論文の内容の要旨

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Molecular Epidemiological Studies on Animal Papillomaviruses

（動物のパピローマウイルスに関する分子疫学的研究）

Introduction and aim of this study

Papillomavirus (PV) is a non-enveloped double-stranded DNA virus, classified into the family, *Papillomaviridae*. Infection of PVs has been associated with cutaneous and/or mucosal tumorigenesis of vertebrae species in both benign and malignant manner. The viral genome is comprised of two capsid-coding genes (L1, L2), several early genes, and a non-coding region called the long control region (LCR). In general, PVs harbor tissue- and species-specific characteristics. PVs are classified based on the nucleotide sequence identity of the L1 open reading frame (ORF). Based on this classification criteria, more than 200 types of human PVs (HPVs) and animal PVs each, have been characterized. In the human medicine field, worldwide molecular epidemiological studies on HPVs contributed to identifying the etiological roles of cervical cancer- and anogenital wart-associated HPVs. Basic studies on molecular epidemiology of HPVs contributed to applied research, leading to the success in HPV vaccine development. In contrast to human medicine, no commercialized animal PV vaccines are currently available in veterinary medicine. In both large (cattle and horses) and small (dogs and cats) domestic animals, various types of PVs have been identified from cutaneous/mucosal neoplasms. Therefore, this study aimed to identify PVs associated with neoplastic lesions in domestic animals and contribute to the development of preventive strategies such as vaccines.

In large animals, *Bos taurus* (Bovine) PVs (BPVs) have been studied for decades, and harbor the most numbers of genotypes. Identification of BPVs have been reported worldwide over the decades, but unlike humans, there is limited information describing viral association with anogenital lesions. Studies on BPVs, especially two *Deltapapillomavirus*-classified BPV types, 1 and 2 (BPV1/2) have been providing the knowledge of genomics, biological, and immunological properties of PVs. Both BPV1/2 have been associated with fibropapilloma development of the natural host (cattle), but BPV1/2 are also considered as unique types showing interspecies infection to equids, causing sarcoid tumors. Previous studies suggest that BPV sequence phylogeny may have an association with either of the two classifiers, host species or geographic distributions, but the significance of these hypotheses has not been fully elucidated.

In small animals, especially cats, PV-associated diseases have become clinical problems since *Felis catus* PV (FcaPV) infection has been characterized from malignant neoplasms such as squamous cell carcinomas (SCCs). Among the six FcaPV types, FcaPV2 has been identified the most from these malignant tumors, but the detection has been limited to

overseas. In particular, aggressive and malignant tumors become clinical problems in domestic animals, so the development of prophylactic vaccines would become valuable. However, due to the limited numbers and geographical origin of the publications, significant association between PV genotype(s) and disease phenotype(s) is uncertain. Based on these backgrounds, this study aimed to clarify the etiological roles of animal PVs in cutaneous/mucosal neoplasms by molecular epidemiological approach, focusing on three topics as follows.

Chapter 1: In animals, information on anogenital-associated PVs is limited in contrast to human studies. Although previous studies have been describing anogenital tumorigenesis in cattle, few attempts have been made to detect BPVs. Therefore, this chapter aimed to evaluate the pathogenicity and genomic features of BPVs in bovine anogenital neoplasms.

Chapter 2: Based on the previous findings that BPV1/2 sequences may be affected by species or geographic classifiers, this study intended to solve this question by comparing BPV1/2 sequences derived from bovine/equine hosts and different geographic origins.

Chapter 3: The significant pathogenicity and type(s) of FcaPV(s) associated with feline SCC is uncertain due to limited publications. Therefore, this study aimed to define the associated type(s) and sequence properties of FcaPVs in SCCs of cats kept in Japan.

Chapter 1: Genomic characterization of Bovine papillomaviruses associated with anogenital tumors of cattle

Since the 1950s, previous studies have been describing anogenital tumor development in cattle. However, few attempts have been made to detect BPVs from bovine anogenital neoplasms. Therefore, this study aimed to clarify the histopathology of bovine anogenital lesions and to characterize genomic features of anogenital-associated BPVs.

Anogenital wart samples collected from five dairy cows were analyzed. Immunohistochemistry (IHC) was conducted to examine the PV antigen within the lesion specimens. BPV DNA was detected by PCR and sequencing. Reverse-transcriptase PCR (RT-PCR) was performed to determine the mRNA expressions of both early and late genes.

Histopathology, PCR, and sequencing revealed that BPV1, BPV2, and two unclassified BPV types were identified in one anal fibropapilloma, two vulval fibropapilloma, and two vulval papilloma lesions, respectively. The L1 sequence homologies of two unclassified BPVs were 75% and 77% against BPV15 and BPV6, respectively, suggesting that these two BPVs should be classified as new genotypes. The novel BPV types were both classified into the genus, *Xipapillomavirus* and designated BPV28 and BPV29. BPV28 was detected in two vulval papilloma lesions excised from one cow. One of the BPV28 isolates harbored frameshift mutation in the L1, resulting in two ORFs. mRNA detection of the early genes, but not the late gene (L1) was confirmed in all of the BPV-detected cases, suggesting the active contribution of BPVs to the lesion development. Immunohistochemically, no positive signals of L1 antigen were observed except for one BPV2-positive case showing PV antigen in a few differentiated keratinocytes. IHC and RT-PCR results suggested that no or very few productions of encapsulated viruses were taken place.

This study confirmed the detection of two classical *Deltapapillomavirus* BPV types, 1 and 2, from anogenital fibropapillomas, and suggests their mucosal tissue-tropisms in addition to the cutaneous sites. Moreover, two novel *Xipapillomavirus*-classified BPV types, 28 and 29, were shown to have an association with bovine vulval papillomas. The

present study extends the knowledge of BPV genomic diversities and anogenital-associated PVs in animals.

Chapter 2: Phylogeny-trait association analyses of Bovine papillomavirus types 1 and 2 identified in cattle and horses kept in Japan

Deltapapillomavirus-classified BPV types, 1 and 2, have been known to show cross-species infection to horses, leading to the development of sarcoids. Equine sarcoids are non-metastatic skin tumors but they could be aggressive and impeditive to equine vital function. The viral mechanism of the interspecies infection has not been revealed, but previous molecular epidemiological studies noted the sequence variants associated with equine sarcoids within the E2, E5, and LCR of BPV1. On the other hand, some studies have discussed that the variants may be associated with geographical origin. This study aimed to clarify whether equine/bovine-derived BPV1/2 sequences collected in Japan, harbor host or geographical trait association. In addition to the three viral regions (E2, E5, LCR), the L1 region was also included, as L1 sequence data become essential information in terms of developing BPV1/2 vaccines.

Ten equine sarcoid-suspected samples and bovine papilloma lesions each, collected in Japan were included in this study. Histopathological diagnosis was demonstrated to characterize the lesion phenotype. BPV1/2 sequences of the four viral regions (E2, E5, L1, LCR) were identified by PCR and sequencing. Obtained sequences were aligned by ClustalW tool, and sarcoid-associated variants were determined. Phylogenetic trees were constructed using Bayesian Markov chain Monte Carlo (MCMC) method including the BPV1/2 sequence available in the GenBank database. The phylogeny-(host/geographical) trait correlation analysis was demonstrated using Bayesian tip-association significance testing (BaTS). Histopathology, PCR, and sequencing showed that seven BPV1 and three BPV2 were identified from equine sarcoids. Equine/bovine-derived samples showed no sarcoid-associated variants in four regions (E2, E5, L1, LCR) of neither BPV1 nor BPV2. The phylogenetic tree of BPV1 E2, L1, and LCR inclined to cluster within its geographical origins. BaTS analysis demonstrated that BPV1 sequence variability may be due to the geographical origin than host species difference. The present study supports the geographic-specific hypothesis of BPV1 sequence variability, indicating that BPV1 may be shared between local equids and bovids. More worldwide-collected BPV1/2 sequence data and biological significance are needed to strengthen this hypothesis.

Chapter 3: Detection and sequence characterization of *Felis catus* papillomavirus types 3 and 4 associated with squamous cell carcinoma of cats in Japan

Geographical dependence of sequence variation has been suggested from the results of BPV1/2 phylogeny-trait analyses in Chapter 2. This phenomenon has also been noted from the studies on HPVs, and are concerned that sequence alterations within the L1 may affect HPV vaccine efficacies. When considering the issue of PV prophylactic vaccine development for animals, a vaccine should be universally applicable and be protective against life-threatening diseases such as malignant neoplasms. In cats, FcaPVs have been described to be one of the causative agents of SCCs, the common malignant tumor in senior aged cats. Therefore, a vaccine targeting FcaPV should become valuable in the veterinary field. FcaPV2 has been identified the most from overseas in feline SCCs. However, geographical discrepancies may also arise in FcaPV type

distributions. This study aimed to identify the etiological association of FcaPV(s) with SCCs and to clarify the sequence characteristics of SCC-derived FcaPV(s) from cats kept in Japan.

Twenty-one feline biopsy samples, diagnosed as SCCs, collected in Japan were analyzed in this study. IHC was carried out to examine the expression of p16 and PV antigen within the SCC specimens. Detection of FcaPV DNA was demonstrated by PCR, applying both consensus and FcaPV L1 type-specific primers targeting FcaPV types, 2, 3, and 4. Sequencing was performed to characterize FcaPV sequences. Moreover, RT-PCR was conducted to determine mRNA expressions of the two oncogenes, E6 and E7 (E6/E7).

Among the 21 analyzed SCC samples, one FcaPV3 and two FcaPV4, but not FcaPV2 were detected by PCR and sequencing. E6/E7 mRNA and p16 expressions were noted in both FcaPV4-positive cases, suggesting that the development of SCC lesion was caused by FcaPV infection. Compared with the FcaPV4 L1 reference sequence, one of the FcaPV4 isolates harbored 95.7% (1465/1536 nt) sequence identity and suggested to be classified as a novel subtype of FcaPV4.

The present study confirmed the involvement of FcaPV3 and FcaPV4 in SCCs of cats owned in Japan. Moreover, a novel subtype of FcaPV4 was characterized. FcaPV2 was not detected in this study, suggesting that geographical differences may have caused these discrepancies. This study extends the knowledge of FcaPV sequence diversities and its etiological roles in feline SCCs.

Conclusion and future contribution

By molecular epidemiological approach, the present study described etiological roles and genomic characteristics of PVs in domestic animals. In Chapter 1, two classical BPV1/2 and two novel BPV28/29 were suggested to have the potential to cause anogenital neoplasms in cattle, providing valuable information on etiological and genomic features of anogenital-associated PVs in animals. Chapter 2 described that BPV1/2 sequence identities were associated with geographical trait rather than the host species, indicating that BPV1/2 is shared between local cattle and horses. This study suggested that a BPV1/2-vaccine is applicable for both species. In Chapter 3, the association of FcaPV types 3 and 4 in feline SCCs were described, and geographical discrepancies of FcaPV type-distribution were suggested. In addition to FcaPV2, inoculating types 3 and 4 needs to be concerned in terms of vaccine development.

These findings enriched our understanding of sequence diversities and etiological roles of animal PVs, contributing to the establishment of PV-associated disease prevention and treatment strategies.