The genetic background of gastric carcinoma in the Belgian Shepherd (Tervueren and Groenendael)

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Informative abstract

The most common neoplasm in the canine stomach is gastric carcinoma (GC). Although the overall incidence is reported to be low, GC has a high prevalence in among others longhaired Belgian Shepherd dogs (Tervueren and Groenendael varieties). Preliminary data by performing Genome Wide Association Study (GWAS) showed significant differences on chromosome 11 and 18 between case and control Belgian Shepherds. These findings suggest a genetic background to the disease in these dogs. In this research, the relevant regions were further investigated by selecting 10 top SNPs on chromosome 11 and 18. KASP analysis was performed to find out if there was any difference in the frequency of these specific alleles in GC cases compared to controls. For this research, 25 new GC cases and 42 control dogs were used. No significance was found on each of the ten top SNPs. Another research goal was to see if RAD50, a gene found on chromosome 11 and responsible for the repair of mutations, had mutations in 8 gastric carcinoma cases. Also, 8 control dogs were used for this research. No mutations were found in the 25 exons of the gene in neither cases nor controls. Further research is recommended because of the high chance of genetic predisposition and poor diagnosis of the disease. When mutations have been identified, a DNA test can be developed to assist breeders in reducing the incidence of this fatal disease.

Introduction

The most common neoplasm in the stomach of the dog is gastric carcinoma (GC),[1–4] accounting for 40-90%.[2,4,5] Although the overall incidence of gastric tumors is reported to be less than 1%, there seems to be a breed predisposition in among others the Belgian shepherd (Tervueren and Groenendael).[2,4,6–8] There is a documented male predisposition for the disease.[1,3,4,9] Also, a higher incidence is known in middle aged to older dogs: the average age for GC at presentation ranges from 8 to approximately 10 years.[1,3,4,9] The etiology of the disease is complex and poorly understood, but it is believed that both genetic and environmental factors play a role.[9]

The most common clinical signs of GC are progressive vomiting, weight loss and anorexia.[2,4] Unfortunately, GC mainly becomes evident at an advanced stage of development: 70-90% of gastric carcinomas have already metastasized by the time of diagnosis.[2,8–10] A possible reason for the high metastasis rate could be the fact that the clinical signs of GC resemble those associated with chronic gastritis.[11] Some patients are symptomatically treated for gastritis for prolonged periods before the proper diagnosis is established.

GC is most often located at the lesser curvature or the pyloric antrum of the stomach.[1–4,9] Extension throughout the stomach is possible.[3,9] The diagnosis is preferably made by means of endoscopy in combination with taking biopsies.[2,4] Mostly seen is a distinct ulcer with thick and irregular walls elevated from the surroundings, but more diffuse changes with loss of rural folds and submucosal vascular pattern have also been described.[7,10] Biopsies are mostly assessed according to WHO classification with major types based on growth pattern: papillary, tubular, signet ring, mucinous or undifferentiated[1].

The only remedial treatment available is surgical removal; chemotherapy is ineffective and radiation therapy is not well tolerated by the adjacent organs.[2,9] Malignancies should be resected with wide margins of 1-2 cm of seemingly non-tumorous tissue.[9] Because CG is mostly diagnosed at an advanced stage, surgical therapy becomes extremely difficult or even impossible.[2,3,9] Also, the extent and location sometimes can preclude dissection.[9] Therefore, GC prognosis is very poor: the disease is usually fatal. [1,2] One case series reported a median survival time of 35 days, with a range of 0 days to 10 months after diagnosis.[12] Palliative surgery may justifiably increase survival times by some months.[3]

Although the incidence of GC in dogs is reported to be low, the disease has an higher incidence in a small number of breeds[2,3]. One of those breeds is the Belgian shepherd.[2,6–8] American veterinary databases show an incidence of gastric carcinoma in the dog of 0.1 percent.[2] A retrospective study in The Netherlands among Belgian shepherds showed an incidence of 1.18%[6]. This incidence is remarkably higher than the American average.[2,6] Another study done by Seim-Wikse et al found a promotional morbidity ratio (PMR) of 56 for the Tervueren shepherd, which indicates that dogs of this breed are 56 times more likely to be diagnosed with gastric carcinoma than the average purebred dogs in their database. The Groenendael variety had a PMR of 35.[4]

Although the etiology of canine GC is complex and poorly understood, the strong breed predisposition and familial occurrence described for among others the Belgian Shepherd (Tervueren and Groenendael), indicates the importance of genetic factors in the cause of this devastating disease. An opportunity exists for research into the genetics of gastric carcinoma in Belgian Shepherds. With the identification of gene mutations that are responsible for the susceptibility for gastric carcinoma in the Belgian shepherd, it will be possible to develop a genetic test to identify dogs at risk at an early age, before they are used for breeding. Implementation of a genetic test in the breeding strategy will aid in diminishing the number of Belgian Shepherds affected with gastric carcinoma. Identification of dogs at risk at a young age will obtain insight in the pathogenesis of the development of gastric carcinoma, which is still unclear. This may be the basis for development of new therapies and breeding advice. Results obtained in these dogs may very well have implications for other breeds and human patients as well.

In this research, we want to achieve an important step forward to unravel the genetic background of gastric carcinoma in Belgian Shepherds and thereby reduce the occurrence of this disastrous disease. Preliminary research has already been performed at the Faculty of Veterinary Medicine of Utrecht University (data unpublished). For genetic analysis, a genome wide association study (GWAS) was performed on 96 Belgian Shepherds (48 cases and 48 controls) with no first degree relationship. The individuals were genotyped with Canine HD BeadChips consisting of 174.000 Single Nucleotide Polymorfisms (SNPs)[13]. Stratification and cryptic relatedness were adjusted to correct sub-structuring between the two different varieties. The analysis demonstrated two peak regions that did differ between cases and controls after 1000 permutations (Figure 2). Figure 1 shows the results of the GWAS before the adjustments.

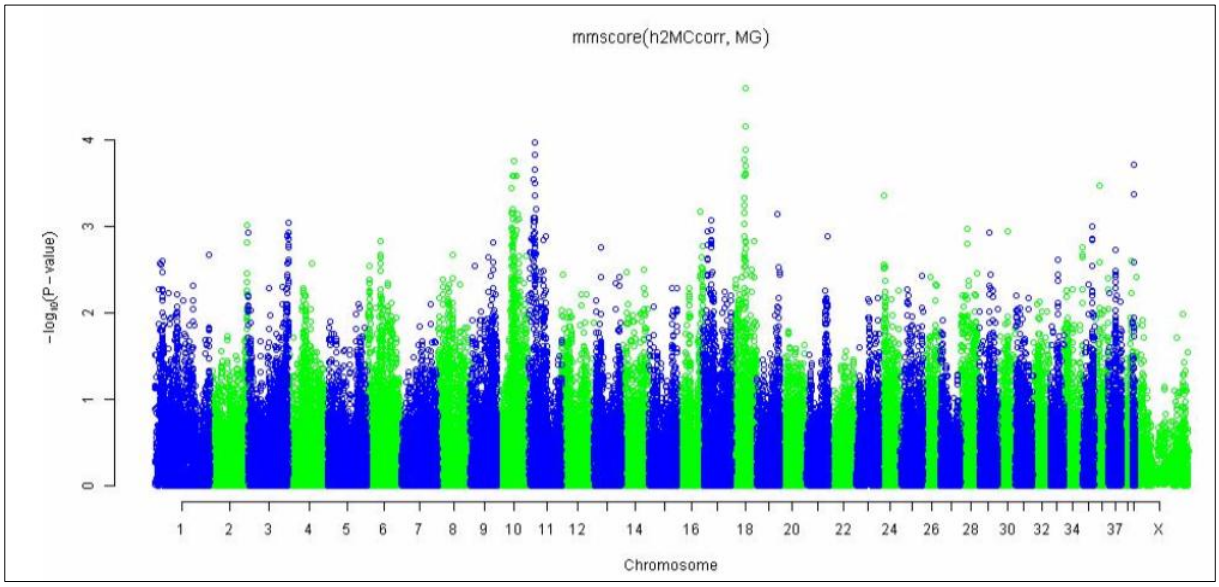

Figure 1: GWAS result before permutations (data unpublished)

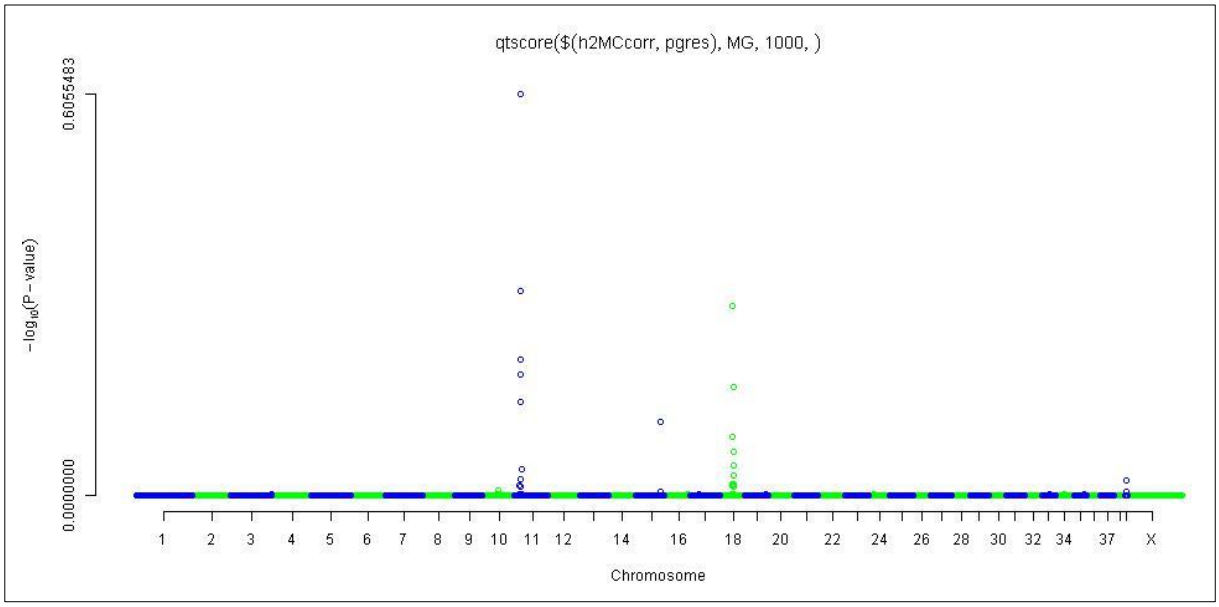

Figure 2: GWAS results after permutations (data unpublished).

The 12 top SNPs that showed the strongest association with the phenotype of gastric carcinoma in the GWAS have been selected for analysis in an extended cohort. KASP analysis[14] was done and statistical analysis had been performed by chi-square statistics. In total, 32 new cases and 62 new controls were included. SNP 1 and 6 did not seem to work. Significance was found for SNP 7,8,9,11 and 12 on chromosome 18 (Table 1).

SNP	P-value	Chromosome location
1. BICF2S23111092	----------	11:14320964
2. BICF2P1319313	0,8465	11:13787547
3. TIGRP2P247540_rs9128313	0,7545	11:15714657
4. BICF2P475373	0,8154	11:18198860
5. BICF2S23060322	0,1740	11:13038995
6. TIGRP2P247891_rs8705452	----------	11:10226566
7. BICF2P784489	**0,0207**	18:28294061
8. BICF2S23450102	**0,0004**	18:31147890
9. BICF2G630294452	**0,0049**	18:28638949
10. BICF2S22957845	0,1153	18:30022198
11. BICF2S23726024	**0,0185**	18:31837102
12. BICF2S23038530	**0,0064**	18:29727234

Table 1: p-values preliminary KASP assay

Research goal

Further investigation will be done by repeating the KASP analysis on a cohort of Belgian Shepherd dogs consisting of 25 new cases and 42 new controls. In the previously performed KASP analysis, significance was found for 5 out of 12 SNPs on chromosome 11 and 18. For this research, the same 10 SNPs that worked will be investigated on the new cohort.

Another goal of this research is DNA sequence analysis of *RAD50*.[15] This is a candidate gene in the critical region of chromosome 11 which could play a role in causing gastric carcinoma in Belgian Shepherds. RAD50 encodes a protein that repairs mutations.[16] If this gene does not function properly, there will be less repairs. This may cause more unfixed mutations which can lead to neoplasia.

The research questions are:

1. *Do regions on chromosome 11 and 18 play a role in the development of gastric carcinoma in Belgian shepherds?*
2. *Are there any mutations in gene RAD50 found which could be associated with the development of gastric carcinomas in Belgian shepherds?*

Materials and methods

KASP assay

The first research goal was to confirm if the 10 SNPs on chromosome 11 and 18 indeed play a role in the development of gastric carcinoma by testing a second cohort of dogs. For this aim, KASP assay (Figure 3) was used[14]. The signals have been detected in a Bio-Rad icycler. The result could be FAM (allele 1), HEX (allele 2) or both of the fluorescent labels (heterozygous) The difference between the groups has been evaluated by Chi-square statistics.

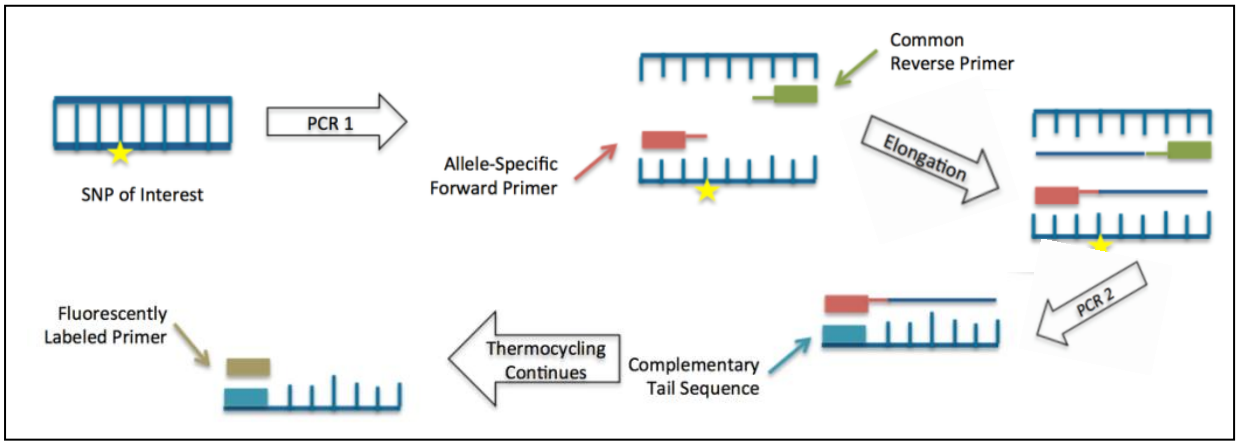

Figure 3:principle of KASP analysis (According to LGC group[14]): the KASP reaction consists of two different allele specific forward primers, common reverse primers, Taq polymerase, FAM labeled fluorescent oligonucleotides, HEX labeled fluorescent oligonucleotides and dNTPs. In PCR 1, denaturation occurs at 94C⁰, followed by annealing and extension of the target specific part of the primers at 60C⁰. The tail of the primers will not extend.PCR 2 starts with denaturation of the newly formed double stranded DNA, followed again by annealing and extension of the strands. This time, the complementary sequence to the allele specific tail sequence (red) is generated (blue). PCR round 3 (after 'thermocycling continues') again starts with denaturation of the double stranded DNA. The presence of the new complement (blue) now allows to bind a FAM or HEX labeled oligonucleotide (brown), depending whether the DNA template is homozygous for either of the allele possibilities or homozygous. The number of PCR products increases exponentially as the denaturation and annealing rounds continue, so more HEX or FAM oligonucleotides become incorporated. After the analysis, signals of the PCR reaction were detected. Results can be FAM, HEX or both (of heterozygous)

gDNA animals

For the KASP assay, the DNA from a newly recruited cohort of 67 Belgian Shepherds, which consisted 25 dogs with confirmed CG (cases) and 42 without CG (controls), was used. Dogs are a suitable 'control' when fulfilling the following criteria: (1) they must be 12 years or older, (2) no clinical signs of gastric problems may be present, (3) there is no first degree relationship to a confirmed GC case and (4) they may have no other neoplasia. For the gDNA extraction, the method 'Salt extraction' by Miller et al.[17] had been used.

KASP assay

KASP assay was performed on all 67 DNA samples. For each SNP reaction (384 qPCR well plate), 2uL KASP V4.0 2x Mastermix (LCG), 0,055 uL KASP assaymix and 2uL gDNA (5-20 ng) of each DNA sample has been utilized. The assaymix consisted of 12uM of each of the SNP specific forward primers and 30uM of one of the common reverse primers (Eurogentec Oligo Centre). The primers for the 10 SNPs had already been designed in collaboration with the University Medical Center Utrecht and were also used for the first cohort of dogs (appendix 1). Notice that the amount of assaymix per well is really low and therefore hard to pipette. The easiest way is to mix the KASParmix and SNP specific assaymix for all the samples together in an Eppendorf tube. Vortex well and spin briefly.

For the thermal cycling reaction, GeneAmp PCR system 9700 had been utilized. Two different PCR protocols were used depending on the SNP: the A-69 protocol or the V4-protocol (Table 2).

Single Nucleotide Polymorphism	Program
2(BICF2P1319313)	A69
3(TIGRP2P247540_rs9128313)	V04
4 (BICF2P475373)	A69
5 (BICF2S23060322)	V04
7 (BICF2P784489)	A69
8 (BICF2S23450102)	A69
9 (BICF2G630294452)	A69
10 (BICF2S22957845)	A69
11 (BICF2S23726024)	A69
12 (BICF2S23038530)	A69

Table 2: protocols used for thermal cycling reaction

A69 protocol

94^{0}C 15' (minutes)

94^{0}C 10'' (seconds)
57^{0}C 5''
72^{0}C 10''
} 20 cycles

94^{0}C 10''
57^{0}C 20''
72^{0}C 40''
} 18 cycles

20^{0}C 5'
20^{0}C 30''
Plate read out

V4-protocol

94^{0}C 15'

94^{0}C 20''
61^{0}C 60''
Drop -0,6^{0}C/cycle
} 10 cycles

94^{0}C 10''
55^{0}C 60''
} 30 cycles

94^{0}C 20''
57^{0}C 60''
} 10 cycles

20^{0}C 5'
20^{0}C 30''
Plate read-out

BIO-Radicycler

Signals of the PCR reaction were detected in a Bio-Rad icycler (CFX 384 Touch). Results can be FAM, HEX or both. For data analysis, CFX manager software (version 3.1) was used (Figure 4).

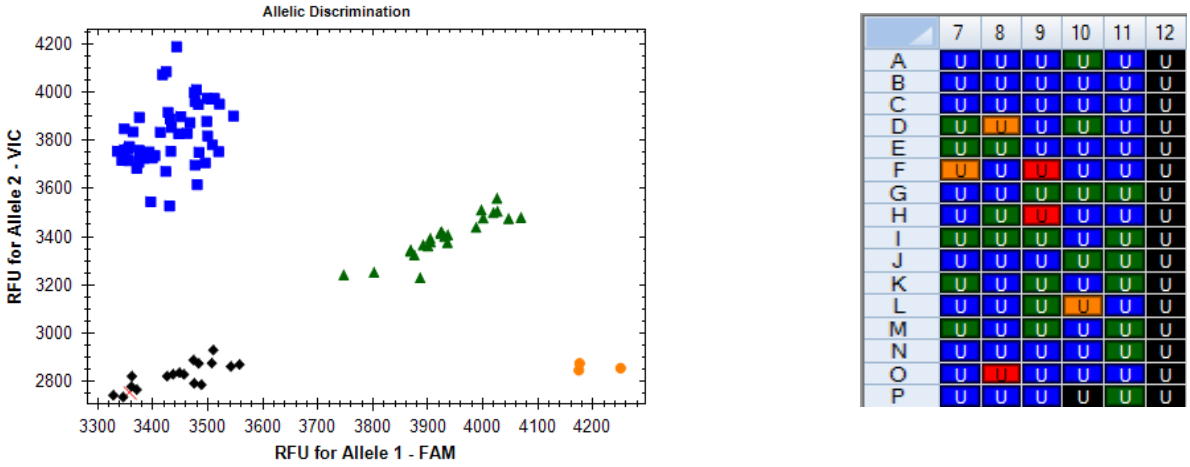

Figure 4: Example of a SNP result. Blue and yellow are homozygous SNPs, green means that the SNP is heterozygous. The red color resembles a false result, the black dots are controls.

Sequencing

The second research goal was to find mutations in *RAD50* that are possibly causative for gastric carcinoma by DNA sequence analysis of this gene. For the subject, DNA of 8 Belgian Shepherds with confirmed gastric carcinoma and that of 8 control dogs was used. Primers had been designed for PCR amplification of the 25 coding exons from *RAD50* (appendix 2). DNA sequence analysis had been performed with the dideoxy chain termination technique/Sanger sequencing. Products were sequenced with BDT 3.1 according to manufacturer protocol with addition of 4M betaine. The DNA sequences are compared with DNA seqman software(SeqMan pro).

Selecting primers

25 forward and reverse primers were designed to sequence all the exonic regions of *RAD50*. The sequence of RAD50 of Canis lupus familiaris[15] was used to design these primers with Perl Primer software (version 1.1.21). The specificity of the primers was validated with BLASTn specifity analysis. The forward and reverse primers were ordered at OligoCentre Eurogentec. The list of primers was added (appendix 2).

PCR + Gel electrophoresis

First, the DNA regions of interest were amplified by performing PCR (GeneAmp PCR system 9700) on each of the 16 DNA samples. The following protocol was used (Table 3). All reagents were put together in a 96-well plate. For primer 6, 8 and 25 the same reagents were used as for the other primers but the annealing temperature was set on 60^0C** instead of 55^0C* for better results. These temperatures were chosen by doing several test PCRs including a gel electrophoresis on the DNA of a non CG dog. Some primers still did not work after doing multiple test PCRs, so new primers had to be ordered. In this case, new primers for exon 1 and 2 have been designed.

Reagent	**uL**	**Concentration**
10X PCR Buffer (Invitrogen™)	1,5	1x
MgCL2 (50mM)(Invitrogen™)	0,6	2mM
dNTPs (10mM) (Biolabs®)	0,3	200uM
Primer F (10mM)	0,75	0,5uM
Primer R (10mM)	0,75	0,5uM
Platinum Taq (Invitrogen™)	0,15	***
gDNA (25ng/uL)	2	25ng
Betaine (4M)	3,75	1M
mQ	5,2	***
Total:	15uL	

Table 3: PCR reagents used.. Thermal cycling program:see below table 3

95 ^{0}C	5’	
95^{0}C	30’’	35 cycles
55^{0}C*/60^{0}C**	30’’	
72^{0}C	30’’	
72^{0}C	10’	
20^{0}C	infinite	

After PCR, gel electrophoresis was done by adding 7,5uL PCR product and 2uL load dye (6X blue/orange, Promega) in a matrix of agarose gel, consisting of 1,2 g agarose and 4uL EtBr dissolved in 100mL 0,5% Tris/Borate/EDTA buffer. Also, a ladder was added, consisting of 3,5uL ladder (100bP, Promega) and 2uL load dye. Settings were put on 100V, 50mA for about 45 minutes. After electrophoresis, the results were visible by using the Bio-Rad Geldoc 2000 in combination with Quantity one pc software (Bio-Rad version 4.6.5.) (Figure 5)

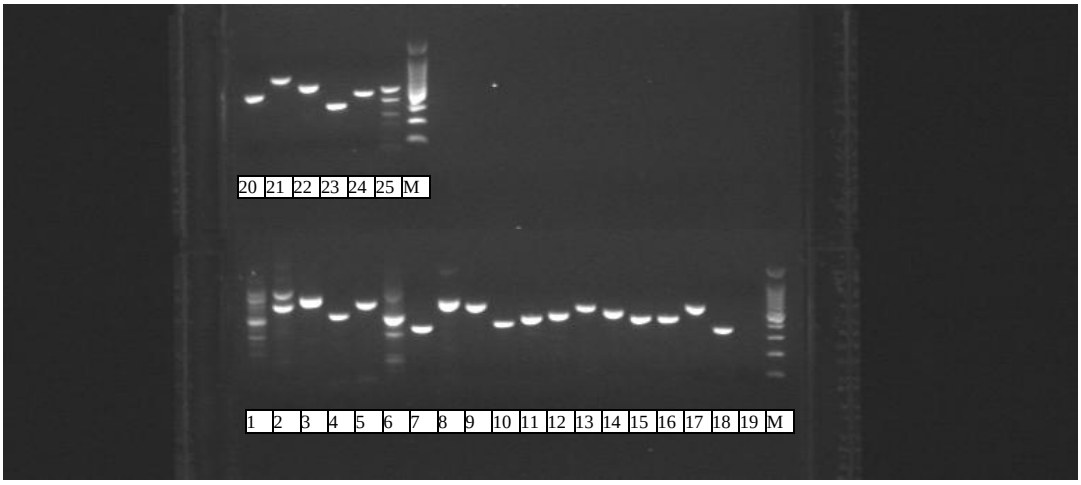

Figure 5: result of the first gel electrophoresis that was done. In this case, the annealing temperature was set on 55 degrees Celsius. For this electrophoresis, the PCR product of DNA from a non GC dag was used to get to know the best annealing conditions and the best program per primer. The bottom row shows exon 1 to 19 (19 is not visible), the upper row shows exon 20 till 25. ‘M’ stands for the ladder that was used (100 bP, Promega). The annealing temperature for primer 6 an 25 was set on 60 degrees Celsius. This change didn’t work for the old primer 1 and 2, so new primers were designed for the exons concerned.

Exo
For purification, exonuclease 1 (BioLabs) was added to every PCR product that showed a positive result on gel electrophoresis. 10 uL of PCR-product and 1uL EXO1 (2units/uL) were added together in a 96-well plate and incubated at 37^{0}C for 45 minutes, 75^{0}C for 15 minutes and then hold at 4^{0}C.

Preparing sequencing reactions
For preparing sequencing, the products shown in table 4 were added in separate tubes (Table 4). The mixtures were put in a thermal cycler (GeneAmp PCR system 9700). In total, 50 reactions, whom 25 forward primer and 25 reverse primer reactions, did take place on all 16 samples.

Reagent	**uL**
Terminator Ready Reaction Mix (ABI PRISM, BigDye Terminator v3.1, Applied Biosystems)	1
PCR product	2
Forward or reverse primer (3.2 pmol)	1
5x sequence buffer	2
mQ	1,5
Betaïne (4M)	2,5
Total volume	10

Table 4: sequencing reagents use. Thermal cycling program: below table

96^{0}C	5’	
96^{0}C	30’’	35 cycles
55^{0}C*/60^{0}C**	15’’	
60^{0}C	30’’	
4^{0}C	hold	

Sephadex purification
Load dry Sephadex plates were constructed by adding Sephadex G-50 to a Column Loader, removing the excess resin off the top of the Column Loader, placing a Multiscreen MAHV plate upside-down on the top of the Column Loader inverting both Multiscreen MAHV plate and the Column Loader, tapping on top of the Column Loader to release the resin followed by adding 300uL mQ water to each well and incubating the plate at room temperature for 3 hours.

After incubating, a Centrifuge Alignment Frame was placed on top of a standard 96-well microplate and the MAHV plate was put on the assembly, without lid. The columns of the MAHV plate were packed by centrifuging the assembly at 1900 rpm for 5 minutes.

For the sequencing reaction, 15uL mQ was added to the PCR-products and everything had been added together to the center of the columns. After this, the MAHV plate was placed on top of a sequencing plate (MicroAmp® Optical 96-well Reaction Plate) and was centrifuged at 1900 rpm for 5 min.

Electrophoresis
Electrophoresis was proceeded on the ABI Prism 3130xl analyzer by using protocol ‘Sequence and Genescan: ABI3130XL.

Seqman Pro
Seqman Pro was used to read the results.

Results

KASP assay

In this research, we were looking for gene varieties which occur more often in dogs with GC compared with healthy Belgian Shepherds. These varieties have come from a common ancestry, visible when looking at the SNP varieties that lay among the gene varieties. KASP analysis was performed on 25 case dogs and 42 control dogs to see whether the SNP genotypes of GC cases differ from the genotypes of control dogs. For each SNP there are two nucleotide options. Thus, three different outcomes could occur: the SNP is either heterozygous for one of the two possible alleles or the SNP is heterozygous. Allele varieties for each SNP were counted afterwards and the results between the case and the control group were compared by performing Chi-square statistics. No significance was found on either of the SNPs. When combining the KASP results of the preliminary group with this second group, significance was found for SNP 8 and SNP 9 (Figure 6 and Table 5).

Chi2-test					
Observed:					
	Control	Case	Total	Percentage	
C	34	18	52	0,393939394	
T	50	30	80	0,606060606	
Total	84	48	132		
Percentage	0,636364	0,363636			
Expected:					
	Control	Case	Total		
C	33,09091	18,90909	52		
T	50,90909	29,09091	80		
Total	84	48			
Chi2					
	Control	Case			
A	0,024975	0,043706		Chi2=	**P-value**
G	0,016234	0,028409		0,113324176	0,736391

Figure 6 (left): an example of the chi-square statistics performed on the allele frequency outcomes of SNP 11. For calculation, Microsoft Excel was used. The observed and expected allele frequencies (C and T) for the case and control group are displayed. KASP results from one case dog had been excluded from statistical analysis because of poor data. The expected outcomes were calculated by using the observed outcomes. The bottom of the figure shows the results of the chi-squared test performed on the observed and expected values.

SNP	P value	P value preliminary Research	P values combined
1. BICF2S23111092	----------	----------	-------
2. BICF2P1319313	0,2953	0,8465	0,427
3. TIGRP2P247540_rs9128313	0,9142	0,7545	0,848
4. BICF2P475373	0,6724	0,8154	0,886
5. BICF2S23060322	0,7683	0,1740	0,481
6. TIGRP2P247891_rs8705452	----------	----------	----------
7. BICF2P784489	0,9218	**0,0207**	0,071
8. BICF2S23450102	0,6322	**0,0004**	**0,020**
9. BICF2G630294452	0,9197	**0,0049**	**0,026**
10. BICF2S22957845	0,4146	0,1153	0,504
11. BICF2S23726024	0,7364	**0,0185**	0,886
12. BICF2S23038530	0,7341	**0,0064**	0,060

Table 5: P values of the preliminary KASP analysis and the actual research combined. Difference in amount of GC case and control dogs per group was corrected for.

DNA Sequencing

The second research question was whether there are any mutations in gene *RAD50* which could be associated with the development of gastric carcinomas in Belgian shepherds. The DNA sequence for all the 25 exonic regions of *RAD50* were compared between GC case and control dogs (all Tervueren or Groenendael). No difference was found on any of the 25 exons for both case and control dogs when compared with the Canis Familiaris sequence of *RAD50*[18]. Unfortunately, primer 19 did not attach on each of the DNA samples. This could be explained by the fact that the primers were designed by using the CanFAm 3.1 assembly of the dog genome produced by the Broad Institute, whereby the DNA of a (female) Boxer was used[19]. It is possible that Belgian Shepherds miss the part of DNA where the primer was supposed to attach. Fortunately, the reverse primer of exon 18 did attach behind exon 19 so the sequence of exon 19 could still be read.

Appendix 3 shows an example of a sequencing result, in this case exon 7. C22144 is a control dog, L14303 is a GC leader dog (Appendix 3).

Discussion

The first research question was about whether chromosome 11 or 18 plays a role in the development of gastric carcinoma. Logistic regression has been done on the KASP assay of this study and the preliminary KASP together. Extra terms have been added to adjust for potential confounders. Based on the logistic regression and the chi2 statistics, the 10 top SNPs do not seem to be associated with the genetic cause of gastric carcinoma among Belgian Shepherds (Tervueren and Groenendael), despite the small significance that was found on SNP 8 and 9 after combining both KASP results. The very high p-values of the KASP analysis performed for this research disproved the first hypothesis. It is presumed that GC is a multifactorial disease: more genes could be responsible for the development of the condition. This makes research on the etiology difficult. Furthermore, not all the SNPs of the dog were checked: this chip only contains 170.000 SNPs, while there are probably millions SNPs located on the whole canine genome. [20,21] Maybe a more important SNP has been missed. The second research question was about whether gene *RAD50* plays a role in the development of gastric carcinoma. No mutations were found on the 25 exons of *RAD50* in either the case nor the control dogs. This suggests that *RAD50* is not associated with the development of the disease.

The reason *RAD50* was selected for sequencing is because *RAD50* might cause less DNA mutation repairs. Also, the gene is located on chromosome 11. On this chromosome, the most top SNPs derived from the GWAS study find its location. Also, relationships have been found between mutations in *RAD50* and the development of cancer in humans. Nevertheless, particularly cases of breast cancer have been described but no relationship between the existence of gastric cancer and *RAD50* have been found to date.[22,23]

Despite the results of this research, there still is a considerable chance that genetic factors play a role in the development of gastric carcinoma, since different studies showed an assuming predisposition in Belgian Shepherds.[4,7] Furthermore, studies confirmed that germ line *CDH1* mutations cause familial gastric cancer in humans.[24–26] This might also be a possible factor of the existence of GC in Belgian Shepherds. Because of these facts, further genetic research is recommended. Next Generation Sequencing will be performed on the genome of 2 new Belgian Shepherd CG cases. Gastric carcinoma tissue and peripheral tissue will be sequenced genome wide. So, *CDH1* will automatically be included. The outcome of this research will determine whether another GWAS study will be performed on a new recruited group of 48 gastric carcinoma cases and 48 control dogs (Tervueren and Groenendael). Follow-up studies can give more insight in the role of genetic factors that could cause canine gastric carcinoma. Subsequently, genetic tests can be created to identify dogs at an early age. Carrier dogs can be prevented from breeding leading to less offspring developing this disastrous disease.

Acknowledgements

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Apppendix 1: Primers used for KASP assay

Primer	Number	
BICF2S23111092 Forward 1 Forward 2 Reverse	1	 GAAGGTGACCAAGTTCATGCTAAATTAGAATATGAACATTAAATAGGTGATGAA GAAGGTCGGAGTCAACGGATTAAATTAGAATATGAACATTAAATAGGTGATGAG ATATAAGCCTTTTCTTTAGTCTACAGGCTA
BICF2P1319313 Forward 1 Forward 2 Reverse	2	 GAAGGTGACCAAGTTCATGCTGGATCTCTGCATGAAATACTCAG GAAGGTCGGAGTCAACGGATTGCTGGATCTCTGCATGAAATACTCAA CCAGCTCAGGAAGTCTTTAAATTTCTGTT
TIGRP2P247540_rs9128313 Forward 1 Forward 2 Reverse	3	 GAAGGTGACCAAGTTCATGCTCAGGCACTTAGCATTAGCTGATATAATA GAAGGTCGGAGTCAACGGATTAGGCACTTAGCATTAGCTGATATAATG ACATTACTTCCATGAGTGTGAAGGGAAAA
BICF2P475373 Forward 1 Forward 2 Reverse	4	 GAAGGTGACCAAGTTCATGCTGATTCCCATTCATATGTTTCAAATGCAC GAAGGTCGGAGTCAACGGATTAGATTCCCATTCATATGTTTCAAATGCAT TCAAAGGTGGGGGCCTAAGGTTTA
BICF2S23060322 Forward 1 Forward 2 Reverse	5	 GAAGGTGACCAAGTTCATGCTAAAATCCTCTAAATCTACAAACTGATACCA GAAGGTCGGAGTCAACGGATTATCCTCTAAATCTACAAACTGATACCG GGATGGTCTCCTCTTGGCAGCTT
TIGRP2P247891_rs8705452 Forward 1 Forward 2 Reverse	6	 GAAGGGTGACCAAGTTCATGCTTTATCTCTGAATTCCCTTTCCAC GAAGGTCGGAGTCAACGGATTGCTTTATCTCTGAATTCCCTTTCCAG CAGATTAAAGATAATTATGATGGCCTTCAA
BICF2P784489 Forward 1 Forward 2 Reverse	7	 GAAGGTGACCAAGTTCATGCTGGGATGAGAAATCCTGCTTCTCC GAAGGTCGGAGTCAACGGATTAGGGATGAGAAATCCTGCTTCTCT CAAACCCTCTCCTTTTTTAAGGCACAATT
BICF2S23450102 Forward 1 Forward 2 Reverse	8	 GAAGGTGACCAAGTTCATGCTCCAGGTGACTTCCCACACATTG GAAGGTCGGAGTCAACGGATTATACCAGGTGACTTCCCACACATTA GGGTGCTTGTCTTTGCAACAGAACAA
BICF2G630294452 Forward 1 Forward 2 Reverse	9	 GAAGGTGACCAAGTTCATGCTATTTATTTTAGTATCATTTAGAGTTACAGTGAC GAAGGTCGGAGTCAACGGATTTATTTTAGTATCATTTAGAGTTACAGTGAT CCTTTGAAAAAGGAAAAGTGTCTTTGAGTA
BICF2S22957845 Forward 1 Forward 2 Reverse	10	 GAAGGTGACCAAGTTCATGCTAGGATTCATGGATCTGAGTCCTC GAAGGTCGGAGTCAACGGATTCTAGGATTCATGGATCTGAGTCCTT TTAGTCTTCCTATGGTCCACGGGTA
BICF2S23726024 Forward 1 Forward 2 Reverse	11	 GAAGGTGACCAAGTTCATGCTCCCTCTGATGGCAAACACCG GAAGGTCGGAGTCAACGGATTCTCCCTCTGATGGCAAACACCA GCCTGGGTCCTGCCTGCCT
BICF2S23038530 Forward 1 Forward 2 Reverse	12	 GAAGGTGACCAAGTTCATGCTAGGAAGAATTTTTCCACATTGGGATTATA GAAGGTCGGAGTCAACGGATTGGAAGAATTTTTCCACATTGGGATTATG TCCCAGAAGAAAAAAAAAATGAGAACTGTT

Appendix 2: Primers used for sequencing *RAD50*

	Forward Primer	Reverse Primer
1	GAATCCGTGAGTGCTCCCTT	CGCCTCGCCTTTCATCCTTA
2	GATCCCTCCTCTCCAGTTAACAC	GTGGTTGTCACTGGGTCCTAA
3	GCAACTTAATCTCAAGTGGTTC	GTACTCAGGAACTCTACTTTCTG
4	TTGTAATGGGATGAATGAGGG	CACTGGTTACTACTGGATGAC
5	ATCTGTCATCCAGTAGTAACCA	GGGCAATTCTAACACTAACCA
6	CTTTGGAGCTAGAAGTGGTG	CCGAAATATGAGTGGCAAATG
7	GTGTGACTTCCATGTATGTGTT	TGGAGATTCATCATTCATGCAG
8	AGCTGCATATGAATGTACAGG	CTGAGGAAAGTTGCTTCCAT
9	CCTCTGATGACCACAAATCTAA	AGCAATAGGCAATATACCTCAG
10	TTCTTGGATAAGGGCAAACAGT	GCACCACCTAAACACTTCAAAG
11	CTTAGTCGGTAGAGGATGTAAC	GGCCCAAATCAATTTCAACA
12	TAGAGTTCAGGCAGAAATGG	AGCCACAAAGTCAGTAAGTT
13	TTAGAACAGTTTCTGGCACAC	ACTGTGGAACACCTACCATAT
14	ACATCATGTACCGCTGATCT	CTCAATTTGGCACCAATTTCAG
15	GAGAGGTTCCAGGTAAGTTTATTG	CTAGGATGTTGAAGTCTTTATGGG
16	TGAGCCTTTGTGAATTCCATAGAG	GACATCTGGTTGGCTAAATCAGT
17	CCCAGACAAATCATTAGTATGCCT	CTTCCCATCACCACCAAGTTT
18	CTTTCTAAATGCCGTGTCCC	AGACCTACCTTCTGTGTATCAA
19	CCCAGGGATCCCTAAACATTAGTA	ACTGTATGCATTTCTACTCGATGG
20	GGCTATCCAGATTCTTCCAA	CTGTCTACCTTCCTGTAAGTC
21	TGGATGATTCAGAGATTTGGGAAG	AGGGTGGTATCAAGAGTACTCTTT
22	TGGTTCCTGTTTCTAGCTCTTA	GCCAAATATGTAAGTCCTGTCA
23	GAATGGTAATGTCAGCTTCAC	AGTTTCTTCCCGTCAATTCA
24	TGACCTCGTGACATTGATCCT	TGCTGTTCACCACCTAGTTCT
25	GCACTGTCTGGAGAGGAGATG	AGAAATAAGCAGTTGCCTGAGGA

Appendix 3: sequencing result example: exon 7