The first genome-wide association study concerning idiopathic epilepsy in Petit Basset Griffon Vendeen

Theis Deschain[1], Jonas Fabricius[1], Mette Berendt[2], Merete Fredholm[1], Peter Karlskov-Mortensen[1,*]

Affiliations

1: University of Copenhagen, Department of Veterinary and Animal Sciences, Animal Genetics, Bioinformatics & Breeding, Gronnegaardsvej 3, DK-1870 Frederiksberg C, Denmark

2: University of Copenhagen, Faculty of Health and Medical Sciences, University Hospital for Companion Animals, Section for Surgery, Neurology & Cardiology, Dyrlægevej 16, DK-1870 Frederiksberg C, Denmark

*: Corresponding author, pkm@sund.ku.dk

Summary

The dog breed Petit Basset Griffon Vendeen has a relatively high prevalence of idiopathic epilepsy compared to other dog breeds and previous studies have suggested a genetic cause of the disease in this breed. Based on these observations, a genome wide association study was performed to identify possible epilepsy-causing loci. The study included 30 unaffected and 23 affected dogs, genotyping of 170K SNPs, and data analysis using PERL and EMMAX. Suggestive associations at CFA13, CFA24 and CFA35 were identified with markers close to three strong candidate genes. However, subsequent sequencing of exons of the three genes did not reveal sequence variations, which could explain development of the disease. This is to our knowledge the first report on loci and genes with a possible connection to idiopathic epilepsy in Petit Basset Griffon Vendeen. However, further studies are needed to conclusively identify the genetic cause of idiopathic epilepsy in this dog breed.

Epilepsy is the most common neurological disorder in dogs. The prevalence in dogs in general has been estimated to around 0.76% (Heske *et al.* 2014). However, certain dog breeds suffer from genetic epilepsy causing a much higher prevalence (Hülsmeyer *et al.* 2015). Among these breeds is the Petit Basset Griffon Vendeen (PBGV), a dog breed originating from the Vendéen region in France and originally bred for rabbit hunting. An epidemiological study previously reported that the Danish population of PBGV suffer from idiopathic epilepsy with a high prevalence (8.9%) and a significant effect on litter prevalence indicating a strong genetic influence (Gulløv *et al.* 2011). Epilepsy in the PBGV is characterized by a relatively early onset around two years of age dominated by focal seizures alone and focal seizures evolving into generalized seizures (Gulløv *et al.* 2011). Unfortunately, a high number of dogs experiencing seizures (13.3%) are euthanized due to reasons related to their epileptic condition.

Here we report the results of a genome wide association study (GWAS) to identify epilepsy-associated genes in PBGV. The GWAS was followed by sequencing of putative candidate genes in the most likely associated regions.

This study was performed using 30 unaffected and 23 affected PBGV dogs identified in a cohort of PBGVs at the University Hospital for Companion Animals, University of Copenhagen. All samples were collected and used for research with the informed consent from the dog owners and the study procedures were approved by the local ethical and administrative committee at the Department of Veterinary Clinical Sciences, University of Copenhagen.

The procedures and criteria for classification of dogs as either cases or controls are described in detail in Gulløv et al. (2011). In brief, an extensive anamnesis was obtained for all dogs based on the dog owners' response to an elaborate questionnaire. This was followed up by telephone interviews with dog owners and finally, clinical examinations of all dogs were performed. This included physical and neurologic examinations, hematology and blood biochemistry. The diagnosis of epilepsy in the individual dog was based on detailed information collected on seizure history, seizure phenomenology and development, seizure duration, and other characteristics of the disorder following the diagnostic guidelines, which are recommended for humans and dogs with epilepsy (De Risio *et al.* 2015).

DNA was isolated from EDTA stabilized blood samples from all dogs with a confirmed case/control status and SNP genotypes were established using the Illumina 170K SNP-chip. Genotype data was cleaned using the PLINK software (Purcell *et al.* 2007) with parameters --maf 0.05, --geno 0.1, --hwe 0.05, --mind 0.1. After that, two GWAS analyses were performed. First, an analysis modelling an autosomal recessive inheritance pattern was performed using PLINK. One thousand permutations were performed using the --mperm option to set a genome wide significance threshold. Secondly, a mixed linear model association analysis was performed using the EMMAX software (Kang *et al.* 2010). The later included the genetic relationship to counter effects of hidden population structures and hereby avoid possible false positive results caused by population stratification.

The PLINK analysis identified one SNP (BICF2G630770657) on CFA35 position 6,342,532 (CanFam3) with a p-value of 0.03 after correction for multiple testing. This marker is located in an exon of the gene *F13A1* and close to the gene *NRN1* (CFA35: 6,070,379-6,078,965). The EMMAX analysis did not recognize any genome-wide significant associations. However, the markers with lowest p-values were located on CFA24 close to the *DOK5* gene and on CFA13 close to the gene *FAM135b* (Table 1).

Table 1: *Summary of EMMAX analysis results*

Marker	CFA	Marker position	p-value	Candidate gene	Gene position
BICF2P474171	24	40,028,928	1.91E-06	*DOK5*	24: 40,130,265-40,282,249
BICF2P1135812	24	39,697,242	1.00E-05	*DOK5*	24: 40,130,265-40,282,249
BICF2P750280	13	33,702,258	2.06E-05	*FAM135b*	33,403,397-33,579,220

Positions refer to base-pair positions in assembly CanFam3.

F13A1 encodes a coagulation factor and is not of interest in relation to epilepsy. On the other hand, *NRN1* as well as *DOK5* are genes that are involved in neurite outgrowth and pruning. *DOK5* (Docking Protein 5) encodes a cell membrane protein, which interacts with phosphorylated receptor tyrosine kinases to mediate neurite outgrowth (Shi *et al.* 2006). *NRN1* (Neuritin 1) encodes a member of the neuritin family, which is expressed in differentiating neurons in the developing nervous system and in structures associated with plasticity in the adult brain. It promotes neurite outgrowth and branching and has a role in promoting neuritogenesis (Naeve *et al.* 1997). Furthermore, it has also been shown that expression of this protein has an indirect effect on neuronal excitability (Yao *et al.* 2016).

There is an emerging realization that genes involved in normal positioning of neurons and cytoarchitectual aspects of brain development may cause epilepsy (Greenberg & Pal 2007; Cowell 2014), and it is not the first time that genes like *DOK5* and *NRN1* are linked to epilepsy in dogs, humans or model organisms (Table 2). Most notably, Seppälä et al. identified a mutation in the *LGI2* gene causing epilepsy in the Lagotto romagnolo dog breed (Seppälä *et al.* 2011). Similarly, several epilepsy causing mutations have been described in *LGI1* (which is very similar to *LGI2*) in humans, and the involvement of this gene in epilepsy has been intensely investigated in human, mouse, rat and zebra fish (Cowell 2014). *LGI1* and *LGI2* have very important functions in neurite outgrowth and pruning just like *DOK5* and *NRN1*. Hence, we

consider those two genes potential candidate genes that might contain mutations, which could cause epilepsy.

The third candidate gene, *FAM135b*, qualifies as a candidate gene due to its importance for neurite integrity and survival (Sheila *et al.* 2019) and due to its interaction with *ZDHHC17* (also known as *HIP14*) and *KAT5* (also known as TIP60) (Stelzl *et al.* 2005; Butland *et al.* 2014; Huttlin *et al.* 2015; Huttlin *et al.* 2017). These genes play roles in neuronal signaling and neural growth, respectively (Huang *et al.* 2004; Pirooznia *et al.* 2012).

All exons in the three candidate genes were amplified by PCR and sequenced using Sanger sequencing. The following transcript sequences were used as reference: ENSCAFT00000049974.2 (*DOK5*), ENSCAFT00000015082.4 (*NRN1*) and ENSCAFT00000001815.4 (*FAM135b*). Primers for PCR and sequencing are listed in Supplementary Table S1. Two cases and two controls were used for PCR and sequencing. Sequences from cases and controls were compared with reference sequences using Seqscape® Software v. 3.0 (Life Technologies, Carlsbad, CA, USA) and/or Clustal Omega (Sievers *et al.* 2011). No sequence variation were found in the coding parts of the three genes. Furthermore, all splice-donor and splice-acceptor sites were intact in both cases and controls.

In conclusion, the present study identifies weak evidence for an association between idiopathic epilepsy in PBGV and loci on CFA13, CFA24 and CFA35. All three loci contain genes, which can be considered good candidate genes for the phenotype, namely *DOK5*, *NRN1* and *FAM135b*. However, the present study rules out genetic variations in the coding parts of those genes as an explanation for the epilepsy in PBGV.

Further studies should be based on a larger cohort in order to increase power of the association study. If similar collections of PBGV epilepsy cases and controls are available in other countries, a joint effort to identify the genetic causes for idiopathic epilepsy in PBGV would be of great value. Confirmation of one or more of the regions identified in the present study would prompt further analyses focused on the mentioned candidate genes and potential regulatory elements in the region(s).

***Table 2**: Epilepsy associated genes with a known effect on neurite growth and pruning*

Gene Symbol	Species	Reference
LGI2	Dog	(Seppälä *et al.* 2011)
LGI1	Human, Mouse, Rat, Zebrafish	(Owuor *et al.* 2009; Cowell 2014; Silva *et al.* 2015)
CTNND2	Human, Mouse	(van Rootselaar *et al.* 2017)
SALM3	Rat	(Li *et al.* 2017)
STXBP1	Rat	(Yamashita *et al.* 2016)
KDM5C	Mouse	(Wei *et al.* 2016)
c-ABL	Human, Rat	(Chen *et al.* 2014)
Ras-GRF1	Human, Rat	(Zhu *et al.* 2013)
CAMSAP1L1	Human	(Zhang *et al.* 2013)
TRPC6	Rat	(Kim & Kang 2015)
PK1	Human, Zebrafish	(Mei *et al.* 2013)

Supplementary Table S1: Primers for PCR and sequencing

Gene	Exon	Forward/Reverse	Primer (5'->3')
FAM135b	1	F	CAGTTGGGCGGTTTTGCCTA
		R	GAGGAAGGGCACAAGTTAGC
	2	F	TGGCCAACCCTACTATCCCT

		R	TTCTCCTTCAACCAGGCTCC
	3	F	TGCAGACAGTGTTTAGGGGC
		R	GTAGGTGTCCACTGACTGGC
	4	F	ACTTCACTCCTGAGCATCGC
		R	GATAGAACCTGCGGCTGACA
	5	F	ACAAAGGGAGTGCTGTCCTG
		R	CAGGGACATGTGGGGACTTC
	6	F	GGAGTTCACCTTGCCCCTAC
		R	ACCAGCATTGGGCTAGGAAC
	7	F	GCCACAAATACCATGTCGCC
		R	ACTCCCCTTAGCAAGCGACT
	8	F	CTAGTGTGGGGTCACAAGGC
		R	CATCATGCTGCTAGACCCCT
	9	F	CAAAAGGCAGTGTGGTGTGG
		R	AAATGCAGGCGAACCAGAGT
	10	F	TTTGCAAACTCTGGTTCGCC
		R	ATGCGTTTCGAGGCTACTGC
	11	F	AGGATGGACAGACAGACGGT
		R	GAGTCTGAACTGAATGCCGC
	12	F1	ACAGTCAGGCTTTGGGATAGT
		F2	GCAAGGTGGTGCTGCTAAGT
		R1	GGCTGTATTTGAGAGATGGGC
		R2	AACCCAGGTCACTGGCATTA
		R3	GGCTGACCTTTCAGCAAGAC
	13	F	AAGCCATGGTAGCCTTGTGG
		R	TCCCTAGACCTAGCACGCTG
	14	F	CCCATCTGAGGGGCTTGATG
		R	TGCATGACAGGGGCTAGATG
	15	F	GGAGTCCACAGGCACATGAA
		R	TCAATGCTCGTCTCACCCAG
	16	F	GGCAGGGCTGCTCTAACAAT
		R	GCTTGCCCTGGCAATGATATG
	17	F	TTTCGGTTCTTCCACGCACT
		R	GACCCCTGTCTCCCTGCTAT
	18	F	CTCTGAGGACGTGGGAACAC
		R	AGGCCAGCGGGATCTAGAGAA
	19	F	ACACACAGGTAAGCCACATT
		R	ATCTCTGTGAGCCAGGGGTA
DOK5	1	F	CCCGGACCTGATTCTCTCTG
		R	TGGAGGTAGGTTGGAATGGG
	2	F	TGGCGTATGAATACTTTACAGGT
		R	ATGTGGGGTTAGAAGGTGGG
	3-4	F	CCCTCCCTTGCTGTGTCTTA
		R	TATGTGCTGGTTTCTGTGGC
	5	F	TCCCGGGATTTTAGACTAACCT
		R	GATGAGGGGCCATTCGTTTC
	6	F	AATGAGAACCCAGTTGCACG
		R	ACTCCGGTACTCAACGTTGT
	7	F	GCTGACACGTGCTTTCCTC
		R	AGAACAGTGGCCTCAGAGAC

	8	F	GTTGCCTTCCGGACTTCTTC
		R	AGCCACCAGGATGACAATGA
NRN1	1	F	AGTGAACCATTCCCAGCTCT
		R	AACTTTGTCATTCACCCGCC
	2	F	AAACGAAGGAGGGAGTGAGG
		R	TCCACTTCCTTGCTCGACTT
	3	F	GGGAGGAGATCTGAGAAGCC
		R	GTTCTTTGGGGACGTTGTGA

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121 Conflicts of interest
122 The authors have no conflict of interest to declare.
123
124 Acknowledgements
125 We want to thank every dog owner who let us use their dog, blood samples and data for this project.
126 Furthermore, we want to thank laboratory technicians Minna Baron Jakobsen and Tina Bahrt Neergaard
127 Mahler for their splendid work in the laboratory. The study was funded by “Agria och SKK Forskningsfond
128 för sällskapsdjur”, project N2018-0013.
129

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