genome of only 3 serovars of *L. interrogans* is available and therefore a comparative genomic analysis is limited.

Materials and Methods
A total of eight *Leptospira interrogans* isolates belonging to six different serogroups were sequenced using the next-generation sequencer. CLC Genomics Workbench 4 program was used to map the reads of each sample to the reference strain Lai str. 56601 and analyzed the genetic background related to pathogenicity, SNP, genomic island, and OMPs. Various programs including Exel, MEGA 5, Genetyx 10, etc. were used for the genomic analysis.

Result
A dendogram of the isolates genome was established. Interestingly, two huge gene missing clusters could be observed. The distribution of the absent genes were clustered in two regions - the *rfb* gene cluster and the Genomic Island. The neighbor joining phylogenic tree was constructed based on concatenated sequence of SNP of the eight isolates (37,651 bp, random 200, 100, 50, 20). The result demonstrated two main clades: the serogroup *pyrogenes* and the other serogroup isolates; same serogroup isolates showed close relation to each other. In case of the SNP aligment of OMP genes revealed similiarities even between same but also different serogroups.

Conclusion
Since the O-antigen cluster is serovar specific, an unmapped gap has occured. A lack of GI is also present in NIID7 and M07-10E. The NJ tree result clarifies the possibility to disgn a PCR method for a rapid typing of serogroups/serovars. The results of the OMPs indicate a possible horizontal tranfer of genes between serogroups. Further study includes the development of gene cluster-specific PCRs for rapid typing of epidemic serogroups and finding new gene candidates for vaccination.

Identification of a new polyomavirus from vervet monkeys in Zambia

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Members of the family *Polyomaviridae* are non-enveloped icosahedral viruses with a circular double-stranded DNA genome approximately 5,000 bp in size. The monkey polyomavirus (PyV), Simian virus 40 (SV40), was accidentally administered to humans through contaminated poliovirus vaccines in the 1960's. Based on the transformation activity of SV40 to human cells, it has been suggested that SV40 infection by its contamination to vaccine may cause tumors in humans. Indeed, SV40 has been reported in a variety of human cancers, such as mesothelioma, central nervous system tumors, and non-Hodgkin's lymphoma. Thus, it might be important to consider the potential threats of zoonotic transfer of PyVs to humans and to perform surveillance of PyVs in wildlife. In this study, we attempted to detect PyVs in non-human primates.

DNAs were isolated from kidneys and spleens of vervet monkeys (VMs, n=50) and baboons (BAs, n=50) in Zambia, and subjected to a nested broad-spectrum PCR. A BLAST search showed that the PCR products had high homology with other PyVs. Thereafter, an inverse PCR was performed to identify whole PyV genomes. We successfully detected PyV genomes from VMs (n=3) and BAs (n=2) out of fifty. Phylogenetic analysis using the neighbor-joining method revealed that these viruses were closely related to B-lymphotropic PyV (87-94%) or Simian agent 12 (88-94%), which are non-human primate PyVs. Only one virus, detected from VM spleen, was related to Chimpanzee PyV with relatively low homology (74%), which has a high homology with human Merkel PyV detected from Merkel cell carcinoma. Phylogenetic analysis further revealed that this PyV is a novel polyomavirus.

To test whether the PyV genome can produce progeny viruses in culture cells, the linearized entire genome was transfected into various cell lines. We detected expression of both mRNA and protein of VP1 in the viral genome-transfected 293T cells.

In this study, we have detected PyVs genomes from VMs (3 / 50) and BAs (2 /50) in Zambia. In addition, we have identified a novel PyV from VM spleen related to Chimpanzee PyV, designated as vervet monkey polyomavirus.

Studies on the transmission route and pathogeneses of Seoul type hantavirus infection among urban rats populatin in Vietnam

Shumpei P. Yasuda
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Graduate School of Medicine, Hokkaido University

Seoul virus (SEOV), one of the serotypes of hantavirus, is a causative agent of hemorrhagic fever with renal syndrome. The natural reservoir of SEOV is the Norway rat (*Rattus norvegicus*) and it is transmitted horizontally from persistently infected rats to uninfected ones. However, the details of this in nature are still unclear.

To study the ecology and mechanisms of persistent infection of SEOV in rat colonies in more detail, wild rats were captured in Ho Chi Minh City, Hai Phong City and Hanoi City, Vietnam. In total, 207 wild Norway rats were collected. IgG and IgM antibodies were detected by ELISA and western blotting, respectively. IgG avidity was calculated in IgG positive rats by avidity assay. Amount of virus genome copy in lung, serum and feces were estimated by real-time PCR method and activities of hantavirus specific cytotoxic T lymphocytes (CTL) were detected by ELISPOT assay. Laboratory rats (WKAH, male, six weeks old) were experimentally infected with SEOV strain SR-11 (6.0×10^4 ffu/rat, intra-peritoneal route). Splenocytes were collected at 6 days post inoculation for ELISPOT assay.

Analyses of antibodies, amount of viruses, and CTL activities indicated following results; 1) Positive rate of IgM was higher at middle to high IgG avidity (>30%) group than in low IgG avidity (<30%) group. According to the results from experimentally infected rats showing transient infection, low IgG-avidity and IgM antibody-positive were indicator for the acute–phase infection. Therefore, antibody responses against hantaviruses in wild rats were different from transient infection. 2) CTL activation was detected in experimentally infected laboratory rats. By contrast, it was not detected in control laboratory rats, wild hantavirus-negative rats and wild hantavirus-positive rats. CTL inactivation might drive persistent infection in natural host, though more samples of wild rats should be collected to discuss about it. 3) In female, there is negative relationship between amount of virus genome and IgG avidity. The relationship may shows female rats recover from the infection. In contrast, there is no relationship in male. This result supports the hypothesis that male have a main role as a reservoir of virus. 4) Rats bearing over 10^6 virus genome copy/mg in their lung tend to have virus in serum and feces. Only 30% of infected rats may shed viruses in their feces. It is a first report that wild hosts shed virus in their feces. It may cause horizontal transmission to susceptible rats and humans.

A novel cell line derived from the kidney of gray red-backed vole (*Myodes rufocanus bedfordiae*) for the isolation and the propagation of hantaviruses

Takahiro Sanada
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Hantaviruses belong to the *Bunyaviridae* family and cause two severe human illnesses, hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS). Although VeroE6 cells originating from African green monkey kidney are widely used for hantavirus research, growth and isolation of hantaviruses in this cell line is very slow and difficult. To develop efficient method to isolate hantavirus, we established new cultured cells, MRK101 C8F4, derived from a kidney of gray red-backed vole (*Myodes rufocanus bedfordiae*), the natural host of Hokkaido virus (HOKV), and used this novel cell line to isolate HOKV.

The cultured cells derived from the kidneys of adult *M. r. bedfordiae* were cloned twice by limiting dilution and has been continuously subcultured for more than 1 year. This cell line was designated as MRK101 C8F4. The susceptibility and replication of each hantavirus in MRK101 C8F4 cells were analyzed by focus forming assay and western blotting. Isolation of HOKV was attempted by inoculation of the supernatant of tissue homogenates from HOKV-infected animals to both MRK101 C8F4 and VeroE6 cells. The virus RNA and nucleocapsid (N) protein in the inoculated cells were detected by RT-PCR and IFA, respectively.

The newly established MRK101 C8F4 cells showed significantly higher susceptibility to Puumala virus hosted by *Myodes glareolus* than VeroE6 cells. Viral N protein in MRK101 C8F4 cells was detected earlier than those in VeroE6 cells. On the other hand, MRK101 C8F4 cells showed no susceptibility to Hantaan virus hosted by *Apodemus agrarius*.

In the attempt to isolate HOKV, both viral RNA and N protein were detected from only in inoculated MRK101 C8F4 cells. The presence of infectious virus in culture fluid was confirmed by inoculation to naïve MRK101 cells. Therefore, HOKV was successfully isolated only in MRK101 C8F4 cells.

In conclusion, MRK101 C8F4 cell line seems to be a useful tool for the isolation and propagation of hantaviruses. It may be used as a good model to analyze the interaction between hantavirus and natural host.

Experimental visceral leishmaniasis in bone marrow chimeric *aly/aly* and *aly/+* mice

Saruda Tiwananthagorn
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Graduate School of Veterinary Medicine, Hokkaido University

Visceral leishmaniasis (VL) is a chronic and fatal disease in humans and dogs caused by the intracellular protozoan parasites, *Leishmania donovani* and *L. infantum* (*L. chagasi*). Reactivation of parasites and relapse of the disease are frequent in the immunocompromised patients, in which the number of VL cases has been increasing recently. However, the mechanisms underlying the parasite persistence in the immunocompromised condition have not been clearly clarified.

Alymphoplastic *aly/aly* mouse, an alternative immunodeficiency murine model, carries a point mutation of NF-κB inducing kinase (*NIK*) gene possessing the structural defects of secondary lymphoid tissues (SLTs). The previous study has employed *aly/aly* mice to clarify the mechanisms of persistent *L. donovani* infection in the liver. While the control *aly/+* mice resolved the hepatic infection by 8 weeks post infection, *aly/aly* mice showed a delayed and impaired granuloma maturation with the long-term parasite persistence in the chronic phase of the infection. Suppressive mechanisms mediated by $CD4^+Foxp3^+$ regulatory T cells (Tregs) are underlying the persistent infection in the liver of *aly/aly* mice.

Bone marrow (BM) chimeric mice, *aly/aly* → *aly/+* and *aly/+* → *aly/aly* mice, were produced to examine the factors underlying the improper T cell-mediated immune response in the liver of *aly/aly* mice. After *L donovani* infection, parasite persistence, delayed and impaired hepatic immune response and expansion of Tregs were demonstrated in the liver of both BM chimeric mice. These results suggest that both structural defects of SLTs and the *NIK gene mutation of hematopoietic cells account for the* diminished hepatic immune response to *L. donovani* infection, but *not for* the induction and suppressive function of Tregs in *aly/aly mice.*

In conclusion, this study offered a novel insight into the involvement of Tregs in *L. donovani* persistence in the liver during murine VL in the *immunodeficient aly/aly mice and BM chimeric aly/aly* → *aly/+* mice *as a model of* immunocompromised condition. Involvement of Tregs in parasite persistence in immunocompromised patients related with VL should be investigated.

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The role of Hokkaido sika deer in the transmission of zoonoses

Kyunglee Lee
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Sika deer (*Cervus Nippon yesoensis*) population in Hokkaido was estimated about 640 thousand in 2010. The government have aimed to control the population with culling and encouraging hunting .The problem caused by overly crowded deer was not limited in the damages of the forest and the cultivation. As the wild animal looking for food broaden their habitat to the urban area and the pasture, consequently, the contacts with cattle increase then a chance of the transmission of disease may rise, too. Deer has been blamed as one of main reservoirs of some infectious disease of cattle and human; such as, Lyme disease in North America, bovine tuberculosis in UK, and Hepatitis E in Eastern Europe. In Hokkaido and other part of Japan, several studies about the infectious disease prevalence in wild deer have been reported; Leptospirosis, Paratuberculosis and Rickettsia, etc. There has been previous research and brief report on the some disease in Hokkaido Sika deer population and the prevalence were not high as being a problem. Also, the outbreaks of zoonosis have been rare in Japan, especially in Hokkaido. Therefore, the follow up on those studies were not held anymore. However, considering the epidemiology of disease is changing quickly and the alteration of the vector involving disease which is beyond our expectation with global warming situation, a close observation on the potential disease is necessary. Indeed, the role of wild deer population in the infectious disease epidemiology in Hokkaido is still not defined well. They may have the responsibility of the propagation of some disease to domestic animal otherwise, may provide a buffering zone. On the other hand, they even can be the victim of spillover the disease from cattle.

In this study, several disease pathogens were chosen for monitoring; *Borrelia* spp., *Leptospira* spp. and *Mycobacterium bovis*, preliminarily.

In total, 159 deer, and 8 bears which were hunted or culled for the nuisance control and caught by accident in Hokkaido were inspected by macroscopic and molecular methods. There was no case which was presenting macroscopic lesions of any suspect disease except liver fluke infestation. No positive in Mycobacterium species detecting PCR in 31 deer sample in total, 1 positive from a bear kidney sample in flab gene detecting PCR, and 5 positive (2 /23 tick on deer, 3/36 deer blood) in Borrelia PCR came up. Further DNA sequencing and serological survey are in schedule. Additionally, 31 deer samples including pulmonary lymph node, kidney and blood are pending for the same procedure of PCR.

Up to now, the tissue sampling was mostly from winter season and summer samples were limited in serum. To monitor the seasonal epidemiological tendency in these diseases, to expand sampling through the year is in need**.**

Host cell factors involved in the synthesis and expression of Borna disease virus glycoprotein

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<Background & Aim>
Borna disease virus (BDV) establishes persistent infection in the central nervous system and causes behavioral disturbances in several animal species including human. BDV glycoprotein (BDVG) expression is extremely low in the cells persistently infected with BDV and in the cells transfected with BDVG cDNA. We previously reported that the expression of avian bornavirus glycoprotein (ABVG) was remarkably higher than that of BDVG in transfected cells, despite the similarities in their polypeptide backbone. We also demonstrated that the amino acid sequence of the transmembrane domain (TM) affected the plasma membrane expression level of BDVG (GCOE progress report, 2010). However, the mechanism for this phenomenon remains unknown. The purpose of the present study was to unravel the mechanism for the restricted expression of BDVG.

<Results>
1) The open reading frame of the BDVG gene contains the splicing elements consisting of the 5'-splice donor site (SD), branch point, polypyrimidine tract (PPT), and 3'-splice acceptor site (SA), while that of the ABVG gene lacks the nucleotide sequence corresponding to the SD. Northern blotting and immunoblotting analyses of the cells transfected with a series of BDVG and ABVG mutants revealed that the splicing event was the primary cause for the BDVG expression at nearly undetectable levels and suggested that the PPT sequence preceding the SA in the BDVG gene would have more beneficial effect in splicing than that in the ABVG gene. These findings may suppose possible roles of cellular splicing factors including U2AF65 in modulating the expression level of BDVG.

2) The FLAG-tagged BDVG possessing the artificially modified the splicing element sequences was expressed in 293T cells and its transport from the ER to the plasma membrane was analysed. The immunoprecipitation of BDVG and ABVG co-precipitated the ER chaperons, calnexin and Bip. Overexpression of SarI-GTP, but not that of ArfI-GTP, resulted in reduced cell surface expression of the furin-cleaved form (GP2) of these proteins as the major population of BDVG and ABVG found in the cell surface.

<Conclusion>
Expression of BDVG in the transfected cells is restricted by the cellular splicing machineries. Once synthesized, BDVG as well as ABVG is transported to the plasma membrane through the conventional secretory pathway.

A decreased expression of pre-synaptic markers in neurons in differentiated neurospheres infected with BSE-derived prion strain

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I have reported that differentiated neurosphere cultures containing neurons, astrocytes, oligodendrocytes, NG2-positive cells, and nestin-positive cells are susceptible to many prion strains and that PrP^{Sc} was preferentially found in GFAP-positive cells and lesser incidence, in neurons. To further characterize the neurosphere cultures infected with prions, I analyzed the intracellular localization of PrP^{Sc} in GFAP-positive cells and expression of pre-synaptic markers in differentiated neurospheres.

Neruosphres were prepared from fetus brains of ICR/Jcl mice at embryonic day 14. Differentiation of neurospheres was initiated by the withdrawal of growthfactors and addition of B27 supplement. Brain homogenates of mice infected with the Chandler, Obihiro, 22L, G1, Fukuoka1 and KUS prion strains were used for the infection of neurospheres. The KUS strains were derived from BSE cattle after serial passage in C57BL6/J mice. Neurosphere cultures were fixed and subjected to multiple immunofluorescent staining with mAb132 for specific detection of PrP^{Sc}, astrocytes or neuronal markers, and various organelle markers or pre-synaptic markers.

In the GFAP-positive cells, PrP^{Sc} were co-localized well with Flotillin and Lamp1, indicating that PrP^{Sc} in GFAP-positive cells were mainly present in late endosomes and lysosomes. Strong PrP^{Sc} infection with neurons was observed in KUS infected neurosphere cultures when KUS prions were inoculated at 30 days after differentiation. Thus I analyzed the expression of pre-synaptic markers in the PrP^{Sc}-positive and PrP^{Sc}-negative neurons in the KUS prion-infected neurospheres. The expression of synaptophysin, α-synuclein, SNAP25 were less in PrP^{Sc}-positive neurons than in PrP^{Sc}-negative neurons. These results suggest that the expression of several pre-synaptic markers may be affected by the infection of KUS prion strain. There is few cell culture models that can reproduce neuronal degeneration by prion infection so far. The decreased expression of pre-synaptic markers in KUS prion-infected neurons in differentiated neurosphere cultures may provide a cell culture model of neurodegeneration caused by prion infection.

Serological evidence of ebola virus infection in Indonesian orangutans

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Ebola virus (EBOV) and Marburg virus (MARV) belong to the filovirus group and cause severe hemorrhagic fever in humans and nonhuman primates. Despite the discovery of EBOV (Reston virus) in nonhuman primates and domestic pigs in the Philippines, information on the reservoirs and potential amplifying hosts for filoviruses in Asia is lacking. In this study, serum samples collected from 353 healthy Bornean orangutans (*Pongo pygmaeus*) in Kalimantan Island, Indonesia, during the period from December 2005 to December 2006 were screened for filovirus-specific IgG antibodies using a highly sensitive enzyme-linked immunosorbent assay (ELISA) with recombinant viral surface glycoprotein (GP) antigens derived from multiple species of filoviruses (5 EBOV and 1 MARV species). Here we show that 18.4% (65/353) and 1.7% (6/353) of the samples were seropositive for EBOV and MARV, respectively, with little cross-reactivity among EBOV and MARV antigens. In these positive samples, IgG antibodies to viral internal proteins were also detected by immunoblotting. Interestingly, while the specificity for Reston virus, which has been recognized as an Asian filovirus, was the highest in only 1.4% (5/353) of the serum samples, the majority of EBOV-positive sera showed exclusive specificity to particular EBOV species such as Zaire, Sudan, Cote d'Ivoire, or Bundibugyo viruses, all of which have been found so far only in Africa. These results suggest the existence of multiple species of filoviruses or unknown filovirus -related viruses in Indonesia, some of which are serologically similar to African EBOVs, and transmission of the viruses from yet unidentified reservoir hosts into the orangutan populations.

1. Searching for a receptor for filovirus
2. Comparison of the immunomodulatory effects of VP35 and VP24 between Zaire and Reston Ebola viruses

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1. Viruses enter host cells by interacting with specific cellular receptors. Inhibition of the virus binding to specific receptors is one of the key mechanisms targeted by neutralizing antibodies and antiviral drugs. However, the molecular mechanism of filovirus entry is largely unknown, although several molecules have been proposed to be filovirus receptors or coreceptors. We produced a monoclonal antibody M224/1 that recognizes a host cell protein and found that it blocks infection of vesicular stomatitis virus pseudotyped with Ebola virus glycoprotein (GP) in Vero E6 cells. We are trying to clarify whether a cellular molecule recognized by M224/1 is a specific receptor for filovirus. First, the expression of the target molecule of M224/1 on the surface of Vero E6 cells was confirmed by using flow cytometory. Then, Vero E6 cells were lysed and cellular components were separated into hydrophobic and hydrophilic proteins. Some protein bands were detected by western blotting (WB) using M224/1. In the future, we will compare the expression levels of these proteins between Vero E6 cells and poorly permissive cells. We will finally identify the target of M224/1 by immunoprecipitation and mass spectrometry.

2. Viruses have many strategies to evade the host immune responses. It is known that Ebola virus VP35, a cofactor of the viral RNA polymerase complex, suppresses the production of type I interferon and that Ebola virus VP24, a minor matrix protein, inhibits type I interferon signaling. These proteins are thought to have an important role in the pathogenesis of Ebola virus infection. But, it is not clear whether the immunomodulatory effects by these proteins contribute to the different pathogenicity among Ebola virus species. Of all known Ebola virus species, Zaire Ebola virus is the most virulent whereas Reston Ebola virus is believed to be less pathogenic for human. For comparative analysis, Zaire and Reston Ebola virus VP35- and VP24-expressing plasmids were created by cloning from cDNA. The expression of these proteins was confirmed by WB using VP35- and VP24-specific antibodies. In the future, we will compare the immunomodulatory effect of VP35 and VP24 on cytokine responses mediated by double stranded RNA and virus-like particle between Zaire and Reston Ebola viruses.

Phosphatidylserine micelle as an immune-enhancing peptide carrier specifically taken by antigen presenting cells

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[Introduction] Although synthetic 8-10 mer peptides are the most desirable material for epitope specific cytotoxic T lymphocyte (CTL) inducing vaccine since they are relatively easy to produce and hardly induce severe side effects, immunization by peptide plus adjuvant do not always induce potent immune response *in vivo*. Epitope peptide should deliver to professional antigen presenting cells (APCs) for subsequent enhancement of T cell responses. Therefore the selection of suitable carrier material is important to develop effective peptide vaccine. In previous studies, liposome has been used as a peptide carrier to induce epitope specific CTL *in vivo*. However, there are several disadvantages (such as high production cost, low stability, difficulty of large-scale production) to continue using it. New carrier candidates were explored to improve these disadvantages in this study.

To narrow down many carrier candidate molecules, chitosan, dendrimer and carboxymethyl cellulose (CMC), which have been researched as a carrier of the drug delivery, were selected as candidates of the peptide carrier from other many carrier candidate molecules. Furthermore we focused on Phosphatidilserine (PS) as new peptide carrier candidate. Because PS is exposed on the surface of apoptotic cells, it works as a trigger of phagocytosis for phagocytes

[Methods] To examine which candidates were the most suitable, *in vivo* cytotoxicity assay was performed. C57BL/6 (B6) mice were immunized subcutaneously with each carrier conjugated peptide plus adjuvant. Seven days after the final immunization, epitope specific killing activity was analyzed.

[Results and Conclusions] In the result, although conjugation of chitosan, dendrimer or CMC did not enhance immune response compared to peptide alone, PS-conjugation enhanced immunogenicity effectively. Moreover the functional mechanism analysis showed that PS-conjugated antigen was delivered to professional APCs selectively.

Therefore this PS carrier strategy can be harnessed to the development of useful CTL peptide vaccine.

The role of mouse oligoadenylate synthetase-1 family

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West Nile Virus (WNV) is an enveloped, positive-sense single-stranded RNA virus, a member of the *flaviviridae* family. The natural transmission cycle of WNV involves birds and mosquitoes of the genus Culex. However, it occasionally involves horses and humans as incidental hosts. In humans, most cases of WNV disease are subclinical. However, a small percentage of patients, less than 1%, develop clinical symptoms ranging in severity from fever to fatal meningoencephalitis. One of the most important immune response proteins to the viral infection is the interferon-induced oligoadenylate synthetase (OAS) family. The double-stranded RNAs produced in virus-infected cells activate OAS enzymes. The 2-5-oligoadenylates produced by activated OAS bind to latent RNase L, leading to the activation of RNase L, followed by degradation of cellular and viral RNA. In mice, the *Oas* gene family locates on Chr 5 and forms *Oas* gene cluster consisting of *Oas1a* through *Oas1h* (8 genes), *Oas2*, *Oas3* and two *OasL* genes. Among the mouse *Oas1* gene family, the *Oas1b* is a critical determinant for genetic susceptibility to the WNV infection in mice. The *Oas1b* gene is shown to possess an anti-flavivirus activity, although it does not possess enzymatic activity. On the other hand, the Oas1a protein is shown to possess the enzymatic activity; however, the anti-viral activity is unknown. Furthermore, the roles of other *Oas1* gene families are little known. In this study, therefore, I will focus on

1-Evaluate the relationship between antiviral and enzymatic activities in both mouse Oas1a and Oas1b.
2-Elucidate the function of other Oas1 families in the mouse.

Results: Flag-conjugated mouse *Oas1a* and *Oas1b* gene constructs were produced by cloning PCR products into mammalian expression vector, IRES-GFP. Accuracy of gene constructs was confirmed by PCR and digestion with several restriction enzymes.

Future plan:

Transfect *Oas1a* and *Oas1b* gene constructs into BHK cells and determine the antiviral activity using WNV -SEAP replicon as well as measure the enzymatic activity of both gene products.

Elucidate the relationship between antiviral and enzymatic activities in both Oas1a and Oas1b by using site-directed mutagenesis technique.

Expression level of the polymerase mRNAs of A/Hong Kong/483/97 (H5N1) was extensively higher than that of a mutant virus with PB2 of a single amino acid substitution at 627 Lys to Glu in mouse cells

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Influenza virus polymerase complex of the PB2, PB1, and PA proteins mediates transcription and replication of the viral genes in the nuclei of the host cells. Since the replication efficiency of the virus determines the pathogenicity in the host, the viral polymerase is responsible for the pathogenicity of the virus. Hatta *et al.* (2001) found that the pathogenicity of A/Hong Kong/483/97 (H5N1) (HK483-K) was higher than that of the mutant virus, HK483-E of which Lys at position 627 of the PB2 was substituted with glutamic acid, in mice. However, it is not well understood why HK483-E lost pathogenicity in mice by a single amino acid substitution of the PB2 protein of HK483-K.

In the present study, pathogenicity of HK483-K and HK483-E generated by using reverse-genetics was compared in mice. The pathogenicity of HK483-K in mice was 1,000 times higher than that of HK483-E. Then, growth of the viruses and expression level of viral RNAs and proteins in mouse NIH/3T3 cells infected with HK483-K were compared with those infected with of HK483-E. The viral growth of HK483-K was higher than that of HK483-E in NIH/3T3 cells. High expression of the mRNAs of the PB2, PB1, and PA were detected in the cells infected with HK483-K. On the other hand, the expression level of the mRNAs of HK483-K except those of the PB2, PB1, and PA were comparable to those of HK483-E. In the cells infected with HK483-K, large amounts of the PB2, PB1, and PA proteins were found in the nuclei. Present findings suggest that extensive expression of mRNA and proteins of the PB2, PB1, and PA induces efficient viral growth, causing severe disease in mice.

Host factors responsible for the pathogenesis of highly pathogenic avian influenza virus in chickens

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Outbreaks of highly pathogenic avian influenza have caused enormous economic losses in poultry industry and public health threat over the world. The reason why chickens suddenly die due to infection with highly pathogenic avian influenza viruses (HPAIVs) is not well understood. The results of experimental infection with 2 HPAIVs, A/turkey/Italy/4580/1999 (H7N1) (Ty/Italy/99) and A/chicken/ Netherlands/2586/2003 (H7N7) (Ck/NL/03), via intranasal route in chickens revealed that 50% chicken lethal dose of Ty/Italy/99 was 10^{-4} of that of Ck/NL/03. A/chicken/Ibaraki/1/2005 (H5N2) (Ck/IB/05), which is a low pathogenic avian influenza virus, caused non-overt infection in chickens. For the better understanding of the basis of the pathogenesis of HPAIVs in chickens, $10^{6.0}$ EID_{50} of each strain was inoculated intranasally into chickens, and then viral growth in and host immune responses of the birds at the early stage of infection were compared.

Ten to the 6.0 EID_{50} of Ty/Italy/99, Ck/NL/03, or Ck/IB/05 were inoculated intranasally into chickens, and 3 chickens per group were euthanized and collected their peripheral blood, lungs, spleen and brain at every 24 hours. All of the chickens inoculated with Ty/Italy/99 and Ck/NL/03 showed typical disease signs such as depression, cyanosis, hemorrhage and conjunctiva. Growth curves of these viruses in each tissue of the chickens indicated that Ty/Italy/99 replicated efficiently, especially in the brain, at the early stage of infection, and Ck/NL/03 replicated more slowly than Ty/Italy/99. The chickens inoculated with Ck/IB/05 did not show any disease signs and viruses were occasionally recovered only from lungs and spleen. Real-time PCR analysis revealed that mRNA level of IFN-α, IL-1β, IL-6, and IFN-γ in each tissue of the chickens inoculated with Ty/Italy/99 significantly increased in corelation with extensive replication of virus, and that of the chickens inoculated with Ck/NL/03 was less extensive. The cytokine response was outstanding in the brain. In the tissues of the chickens inoculated with CK/IB/05, no increase of mRNA of the cytokines was observed.

The present results indicate that rapid replication of virus induces hyper production of cytokines, leading multiple organ disorder followed by sudden death of chickens.

Roles of ubiquitin and heat shock protein 70(Hsp70) in the development of rabies virus (RBV) inclusion

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[Background and Hypothesis]

RBV causes fetal neural disorder called rabies. Although rabies is important issue in global public health, the biology of RBV is poorly understood and the unaware on RBV impedes the development of effective therapeutics other than immunization.

Replication of genome and transcription of mRNA are essential events for viruses. In the case of RBV, these events are reported to be taken place in inclusion body (IB) in which viral components aggregate. Thus, the formation of IB in host cell is critical for RBV. However, the mechanism how the IB develops has been still unveiled.

We hypothesized ubiquitin and Hsp70 play roles in gathering viral protein to form IB because these proteins function in protein transport system and were found in IB.

[Results]

To confirm this hypothesis, inhibitor of ubiquitination (E1 ligase), UBEI41 and two inhibitors for Hsp70, Quercetin and Pifithrin-μ, were used respectively. Treatment of each inhibitor to RBV-infected cells significantly reduced the spread of infection and the number of infective viral particle in the medium. In non-treated cells, IBs are firstly noted as small spots (early IBs), gradually grow in size (middle-IBs) and finally segregated from middle-IBs as numerous small spots (late-IBs) which seems responsible for transmission because only late-IB can be found in the axon. UBEI41 inhibited the formation of middle-IB, while Hsp70-inhibitors suppressed the segregation of late-IB. PCR assay detected the increase of viral genome byHsp70 inhibitor and decrease by UBEI41. The amount of viral mRNA was not changed significantly.

Further, correlation of IBs and endosome were investigated. As a result positive signals of Rab9, a marker for late endosome, were docked with nucleoprotein, while early or recycling endosome marker has no contact with nucleoprotein.

[Conclusion]

Ubiquitin and Hsp70 play essential role in the development of IB in different way and inhibition of these proteins can prevent the viral transmission. Thus, Hsp70 is a possible target for anti-RBV drug. However, further investigation is needed to clarify how the IB develops and to find more specific drug target molecules for RBV.

Characterization of protooncogene TCL1b as an Akt kinase co-activator

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The intracellular Akt signaling pathway is activated by various cytokines or growth factors and mediates intracellular signals to regulate a wide variety of cellular responses, including anti-apoptosis, proliferation, cell cycling, protein synthesis, glucose metabolism, and telomere activity. Genomic mutations, alterations of the Akt regulatory signaling pathway underlie various human diseases such as cancers, glucose intolerance (or diabetes mellitus), schizophrenia, and/or autoimmune diseases.

Recently, in addition to direct involvement in tumorigenesis by genetic alterations of human cancers, the Akt signaling network also underlies the clinical manifestation of various stages of tumorigenic viral infection, such as latent infection, chronic infection, and malignant transformation of the Epstein-Barr virus, the Hepatitis C virus, the Hepatitis B virus, or the Human Immunodeficiency Virus (HIV).

We have previously demonstrated that protooncogene TCL1 (T cell leukemia 1), translocated in human T cell prolymphocytic leukemia (T-PLL), is an Akt kinase co-activator. Three TCL1 isoforms have been identified, namely TCL1, TCL1b, and MTCP1 with predicted molecular weight of 13-15 kDa. The TCL1 families share a relatively high degree of amino acid homology (30-50%) and a unique, symmetrical β-barrel structure. In human T-PLL, both TCL1 and TCL1b genes are activated by juxtaposition onto the T-cell receptor α or β loci. Therefore, it remains unclear whether TCL1b itself bears oncogenicity. In this study, we investigated whether and how TCL1b might have similar function as Akt kinase co-activator. In co-immunoprecipitation assays, TCL1b co-immunoprecipitated with Akt. In vitro Akt kinase assays, TCL1b enhanced Akt kinase activity. Moreover, by Agilent Expression Array analysis, both TCL1 and TCL1b showed highly significant homologous gene induction pattern as myr-Akt, a constitutive active form of Akt. The observations are consistent in Gene Ontology, KEGG pathway, and cluster analysis. These observations together suggested that analogous to TCL1, TCL1b also functions as an Akt kinase co-activator, and possibly plays an active role in oncogenicity in human T-PLL. And we comfirmed oncogenicity of TCL1b by colony transformation assay.

Furthermore, anti-viral activity by suppression of Akt activity may provide alternative therapeutic opportunities that can prevent malignant transformation by suppression of tumorigenic viral infection rather than directly targeting anti-cancer activities via oncogene.

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