

Genetic Test Development for Optic Nerve Hypoplasia, Micropapilla and Juvenile Cataracts in Poodles

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Objective:

Poodle breeds may be affected by a variety of ophthalmic diseases. Among these, we find Optic Nerve Hypoplasia (ONH), Micropapilla, juvenile Cataracts, and 'Achromatopsia' as the most relevant. Since those diseases are likely of genetic cause, our work focuses on the analysis of the Poodle population to identify the genes and mutations responsible for the development of these eye related conditions, as well as to predict their possible mechanisms of inheritance.

Once a causative mutation is identified and its disrupting effect validated, the subsequent step would be the development and implementation of genetic tests that can identify genetically normal, affected and carrier dogs. The identification of the latter would be absolutely vital for the health of the Poodle population. In fact, a quick identification of carriers is crucial for monitoring the population, and for a proper and conscious planning of the breeding.

Background:

As with other dog breeds, Poodles are affected by a variety of ophthalmic problems, some of these common among all Poodle breeds, and some specific to one Poodle variety. In our studies we have concentrated on the most common ophthalmic diseases affecting standard and Poodles.

Our initial research focused are on Optic Nerve Hypoplasia (ONH), Micropapilla, and juvenile cataracts, and subsequently was expanded to include Polymicrogyria and Achromatopsia (now known as Day Blindness/Retinal Degeneration-DB/RD).

ONH is a congenital condition in which the optic nerve is abnormal/underdeveloped, and because of its smaller size, depending on the degree of underdevelopment (hypoplasia) the vision can be partially or fully impaired (that is, the condition leads to blindness). It is thought that ONH has a genetic cause in dogs, since it does in humans where associated genetic mutations have been found. We discovered ONH in a number of Poodle families.

On the other hand, Micropapilla is characterized by a smaller than normal optic disc diameter, precisely at the point where the optic nerve enters the posterior part of the eye. Micropapilla rarely results in blindness, or even vision loss, though the way this phenotype is expressed may vary. At the moment, there is no clear evidence of how the inheritance for Micropapilla works.

We detected the presence of Micropapilla in dogs related to the ONH cases, sometimes half-siblings. At the moment we cannot exclude the chance of a presence of the same disorder with variable phenotypes, or two different disorders present in the same extended family, or any other possibility. In other words, Micropapilla is possibly a stage between normal and ONH, present in Poodles.

Cataracts are the most common cause of vision impairment in humans and are often observed in animal models such as mice, sheep, cattle and swine. Age, poor nutrition, UV light exposure, trauma, as well contact with toxic substances can lead to the development of cataracts. However, cataracts are often an inherited condition, and cataracts detected in younger dogs are more likely to be such. Dogs with inherited cataracts are born with normal lenses, which then proceed to increase in opacity over time, which may lead to blindness. As shown in human, livestock and dogs, mutations in many genes can lead to cataract development, and the inheritance mechanism can be dominant, recessive, or related to the sex of the animal. We have detected a number of cataract cases of probable genetic origin both the Miniature and Toy

Poodle. Based on the population structure of dogs with juvenile cataracts, our working hypothesis is that in the Poodle, cataracts are inherited as autosomal recessive.

In addition to these starting projects, we have also included in our studies two other inherited diseases affecting vision in Poodles. These are Polymicrogyria and Achromatopsia. The former is an abnormal development of the “gyri” (the ridges of the brain) and is characterized by a variable number of effects related to brain abnormality, included seizures, palsy, and the dogs are centrally blind. The latter is a progressive form of color blindness that leads to day blindness, characterized by visual deficits in daylight and subsequently complete blindness. Through support of the Poodle Club of America Foundation, research completed in the past year identified the gene and mutation responsible for the disease, and developed a DNA-based test for diagnosis of affected and carrier dogs.

Methods (where applicable, the Methods section provides expanded details from original proposal and previous progress reports):

Sample collection: We put every effort to have Dr. Aguirre examine all the study dogs (thus, counting on a reliable phenotype assessment consistent between samples). Nonetheless, we realized early on that this would be impractical, thus dogs examined by other ACVO certified veterinary ophthalmologists from the US, and ECVO certified veterinary ophthalmologists from different centers in Europe were included in the study.

In order to avoid any misinterpretation of the data provided by the examining ophthalmologist, a standardized eye exam form was developed. This is a standard procedure for our group and is carried out in order to grant the greatest clarity in the dataset, particularly crucial if the analysis of the data and the genetic investigation demands the use of statistical tools.

Blood samples and eye exam forms were collected from Poodles with above mentioned conditions, as well as their non-affected relatives. All of the blood samples have been sent to us in EDTA lined tubes, to prevent clotting. DNA was isolated from the blood samples, for further analysis. The standardized eye exam forms were used to assess the dog’s phenotype (case vs. control).

Pedigree analysis: We investigated the family trees of the affected dogs using pedigree information obtained from an online database <http://www.Poodledata.org>. Plotting the family tree of the cases helped us in identifying common ancestors among the affected dogs, and the possible mechanism of inheritance. Such analysis guided us in any further sample collection in

order to gather genetic material from appropriate controls within the family. Pedigree analysis is vital for many Family Analysis based on SNP chips (see below).

Candidate gene analysis: Since many of the conditions investigated have genes and mutations that been found in humans, or in the very least, genes suggested as possibly associated in humans and animals, we proceeded to carry out a number of gene analysis targeted at genes already known in literature (candidate genes) causing the disease investigated. This is a standard procedure to attempt a quick (and more economic) resolution of the case if there is a clearly disruptive mutation detected in a candidate gene.

Population and Family mapping analysis: We have analyzed data obtained from SNP chip genotyping, which is an instrument able to give us vital information on a dog genome through markers assigned to a specific position on the chromosomes. This technique gives us a higher probability of knowing more about the possible inheritance of genetic disease.

GWAS: Genome Wide Association Studies are a statistical analysis based on cases and controls within a population. The aim of such studies is to associate a specific genomic region and its markers to a cohort of study cases. This can be achieved by variation comparison of cases against a similar-sized cohort of controls. We carried out the GWAS with the GenABEL (R package) software. Because of sample size limitations, the experimental power was not always sufficient to obtain a clear signal; however it was possible to at least obtain some suggestive association peaks which now are under investigation. Nonetheless, a perfect assessment of the phenotype and further gathering of samples would help immensely this kind of analysis, and we are actively recruiting additional dogs for the study.

Homozygosity mapping: SNP marker data has been used to carry out homozygosity mapping on cases and controls, in order to find a homozygous region identical by descent (IBD) among the cases and not in the controls. Plink software was used for this mapping strategy. Note that failing to obtain such result does not completely exclude the regions since the alleles could be present and diffused in the population. However, a very recent causative mutation could be present only in a sub-group of such haplotypes. This has been reported, documented, and demonstrated in animal genetics, and we consider this possibility in all ongoing analyses.

Linkage: Genetic linkage is the study of the DNA tendency regions that are close enough together to be inherited. Our analysis uses the markers obtained through SNP chip genotyping to analyze the possible inheritance patterns of such markers and the genome regions they are

linked to. An in-depth analysis of the genes present in such regions was carried out for possible causative mutations.

Phasing: Phasing is a method used to observe which haplotypes individuals share. Such method is particularly useful as an integration of the methods described above, because it can be helpful in case non-simple Mendelian methods of inheritance are involved- to sort out flanking regions of homozygous haplotypes, to hunt for compound heterozygous, and so on. Mach1, a phasing software, was used to phase the chromosomes of the population. It is possible to carry out chromosome phasing using just family information, but some of the projects involve samples gathered from the whole population of unrelated individuals. For this reason, population-based phasing is helped immensely by a wide sample pool. During the current period, Dr. Murgiano has developed specific computer algorithms that permit phasing of the study population. As described in the *GWAS* section, an increase in the study population size will contribute greatly to this analysis, and recruitment is ongoing.

Whole Genome Sequencing: In order to gather a complete number of variants present in the genome of a selected case, one or more samples can be sequenced entirely through whole-genome sequencing (WGS) methods. The whole list of variants present in the dog is analyzed by a researcher, which looks for variants in the candidate genes at once, and if possible, focus on variants present in regions selected through the mapping experiments described above. Often an alleged carrier and/or controls are sequenced to help sort which mutations can be responsible for the disease and which can be excluded because present in healthy individuals, or partially in carriers.

Current state and future developments:

Micropapilla/Optic Nerve Hypoplasia

Samples:

138 Miniature Poodles of which 11 have ONH and/or Micropapilla, unilateral and bilateral.

Actions:

We established pedigree connecting all Micropapilla and ONH cases. As stated in a previous report, we were able to connect all dogs diagnosed with ONH and/or Micropapilla in one pedigree with a possible common founder born more than 30 years ago. The pedigree suggests an autosomal recessive inheritance pattern.

The available samples and their close family member were genotyped creating a dataset of 5 sub-families. Some of the family members had unilateral or bilateral ONH, other were affected by Micropapilla. It should be noted that the presence of a given affected phenotype described above is not exclusive of a specific family. In fact, the sub-families studied can contain exclusively ONH or Micropapilla cases, or a combination, and these can be unilateral or bilateral. This could suggest that the disease has the same origin but a different expressivity, but there is no evidence at this moment. As a first step, as reported previously, we carried out an analysis of 14 candidate genes that are associated with ONH and/or ocular malformations in humans and other species. Additionally, several new candidate genes we included in the current analysis and included: *BMF*, *FNDC11*, *POU6F1*, *ZDHHC17*, and *EBHB6* genes. No significant association was found. We then proceeded to do GWAS by SNP chip genotyping in order to carry out all the analysis that such technique allows.

A GWAS analysis was carried out using cases and controls within and outside the population. Since no significant hit was scored, we are now recruiting additional dogs for the study to increase the power of the analysis. To this end, we continue to publicize the 'need' for additional samples through the Optigen web page and through direct contacts with veterinary ophthalmologists in US, Canada and Europe. Any assistance that the PCA Foundation can provide in this regard for ONH as well as other diseases under study will be much appreciated.

Homozygosity mapping was carried out using the data set generated by GWAS. Albeit seven regions of homozygosity were detected, a careful comparison with additional controls excluded such regions since these were present in the additional controls as well. We proceeded to carry out a linkage analysis. The analysis carried out with Merlin did not show suitable candidate regions in any of the two possible inheritance mechanisms (simple recessive and simple dominant). The analysis at first focused on ONH but it was repeated with different assumption on the second iteration (the assumption that ONH and Micropapilla are the same phenomenon with a different phenotype expressivity, as opposed to being two different conditions).

We suspect that the inheritance mechanism could be complex, involving different loci or a compound heterozygous. We proceeded to phase the haplotypes in order to evaluate this option and to analyze the flanking region of the homozygous regions common between cases and controls (to observe whether their exclusion was unjustified because they are conserved in the breed but the fact is different haplotypes). The development of the phasing algorithm was

done during the current budget period, and now we are analyzing the haplotypes that resulted from haplotype phasing.

In four samples we have performed whole genome sequencing: two cases and 2 controls. Analysis of this newly acquired data identified 3 genes whose sequence changes are predicted to cause disease in susceptible populations, but genotyping of such variants did not segregate perfectly within the study population. No other high-impact variant was detected. We now are analyzing the whole genome sequence using big-indel detection softwares in order to consider the option of a greater chromosomal alteration. The analysis of the output is yet to be completed. A revised and expanded manuscript describing the preliminary genetic and molecular studies of ONH has been completed, and submitted to a scientific journal for consideration for publication. Once the manuscript is accepted for publication, a copy will be sent to the PCA Foundation.

Cataract

Samples:

Samples: 22 Toy Poodles (3 cases), 138 Miniature Poodles (several cases, but not all are well phenotyped, 3 cases of a specific form of plaque cataract with good clinical characterization), 28 Standard Poodles (1 case).

Actions:

We established a family tree analysis to track the ancestors, but the number of samples received and the unclear phenotype, particularly for Miniature Poodles did not allow a thorough pedigree analysis of the whole dataset. For any genetic investigation it is essential to identify dogs that have the same 'type' of cataracts on clinical examination as these cataracts are likely to have been caused by the same genetic defect. Although it is possible that a single gene/mutation causes cataracts that are clinically different, the most likely scenario is that a single gene/mutation will result in a cataract phenotype that is relatively consistent between affected dogs. As reported previously, we detected a peculiar type of cataract in a low number of Miniature Poodle, and referred to it as a plaque type cataract; this was a consistent phenotype among the cases and divergent from the usual Poodle cataract. As a start, we decided to focus on this one type of cataract, and have tested and excluded 23 candidate genes expressed in the lens and associated with inherited cataracts in humans and other species (manuscript is currently under preparation and will be provided to PCAF once it is sent out to a

scientific journal). Since the possible candidate gene for cataracts in dogs and other mammals are in the order of hundreds, we opted for an alternative approach.

We decided to have 2 animals whole-genome sequenced as cases and two unaffected Miniature Poodles sequenced as controls. We identified a number of variants of different predicted impact that could be possibly responsible for this specific cataract. Validation of these variants (especially two variants situated on coding regions) within a population of cases is ongoing.

For the rest of the cases, we opted to send Miniature and Toy Poodle samples for SNP chip genotyping. Initial GWAS attempts were too weak in power because of the low number of cases. We plan to sensitize the breeders toward the collection of further samples in order to have a better comparison within and between different cataract types in the breed and to reach an acceptable level of samples for a population-based statistical analysis (and possibly a family-based one). For the moment, we are phasing the whole population of samples gathered in order to do not exclude the chance to spot shared haplotypes.

As previously reported, we developed a research form and publicized the research goals in several publications and newsletters (Fall Newsletter – The Poodle Club of Canada, Poodle Variety, Purina Pro Club Poodle Update, etc). This has generated considerable interest among breeders, and veterinary ophthalmologists, and has prompted submission of samples from Miniature and Toy varieties from US, Canada and Western Europe. We wish to thank the breeders for all the contribution, however the number of samples gathered is low in comparison with the variety of the phenotypes. At the moment, is absolutely vital the gathering of additional samples along with proper phenotyping data about the dogs.

Polymicrogyria Standard Poodle

Samples:

Tissue blocks and blood from case diagnosed by Dr. Aguirre.

Actions:

The disease has been recognized in the Standard Poodle variety since Aguirre and colleagues reported the disease in a family of related dogs (Van Winkle et al., 1994). The prevalence of the disease is unknown although isolated cases occur and are reported by

veterinary neurologists and/or ophthalmologists. Tracing of a pedigree linking the two known cases available has identified one common founder.

As reported previously, we carried out a candidate gene analysis (13 genes, known in literature for being associated to polymicrogyria or to syndromes that had polymicrogyria among the symptoms), but no association was found using haplotype-based analysis. We excluded any variant present in the candidate genes. The dogs were genotyped, but after an initial homozygosity mapping of both of the samples, and controls, we detected a high-rate of missing SNPs in the data obtained from the tissue-block sample. For this reason, after the initial homozygosity run, we decided to re-run the mapping only for one of the samples and used this case as the informative one. This left us with 39 homozygous regions present in the single dog. In parallel, we sequenced the whole genome of the same informative dog in order to detect a suitable variant, that possibly could be disease-associated, with the aim of comparing the SNP and whole genome sequence data. The WGS data analysis resulted in two SNPs altering the coding variants falling within the homozygous regions. We are now verifying the prevalence of those SNPs in homozygous state within the population. As an additional measure, we are analyzing the whole genome sequence data using big-indel detection softwares in order to consider the option of a greater chromosomal alteration. Any larger insertion, deletion duplication, inversion affecting coding sequences within candidate regions will be verified as well.

Day blindness/retinal degeneration

In the previous funding period, we identified the gene and disease causing mutation responsible for the disease, and implemented a DNA-based test to identify genetically normal, carrier and affected dogs. Awareness of this disease is increasing among veterinary ophthalmologists, and we are receiving questions and doing tests in Standard Poodles from several regions in the country and Canada. In the last 2-3 months, affected dogs have been diagnosed clinically and confirmed by DNA testing from Eastern and Western Canada, Delaware and Georgia. In parallel, the disease appears to be increasing in severity in "Golden Doodles", and the mutation present is of Standard Poodle origin. Affected dogs are being produced by intercrossing the first generation PoodleXGolden progeny, or backcrossing these dogs to Standard Poodles who are carriers.

Although not part of the PCA Foundation funded project, we are interested in examining the affected gene, and seeing how the mutation causes disease. Progress in this area has identified that the primary gene involved (Gene 1) has the terminal 2 protein coding regions (exons)

deleted. At the same time, an adjoining gene (Gene 2), which is oriented in the opposite direction as Gene 1, has the 4 terminal exons deleted. Thus the disease is caused by a very large genomic deletion. Determining what is the primary causative gene is not critical for DNA-based diagnostics as the test is 100% specific. However, as one goal of our studies is to develop a gene therapy treatment for the disease in dogs, determining if Gene 1 or Gene 2 are the culprits is critical for this work. These studies are continuing.

Conclusions:

Candidate gene analysis and sequencing for all the projects will continue. Genomic regions of interest will be screened for associated variants, which will help develop future tools/tests for identifying carriers. Whole genome sequencing data will be analyzed for variants individually present in cases. We will test the association of identified variants with disease by sequencing cases and controls.

We are still recruiting subjects for all of our Poodle-related projects, and collecting eye exam records/blood samples. We are mostly interested in samples from affected dogs with a reliable clinical diagnosis, as well as samples from their close relatives. We cannot stress enough that for the sake of our research and of the long-term health and welfare of these dogs, more samples are needed, especially in the case of the diverse group of cataract conditions.

We are grateful for all the publicity our research received, which we found to be significant in educating owners about Poodle ophthalmic disorders, as well as encouraging them to get involved in our study. It is important to note that any information we are provided with will be kept strictly confidential, and will only be used for informational and research purposes.

Lastly, we thank the owners, breeders, and the Poodle Club of America Foundation for the continuous support throughout our study. Our research would not be possible without their much appreciated dedication and help. We hope to continue our ongoing positive collaboration with the breeders and regional Poodle clubs.