Risk Factors and Treatments of Idiopathic Epilepsy in Dogs

犬の特発性てんかんの発症の素因および治療に関する研究

2019 年 2 月

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Abstract

Introduction

Epilepsy is one of the most common chronic neurological disorders that affects both dogs and humans. The type of epilepsy affecting about half of the epileptic dogs is idiopathic epilepsy (IE). There is no standardized definitive treatment for IE, and most of the dogs affected by this disease need to be medicated with an anti-epileptic drug (AED) throughout their lifespans to prevent epileptic seizures. Despite being administered adequate doses of AEDs, 20–30% of the epileptic dogs remain inadequately treated for seizure control. Seizures deteriorate the quality of life of both the affected animals and the owners of these animals. Therefore, in Chapter 1, the author retrospectively investigated the clinical data corresponding to dogs with IE in Japan to search for predisposing factors to this condition and to identify problem areas in the treatment of IE that have the potential to be improved upon for further investigation. In Chapter 2, the author focused on potassium bromide (KBr), which is an effective AED to control seizures in dogs; however, there are problems associated with administering this treatment in some clinical cases. Next, in Chapter 3, a search was

conducted for the risk loci of IE in a dog breed that was overrepresented in the retrospective study (Chapter 1) using a genome-wide association study (GWAS) and a direct sequence method.

Chapter 1: A retrospective study of canine idiopathic epilepsy in referral centers in Japan

The aim of this chapter was to describe the clinical data associated with dogs affected by IE in Japan and to search for predisposing factors responsible for this condition. Furthermore, another purpose was to address the problems encountered with the AED treatments administered to dogs with epilepsy. The medical records and clinical information corresponding to dogs diagnosed with IE at 2 referral centers between April 2013 and March 2016 were retrospectively reviewed. The review was conducted according to the consensus statements published by the International Veterinary Epilepsy Task Force. The data of a total of 70 dogs that had a median (minimum–maximum) weight of 5.15 (1.85–79.85) kg and were at a median age of 4.2 (0.3–11.8) years at the initial onset of epileptic seizures were used. Forty-four of these dogs were male, and 26 were female. Toy poodles were overrepresented in the present study

indicating that this breed may be predisposed to IE and would be a good candidate for a gene study to elucidate the cause of IE in dogs. Moreover, the cases suspected to be refractory among these were being administered 2 or more AEDs. Since 67 % of the dogs with a poor therapeutic outcome were on potassium bromide (KBr), it was hypothesized that optimizing the KBr treatment would contribute to the improvement of the therapeutic outcome.

Chapter 2: The effects of chloride in the diet on serum bromide concentrations in dogs

Since it is known that a high chloride intake decreases the serum bromide concentration, it was considered that elucidating the relationship that exists between these parameters would provide useful data. Therefore, in this chapter, the author clarifies the relationship between the dietary chloride intake and serum bromide concentrations. Steady-state serum bromide concentrations were measured using a gold chloride method in 23 dogs treated with KBr. The content of dietary chloride (per 1 g) was measured using the Mohr's method or calculated from the ingredients label provided by the manufacturer of the dog food. A regression analysis was used to evaluate the

relationship between dietary chloride intake (mg/kg/day) and the serum bromide concentration per dose (μg/ml per mg/kg). Dogs with a higher chloride intake had lower serum bromide concentrations. A strong negative correlation was observed between the dietary chloride intake (mg/kg/day) and the serum bromide concentration per dose (μg/ml per mg/kg) ($P < 0.01$). These results suggest the importance of considering the dietary chloride content for KBr-treated dogs and may also assist clinicians with the selection of KBr doses and appropriate diets for dogs treated with KBr.

Chapter 3: The investigation of the candidate gene associated with IE in toy poodles

Since toy poodles were overrepresented in the investigation conducted in Chapter 1, it was indicated that this breed may be predisposed to developing IE and, hence, would be a candidate for the gene study to elucidate the cause of IE in dogs. Therefore, in this chapter, the author tried to locate the loci that are related to IE in toy poodles. Using 10 and 22 toy poodles with and without IE, respectively, a genome wide association study

(GWAS) was performed. The identified candidate variant was validated using a standard PCR, followed by Sanger sequencing in an additional sample of 39 toy poodles (23 affected and 16 unaffected). The GWAS and confirmation study revealed a significant variant single nucleotide polymorphism (SNP), rs22063361, in chromosome 10. Exonic sequencing was performed on 1 of the candidate genes, *ELFN2,* and its potential to be involved in the pathogenesis of IE in dogs was evaluated. The analysis of the coding region of *ELFN2* did not reveal a causative mutation responsible for IE. Therefore, further investigation is needed to elucidate the relative gene responsible for IE in this breed.

Conclusion

The author described the clinical data corresponding to canine IE in Japan, elucidated the relationship that exists between the dietary chloride intake and serum bromide concentration. Although the present study could not reveal the association between specific candidate gene and toy poodle with IE, these findings of the present study act as a resource to assist clinicians in treating dogs with IE and provide multiple basic data to facilitate further investigations of canine IE.

Content

Abbreviations

AED, anti-epileptic drug

AUVTH, Azabu University Veterinary Teaching Hospital

CAMIC, Companion Animal Medical Imaging Center

CLP, clorazepate.

CS, cluster seizure

DNA, deoxyribonucleic acid

EEG, electroencephalography

FES, focal epileptic seizure

FEvG, focal epileptic seizure evolving into generalized epileptic seizure

GES, generalized epileptic seizure

GWAS, genome-wide association study

IE, idiopathic epilepsy

IVETF, nternational Veterinary Epilepsy Task Force

KBr, potassium bromide

LEV, Levetiracetam

NaBr, sodium bromide

ORs, odds ratios

PB, Phenobarbital

QOL, quality of life

SE, status epilepticus

SNP, single nucleotide polymorphism

StE, structural epilepsy

ZNS, Zonisamide

General Introduction

Epilepsy is one of the most common chronic neurological disorders that affects both dogs and humans. Its prevalence has been reported to be 0.6–0.75% in dogs [1,2]. Epilepsy can be classified into 2 types based on its etiology: structural epilepsy (StE) and idiopathic epilepsy (IE). StE is defined as epileptic seizures that are provoked by intracranial or cerebral pathologies including vascular, inflammatory/infectious, traumatic, anomalous/developmental, neoplastic, and degenerative diseases confirmed by diagnostic imaging, cerebrospinal fluid examination, deoxyribonucleic acid (DNA) testing, or post mortem findings [3]. On the other hand, IE is defined as an epilepsy of a predominantly genetic or presumed genetic origin and in which there are no gross neuroanatomical or neuropathological abnormalities nor any other relevant underlying diseases causing the seizure activity [3]. The epilepsy in about half of the epileptic dogs is classified as IE [3]. In dogs with IE, the onset of epileptic seizures usually begins at a young age, often developing when they are between 6 months and 6 years old [4].

The mainstream treatment for IE involves controlling the seizures by using anti-epileptic drugs (AEDs). To date, several AEDs are available for dogs worldwide. These include phenobarbital (PB), bromide, zonisamide (ZNS), imepitoin, and levetiracetam (LEV) [5, 6]. Each of these AEDs have their advantages and drawbacks. For example, PB is an effective and well-tolerated drug that has been used for a long time in veterinary medicine. Among the AEDs, it has the highest evidence level supporting its efficacy [5]. However, PB is an auto-inducer of hepatic microsomal enzymes (p450 system), which can progressively decrease its own elimination half-life and that of other drugs with chronic dosing [7–9], and in less common cases, usually in cases involving the administration of high doses of this drug, it can lead to the development of hepatotoxicity. Meanwhile, bromide is another AED that has a long history of use in dogs and a high evidence level supporting its efficacy. Some disadvantages of this drug are that the efficacy of PB was reported to be better than that of bromide in 1 report [10] and that its excretion is influenced by the systemic chloride concentration since the amount of bromide excreted depends on the total body halide

concentration [11]. Therefore, it is recommended to keep the chloride intake stable while administering bromide.

Although several treatments are available and have proven their effectiveness on seizure control in dogs, to date, there is no standardized definitive treatment for IE. Most of the dogs with this disease need to be medicated with AEDs throughout their lifespan to prevent epileptic seizures. Nevertheless, despite being administered adequate doses of AEDs, 20–30% of the epileptic dogs remain insufficiently treated for seizure control [12–14]. Reducing the seizure frequency is a critical factor in the treatment of IE dogs since a seizure frequency of fewer than 0.3 times/month has been reported to be associated with the epileptic dogs surviving for a longer time [15]. Moreover, the same seizure frequency was proposed as the cutoff value for an acceptable seizure frequency for the comfort of the owners of the epileptic dogs based on a study on the quality of life (QOL) of the owners and their epileptic dogs [16]. Insufficient seizure control deteriorates the QOL of both the affected animals and the owners of these animals [17–19].

The cause of IE is suspected to be genetic although the genes associated with IE are poorly understood in dogs. In humans, although the significance of many of the genes and their genetic variations on epilepsy is still unclear, more than 500 genes are reported to be associated with epilepsy including IE [20]. However, in dogs, there are only 3 genes, *LGI2*, *ADAM23*, and *DIRAS1*, reported to be associated with IE in specific breeds (*LGI2*, lagotto Romagnolo; *ADAM23,* Belgian shepherd; *DIRAS1*, Rhodesian ridgebacks) or specific phenotypes [21–23], but the causative gene responsible for IE in the other breeds remains unknown. Therefore, further investigation is required to identify the causative gene responsible for IE in other popular breeds. The discovery of a novel gene responsible for IE in dogs would potentially result in many benefits, such as it can aid in understanding the etiology of the disease further, in developing new treatments, and in establishing a DNA test to detect IE for breeding purposes. Moreover, it may introduce a novel candidate gene for human IEs since there has been a keen interest in dog breed models of inherited epilepsy as large animal models for epilepsy in humans [24].

In this study, the author performed studies using multiple approaches to contribute to the current treatments and to reveal the pathogenesis of IE in dogs. In Chapter 1, the author conducted a retrospective study to highlight the disease distribution of IE among dogs in Japan and to search for any subject that warranties the need for further investigation. Next, in Chapter 2, the author focused on KBr administration and clarified the relationship that exists between the dietary chloride intake and serum bromide concentration in detail. Finally, in Chapter 3, the genetic study conducted to identify the causes of IE using a breed that is potentially predisposed to this condition is described.

Chapter 1

Retrospective study of canine idiopathic epilepsy in referral centers in Japan

Introduction

Recently, the International Veterinary Epilepsy Task Force (IVETF) was organized and a series of consensus statements on canine epilepsy were published in 2015 [3, 4, 6, 25–28]. According to the IVETF consensus statements, IE is defined as an epilepsy of a predominantly genetic or presumed genetic origin and in which there are no gross neuroanatomical or neuropathological abnormalities nor any other relevant underlying diseases causing the seizure activity [4]. Today, there are 3 genes reported to be associated with IE in specific dog breeds [21–23], but the cause behind the development of IE remains unknown. A further genetic study is needed to elucidate the cause of IE.

To date, there were several epidemiological studies conducted on epilepsy in dogs. However, studies that describe detailed data associated with IE are limited [15, 29–31]. Some of the predisposing factors responsible for the development IE were described consistently throughout the previous studies; however, many of the others were not. For example, male dogs were identified as being more likely to develop IE in all of the studies whereas the overrepresented breeds differed among the studies. In reports from

Western countries, the population of dogs with epilepsy included a large proportion of large-breed dogs [29–31] whereas in the report from Japan, small-breed dogs were predominantly affected [15]. Considering these previous reports, the epidemiological distribution of this condition seems to vary from region to region due to the differences in the populations of the dogs breeds between Western countries and Japan suggesting that a concise understanding of the disease distribution in each region would be a critical factor to consider when performing an investigation on IE. Nevertheless, there is only 1 epidemiological study recently reported from Japan that was conducted on epilepsy in dogs [15]. In this study, the epidemiological distribution, risk factors, survival time, and lifespan associated with 172 dogs with epilepsy, including both structural and idiopathic epilepsies, through the period between 2003–2013 were retrospectively investigated. Since some of the results of this study differed from those of previous reports and no predisposed breed was identified, a further study on IE is required to understand the clinical data of the disease and to conduct an efficient study on IE in our country. Therefore, we focused on IE in dogs and aimed to describe the clinical data of the disease associated with the recent dog breeds that are popular among

the population of Japan in order to search for any subject whose data warranties further investigation.

Materials and Methods

Data collection and analysis

The medical records of dogs diagnosed with IE at the Neurology Service of the Azabu University Veterinary Teaching Hospital (AUVTH) and the clinical information of the dogs diagnosed with IE at the Companion Animal Medical Imaging Center (CAMIC) that was sent to the Neurology Service of the AUVTH between April 2013 and March 2016 were retrospectively reviewed. The diagnosis of IE was made according to the IVETF consensus statements [4]. The collected information included the signalment (body weight, the age at which the initial onset of the epileptic seizures occurred, sex, and breed) and seizure type. According to the IVETF consensus statements [4], the types of seizures were classified into focal epileptic seizures (FES), generalized epileptic seizures (GES), and focal epileptic seizures evolving into generalized epileptic seizures (FEvG). To analyze whether the dog breed is a risk factor for IE, the control

population consisted of all the dogs that were evaluated at the AUVTH between April 2013 and March 2016 and those that were not diagnosed with IE at the Neurology Service.

Further investigation was performed on the data associated with the dogs diagnosed with IE at the Neurology Service of the AUVTH. These data included information on the duration of seizures, whether the dog had ever had an episode of cluster seizures (CS) or status epilepticus (SE), autonomic signs during the seizure, prodrome or postictal phenomena, short-term (6 months) therapeutic outcomes, and the QOL of the dogs and their owners. According to the IVETF consensus statements [26], the dogs were evaluated based on the therapeutic outcomes that they achieved; the therapeutic goals were categorized into the following 3 levels: primary goal (seizure free or seizure frequency decreased by one-third and seizure frequency is < 0.3 times/month), secondary goal (cluster seizure/status epilepticus free or relatively decreased seizure frequency or decreased seizure severity), and no responder (outcomes other than the primary and secondary goals). To evaluate these goals, the owners were asked to answer

a questionnaire [17] during their visit to the AUVTH or to send the questionnaire in by mail. Informed consent for research participation was obtained from all the owners.

Statistical analysis

The Kruskal-Wallis test was used to evaluate the differences in the duration of seizures among the seizure types. The breed distribution of dogs with IE (the breed that had 5 or more dogs diagnosed with IE) was compared to the breed distribution of the control dogs examined at the AUVTH. *P* values, odds ratios (ORs), and their 95% confidence intervals (CIs) were calculated corresponding to each of the comparisons made. P values were calculated by means of Fisher exact tests. The value of $P < 0.05$ was considered significant. Statistical analyses were performed using JMP®7.0.1 (SAS Institute Inc.).

Results

Twenty-eight dogs were diagnosed with IE at the Neurology Service of the AUVTH; in addition, the clinical information of 42 dogs diagnosed with IE at the CAMIC was sent

to the Neurology Service of the AUVTH. The data corresponding to a total of 70 dogs with IE that had a median (minimum–maximum) weight of 5.15 (1.85–79.85) kg and were at a median age of 4.2 (0.3–11.8) years at the initial onset of epileptic seizures were used in this study. Forty-four of the dogs were male (27 castrated), and 26 were female (15 spayed). The breeds included 16 toy poodles; 10 Chihuahuas; 6 miniature dachshunds; 4 each of Italian greyhounds and mixed-breed dogs; 3 each of Cavalier King Charles spaniels and Yorkshire terriers; 2 each of American cocker spaniels, beagles, Pekingeses, Boston terriers, Pomeranians, and miniature schnauzers; and 1 dog from each of the following breeds: Pembroke Welsh corgi, cairn terrier, Shih-Tzu, Shetland sheep dog, Jack Russell terrier, Shiba, pug, Saint Bernard, papillon, French bulldog, miniature pinscher, and wire fox terrier. The 4,779 dogs examined at the AUVTH were included in the study as control dogs for the analysis of breed as a risk factor in developing IE. Among the control dogs, there were 423 toy poodles, 400 Chihuahuas, and 770 miniature dachshunds. The toy poodles had a higher probability of having IE ($P = 0.03$; OR = 3.08; 95% CI, 1.24–7.64) compared to the Chihuahuas ($P = 0.80$; OR = 0.44; 95% CI, 0.06–3.23) and the miniature dachshunds ($P = 0.73$; OR =

1.13; 95% CI, 0.43–2.99). Twenty-one (30%) dogs were affected by FES, 37 (53%) by GES, and 9 (13%) by FEvG. Two (2.7%) dogs had both GES and FEvG, and the seizure type affecting 1 (1.3%) of the dogs was unknown.

The median (minimum–maximum) duration of the seizures was 150 (15–900) seconds. The durations of the seizures of each of the seizure types are summarized in Table 1. There were no significant differences observed in seizure duration among the 3 types of seizures ($P > 0.05$). Fifty-seven percent and 39% of the dogs had CS and SE, respectively. Twenty-five percent of the dogs experienced both CS and SE. Autonomic signs were observed in 68% of the dogs. Urination was the most frequently (50%) observed autonomic sign. Fourteen percent and 68% of the dogs displayed prodrome and postictal phenomena, respectively. The statuses of the CS, SE, autonomic signs, prodrome, and postictal phenomena corresponding to each of the seizure types are provided in Table 2. We were able to evaluate the therapeutic outcome in 20 (71%) dogs. Sixty percent of these dogs were administered a monotherapy whereas the remaining 40% were administered 2 or more AEDs. Eight dogs were evaluated as

having achieved the primary goal; 6, the secondary goal; and the remaining 6 were no responders. The AEDs administered, seizure frequency prior to and after starting the AED treatment, and therapeutic outcomes corresponding to each of the dogs are provided in Table 3. Seven owners responded to the questionnaire on QOL. The answers to the questionnaire are summarized in Table 4. Although 7 (25%) of the owners responded to the questionnaire, none of them answered the question on whether their dog's seizure levels were unacceptable or severe.

Discussion

Toy poodles were overrepresented in our study. Toy poodles, Chihuahuas, and miniature dachshunds were the top 3 breeds that were most frequently diagnosed with IE per the findings of our study. Although these are popular breeds in Japan, the high ORs in toy poodles compared to in the other 2 breeds suggest that this breed might have a higher risk of developing IE. Several breeds were reported to be overrepresented in studies on IE from various countries. These breeds include beagles [31, 32], Labrador retrievers [33, 34], golden retrievers [25], German shepherds [29, 30], and border

terriers [29]. However, these breeds were not overrepresented in our study. This was possibly due to the differences in the breed populations between Western countries and Japan since the majority of our patients were small-breed dogs. One descriptive epidemiological study conducted on IE in dogs, in which more than half (51.1%) of the cases involved small-breed dogs, reported a high prevalence of IE in toy poodles [35]. However, our current study is the first study to report that the toy poodle is overrepresented among the breeds that develop IE. Therefore, toy poodles have the potential to be predisposed to developing IE, and hence, can be considered as 1 of the candidates among dog breeds that can be studied in order to investigate the association between genetic factors and the development of IE.

There are many studies on canine epilepsy; however, epidemiological studies or data on IE in the literature are less commonly available. There is a significant association between the age at the onset of epilepsy and cause of this condition in dogs. In the IVETF consensus statements, it was reported that dogs between the ages of 6 months and 6 years were more likely to be affected by IE [4]. One study on epilepsy from

Europe reported that 4.2 years old was the median age at the initial seizure onset in 115 dogs with IE [31], which is consistent with our current results. However, the median age of initial seizure onset was reported to be 2.5 years old in the previous report on IE from Japan [15]. One possible explanation for the previous study from Japan reporting the initial seizure onset occurring at a younger age [15] is that MRI and CSF analyses were required as part of the inclusion criteria for dogs with an onset age of more than 6 years old in this study, but this was not required in the current study or the study from Europe [31]. This criteria of the previous study from Japan [15] made it difficult to recruit dogs that were more than 6 years old in age. The percentage of male dogs (63%) diagnosed with IE was higher compared to that of female dogs (37%) in our study. This was consistent with several previous reports [15, 30–33] indicating that male dogs are prone to IE.

The dogs that experienced the episodes of CS (57%) and SE (39%) were observed in this study. In a 2001 study, it was reported that 19 of the 32 (59%) dogs with IE that were being studied had 1 or more episodes of SE [33]. The proportion of dogs that had

1 or more episodes of SE are low in our study compared to in this previous report [33]. This may be due to the increase in the number of effective AEDs in the veterinary field during the past 20 years. Although our results are not pertaining to the general population but to the population in the referral hospital, these data encourage the veterinarians to inform the owners about the risk of the development of CS and SE in dogs affected by IE. Most of the dogs experiencing the GES (92%) and FEvG (86%) seizure types displayed autonomic signs associated with their seizures whereas only 30% of the dogs experiencing FES displayed an autonomic sign. The differentiation between epileptic seizures and other episodic events are challenging in some situations. In general, it is known that autonomic signs are 1 of the indicators to suspect the onset of an epileptic seizure, and our results on an evaluation of generalized seizures are consistent with this observation. However, the differentiation of FES and other episodic events remains challenging since autonomic signs were observed only in a small proportion of dogs affected by FES (30%) in this study.

In human medicine, AEDs are selected based on the patient's seizure type. However, in dogs, there is no evidence to support selecting particular AEDs for specific seizure types. Therefore, an evaluation of the efficacy of AEDs on the specific types of seizures in dogs is required, but it is difficult to perform this type of study in a single institution. To address this problem, the IVETF advocated using a measure that is outlined in their consensus statements called the "evaluation of therapeutic outcome" in order to draw comparisons among institutions [25, 26]. Although this current study was not focused on only evaluating the therapeutic outcomes experienced by the dogs and although we could not evaluate the efficacy of different AEDs on the different seizure types, we utilized the "evaluation of therapeutic outcome" measure according to the IVETF consensus statements, and this makes it possible for our data to be utilized in future investigations conducted to evaluate the efficacies of different AEDs on the specific seizure types.

A certain number of the cases that were potentially refractory were being administered 2 or more AEDs (40%), and 4 (67%) out of the 6 dogs whose therapeutic outcomes were

evaluated as “no responder” were medicated with KBr. These findings might raise the possibility that optimizing the KBr treatment could improve the therapeutic outcomes. Therefore, since it is known that high contents of chloride in the diet decrease serum bromide concentrations in dogs [14,17], a detailed clarification on the relationship that exists between the dietary chloride intake and serum bromide concentration is needed to contribute to the improvement of the treatment of epilepsy in dogs being administered KBr.

A seizure frequency of fewer than 0.3 times/month is reported to be acceptable for the dog owners in Europe [19]. In our study, none of the owners indicated whether their dog’s seizure levels were unacceptable or severe. Though this could not be concluded from our observations, this might imply that there is a difference in the perception of seizures between the regions. Although the exact reasons were not known, the low percentage of owners answering the questionnaire (25%) might have been due to their dissatisfaction with the therapeutic outcomes of their epileptic dogs. Moreover, a seizure frequency of more than 0.3 times/month is reported as a risk factor in the

decrease of the survival time of dogs with epilepsy [15]. Therefore, it is important to inform the owners about the risks that can arise due to seizure frequency, especially in Japan. The questionnaire revealed objective data on the owners' thoughts about their dog's seizures and the problems that they encounter with the treatment of their epileptic dogs. Since it is said that the questionnaire on QOL provides useful data for improving the management of the epileptic dogs and for communicating with their owners [17], a clinician must be aware of the usefulness of the questionnaire on QOL when treating dogs with epilepsy.

A limitation of this study is that the control population consisted of all the dogs evaluated at the AUVTH that were not diagnosed with IE at the Neurology Service. Since this study was conducted at referral centers, the control population does not represent the general population in Japan. Moreover, there is a possibility that dogs that were diagnosed with IE at different services of the AUVTH were included in the control population. A further investigation on IE in dogs that involves the general population is needed in order to evaluate the validity of the dog breed being a risk factor for

developing this disease. Another limitation is that both the AUVTH and CAMIC are located in a closed region of Japan. Thus, further investigation involving IE dogs diagnosed at multiple places is required to reveal the clinical data associated with IE in the general population of Japan.

In conclusion, the author has described the clinical data associated with dogs affected by IE diagnosed in 2 referral centers in Japan. Toy poodles were overrepresented in the present study suggesting that this breed would be a good candidate for a genetic investigation. Moreover, the need to optimize the KBr treatment was raised in order to contribute to the improvement of therapeutic outcomes in dogs medicated with KBr. Since the data acquisition was performed in accordance with the IVETF consensus statements, this study can provide useful data to aid further investigations on IE in dogs.

Figures and Tables

Table 1 The duration of seizures (seconds) of each seizure type (median, range).

Seizure type	Duration of seizure (seconds)
FES (n = 10)	180 (15–900)
GES (n = 12)	135 (20–270)
FEvG (n = 7)	90 (25–150)
All cases (n = 29)*	150 (15–900)

*Twenty-nine seizures affecting 28 dogs were analyzed because 1 dog experienced 2 types of seizures.

Table 2 The status of cluster seizures (CS) and status epilepticus (SE), autonomic signs, and the status of the prodrome and postictal phenomena corresponding to each seizure type.

	Seizure type			
	FES (n = 10)	GES (n = 12)	FEvG (n = 7)	All cases (n = 28)*
CS	2 (20%)	10 (83%)	5 (71%)	17 (57%)
SE	4 (40%)	5 (42%)	2 (29%)	11 (39%)
CS + SE	1 (10%)	5 (42%)	1 (14%)	6 (21%)*
Autonomic signs	3 (30%)	11 (92%)	6 (86%)	20 (71%)
Prodrome	1 (10%)	3 (25%)	0 (0%)	4 (14%)
Postictal phenomenon	4 (40%)	9 (75%)	6 (86%)	19 (68%)

*One dog had both GES and FEvG.

Table 3 The seizure type, AED type, seizure frequency (times/month) prior to and after starting the AED treatment, and therapeutic outcome of each of the dogs.

Table 3

			Seizure frequency (times/month)	
Therapeutic outcome	AED	Seizure type	Prior	After
Primary goal	PB	GES	3.1	0
Primary goal	LEV	GES	0.2	0
Primary goal	KBr, ZNS, LEV	FES	0.3	0
Primary goal	ZNS	FES	1	0
Primary goal	ZNS	FES	0.2	0.2
Primary goal	PB	FEvG	several times	0
Primary goal	ZNS	FEvG	1.1	0
Primary goal	ZNS	FES	1.6	0
Secondary goal	PB	GES	1.3	0.3
Secondary goal	ZNS, LEV	GES	4	3.8
Secondary goal	ZNS, LEV, CLP	FES	NA	NA
Secondary goal	ZNS	GES+FEvG	0.9	0.83
Secondary goal	ZNS	GES	1	1.7
Secondary goal	ZNS	FEvG	2	0.3
No responder	PB, KBr,ZNS	GES	0.5	1
No responder	PB, KBr, ZNS, LEV	GES	0.5	2.2
No responder	PB	GES	0	0.8
No responder	PB, ZNS, LEV	FES	2	6.7
No responder	PB, KBr, ZNS, LEV	FEvG	1.8	2.4
No responder	PB, KBr, ZNS, LEV	GES	4	10

PB, phenobarbital; KBr, potassium bromide; ZNS, zonisamide; LEV, levetiracetam; CLP, clorazepate; NA, not available.

Table 4 The answers to the questionnaire [17].

Score	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Seizure severity and frequency	1–5 (strongly agree–strongly disagree)						
In the last 3 months, the frequency of the fits in my dog was acceptable	2	4	1	0	0	-	-
In the last 3 months, the severity of the fits in my dog was acceptable	3	3	1	0	0	-	-
In the last 3 months, overall, the fits in my dog were managed successfully	3	2	2	0	0	-	-
	1–7 (very mild–very severe)						
Overall, how severe were your dog's fits in the past 3 months?	3	2	2	0	0	0	0
Adverse effects of AED	1–5 (strongly agree–strongly disagree)						
In the past 3 months, the adverse effects of the medication to control the fits in my dog were acceptable	4	3	0	0	0	-	-
In the past 3 months, how severe was the following adverse effect:						-	-
Eating more/would like to eat more	0	1	1	0	0	-	-
Gaining weight	0	0	1	0	0	-	-
Drinking more	0	1	0	0	0	-	-
Urinating more	0	1	0	0	0	-	-
Sleeping more than before	0	2	0	0	0	-	-
Wobbly/not coordinated when walking	0	1	0	0	0	-	-
Restlessness/pacing	0	1	0	0	0	-	-
Itchiness or skin rash	0	0	0	0	0	-	-
Vomiting	0	1	0	0	0	-	-
Diarrhea	0	0	0	0	0	-	-
Coughing	0	0	0	0	0	-	-

Restrictions on the carer's life (related to caring for a dog with IE)	1–5 (never–very often)						
In the past 3 months, how often did you feel that your dog's epilepsy caused a conflict with your work, education, or day-to-day activities?	1	6	0	0	0	-	-
In the past 3 months, how often did you feel that your dog's epilepsy limited your social life?	2	4	0	1	0	-	-
In the past 3 months, how often did you feel that your dog's epilepsy limited your independence?	2	4	0	1	0	-	-
Frustrations over caring for a dog with IE	1–5 (not at all bothersome–extremely bothersome)						
My limitations in work, education, or day-to-day activities because of my dog's fits	2	2	1	2	0	-	-
My social limitations because of my dog's fits	1	4	1	1	0	-	-
Overall, the limitations on my life due to caring for my epileptic dog	1	3	3	0	0	-	-

Owner distaste of AED adverse effects	1–5 (not at all bothersome–extremely bothersome)						
How bothersome are the physical effects of the medication on my dog?	4	1	1	0	0	-	-
How bothersome are the mental effects of the medication on my dog?	4	1	0	1	0	-	-
In the past 3 months, how much did you dislike the following adverse effects:						-	-
Eating more/would like to eat more	0	1	0	1	0	-	-
Gaining weight	0	1	0	1	0	-	-
Urinating more	0	0	0	1	0	-	-
Sleeping more than before	1	0	0	0	0	-	-
Wobbly/not coordinated when walking	0	0	0	1	0	-	-
Restlessness/pacing	0	1	0	0	0	-	-
Coughing	0	0	0	0	0	-	-
Carer anxiety around the seizure event (and its effects on the dog)	1–5 (strongly disagree–strongly agree)						
In the last 3 months, I worried about the frequency of the fits in my dog	1	2	0	3	1	-	-
In the last 3 months, I worried about the severity of the fits in my dog	1	2	0	4	0	-	-
Perceptions of rectal diazepam use	1–5 (never–always)						
Have you ever been uncertain when to give rectal diazepam?	0	0	0	2	0	-	-
Have you ever been worried how much or how often you are supposed to give rectal diazepam	0	1	0	1	0	-	-

Chapter 2

Effects of Chloride in the Diet on Serum Bromide Concentrations in Dogs

Introduction

Bromide is an antiepileptic drug (AED) that is widely used for seizure control in dogs since the early 1990s [14, 36–38]. It is commonly administered as potassium bromide (KBr) or, in some situations, as sodium bromide (NaBr). Bromide is reportedly effective both as a monotherapy [10] and as an add-on treatment [14, 38, 39–42]. Today, bromide is widely used as a first- and second-line AED for dogs because of its efficacy, wide safety margin, and infrequency of dosing.

Bromide is excreted via the urine without being metabolized in the liver because its molecule size is very small [43–46]. This pharmacokinetic property makes bromide especially useful for seizure control in animals with hepatic dysfunctions. Since the amount of bromide excreted depends on the total body halide concentration [47], bromide excretion is influenced by the systemic chloride concentration. Recently, it was reported that the serum bromide concentration of dogs decreases with the administration of higher sodium and chloride contents via infusion fluids [48]. Also, in both an experimental study and a clinical setting, diets with high contents of chloride were

shown to decrease serum bromide concentrations in dogs [49, 50]. Hence, the chloride levels of animals on bromide should be kept stable by monitoring their dietary chloride intake. However, there are various diets including commercial diets and prescription diets with various contents of chloride. Thus, the individual dietary chloride intake varies among dogs, and some of them have a higher dietary chloride intake. For example, a diet with a high chloride content may be given to dogs with urolithiasis as part of their treatment. Urolithiasis is a common urinary tract disease that can occur in dogs with epilepsy that are being administered with bromide. In such cases, considering the dietary chloride intake is critical because a high chloride intake decreases the serum bromide concentration and makes seizure control difficult [50].

Optimizing the KBr treatment was considered to have the potential to have improved the therapeutic outcomes in a certain number of cases from the findings in Chapter 1. Therefore, the author hypothesized that quantifying the serum bromide concentrations in dogs that are being fed diets with varying contents of chloride can provide detailed clarifications on the relationship that exists between serum bromide concentrations and

the dietary chloride intake and thereby, contribute to the treatment of epilepsy in dogs being administered bromide. The aim of this chapter was to clarify the relationship between the dietary chloride intake and serum bromide concentrations in order to provide useful data that can aid in improving seizure control in dogs that are being administered KBr.

Materials and Methods

Animals

Twenty-three client-owned dogs being treated with KBr and that met either of the inclusion criteria (a or b) were enrolled in the study. The inclusion criteria were (a) serum bromide concentration at steady-state or (b) the amount of KBr administered at the same dose for at least 4 months. Thirteen of the dogs were presented to the AUVTH while the remaining 10 dogs were presented to the Watanabe Animal Hospital. Informed consent for research participation was obtained from all owners.

Information collection and questionnaire for the owner

The dose of KBr, body weight, and cause of epilepsy were investigated via data from the medical records. The owners were questioned about their dog's diet via a questionnaire. The questionnaire recorded information on the type of diet, amount of diet (g/day), the number of meals (/day), and any foods given besides the main diet.

Measurement of the concentrations of serum bromide and dietary chloride

A gold chloride method was applied for the measurement of serum bromide [48]. Dietary chloride was measured using the titration methods of Mohr [51, 52] or calculated from the ingredient labels provided by the manufacturer of the dog food.

Data analysis

To quantify the relationship between the dietary chloride intake and serum bromide concentration, the 2 parameters that are mentioned below (a and b) were calculated and their numerical relationship was examined.

(a) Dietary chloride intake (mg/kg/day)

Dietary chloride intake (mg/kg/day) = Daily amount of diet (g/kg/day) × chloride content of the diet (mg/g)

Daily amount of diet (g/kg/day) = Amount of diet (g/time) × Times (/day) / Body weight (kg)

(b) Serum bromide concentration per KBr dose (μg/ml per mg/kg/day)

Serum bromide concentration per KBr dose = Serum bromide concentration (μg/ml) / KBr dose (mg/kg/day)

Based on the parameters above, a regression analysis was performed in order to obtain the formula that outlines the relationship between the dietary chloride intake and serum bromide concentration.

Clinical application

The formula described above was applied to 2 clinical cases that were being medicated with KBr, and its usefulness was evaluated.

Statistical analysis

The Spearman's rank correlation coefficient was utilized to evaluate the relationship between the parameters, the dietary chloride intake by the body weight (mg/kg/day) and serum bromide concentration per KBr dose (μg/ml per mg/kg/day). The relationships between each of these parameters, i.e., the dietary chloride intake by the body weight (mg/kg/day), serum bromide concentration per KBr dose (μg/ml per mg/kg/day), daily amount of diet (g/kg), chloride content in the diet (mg/g), and body weight (kg) were also examined using the Spearman's rank correlation coefficient. A value of $P < 0.05$ was considered significant. Statistical analyses were performed using JMP®7.0.1 (SAS Institute Inc.) and IBM SPSS Statistics ver. 24.0 (IBM Institute Inc.).

Results

Twenty-three client-owned dogs with a mean (± SD) body weight of 9.7 ± 8.8 kg (minimum–maximum, 0.74–40.2 kg) and a mean age of 8.0 ± 2.8 years (3–12 years) were enrolled in this phase of the study. There were 5 sexually intact females, 10 spayed females, 2 sexually intact males, and 6 castrated males. The breeds of dogs represented

in the study included pugs (n = 4), toy poodles (n = 2), Shetland sheepdogs (n = 2), border collies (n = 2), beagles (n = 2), mixed-breed dogs (n = 2), and 1 each of the following breeds: miniature dachshund, Yorkshire terrier, French bulldog, American cocker spaniel, miniature schnauzer, Pekingese, Boston terrier, Italian greyhound, and Great Pyrenees. The dogs represented in the study were diagnosed with the following conditions: IE (n = 20), meningoencephalitis of unknown origin (n = 2), and hydrocephalus (n = 1). The diet being fed to all the dogs was a dry type. All of the dogs had little or no treatment added to their feed besides the main diet. The Mohr's method was used to measure the dietary chloride intake of 19 dogs, and the ingredients label provided by the food manufacturer was used for the remaining 4 dogs. The KBr dose, serum bromide concentration, and the dietary chloride intake corresponding to each of the dogs are provided in Table 5.

A significant strong negative correlation was observed between the dietary chloride intake (mg/kg/day) and serum bromide concentration per KBr dose (μg/ml per mg/kg/day) ($r = 0.81$, $P < 0.01$) (Fig. 1). The numeric relationship between the 2

parameters can be expressed by the KBr-chloride intake formula: y = −0.2 (± 0.06) x + 87 (± 12.8) (x: dietary chloride intake, y: serum bromide concentration per KBr dose). A significant positive correlation was observed between the serum bromide concentration per dose (μg/ml per mg/kg/day) and body weight ($r = 0.65$, $P < 0.01$). A significant negative correlation was observed between the dietary chloride intake by body weight (mg/kg/day) and body weight ($r = -0.52$, $P < 0.01$). There was no significant correlation observed between the body weight and daily amount of diet (g/kg) or the chloride content in the diet (mg/g).

Applications of the derived equation for clinical cases

Case 1 was a 9-year-old neutered male mixed-breed dog evaluated for poor seizure control. The seizure frequency was 4 times/month at a KBr dose of 30 mg/kg/day. The dog was being administered a prescription diet for the prevention of urolithiasis. Calculated from the ingredients label, its dietary chloride intake was 543.7 mg/kg/day. Measured using the method described above, the serum bromide concentration was less than 0.1 mg/ml. The formula was applied to facilitate decision-making in relation to

KBr doses and the appropriate diet needed to gain better seizure control. Utilizing the KBr-chloride intake formula, the expected serum bromide concentration calculated in relation to the current diet was less than 0 mg/ml. After checking that the dog did not have urolithiasis by a urine analysis and an abdominal ultrasound, its diet was changed to a commercial regular maintenance diet. Four months after changing the diet, the serum bromide concentration had increased to 1.2 mg/ml. At this point, the seizure frequency decreased to once a month. Case 2 was a 3.5-year-old neutered male miniature dachshund, presenting with newly occurring seizures. The dog was being administered KBr at a dose of 20 mg/kg/day for 4 months. The dog was receiving a prescription diet for a possible kidney disease. Calculated from the ingredients label, the dietary chloride intake was 70.9 mg/kg/day. The corresponding serum bromide concentration was 1.3 mg/ml. At this serum bromide concentration, the seizures continued to occur. Utilizing the KBr-chloride intake formula, the expected serum bromide concentration was calculated to be 1.47 mg/ml. The difference between the actual serum bromide concentration and expected bromide concentration from the formula was 12%. To improve the seizure control and prevent severe seizures, the KBr

dosage was increased in order to achieve a target serum bromide concentration of 2 mg/ml. By using the KBr-chloride intake formula, an appropriate KBr dose of 25 mg/kg/day could be calculated to achieve the target concentration. In order to correct the difference between the actual bromide concentration and expected bromide concentration (12%) measured previously, the KBr dosage was increased to 28 mg/kg/day (25 × 1.12 = 28). Three months after changing the dose, the serum bromide concentration was 2.2 mg/ml, and the dog was seizure free.

Discussion

In this chapter, our study provides the KBr-chloride intake formula that describes the numeric relationship between the dietary chloride intake and serum bromide concentrations in dogs. To the authors' knowledge, this is the first study to report on an actual numeric relationship between the dietary chloride intake and serum bromide concentrations in dogs. The KBr-chloride intake formula was applied effectively to 2 clinical cases in this study. Our results suggest that it is important to consider the dietary content of chloride for KBr-treated dogs. The KBr-chloride intake formula can

contribute to improving seizure control by aiding clinicians in their selection of KBr doses and appropriate diets for dogs that need to be treated with KBr.

In this study, a positive correlation was observed between the body weight and serum bromide concentration per dose whereas a negative correlation was observed between the body weight and dietary chloride intake. Since there was no correlation between the body weight and daily amount of diet (g/kg) or chloride content in the diet (mg/g), these results indicate that dogs with lower body weights had slightly lower serum bromide concentrations than dogs with greater body weights regardless of their diet. One possible explanation for this is that there might be an endogenous factor affecting bromide excretion among dogs of different sizes. Another possible explanation is that the results were influenced by the fact that only 1 dog with a higher body weight, having a high serum bromide concentration and a low chloride intake, was included in this study. From these findings, it might be suspected that chloride has a stronger influence on dogs with lower body weights or that it increases bromide excretion when compared to in dogs with higher body weights. However, we could not confirm this

from our results as there was only 1 dog weighing more than 20 kg included in this study. Ideally, including more dogs that are heavier than 20 kg would potentially have allowed for an evaluation of the influence of body weight differences on the relationship between dietary chloride intake and serum bromide concentration.

In case 1, the KBr-chloride intake formula was applied to examine the influence of the dog's chloride intake, revealing an expected serum bromide concentration of less than 0 mg/kg corresponding to the intake. This value suggested that a high dietary chloride intake increased the serum bromide excretion and that the serum bromide concentration was not maintained. In this case, changing the dog's diet resulted in an increased serum bromide concentration and facilitated the reduction of seizure frequency. It is important to note that the KBr-chloride intake formula cannot be applied to dogs with a high dietary chloride content of more than 435 mg/kg/day because this would result in the right side of the KBr-chloride intake formula being equal to less than 0. Some dogs cannot discontinue their high chloride diets because of concurrent conditions, for example, urolithiasis. Based on our results, clinicians must use caution when treating

such dogs for seizure control with KBr. In case 2, the KBr-chloride intake formula was utilized to select the appropriate KBr dose in order to achieve better seizure control. Based on the results of applying this formula to the previous serum bromide concentration measurement of this dog, an adjustment was made to the KBr dose that resulted in better seizure control. Considering the benefits that were observed in these 2 clinical cases being medicated with KBr, the KBr-chloride intake formula was useful in the clinical setting.

The formulae for calculating the adequate dose of KBr for a concomitant phenobarbital-KBr treatment and a KBr monotherapy in dogs have been reported on previously [6]. These formulae are useful in assisting with the decision-making process associated with most cases of dogs treated with KBr, but they cannot be applied in all of the cases. The problem encountered is that the dietary chloride intake has not been considered despite the evidence that a high chloride content in the diet decreases the serum bromide concentrations in dogs. Hence, if data regarding the dietary chloride intake is available, using the KBr-chloride intake formula is more useful that the

conventional formulae for selecting the KBr dose. However, the formulae reported by Bhatti et al. [6] remain useful because the measurement of the amount of chloride intake is not available in every case. Our 2 cases emphasized the importance of considering the dietary chloride intake in dogs being administered KBr and the usefulness of the KBr-chloride intake formula.

One of the limitations of this study was the small sample size. There was only 1 dog that weighed more than 20 kg that was included in this study. Including more dogs with greater body weights might have allowed for the evaluation of the influence of body weight differences on the relationship between the chloride intake and serum bromide concentration. Another limitation of our investigation is that the accuracy of the KBr-chloride intake formula for cases involving renal failure and the administration of diuretics is unknown. Although the excretion pathway of bromide is not fully elucidated, diuretics might increase bromide excretion by inhibiting bromide reabsorption by the chloride transporters in the renal tubules. In 1 case report, diuretics were administered to dogs with KBr intoxication in order to reduce serum bromide concentrations [53].

Additional research is needed to evaluate the applicability of the KBr-chloride intake formula in cases involving dogs with renal failure that are being medicated with diuretics.

In conclusion, a strong negative correlation was observed between the dietary chloride intake (mg/kg/day) and serum bromide concentration per dose (μg/ml per mg/kg) (P <0.01). The KBr-chloride intake formula was established based on these 2 parameters. Our results suggest the importance of considering the dietary content of chloride for KBr-treated dogs and may also aid clinicians in the selection of KBr doses and appropriate diets for dogs treated with KBr.

Figures and Tables

Table 5

The KBr dose, serum bromide concentration, and dietary chloride intake of each dog.

Dog No.	KBr dose (mg/kg/day)	Serum bromide concentration (mg/mL)	Dietary chloride intake (mg/kg/day)
1	38	2.3	129.2
2	60	4.8	135.5
3	34	2.6	82.3
4	60	3.8	127.8
5	50	3.3	90
6	22	2.4	37.3
7	60	4	198.8
8	36	2.8	133.2
9	48	2.4	163.6
10	70	4.2	150
11	40	1.5	279
12	40	1.1	196.5
13	110	3.4	148.6
14	54	3.6	113.3
15	30	2.4	125.5
16	30	0.1	293.1
17	70	5.4	120.6
18	80	1	385.9
19	44	2	205.4
20	30	0	534.6
21	30	1.6	358.7
22	30	1.3	116.7
23	25	1.2	111.1

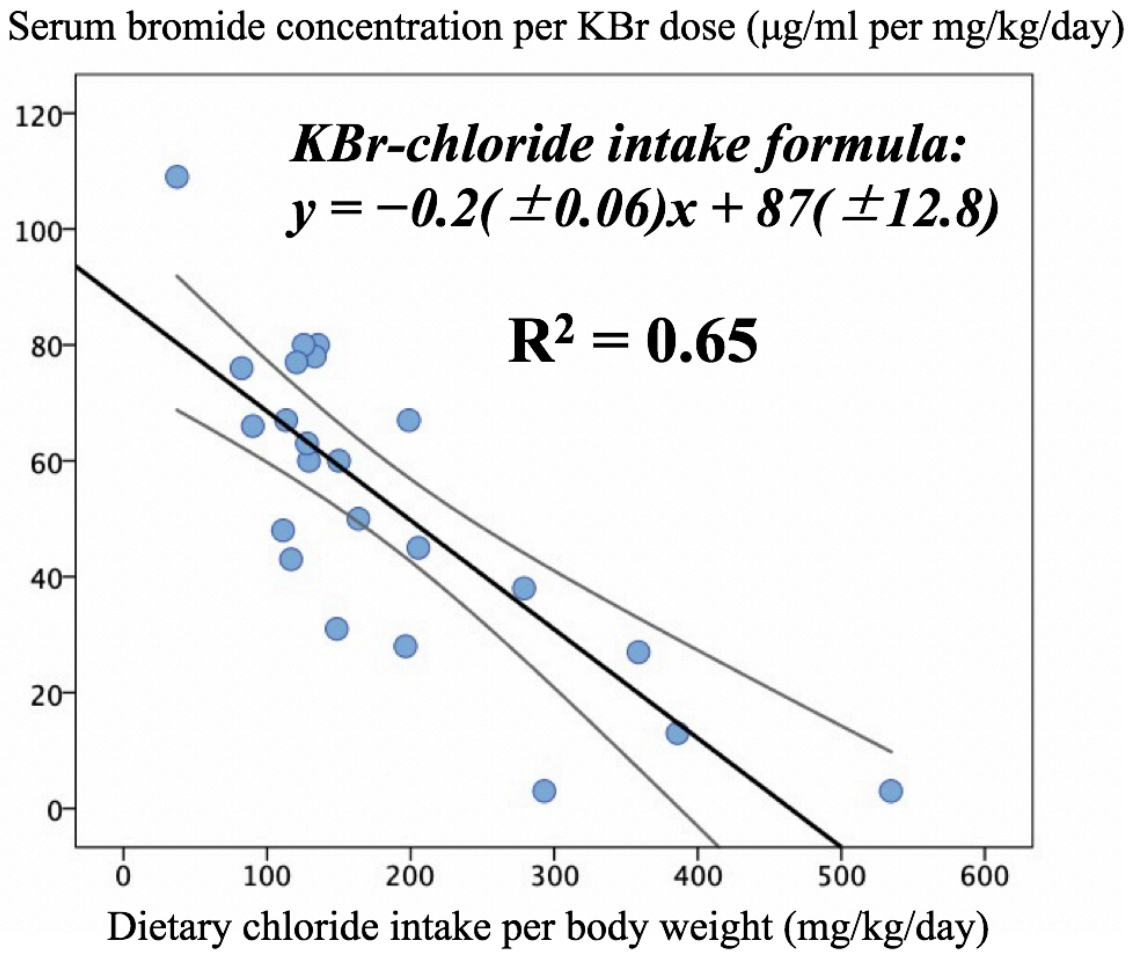

Fig. 1. The relationship between the dietary chloride intake (mg/kg/day) and serum bromide concentration per KBr dose (μg/ml per mg/kg/day). A strong negative correlation was observed between the parameters (P <0.01). The numeric relationship between these 2 parameters was demonstrated via the KBr-chloride intake formula. The upper and lower limit of the 95% confidence interval was represented by the curved lines.

Chapter 3

The genetic investigation in toy poodles with idiopathic epilepsy

Introduction

Investigations into the genetic factors that influence the development of IE have led to the consideration that the genetic causes are probably complex and associated with multiple genes, involving interactions between genes and between genes and the environment [54]. The cause behind the onset of IE is suspected to be genetic though the genes associated with IE are poorly understood in dogs. To date, there are 3 genes, *LGI2*, *ADAM23*, and *DIRAS1*, reported to be associated with IE in specific breeds of dogs with the specific phenotype [21–23], but the causative gene responsible for IE in the majority of breeds remains unknown. *LGI2* was the first gene to be reported in 2011 that is associated with remitting focal epilepsy in the breed, lagotto Romagnolo [21]. The next gene to be reported was the *ADAM23* gene in 2012, which was identified as a novel IE locus in Belgian shepherd dogs [22]. Moreover, further investigation revealed that this gene is also involved in the etiology of IE in several other breeds, and a corresponding risk haplotype was reported on [55, 56]. The most recently discovered gene is *DIRAS1* in 2017; its association with generalized myoclonic epilepsy with photosensitivity in juvenile Rhodesian ridgebacks was reported on [23].

Toy poodles were overrepresented in our retrospective study outlined in Chapter 1 suggesting that this breed might be a good candidate for the genetic study on IE. Therefore, the aim of this chapter was to locate the genome loci associated with IE in toy poodles and to investigate the potential gene responsible for IE in this breed. In this chapter, the author investigated the 3 genes that are already known to be associated with IE in toy poodles affected by this condition. Moreover, by using this breed, the author performed a GWAS to reveal the risk loci of IE. Subsequently, the risk loci were evaluated by their validity assessed in the validation study using the direct sequence method. Finally, 1 potential candidate gene, *ELFN2*, was identified in toy poodles, and its potential to be involved in etiology of IE was evaluated.

Materials and Methods

Sample collection and the study population

Samples from toy poodles with and without IE were collected between April 2015 and May 2018 from multiple centers, including the AUVTH (Kanagawa, Japan), CAMIC

(Tokyo, Japan), Watanabe Animal Hospital (Shizuoka, Japan), Mori Animal Hospital (Shiga, Japan), and Kojima Animal Hospital Animal Wellness Center (Niigata, Japan). The diagnosis of IE was made in accordance with the IVETF consensus statements [4], however, some of the dogs did not undergo urinalysis, which is 1 of the recommended tests. The inclusion criteria for the unaffected dogs were as follows: 1) dogs that never had an epileptic seizure and 2) dogs that were older than 6 years old. The DNA samples that were restored in the gene bank (Biobank, Azabu University, Kanagawa, Japan) that met the inclusion criteria were also used. Informed consent for research participation was obtained from all the owners.

Already-known IE associated genes: LGI2, ADAM23, DIRAS1

The exons of *LGI2* and *DIRAS1* have already been investigated whereas the risk haplotype associated with *ADAM23* was sequenced. The exons (*LGI2* and *DIRAS1*) or risk SNPs (*ADAM23*) were amplified by a PCR assay with the primers listed in Table 6. The PCR products were purified with an ExoSAP-IT kit (USB Corporation, Cleveland,

Ohio), and the sequence was entrusted to a commercial facility (Fasmac Co., Ltd., Kanagawa, Japan).

The *GWAS*

Genotyping was performed with the Illumina Canine SNP Array containing approximately 170K markers (Illumina, San Diego, California, USA). The SNP association analysis was performed with the GEMMA software [57] with the following criteria: a macrophage-activating factor (MAF) of < 0.05, call rate > 95%, and < 5% of missing genotypes in individual dogs. After applying these filters to the SNPs, 12,195 of them remained in the analysis for all the dogs.

Validation study

Eight SNPs were selected for the validation study based on the GWAS results. The DNA products were amplified by PCR assays with the primers listed in Table 7. The PCR products were purified with the ExoSAP-IT kit (USB Corporation), and the sequence was entrusted to a commercial facility (Fasmac Co., Ltd.). The Fisher's exact

test was performed to evaluate the differences in the proportion of each of the SNPs between the affected and unaffected dogs. $P < 0.05$ was considered significant.

Candidate gene sequencing

One candidate gene, *ELFN2*, and its exon were sequenced. Samples from 33 affected and 38 unaffected dogs were used. The protein coding region was amplified by PCR assays with the primers listed in Table 8. The PCR products were purified using the ExoSAP-IT kit (USB Corporation) or QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), and the sequence was entrusted to a commercial facility (Fasmac Co., Ltd.).

Results

Sample collection and the study population

DNA samples from a total of 33 toy poodles affected by IE and 38, unaffected by IE were used in this study. Further, samples corresponding to 10 affected and 22 unaffected dogs that were restored in the Biobank were used in the GWAS. Additionally, samples of 23 affected and 16 unaffected dogs were collected and used for

the validation study. Eight affected and 2 unaffected dogs were selected for the investigation into the association between the already-known IE associated gene and the toy poodle breed.

Already-known genes: LGI2, ADAM23, and *DIRAS1*

A variant in exon 12 of *LGI2* (chr10: 272172210) was observed in 3 of the affected and 1 of the unaffected dogs. However, this mutation was a silent mutation. The SNP variants were not consistent with the previously reported *ADAM23* risk haplotype [55]. The sequence analysis of *DIRAS1* did not reveal any coding variants.

The *GWAS*

The genome-wide significance plot of logged P values for the GWAS analysis is shown in Figure 2. From the plot, 1 of the SNPs was over the $\log_6$ threshold, and 29 of the SNPs were over the $\log_4$ threshold, suggesting that these SNPs are significantly associated with the development of IE. The confidence intervals and odds ratios

corresponding to these results were used to select the top 8 SNPs for the validation study (Table 9).

Validation study

The top 8 SNPs selected from the GWAS were further investigated. The results of the validation study are summarized in Table 10. A significant difference between the affected and unaffected dogs was observed in 1 SNP. Although their association with IE was indicated from the GWAS, the remaining SNPs did not differ between the affected and unaffected dogs.

Candidate gene sequencing

Exon 1 was successfully sequenced using 5 consecutive sub-regions (CDS1-1, CDS1-2, CDS1-3, CDS1-4, and CDS1-5). CDS1-2 through CDS1-5 did not reveal any significant coding variants. However, there was an insertion mutation in the CDS1-1 region in both the affected and unaffected dogs.

Discussion

In the study of the already-known 3 genes, the variant that was reported to be associated with IE was not identified in toy poodles. An SNP in exon 12 of *LGI2* (A/T, Chr. position: 85210442), which replaces the codon with a stop codon, is reported to be a causative mutation responsible for the development of IE in the breed, lagotto Romagnolo [21]. There was no mutation in this SNP corresponding to the toy poodles investigated in our study. A risk haplotype (T-C-del-del-G-G at Chr. position: 15085438, 15106446, 15108593, 15108802, 15111724, and 15113325) in an intron of *ADAM23* is reported to be associated with IE in several breeds such as the Belgian shepherd, Schipperke, Finnish spitz, and beagle [55]. However, consistent variants associated with this risk haplotype were not observed in our study population. There was no coding variant in the exon of *DIRAS1* in toy poodles, indicating that this gene is not associated with IE in toy poodles. Therefore, neither the *LGI2* and *DIRAS1* mutations nor the risk haplotype of *ADAM23* is associated with IE in toy poodles. Since the exons of *ADAM23* were not sequenced, there is a possibility that toy poodles with IE have mutations in this coding region.

The GWAS and validation study successfully narrowed down the locus associated with IE in toy poodles. Several potential SNPs that are associated with IE in toy poodles were discovered via the GWAS. The subsequent validation study revealed that most of the SNPs were false positives with regard to their relationship to the disease and that only 1 SNP (chr10: 27217210) mutation was significantly different between the affected and unaffected dogs. This was possibly due to the small sample size of 10 affected dogs used in the GWAS. One of the most important factors in conducting a successful GWAS is the size of the sample; however, including a large enough sample size is challenging in veterinary medicine since sample collection is difficult compared to in human medicine. Therefore, conducting further validation studies involving additional cases would clarify the validity of the SNPs discovered in the GWAS. Another possible reason that could be behind the false positive SNPs in the GWAS might be the quality of the diagnosis of the affected dogs. We consider the accuracy of the diagnosis of IE to be appropriate since the diagnosis was performed and confirmed in accordance with the IVETF consensus statements and by a neurology specialist. Moreover, the dogs that

were highly suspected to be affected by IE but that had insufficient clinical data were excluded from the study in order to avoid errors due to data contamination by data corresponding to unaffected dogs. On the other hand, there is a possibility that affected dogs were included in the unaffected dog group since the information regarding the occurrence of the epileptic seizures was based on the owner's observations. Tightening the inclusion criteria will improve the quality of recruitment for future studies. For example, adding an electroencephalography (EEG) test as a prerequisite as 1 of the inclusion criteria will ensure the accuracy of a dog's epilepsy diagnosis. However, this solution is not practical because there seldom are chances to perform an EEG on non-epileptic dogs.

The candidate gene, *ELFN2*, investigated based on the risk locus of IE in toy poodles did not have significant variants normally associated with IE dogs in its coding region. This was the gene closest to the risk locus that was identified (chr10: 27217210), and there were no reports associating it with IE or epilepsy in any species. However, *ELFN1*, a gene which is in the same gene family, is reported to be associated with seizures and

hyperactivity in mice and humans [58, 59]. The *ELFN1* protein is a critical postsynaptic component that induces the metabotropic glutamate receptor 7 (mGluR7)-positive presynaptic structures in excitatory neurons [59]. Thus, the possibility that the dysfunction of the ELFN1–mGluR7 complex is a critical contributor to neurological diseases was proposed by Tomioka et al. [59]. These pieces of evidence support the probability of the involvement of the *ELFN2* gene in the IE of toy poodles since this protein has a similar molecular structure to the ELFN1 protein. Therefore, further investigation of the introns and gene expression of *ELFN2* might reveal an association with IE in toy poodles.

Although it was an incidental finding, interestingly, an insertion mutation was found in the coding region of *ELFN2* in both the affected and unaffected dogs. This region was unidentified or considered as an intron by a comparison with the reference genome (Fig. 3). Thus, this insertion mutation found in toy poodles was highly suspected of not being associated with IE since it does not influence the coding region (a frameshift would not occur due to this mutation). Although the nucleotide is not observed in the reference

genome, the annotation of the gene in the NCBI and Ensembl databases described it as an unidentified nucleotide that codes some sort of amino acid and an intronic nucleotide, respectively. Thus, the insertion mutation would actually exist in almost all dogs, and the reference genome would be wrong in this part.

In conclusion, the author revealed a locus in chromosome 10 that is associated with the IE of toy poodles. However, further investigation is needed to elucidate the relative gene responsible for IE in this breed. Moreover, the author found an insertion mutation in a candidate gene, *ELFN2*, that is unidentified by the reference genome though it is not suspected to be associated with the disease.

Figures and Table

Table 6 The PCR primer sequences of *LGI2*, *ADAM23*, and *DIRAS1*.

	Target	Forward primer sequences	Reverse primer sequences	Product length (bp)
LGI2	Exon 5	gattccgagcctcgtgcg	caggcactcacagggagc	152
	Exon 6	cccatcctgcaatcacttct	gaaggagtgacgccaaagag	245
	Exon 7	tgtacatcactgcgctgaaa	cctaagagatgccgttcctg	200
	Exon 8	ggttttgttccgagtatcgtg	agggaccaggaagtggatct	227
	Exon 9	gagtgacagggcatgaggat	gattaactggagcccaacga	180
	Exon 10	ggcatatctgtttctccgtct	ccattccctgtggatgttct	292
	Exon 11	cgaagggaagcaggttactg	ttgaggccacaatgaaatga	298
	Exon 12—5'-end	cattcttacctaatcccctctcg	gcctttgctgttccatttatacac	300
	Exon 12—middle	atcgctgacagctccaaag	aaagggctgcagggtcat	387
	Exon 12—3'-end	gggtcatgaggtggaacagt	ttggctttccatttgcttct	369
ADAM23	Chr37: 15,085,438	gtgaagcaatacctttgagtgc	agtgagcaaggcaacaggaa	213
	Chr37: 15106446	agggttgtaattgttcaggaagga	agtgaatgtaggcaaaatgtttct	177
	Chr37: 15108593, 15108802	ccacaccagagggaactttct	agtcgcaaccttaagtcccttt	354
	Chr37: 15111724	tctgtctttgaaacagtccccc	accaggtgtaaactggcttt	150
	Chr37: 15113325	ccatcctccaggtcaactcat	gtgcagcatgtacccaagag	201
DIRAS1	Exon 1—5'-end	tccccaggacacaggagaaa	cgtcggccaagatgaactaca	664
	Exon 1—3'-end	cacgccttcatcctggtctac	ctccagcctggctctgc	586

Table 7 The PCR primer sequences of the validation study

Target	Forward primer sequences	Reverse primer sequences
chr22: 21445585	gcccagactaaaaggcccaa	tgtggagaattgactaactattgctt
chr22: 21551991	gtccactacaggtttggggt	tggagtttgtgtagagggagaa
chr22: 19227532	acctgaacagcatcttgggc	atgggtctagggccatctaca
chr1: 99538302	gccacactccccacacataa	gcaaccaccacggtacctat
chr10: 27182448	ctaggaagggagtgcacagg	tcctatccaagagcctcgct
chr10: 27217210	tacaatggggttggcatccg	aaaaagccaactctccgggt
chr10: 27220899	tgcctttgttcccaagatgc	gaccgaatttgtatgcgggt
chr34: 40853291	actacccattgtctccctgc	ccgacggatgagggcagaa

Table 8 The PCR primer sequences of *ELFN2*.

Target	Forward primer sequences	Reverse primer sequences	Product length (bp)
Exon 1—5'-end	gtcgcccccaagatggtg	cagttcctcttcctgcagca	537
Exon 1—5'-end-middle	ctcttacatcgaggacggcg	ctcgtccaccacagatgcat	608
Exon 1—middle	tgagaactcgggcttcaacc	atcccctccatgattgggga	627
Exon 1—middle-3'-end	agcagaagtcggtcaaggtc	gtacatcgagaagggcagc	728
Exon 1—3'-end	aagagcgccaaggtcttcag	acacactgataccgaaaccagt	799

Table 9 The 10 case × 22 control GWAS results of the top 8 SNPs.

Chromosome	SNP Position	F_A	F_U	Odds Ratio (95 % CI)	*P* Value*
22	21445585	0.4	0.02273	28.67 (3.3-252)	1.61×10^{-7}
22	21551991	0.3	0	N/A	4.77×10^{-6}
22	19227532	0.4	0.04545	14 (2.4-74.9)	5.55×10^{-6}
1	99538302	0.3	0.18182	1.9 (0.56-6.57)	7.0×10^{-6}
10	27182448	0.35	0.02273	23.15 (2.6-205)	8.0×10^{-6}
10	27217210	0.35	0.02273	23.15 (2.6-205)	8.0×10^{-6}
10	27220899	0.35	0.02273	23.15 (2.6-205)	8.0×10^{-6}
34	40853291	0.35	0.02273	23.15 (2.6-205)	8.0×10^{-6}

F_A: Frequency of the minor allele in affected individuals.

F_U: Frequency of the minor allele in unaffected individuals.

N/A: Not available.

**P* values were collected using the Bonferroni's method.

Table 10 The results of the 23 case × 16 control validation study.

Chromosome	SNP Position	F_A	F_U	Odds Ratio (95% CI)	*P* Value
22	21445585	0.24	0.19	1.36 (0.45–4.16)	0.74
22	21551991	0.044	0.031	1.4 (0.12–16.2)	0.77
22	19227532	0.079	0.13	0.34 (0.078–1.51)	0.35
1	99538302	0.26	0.16	1.6 (0.55–4.63)	0.25
10	27182448	0.43	0	N/A	0.5
10	27217210	0.24	0.063	7.64 (1.41–41.49)	0.037
10	27220899	0.065	0.031	2.16 (0.22–21.79)	0.63
34	40853291	0.13	0.13	0.61 (0.17–2.25)	0.83

F_A: Frequency of the minor allele in affected individuals.

F_U: Frequency of the minor allele in unaffected individuals.

N/A: Not available.

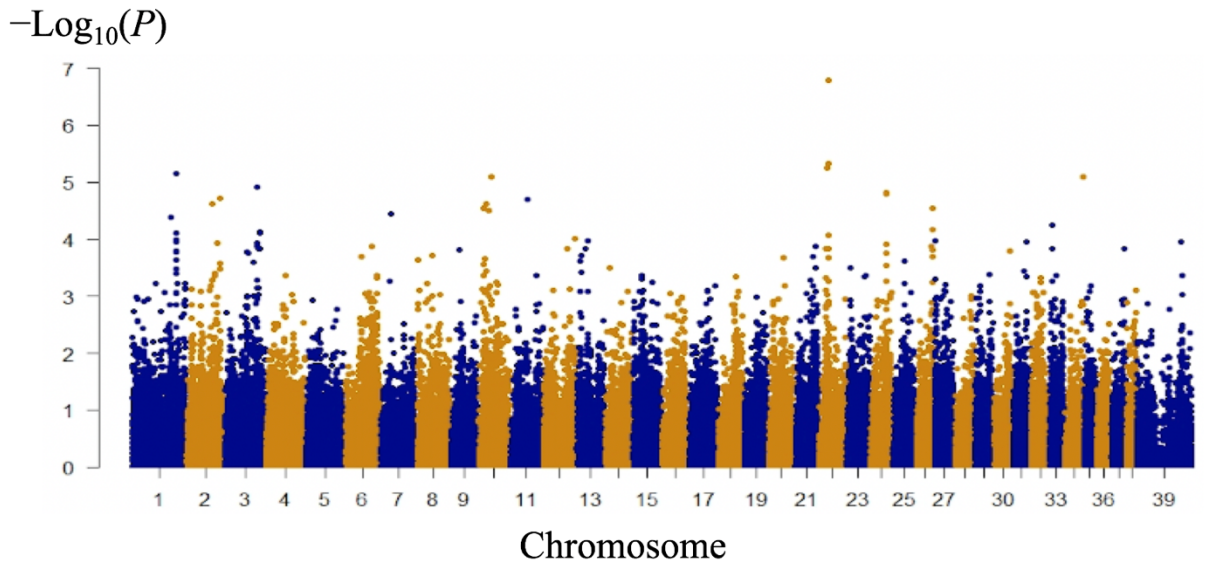

Fig. 2. A Manhattan plot of $-\mathrm{Log}_{10}(P)$ values from a 10 case × 22 control GWAS for the IE in Toy Poodle.

Fig. 3. A reference genome sequence of chr10: 27,185,360–27,185,370. The mutation found in this study was an insertion of adenine between chr10: 27,185,366 and 27,185,367.

Conclusion

The author revealed the clinical data corresponding to dogs affected with IE in Japan and investigated on several topics related to IE further using clinical and genetic approaches. The new findings on IE from the results of this study are summarized as follows.

1. The toy poodle was overrepresented in the retrospective study in Japan. This result supported the validity of conducting a further genetic investigation using this breed.

2. Optimizing the KBr treatment was expected to improve the therapeutic outcomes in dogs with suspected refractory epilepsy. Thus, the importance of elucidating the relationship between the serum bromide concentration and dietary chloride intake in detail was emphasized in order to improve the management of epileptic dogs that are being administered bromide. A strong negative correlation was observed between the dietary chloride intake (mg/kg/day) and serum bromide concentration per dose (μg/ml per mg/kg). The relationship between the serum bromide

concentration and dietary chloride intake was described in the KBr-chloride intake formula: $y = -0.2x + 87$ (x: dietary chloride intake, y: serum bromide concentration per KBr dose). The application of this equation to 2 clinical cases involving uncontrolled seizures contributed to improving the seizure control. This newly derived equation assists clinicians in selecting KBr doses and/or appropriate diets for dogs treated with KBr.

3. The risk genome locus of IE in toy poodles was revealed in the GWAS and validation study. This finding proposed the candidate gene, *ELFN2*, of IE in this breed.

4. The analysis of the coding region of *ELFN2* did not reveal a causative mutation responsible for IE. Though no association with the disease was suspected, the mutation was found in both affected and unaffected dogs in the coding region of *ELFN2*.

In summary, the author revealed the clinical data associated with dogs affected by IE

in Japan, detailed clarifications on the relationship between the serum bromide concentration and dietary chloride intake, and the potential genetic locus of IE in toy poodles. Although the present study could not reveal the association between specific candidate gene and toy poodle with IE, these findings of the present study provide useful basic data for both clinical and genetical studies and act as a tool to assist in improving the treatment of epilepsy in dogs being administered with KBr.

References

1. Kearsley-Fleet L, O'Neill DG, Volk HA, Church DB, Brodbelt DC. 2013. Prevalence and risk factors for canine epilepsy of unknown origin in the UK. *Vet Rec.***172**: 338.

2. Heske L, Nodtvedt A, Jaderlund KH, Berendt M, Egenvall A. 2014. A cohort study of epilepsy among 665,000 insured dogs: incidence, mortality and survival after diagnosis. *Vet J.* **202**: 471–476.

3. Berendt M, Farquhar RG, Mandigers PJ, Pakozdy A, Bhatti SF, De Risio L, Fischer A, Long S, Matiasek K, Muñana K, Patterson EE, Penderis J, Platt S, Podell M, Potschka H, Pumarola MB, Rusbridge C, Stein VM, Tipold A, Volk HA. 2015. International Veterinary Epilepsy Task Force Consensus report on epilepsy definition, classification and terminology in companion animals. *BMC Vet Res.* **11**: 182.

4. De Risio L, Bhatti S, Muñana K, Penderis J, Stein V, Tipold A, Berendt M, Farqhuar R, Fischer A, Long S, Mandigers PJ, Matiasek K, Packer RM, Pakozdy A, Patterson N, Platt S, Podell M, Potschka H, Batlle MP, Rusbridge C, Volk HA. 2015. International Veterinary Epilepsy Task Force Consensus Proposal: diagnostic approach to epilepsy in dogs. *BMC Vet Res.* **11**: 148.

5. Podell M, Volk HA, Berendt M, Löscher W, Muñana K, Patterson EE, Platt SR. 2016. 2015 ACVIM Small Animal Consensus Statement on Seizure Management in Dogs. *J Vet Intern Med*. **30**: 477–90.

6. Bhatti SFM, De Risio L, Muñana K, Penderis J, Stein VM, Tipold A, Berendt M, Farquhar RG, Fischer A, Long S, Löscher W, Mandigers PJJ, Matiasek K, Pakozdy A, Patterson EE, Platt S, Podell M, Potschka H, Rusbridge C, Volk HA. 2015. International Veterinary Epilepsy Task Force consensus proposal: medical treatment of canine epilepsy in Europe. *BMC Vet Res.* **11**: 176.

7. Ravis WR, Pedersoli WM, Wike JS. 1989. Pharmacokinetics of phenobarbital in dogs given multiple doses. *Am J Vet Res.* **50**, 1343–1347.

8. Pedersoli WM, Wike J, Ravis WR. 1987. Pharmacokintics of single doses of phenobarbital given intravenously and orally to dogs. *Am J Vet Res.* **48**, 679–683.

9. Al-Tahan F, Frey HH. 1985. Absorption kinetics and bioavailability of phenobarbital after oral administration to dogs. *J Vet Pharmacol Therap*. **8**, 205–207.

10. Boothe DM, Dewey C, Carpenter DM. 2012. Comparison of phenobarbital with bromide as a first-choice antiepileptic drug for treatment of epilepsy in dogs. *J Am Med Assoc.* **240**, 1073–1073.

11. Rauws AG. 1983. Pharmacokinetics of bromide ion: an overview. *Food Chem Toxicol.* **21**: 379–382.

12. Trepanier LA, Van Schoick A, Schwark WS, Carrillo J. 1998. Therapeutic serum drug concentrations in epileptic dogs treated with potassium bromide alone or in combination with other anticonvulsants: 122 cases (1992-1996). *J Am Vet Med Assoc.* **213**, 1449–53.

13. Schwartz-Porsche D, Löscher W, Frey HH. 1985. Therapeutic efficacy of phenobarbital and primidone in canine epilepsy: a comparison. *J Vet Pharmacol Ther.* **8**, 113–119.

14. Podell M, Fenner WR. 1993. Bromide therapy in refractory canine idiopathic epilepsy. *J Vet Intern Med.* **7**, 318–327.

15. Hamamoto Y, Hasegawa D, Mizoguchi S, Yu Y, Wada M, Kuwabara T, Fujiwara-Igarashi A, Fujita M. 2016. Retrospective epidemiological study of canine epilepsy in Japan using the International Veterinary Epilepsy Task Force

classification 2015 (2003–2013): etiological distribution, risk factors, survival time, and lifespan. *BMC Veterinary Research.* **12**, 248.

16. Chang Y, Mellor DJ, Anderson TJ. 2006. Idiopathic epilepsy in dogs: owners' perspectives on management with phenobarbitone and/or potassium bromide. *J Small Anim Pract.* **47**, 574–581.

17. Wessmann A, Volk HA, Parkin T, Ortega M, Anderson TJ. 2014. Evaluation of Quality of Life in Dogs with Idiopathic Epilepsy. *J Vet Intern Med.* **28**, 510–514.

18. Wessmann A, Volk HA, Packer RM, Ortega M, Anderson TJ. 2016. Quality-of-life aspects in idiopathic epilepsy in dogs. *Vet Rec.* **179**, 229.

19. Chang Y, Mellor DJ, Anderson TJ. 2006. Idiopathic epilepsy in dogs: owners' perspectives on management with phenobarbitone and/or potassium bromide. *J Small Anim Pract.* **47**, 574–581.

20. Weber YG, Biskup S, Helbig KL, Von Spiczak S, Lerche H. 2017. The role of genetic testing in epilepsy diagnosis and management. *Exp Rev of Mole Diag*. **17**: 739–750.

21. Seppälä EH, Jokinen TS, Fukata M, Fukata Y, Webster MT, Karlsson EK, Kilpinen SK, Steffen F, Dietschi E, Leeb T, Eklund R, Zhao X, Rilstone JJ, Lindblad-Toh K, Minassian BA, Lohi H. 2011. LGI2 truncation causes a remitting focal epilepsy in dogs. *PLoS Genet.* **7**, e1002194.

22. Seppälä EH, Koskinen LL, Gulløv CH, Jokinen P, Karlskov-Mortensen P, Bergamasco L, Baranowska Körberg I, Cizinauskas S, Oberbauer AM, Berendt M, Fredholm M, Lohi H. 2012. Identification of a novel idiopathic epilepsy locus in Belgian Shepherd dogs. *PLoS One.* **7**, e33549.

23. Wielaender F, Sarviaho R, James F, Hytönen MK, Cortez MA, Kluger G, Koskinen LL, Arumilli M, Kornberg M, Bathen-Noethen A, Tipold A, Rentmeister K, Bhatti

SF, Hülsmeyer V, Boettcher IC, Tästensen C, Flegel T, Dietschi E, Leeb T, Matiasek K, Fischer A, Lohi H. 2017. Generalized myoclonic epilepsy with photosensitivity in juvenile dogs caused by a defective DIRAS family GTPase 1. *Proc Natl Acad Sci U S A.* **114**, 2669–2674.

24. Ekenstedt KJ, Patterson EE, Michelson JR. 2012. Canine epilepsy. *Mam Genome.* **23**: 28–39.

25. Volk HA. 2015. International Veterinary Epilepsy Task Force consensus reports on epilepsy definition, classification and terminology, affected dog breeds, diagnosis, treatment, outcome measures of therapeutic trials, neuroimaging and neuropathology in companion animals. *BMC Vet Res.* **11**, 174.

26. Potschka H, Fischer A, Löscher W, Patterson N, Bhatti S, Berendt M, De Risio L, Farquhar R, Long S, Mandigers P, Matiasek K, Muñana K, Pakozdy A, Penderis J, Platt S, Podell M, Rusbridge C, Stein V, Tipold A, Volk HA. 2015. International Veterinary Epilepsy Task Force Consensus Proposal: outcome of therapeutic interventions in canine and feline epilepsy. *BMC Vet Res.* **11**, 177.

27. Rusbridge C, Long S, Jovanovik J, Milne M, Berendt M, Bhatti SF, De Risio L, Farqhuar RG, Fischer A, Matiasek K, Muñana K, Patterson EE, Pakozdy A, Penderis J, Platt S, Podell M, Potschka H, Stein VM, Tipold A, Volk HA. 2015. International Veterinary Epilepsy Task Force recommendations for a veterinary epilepsy-specific MRI protocol. *BMC Vet Res.* **11**, 194.

28. Matiasek K, Pumarola I, Batlle M, Rosati M, Fernández-Flores F, Fischer A, Wagner E, Berendt M, Bhatti SF, De Risio L, Farquhar RG, Long S, Muñana K, Patterson EE, Pakozdy A, Penderis J, Platt S, Podell M, Potschka H, Rusbridge C, Stein VM, Tipold A, Volk HA. 2015. International Veterinary Epilepsy Task Force recommendations for systematic sampling and processing of brains from epileptic dogs and cats. *BMC Vet Res.* **11**, 216.

29. Kearsley-Fleet L, O'Neill DG, Volk HA, Church DB, Brodbelt DC. 2013. Prevalence and risk factors for canine epilepsy of unknown origin in the UK. *Vet Rec.* **172**, 338.

30. Short AD, Dunne A, Lohi H, Boulton S, Carter SD, Timofte D, Ollier WE. Characteristics of epileptic episodes in UK dog breeds: an epidemiological approach. *Vet Rec.* **169**, 48

31. Pákozdy A, Leschnik M, Tichy AG, Thalhammer JG. 2008. Retrospective clinical comparison of idiopathic versus symptomatic epilepsy in 240 dogs with seizures. *Acta Vet Hung* **56**, 471–483.

32. Bellumori TP, Famula TR, Bannasch DL, Belanger JM, Oberbauer AM. Prevalence of inherited disorders among mixed-breed and purebred dogs: 27,254 cases (1995-2010). 2013. *J Am Vet Med Assoc.* **242**, 1549–1555.

33. Saito M, Muñana KR, Sharp NJ, Olby NJ. Risk factors for development of status epilepticus in dogs with idiopathic epilepsy and effects of status epilepticus on outcome and survival time: 32 cases (1990–1996). *J Am Vet Med Assoc.* **219**, 618–623.

34. Jaggy A, Bernardini M. 1998. Idiopathic epilepsy in 125 dogs: a long-term study. Clinical and electroencephalographic findings. *J Small Anim Pract* **39**, 23–29.

35. Grayzel S, Lefebvre S. 2014. Descriptive epidemiology of idiopathic seizures in dogs. *Vet focus*. **24**, 25–27.

36. Parent JM. 1988. Clinical management of canine seizures. Veterinary Clinic of North America Small Animal Practice **18**, 947–965.

37. Lane SB, Bunch SE. 1990. Medical management of recurrent seizures in dogs and cats. *J Vet Intern Med.* **4**, 26–39.

38. Pearce LK. 1990. Potassium bromide as an adjunct to phenobarbital for the management of uncontrolled seizures in dogs. *Prog Vet Neuro.* **1**, 195–101

39. Trepanier LA, Van Schoick A, Schwark WS, Carrillo J. 1998. Therapeutic serum drug concentrations in epileptic dogs treated with potassium bromide alone or in combination with other anticonvulsants: 122 cases (1992–1996). *J Am Vet Med Assoc.* **213**, 1449–1453.

40. Löscher W, Potschka H, Rieck S, Tipold A, Rundfeldt C. 2004. Anticonvulsant efficacy of the low-affinity partial benzodiazepine receptor agonist ELB 138 in a dog seizure model and in epileptic dogs with spontaneously recurrent seizures. Epilepsia **45**, 1228–1239.

41. Rieck S, Rundfeldt C, Tipold A. 2006. Anticonvulsant activity and tolerance of ELB138 in dogs with epilepsy: a clinical pilot study. *Vet J.* **172**, 86–95.

42. Royaux E, Van Ham L, Broeckx BJ, Van Soens I, Gielen I, Deforce D, Bhatti SF. 2017. Phenobarbital or potassium bromide as an add-on antiepileptic drug for the management of canine idiopathic epilepsy refractory to imepitoin. *Vet J.* **220**, 51–54.

43. Soremark R. 1960. Distribution and kinetics of bromide ions in the mammalian body. *Acta Radiol Suppl.* **190**, 114.

44. Pozuelo-Utonda, J, Crawford DC, Anderson JC. 1966. Bromism and epilepsy. *Int J Neuropsychiatry.* **2**, 90-97.

45. Woodbury DM, Pippenger CE. 1982. Other antiepileptic drugs, bromides. In: Antiepileptic Drugs, Second Ed. Raven Press, New York, USA, pp. 791–801.

46. Bhatti SF, De Risio L, Munana K, Penderis J, Stein VM, Tipold A, Volk HA. 2015. International veterinary epilepsy task Force consensus proposal: Medical treatment of canine epilepsy in Europe. *BMC Vet Res*. **11**, 176.

47. Rauws AG. 1983. Pharmacokinetics of bromide ion: an overview. *Food Chem Toxicol.* **21**, 379–382.

48. Fukunaga K, Matsumoto H, Wate M, Misawa K, Saito M, Matsuda H, Orito K. 2018. Effects of three infusion fluids with different sodium chloride contents on steady‐state serum concentrations of bromide in dogs. *J Vet Pharmacol Ther*. **41**, 684–690.

49. Trepanier LA, Babish JG. 1995. Effect of dietary chloride content on the elimination of bromide by dogs. *Res Vet Sci.* **58**, 252–255.

50. Shaw N, Trapenier LA, Center SA, Garland S. 1996. High dietary chloride content associated with loss of therapeutic serum bromide concentrations in an epileptic dog. *J Vet Med Assoc.* **208**, 234–236.

51. Sheen HT, Kahler HL. 1938. Effect of ions on Mohr method for chloride determination. *Ind Eng Chem. 10*, 628–629.

52. Kamiya Y, Kamiya M, Hattori I, Hayashi Y, Funaba M, Matsui T. 2017. Effect of feeding sweet-potato condensed distillers solubles on intake and urinary excretion of minerals in Japanese Black steers. *Anim Sci J.* **88**, 79–85.

53. Steinmetz S, Tipold A, Bilzer T, Schenk HC, 2012. Transient neuromyopathy after bromide intoxication in a dog with idiopathic epilepsy. *Ir Vet J.* **65**, 19.

54. horvon S. 2014. The concept of symptomatic epilepsy and the complexities of assigning cause in epilepsy. *Epilepsy Behav*. **32**, 1–8.

55. Koskinen LL, Seppälä EH, Belanger JM, Arumilli M, Hakosalo O, Jokinen P, Nevalainen EM, Viitmaa R, Jokinen TS, Oberbauer AM, Lohi H. 2015. Identification of a common risk haplotype for canine idiopathic epilepsy in the ADAM23 gene. *BMC Genomics*. **18**,16:465.

56. Koskinen LL, Seppälä EH, , Weissl J, Jokinen TS, Viitmaa R, Hänninen RL, Quignon P, Fischer A, André C, Lohi H. 2017. ADAM23 is a common risk gene for canine idiopathic epilepsy. *BMC Genet.* **18**, 8.

57. Wang Q, Wei J, Pan Y, Xu S. 2016. An efficient empirical Bayes method for genomewide association studies. *J Anim Breed Genet.* **133**, 253-63.

58. Jackie Dolan, Kevin J. Mitchell. 2013. Mutation of Elfn1 in Mice Causes Seizures and Hyperactivity. *PLoS One.* **27**, e80491.

59. Tomioka NH, Yasuda H, Miyamoto H, Hatayama M, Morimura N, Matsumoto Y, Suzuki T, Odagawa M, Odaka YS, Iwayama Y, Won Um J, Ko J, Inoue Y, Kaneko S, Hirose S, Yamada K, Yoshikawa T, Yamakawa K, Aruga J. 2014. Elfn1 recruits presynaptic mGluR7 in trans and its loss results in seizures. *Nat Commun*. **22**, 5: 4501.

Acknowledgements

The author wishes to express his sincere appreciation and gratitude to Dr. Yoko Fujii, Professor, Laboratory of Veterinary Surgery I, Azabu University, for her valuable guidance and encouragement during the past 4 years, as well as for her critical review of this thesis as a supervisor.

The author thanks Dr. Kensuke Orito, Professor, Laboratory of Veterinary Physiology II, Azabu University, for his valuable advice and technical teachings associated with all the experiments. The author also sincerely appreciates Dr. Masahiro Sakaguchi, Professor, Laboratory of Veterinary Microbiology I, Azabu University, for his valuable teaching on manipulation and techniques related to the genetic study. The author sincerely expresses his thanks to Dr. Keijiro Mizukami, Laboratory of Veterinary Microbiology I, Azabu University, for their valuable guidance.

This study was supported by many veterinary clinicians and students, and the author is especially thankful to Dr. Yumi Onishi and Dr. Hinako Yamazoe and their co-workers (Companion Animal Medical Imaging Center, Tokyo, Japan), Dr. Naoyuki Watanabe

(Watanabe animal hospital, Shizuoka, Japan), Dr. Kenji Mori (Mori animal hospital, Shiga, Japan), and Dr. Daisuke Kojima (Kojima Animal Hospital Animal Wellness Center, Niigata, Japan), Shinsuke Iwata and Itsuki Kamibayashi (Azabu University, Kanagawa, Japan).

Finally, the author expresses a special thanks to his family for their careful support in his daily life.

A part of this thesis was published as the following articles.

1. Togawa G, Saito M, Uebayashi I, Onishi Y, Yamazoe H. 2018. A Retrospective study of canine idiopathic epilepsy in referral centers in Japan. *J Azabu Univ* (In Press).

2. Togawa G, Saito M, Iwata S, Watanabe N, Orito K. 2018. Effects of chloride in the diet on serum bromide concentration in dogs. *Inter J Appl Res Vet Med* **16** 197–202.