

Association of A31P and A74T Polymorphisms in the Myosin Binding Protein C3 Gene and Hypertrophic Cardiomyopathy in Maine Coon and Other Breed Cats

G. Wess, C. Schinner, K. Weber, H. Küchenhoff, and K. Hartmann

Background: Hypertrophic cardiomyopathy (HCM) is an inherited autosomal dominant trait in cats. The A31P single nucleotide polymorphism (SNP) in the myosin binding protein C 3 gene is thought to be the causative mutation in Maine Coon cats. Additionally, the A74T SNP is offered as a genetic test for HCM.

Objectives: To evaluate the genetic association between the above-mentioned SNPs and phenotypes.

Animals: Eighty-three Maine Coon cats and 68 cats of other breeds.

Methods: The study was performed prospectively. Cats were phenotyped as healthy or HCM with echocardiography. Taqman genotyping assays were used for genotyping; results were confirmed by sequencing analysis.

Results: A31P was found in 18/83 (22%) Maine Coon cats. Fifteen of 18 Maine Coons (83%) with the A31P mutation were healthy on echocardiographic examination (mean age 65 months). A74T was present in 28/79 (35%) of Maine Coons and in 42/68 (62%) of other cat breeds. Twenty-two of 28 (79%) of Maine Coons and 21/42 (62%) of other breed cats with the A74T mutation were healthy at a mean age of 72 months and 91 months, respectively. Of 12 Maine Coons with HCM, 9 (75%) were genotype-negative for A31P and 6 (50%) for A74T. Allele frequencies did not differ significantly ($P = .47$) between phenotype groups. None of the evaluated genetic tests was able to provide useful predictive information of disease outcome.

Conclusions and Clinical Importance: The value of currently available genetic tests is low in the cats of this study. The mutations analyzed appear to have a low penetrance, and even homozygote cats can remain healthy.

Key words: Animals; Carrier proteins/genetics; Genetic tests; HCM.

Hypertrophic cardiomyopathy (HCM) is the most common familial genetic heart disease in humans, affecting one in 500 individuals.^{1,2} In 60% of cases it is inherited as an autosomal dominant trait, while exhibiting an enormous phenotypic and genotypic heterogeneity. De novo mutations are also considered to be a cause of sporadic HCM.^{3,4} For many years, HCM in humans has been suspected of being a genetic disease of the sarcomere. To date, >450 different mutations considered responsible for familial HCM have been identified within 13 sarcomere- and myofilament-related genes.^{5–7}

HCM is the most common cardiac disease in cats. Autosomal dominant inheritance with a heterogeneous disease outcome has been documented in a family of Maine Coon cats.^{8,9} One point mutations in the cardiac myosin binding protein C 3 (MYBPC3) gene (A31P) leading to amino acid changes in the protein occur in Maine Coon cats with HCM and one point mutation in the same gene (R820W) is thought to cause the same disease in Ragdoll cats.^{9,10} Another single nucleotide polymorphisms (SNP) (A74T) of the MYBPC3 has been

Abbreviations:

CV	coefficient of variation
HCM	hypertrophic cardiomyopathy
IVS	interventricular septum
IVSd	interventricular septum at end-diastole
LV	left ventricular
LVPW	left ventricular posterior wall
LVPWd	left ventricular posterior wall at end-diastole
MYBPC3	myosin binding protein C 3
PCR	polymerase chain reaction
SNPs	single nucleotide polymorphisms
TDI	tissue Doppler imaging

suspected to cause HCM in Maine Coon cats.^a Several commercial laboratories currently provide genetic tests for the above-mentioned mutations. The A31P mutation is present in about 34% of all Maine Coon cats worldwide, although studies evaluating the relationship between genotype and clinical outcome are lacking.¹¹ The goal of this study, therefore, was to evaluate the genetic association of A31P and A74T SNPs with the phenotype of HCM in Maine Coon cats in Germany. Clinical validation (sensitivity, specificity) of both genetic tests was assessed as well. In addition, the presence of the 2 above-named SNPs was evaluated in other breeds besides Maine Coon cats.

Materials and Methods

Animals

Eighty-three Maine Coon cats and 68 cats of various other breeds (Norwegian Forest cats, Persian cats, Domestic Shorthair cats) were included in this prospective study over a period of 2 years (August 2005–August 2007). All cats were from owners living in

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Submitted September 5, 2009; Revised February 5, 2010; Accepted March 1, 2010.

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10.1111/j.1939-1676.2010.0514.x

Germany, Switzerland, or Austria and patients presented to the cardiology service of the Clinic of Small Animal Medicine or cats participating in an HCM screening program. Pedigree sheets and Internet databases were used to determine the origin of the Maine Coon cats. The majority of the Maine Coon cats originated from Germany ($n = 58$) and Austria ($n = 11$), and 8 cats were from the United States, 3 from Switzerland, and 2 from Canada and Denmark each. Looking at the parents level of these cats, 48.2% ($n = 80$) were from Germany, 21.1% ($n = 35$) came from United States, 8.4% ($n = 14$) were from Austria, 6.0% ($n = 20$) from Canada, 3.6% ($n = 6$) from Italy, 3.0% ($n = 5$) from Denmark, 3.0% ($n = 5$) from Switzerland; 3 cats were from United Kingdom, 2 from Poland, 2 from France, 2 from the Netherlands, and 1 from Spain. Cats were classified into 2 breed groups: "Maine Coon" and "other breeds." They were then further classified into the groups "healthy" or "HCM" according to echocardiographic phenotype results. In the control group, female and male cats had to be a minimum age of 36 and 24 months, respectively, based on the fact that HCM can usually be detected echocardiographically by this age in affected cats.⁸ Cats were considered genotype-positive for A31P or A74T if at least 1 mutated allele was detected. Cats displaying only the wild-type allele were considered genotype negative.

Phenotyping

Cats were prospectively phenotyped by clinical examination and echocardiography. Animals with other diseases causing ventricular concentric hypertrophy were excluded from the study. All echocardiographic studies were performed by 1 experienced examiner (G.W.), with an ultrasound unit equipped with a 5.5–7.5 MHz phased-array transducer with continuous ECG monitoring and echocardiographic loops were stored digitally.^b Ultrasound examinations were performed without sedation in gently restrained cats in lateral recumbency. Standard echocardiographic views were obtained in right and left lateral recumbency.¹² End-diastolic measurements of left ventricular (LV) wall thickness were performed with two-dimensional echocardiography in the right parasternal short-axis view at the level of the papillary muscles, and in the right parasternal long-axis view in the basal and mid-ventricular myocardial segments of the left ventricular posterior wall at end-diastole (LVPWd) and the interventricular septum at end-diastole (IVSd). HCM was defined as regional or generalized hypertrophy with a diastolic wall thickness ≥ 6 mm of the LVPWd or of the IVSd. At least 3 measurements were performed in each of the myocardial segment described above of the left ventricular posterior wall (LVPW) and interventricular septum (IVS) and the mean value of at least 3 measurements of the thickest segment was calculated. Hyperthyroidism and hypertension were excluded as secondary causes of hypertrophy by measurement of basal serum T4 concentration and blood pressure by Doppler technique.^c Blood pressure was considered normal if systolic blood pressure was < 150 mmHg. Healthy genotype-positive cats were re-examined after 1 year. In addition, cats with equivocal measurements (only prominent papillary muscle, or wall thickness between 5.5 and 6.0 mm) were excluded from the study if ultrasound findings remained equivocal after 1 year. Measurement reliability was determined for systolic and diastolic LV chamber diameter and for diastolic wall thickness of the LVPW and IVS. Ten echocardiograms were randomly selected to be subjected to 3 repeated analyses within 1 week by 1 investigator (G.W.) to determine intraobserver measurement variability. The investigator was unaware of the results of the prior echocardiographic analyses.

Genotyping

For genotyping, DNA was extracted from peripheral blood leucocytes with the QIAamp DNA Mini Kit.^{d,13} The quantity of

DNA was assessed by photometric measurement. A 250-bp fragment of the feline MYBPC3 gene including both polymorphic sites was amplified by polymerase chain reaction (PCR) from 4 cats with known A31P SNP genotype to confirm the feline sequence. Amplification primers were obtained from a commercial laboratory.^e The forward primer was 5'-AGT CTC AGC CTT CAG CAA GAA GCC-3', and the reverse primer 5'-GGT CAA ACT TGA CCT TGG AGG AGC C-3'.

Standard PCR amplification was carried out with the HotStarTaq PCR Master Mix^f according to the manufacturer's instructions,¹⁴ with 30 cycles on an Eppendorf thermal cycler, using 60°C as annealing temperature. The PCR product was visualized by gel electrophoresis and purified with the MinElute PCR Purification Kit^g according to the manufacturer's instructions.¹⁵ The PCR product was sequenced by a commercial laboratory^d performing a single read of each sample with forward and reverse primers. Using this sequence as a template, TaqMan Genotyping Assays for the A31P and A74T SNPs were produced by a commercial Assay-by-Design-Service.^h These assays use a primer pair and allele-specific minor groove binding probes with either VIC or 6-FAM as fluorescent reporter dye. For allelic discrimination of the A31P SNP, the wild-type G-allele was labelled with VIC and the mutated C-allele with 6-FAM. For discrimination of the A74T SNP, the wild-type A-allele was labelled with 6-FAM and the mutated G-allele with VIC.

The allelic discrimination assays were run in 96-well reaction plates on a 7,500 Real-Time PCR System.^h 12.5 μ L of 2 \times TaqMan Universal Master Mix, no AmpErase UNG^h and 1.25 μ L 20 \times SNP Assay Mix^h were mixed with 11.25 μ L DNA, diluted in nuclease-free water to a final reaction volume of 25 μ L. On each plate, no template controls (reaction mix without DNA) were included as negative controls. DNA samples from cats with known homo- and heterozygous genotypes were included as positive controls. Allelic discrimination analysis was performed by 7,500 SDS software. For all cases assigned to either the G/C or C/C genotype of the A31P SNP with the TaqMan assay, standard PCR reactions were performed. PCR products were then sent to a commercial laboratory^d for sequencing analysis to confirm specificity of the TaqMan assays.

In silico analysis of A31P and A74T amino acid variations in the MYBPC3 protein.

The possible impact of the SNPs on the protein was evaluated by PolyPhen.ⁱ This tool is designed to calculate the likelihood that an amino acid substitution resulting from a genetic mutation changes structure and function of a human protein by comparing the allelic variants with homologous proteins.¹⁶

Statistics

The prevalence of HCM in Maine Coon cats was calculated from randomly screened Maine Coon cats in the HCM screening program. Allele frequencies were calculated for all phenotype groups. Fisher's exact test was used to compare allele frequencies of the phenotype groups "healthy" and "HCM" ($P < .05$). Odds ratios for having HCM were generated for all genotype-positives and homozygotes separately. Clinical validity was evaluated for both genetic tests by calculating sensitivity and specificity.^{17,18} The intra-observer coefficients of variations (CV) were calculated by a variance component analysis. The CV were obtained by dividing the root of the variance error by the mean of the repeated measurements, times 100.

Results

The prevalence of HCM in cats in the present study was 15% (95% CI = 7–22%). The A31P SNP was found in 22% ($n = 18$) of the Maine Coon cats, but not in any of the other breeds. Minor allele frequency was 0.13 for

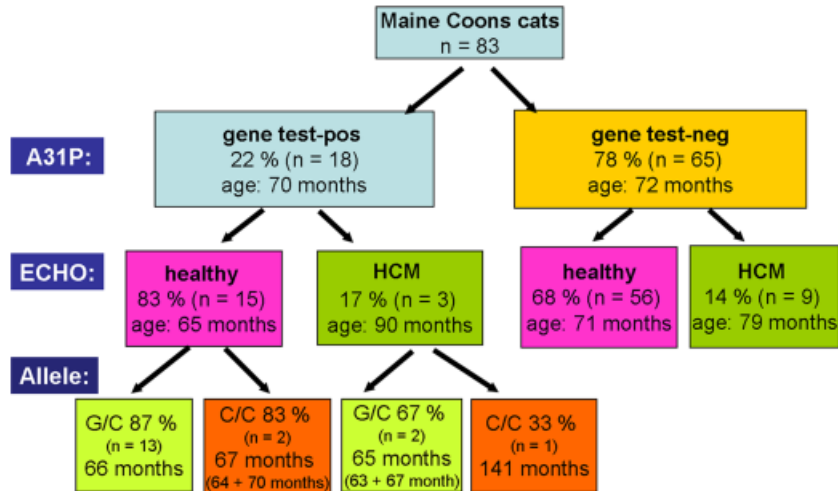


Fig 1. Phenotypes and genotypes of Maine Coon cats with the A31P mutation. G/C represents heterozygous state and C/C the homozygous state. Age is displayed as mean age of the selected group.

the C-allele. Of the genotype-positive cats, 83% (n = 15) were classified as normal (healthy) on echocardiographic examination (LVPWd, mean 4.51 mm, range 3.0–5.5 mm; IVSd mean 4.57 mm, range 3.2–5.4 mm) (Fig 1). Mean age of genotype-positive cats with healthy phenotype was 65 months (range 24–146 months). Two of these cats were homozygous (C/C) for the A31P SNP (58 and 64 months old). On echocardiographic examination, the phenotype HCM was found in only 3 cats with the A31P-SNP mutation. One of these cats was homozygous and 2 cats were heterozygous for the A31P SNP. All heterozygous and homozygous cases (G/C and C/C) identified with the TaqMan assay were confirmed as heterozygous G/C and homozygous C/C by sequencing analysis.

Sixty-five Maine Coon cats (78%) were genotype-negative. Of those cats, 68% (n = 56) had a normal phenotype (mean age 71 months); 9 of the cats (14%) in this group had HCM. In total, 9/12 phenotype-positive Maine Coon cats were genotype-negative (75%). The

echocardiographic examination of the phenotype-positive cats included 8 cats with concentric hypertrophy and 4 cats with regional hypertrophy (LVPWd, mean 7.1 mm, range 6.1–10.0 mm; IVSd mean 7.0 mm, range 6.2–8.7 mm).

The A74T SNP was found in 28 (35%) of the Maine Coon cats studied (Fig 2). Minor allele frequency was 0.22 for the A-allele. Of the genotype-positive Maine Coon cats, 79% had a “healthy” phenotype (n = 22) at a mean age of 72 months. Four of these cats were homozygous (A/A) for the A74T SNP, and 18 cats (82%) were heterozygous (G/A). The A74T SNP was identified in 21% (n = 6) of Maine Coon cats with HCM, 2 of which were homozygous and 4 of which were heterozygous for the mutation.

The A74T SNP was present in 42 (62%) cats of other breeds (Persian, Norwegian Forest cats, and Domestic Shorthair cats) and in 26 (38%) other breed cats the SNP was not found (G/G). Of the 21 “healthy” phenotype

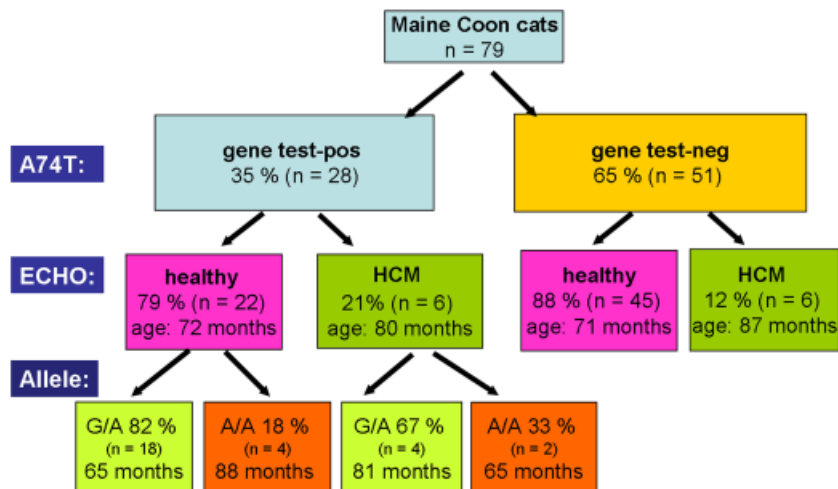


Fig 2. Phenotypes and genotypes of Maine Coon cats with the A74T mutation. G/A represents the heterozygous state and A/A the homozygous state. Age is displayed as mean age of the selected group.

cats (mean age 91.4 months), 9 cats were heterozygous (G/A), 4 cats were homozygous (A/A) for the A74T SNP alleles, and in 8 cats the SNP was not found (G/G). HCM was diagnosed in 40/68 cats of other breeds (59%). The echocardiographic examination of the phenotype-positive cats included 28 cats with concentric hypertrophy and 12 cats with regional hypertrophy (LVPWd, mean 7.2 mm, range 6.1–9.8 mm; IVSd mean 6.8 mm, range 6.2–8.9 mm).

Of the 40 cats with HCM (mean age 108 months), 35% (n = 14) were heterozygous and 25% (n = 10) were homozygous for the A74T SNP. The SNP was not found in 40% (n = 16) of the HCM cats (G/G). Seven cats were equivocal on the echocardiographic phenotype (2 were homozygous, and 3 were heterozygous for the A74T SNP).

There was no statistically significant difference observed in allele frequencies between cats with HCM and healthy controls for both SNPs, neither in Maine Coon cats nor in the other breeds. No statistically significant association between genotype and HCM was detected (Table 1).

Sensitivity (25% for A31P; 50% for A74T) was very low for both genetic tests in the examined population. None of the genetic tests were able to reliably predict the echocardiographic phenotype (Tables 2 and 3). Evaluation of the potential impact of the amino acid substitutions caused by the A31P and A74T SNPs by PolyPhen did not suggest damaging effects on the protein. Results of the echocardiographic repeatability study revealed very good findings: CV for diastolic LVPW was 3.3%, for diastolic IVS 2.6%, for diastolic LV diameter 2.0% and for systolic LV diameter 4.2%.

Discussion

The current study found that a positive test result in the AP31 SNP or A74T SNP test does not predict that a

Table 1. Odds ratios for the A31P and A74T SNPs in the MYBPC3 gene in Maine Coon cats (MC).

MC	Phenotype	Genotype 1	Genotype 2	N	OR	95% CI
A31P		G/G + G/C	C/C		3.14	0.20–5.96
	Healthy	69	2	71		
	HCM	11	1	12		
		G/G	G/C + C/C		1.24	0.29–5.18
A74T	Healthy	56	15	71		
	HCM	9	3	12		
		G/G + G/A	A/A		3.15	0.51–9.51
	Healthy	63	4	67		
	HCM	10	2	12		
		G/G	G/A + A/A		2.04	0.59–7.07
	Healthy	45	22	67		
	HCM	6	6	12		

Genotype 1 and Genotype 2 show which combinations of genotypes were used to calculate the odds ratios. For the A31P SNP G/C, heterozygous; C/C, homozygous cases and G/G, wildtype; for A74T G/A, heterozygous; A/A, homozygous cases and G/G, wildtype.

n, number of animals; OR, odds ratio; CI, confidence interval.

Table 2: Validity of the genetic test for the A31P SNP in Maine Coon cats.

	Scenario 1 G/C + C/C = Genotype Positive		Scenario 2 C/C = Genotype Positive	
	95% CI		95% CI	
A31P				
Sensitivity	0.25	0.08–0.55	0.08	0.01–0.41
Specificity	0.79	0.68–0.87	0.97	0.89–0.99

Scenario 1, heterozygous (G/C) and homozygotes (C/C) are counted as genotype positives; Scenario 2, only homozygotes are counted as genotype positives; 95% CI, 95% confidence interval.

cat has echocardiographic changes or that it will develop HCM later in life, and that a negative AP31 SNP or A74T SNP test does not determine whether a cat has or will develop HCM.

HCM is the most common cardiac disease in cats. It is considered an autosomal dominant disease in humans as well as in cats. A penetrance of 100% was reported for a family of inbred Maine Coon cats, with the stillborns representing lethal homozygotes that died in utero.⁸ The A31P mutation was first detected in this colony of Maine Coon cats,⁹ most of which had echocardiographic evidence of HCM by an age of 24 months in males and 36 months in females.⁸ Therefore, only males older than 24 months and females older than 36 months were included into the healthy control group in the current study. HCM cats were included in the present study at any age. The possibility that cats in the control group will develop HCM later in life cannot be excluded, and follow-ups in this group are desirable. However, as the mean age of the cats in the control group of the present study was 65 months, it is unlikely that many cats in this group will develop HCM later in life.

Two cats with a normal phenotype at an age of 58 and 65 months were homozygous for the AP31 SNP. Maine Coon cats carrying the homozygous mutation were suspected to represent stillborn cats in the Maine Coon breeding study, but homozygous cats for the A31P mutation were found to be alive not only in the present study, but also in the UC Davis Maine Coon colony.¹⁹ Therefore, either the assumption that cats carrying a homozygous mutation will be stillborn cats is not correct, or the A31P mutation alone is not the only mutation causing HCM in Maine Coon cats. At least, the A31P

Table 3: Validity of the genetic test for the A74T SNP.

	Scenario 1 G/A + A/A = Genotype Positive		Scenario 2 A/A = Genotype Positive	
	95% CI		95% CI	
A74T				
Sensitivity	0.50	0.24–0.76	0.17	0.04–0.48
Specificity	0.67	0.55–0.73	0.94	0.85–0.98

Scenario 1, heterozygous (A/C) and homozygotes (A/A) are counted among genotype positives; Scenario 2, homozygotes are counted among genotype positives; 95% CI, 95% confidence interval.

mutation does not appear to be a prenatal lethal factor, neither in the present study population nor in the UC Davis Maine Coon colony and another study evaluating the A31P mutation in Maine Coon cats.^{8,19,20} Nevertheless, in the inbred Maine Coon cat colony, the A31P mutation appears to be associated with cardiac changes.¹⁹ A recent study demonstrated that the heterozygous manifestation of the MYBPC3 A31P mutation is not associated with occurrence of LV hypertrophy and major myocardial dysfunction in Maine Coon cats.²⁰ Only inconsistent, minor regional diastolic myocardial dysfunction were detected in a study using tissue doppler imaging (TDI) in cats with the A31P mutation. A reduced diastolic function was identified in only a few LV wall segments, whereas other segments had normal TDI values.²⁰ A diastolic dysfunction detected using TDI could be an early marker of HCM, but this remains to be proven in future studies. In the present study, TDI was not used and therefore we potentially might have missed early diastolic dysfunction. However, if diastolic dysfunction is truly an early indicator of HCM, than the diastolic dysfunction should progress to a more obvious HCM picture over time. The cats in the present study were almost twice as old compared with the cats in the TDI study population, in which the inconsistent regional diastolic dysfunction was reported.²⁰ Therefore, even if we would have missed diagnosing a diastolic dysfunction, the disease should have progressed to a more obvious HCM picture in the cats of the present study. As this was not the case, the clinical implication of a potentially missed TDI abnormality seems to be low, with >80% of the cats in the present study still being normal based on echocardiography at an advanced age.

The A31P mutation might be the cause of HCM in the Maine Coon cats in the UC Davis breeding colony. However, it could also only be a marker for an unknown mutation with pathogenic potential. In the Maine Coon cats tested in this study, the A31P SNP was not associated with HCM, which could possibly be because of a varying genetic background. The analysis of the pedigrees revealed that Maine Coon cats used in this study were from catteries throughout the world, with the majority of the cats originating from Germany, Austria, the United States, and Canada. As the Maine Coon breed is a comparatively young breed founded in the 1960s it is not surprising that there are still many cats imported from the United States and Canada and that cats in this breed have a close genetic relationship. A study evaluating the prevalence of the A31P mutation found that this mutation exists in about one third of the Maine Coon cats throughout the world, and that the prevalence of the mutation (heterozygous or homozygous) was very similar among countries of submission.¹¹ Therefore, the results of this study could be representative also for other countries and not only for the selected population. However, further studies are necessary on this subject.

Basing breeding recommendations for Maine Coon cats on the A31P or A74T, or both gene tests appears questionable unless cats that are related to the UC Davis family are used for breeding. Another reason for the observed variations in field and experimental breeding

conditions could be modifier genes that cause HCM in combination with the A31P mutation. However, to date no modifier gene has been identified in cats. In humans, >400 mutations have been detected in 24 genes encoding for various forms of HCM.^{2,21,22}

The other SNP (A74T) suspected to cause HCM in Maine Coon cats and other breed cats is incompletely reported, yet the test is already being offered by commercial laboratories.⁴ Therefore, the A74T SNP was also investigated in this study. As with the A31P mutation, HCM cats negative for the mutation were identified. Consequently, it appears highly likely that other or additional mutations causing HCM exist in Maine Coon cats. Similar to the A31P mutation, 79% of the Maine Coon cats with a positive gene test were normal on echo at a mean age of 72 months. Of these cats, 4 cats were homozygous for the mutation at a mean age of 88 months. As with the A31P mutation, no statistical difference in the percentage of affected cats was found between gene test-positive and -negative cats, nor was a correlation detected between phenotype and genotype. In contrast to the A31P mutation, which was specific for Maine Coon cats, the A74T mutation was also detected in other breeds (in which also no correlation was present between genotype and phenotype). The A74T SNP, therefore, appears to be a mutation that is neither specific for Maine Coon cats nor causes HCM.

An Internet-based software program (Polyphen) from Harvard University that tries to predict whether a mutation is likely to affect protein function, classifying the changes as “benign” or “malignant,” was used in this study. The feline genome was used in the present study as a reference to let the software predict if the SNPs are suspected to be benign or malignant changes. For both the A31P and A74T SNP, the program predicted that the SNP probably are benign changes, which is in contrast to a previous study that used the human genome and this explains the different findings.⁹ Although this might support the findings of this study, the results are only calculations by a software and the value of a computational method should not be overestimated. Only functional tests of the mutated protein would be able to prove this assumption. A limitation of this study is that, as all cats were client-owned cats, no necropsy was performed on the phenotypically healthy, but positively tested cats, and therefore no necropsy results could be compared with the genotype. Another limitation is that some cats may have been too young for the detection of disease on echocardiography and may develop HCM later in life. However, as mentioned earlier, the mean age of the healthy group of cats was 65 months and, thus, quite old, so that the likelihood of developing the disease later on is very low. Certainly, long-term follow-up studies would help answer this question.

This study proves that other mutations or genetic influences causing HCM must exist as most cats with HCM were negative for the AP31 SNP.

Therefore, it can be concluded that:

1. A negative AP31 SNP or A74T SNP test does not determine whether a cat has or will develop HCM, as

- most of the cats with HCM in this study did not have either SNP;
- A positive test result does not implicate that a cat has echocardiographic changes or that it will develop HCM later in life, at least in the selected population, as most of the cats with a positive gene test in this study did not exhibit echocardiographic changes at a mean age of 65 months;
 - Breeding decisions or recommendations should not be based solely on A31P or A74T testing.

Footnotes

- ^a Nyberg MT, Koch J, Christiansen M. Intra-allelic Genetic Heterogeneity of Hypertrophic Cardiomyopathy in the Maine Coon Cat. Hugo Human Genome Meeting HGM2007, Montreal, Canada, 2007; 199 [poster abstract].
- ^b Vivid 7, GE, Horten, Norway
- ^c Parks 811-BT, Parks Medical Electronics Inc, Aloha, OR
- ^d Qiagen, Hilden, Germany
- ^e Metabion GmbH, Martinsried, Germany
- ^f HotStarTaq PCR Master Mix, Qiagen
- ^g MinElute PCR Purification Kit, Qiagen
- ^h Applied Biosystems, Foster City, CA
- ⁱ PolyPhen, Harvard University, Cambridge, MA: <http://genetics.bwh.harvard.edu/pph/index.html>
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