Evaluation of the cardiac actin gene in Doberman Pinschers with dilated cardiomyopathy

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Objective—To evaluate the coding region of the cardiac actin gene in Doberman Pinschers with dilated cardiomyopathy (DCM) for mutations that could be responsible for the development of the condition

Animals—28 dogs (16 Doberman Pinschers with DCM and 12 mixed-breed control dogs).

Procedure—Ten milliliters of blood was collected from each dog for DNA extraction.

Polymerase chain reaction (PCR) primers were designed to amplify canine exonic regions, using the sequences of exons 2 to 6 of the cardiac actin gene. Single-stranded conformational polymorphism analysis was performed for each exon with all samples. Autoradiographs were analyzed for banding patterns specific to affected dogs. The DNA sequencing was performed on a selected group of affected and control dogs.

Results—Molecular analysis of exons 2 to 6 of the cardiac actin gene did not reveal any differences in base pairs between affected dogs and control dogs selected for DNA evaluation.

Conclusions—Mutations in exons 5 and 6 of the cardiac actin gene that have been reported in humans with familial DCM do not appear to be the cause of familial DCM in Doberman Pinschers. Additionally, evaluation of exons 2 to 6 for causative mutations did not reveal a cause for inherited DCM in these Doberman Pinschers. Although there is evidence that DCM in Doberman Pinschers is an inherited problem, a molecular basis for this condition remains unresolved. Evaluation of other genes coding for cytoskeletal proteins is warranted. (*Am J Vet Res* 2001;62:33–36)

Dilated cardiomyopathy (DCM) is a primary condition of cardiac muscle characterized by ventricular dilation and systolic dysfunction.[1] By definition, the condition is one of unknown cause, although it has been determined in human beings to be of familial origin in at least 30% of cases.[2] In humans affected with DCM of familial origin, the condition has substantial genotypic heterogeneity with patterns of inheritance described as autosomal dominant (most common), autosomal recessive, X-linked, and mitochondrial.[3,4] Causative mutations have been observed within genes encoding for the cytoskeletal proteins dystrophin, α sarcoglycan, β dystroglycan, desmin, and metavinculin.[5-10] Recently, 2 causative mutations have been identified in the gene encoding for the cytoskeletal protein, actin.[11] It has been suggested that mutations in the genes encoding for cytoskeletal proteins may be the common factor that lead to the development of familial DCM.[2]

Dilated cardiomyopathy in dogs has many similarities to the condition in humans, including severe dysfunction and dilatation of the left ventricle.[12] Some breeds appear to be affected more often, including Doberman Pinschers, Boxers, and Cocker Spaniels.[12-16] In Doberman Pinschers, a strong breed predisposition and increased prevalence of the disease within certain families suggests that the disease may be familial.[17,a] The mode of inheritance is uncertain, but it was suggested in 1 report[a] to be autosomal dominant transmission. The condition in Doberman Pinschers is particularly severe, because systolic dysfunction and arrhythmias lead to the death of most affected dogs within 2 months after the condition is diagnosed.[12,18] There is not a definitive treatment, and therapy is palliative at best. The inability to successfully treat or slow the progression of the condition has led to an increased interest in determining its cause. The objective of the study reported here was to evaluate the coding region of the cardiac actin gene in Doberman Pinschers with DCM and determine whether it contained a causative mutation.

Materials and Methods

Animals—Sixteen Doberman Pinschers were selected for DNA evaluation on the basis of diagnostic criteria for DCM that included left ventricular dilation and myocardial systolic dysfunction. Left ventricular dimensions were assessed relative to body size, as described elsewhere.[19] Systolic dysfunction was defined as fractional shortening < 25% and end-systolic volume index > 30 ml/m^2 without congenital heart disease or clinically important acquired valvular disease.[19,20] A pedigree was collected and evaluated on each animal to determine whether dogs were related. Twelve mixed-breed control dogs with normal results of physical examinations and echocardiography also were selected for DNA evaluation.

Collection and Extraction of DNA—A 10-ml blood sample was obtained from each dog for DNA isolation. The DNA was extracted manually by lysing the cells with a hyperosmotic solution (2X sucrose-Triton, tris-NH_4Cl), centrifugation[b] (20 minutes at 4 C), and decanting the supernatant. The pellet was resuspended in 5 ml of 0.5*M* saline (NaCl)-EDTA with 1/20 volume of a solution of 20%

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sodium dodecyl sulfate and 15 µl of proteinase K[c] (10 mg/ml). The mixture was incubated overnight at 56 C. The DNA was isolated, using 2 successive extractions of a phenol:chloroform:isoamyl alcohol mixture (25:24:1, pH 8) and 1 extraction with pure chloroform. After precipitation with ethanol, the final pellet was washed with 75% ethanol and resuspended in 250 µl of TE buffer (10 m*M* tris-HCl, 1 m*M* EDTA, pH 7.5).

Polymerase chain reaction (PCR) and single-stranded conformational polymorphism (SSCP) analysis—Primers for PCR were designed on the basis of known sequences of the human cardiac actin gene (**Appendix**). Five hundred nanograms of canine genomic DNA was added to each PCR reaction (total volume of 25 µl that consisted of 14.3 µl of water, 2.5 µl of 10 m*M* deoxynucleotide triphosphate mix, 2.5 µl of 10× PCR buffer, 2.5 µl of each primer (20 µ*M*), 2 µCi ^{33}P α-dATP, and 0.2 µl of *Taq* polymerase.[d] Amplification conditions for all exons were optimized to an initial 94 C denaturation (5 minutes), followed by 30 cycles of 94 C (30 seconds), 60 C (35 seconds), and 72 C (35 seconds), which was followed by 72 C for 10 minutes.

After PCR amplification, samples were diluted with an equal volume of formamide buffer (95% formamide, 10 m*M* NaOH, 0.25% bromphenol blue, 0.25% xylene cyanol FF) and denatured for 2 minutes at 92 C. Four microliters of each sample was loaded onto a 0.5X MDE nondenaturing polyacrylamide gel.[e] The gel was electrophoresed at 6 to 8 watts for 8 hours, dried on filter paper, and exposed to autoradiographic film for 18 to 24 hours. Autoradiographs were analyzed for banding patterns specific to affected dogs.

DNA sequencing—Ten samples from the 16 affected Doberman Pinschers and 2 samples from the 12 control dogs were randomly selected for sequencing of each exon. The PCR for each exon was performed as described previously for each of the 12 samples. Fifteen microliters of each reaction mixture was analyzed by electrophoresis on a 1% agarose gel and developed with ethidium bromide. The PCR product then was isolated, purified by use of a gel extraction kit,[f] and sequenced by use of an automated sequencer. Forward and reverse primers were used to evaluate each sample for each exon.

Results

Pedigree analysis was performed. It revealed that the 16 Doberman Pinschers were not related to each other within the 4 most recent generations.

Electrophoretic mobility shifts indicative of differences in base pairs were not observed when samples from affected Doberman Pinschers were compared with those of control dogs by use of SSCP analysis for exons 2 to 6 (**Fig 1**). Sequencing of the PCR products for these regions did not reveal changes in base pairs between the affected dogs and control dogs.

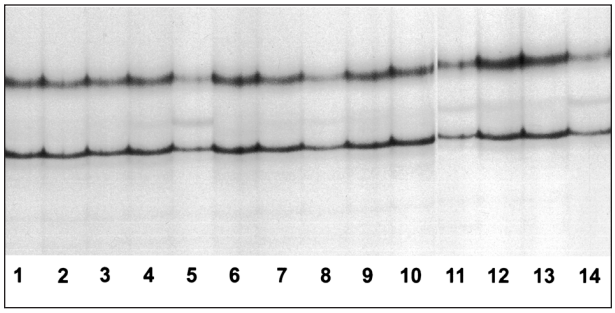

Figure 1—Autoradiograph of single-stranded conformational polymorphism analysis of exon 6 of the canine cardiac actin gene. Lanes 1 to 10 are samples of DNA from Doberman Pinschers with dilated cardiomyopathy. Lanes 11 to 14 contain DNA from healthy mixed-breed control dogs. Notice the identical banding pattern in affected and unaffected (control) dogs.

Discussion

Development of DCM of familial origin in humans has been associated with mutations within several genes that encode for cytoskeletal proteins, including dystrophin, α sarcoglycan, metavinculin, β dystroglycan, desmin, and actin.[5-11] Cytoskeletal proteins have important structural functions in cells including maintaining the structural integrity of a cell, preserving cell shape, organizing the contractile apparatus, and enabling cells to withstand mechanical stress.[8] It has been suggested that an abnormality of a cytoskeletal protein may be a common factor in the development of DCM, because without the structural support provided by these proteins, a dilated, dysfunctional heart will develop.[2] The apparent familial nature of DCM in Doberman Pinschers and noticeable similarities between the condition in dogs and humans warrants evaluation of the role of known human cytoskeletal defects in the development of the condition in dogs.

The cardiac actin gene is a 6-exon gene that encodes for actin protein, the main component of the thin filament of the sarcomere. Actin crossbridges with myosin and a Z band or intercalated disc and transmits force between adjacent sarcomeres and neighboring myocytes to effect coordinated contraction of the heart.[11] The importance of actin mutations in the development of DCM is emphasized by 2 studies.[11,21] First, cardiac actin-deficient fetal mice have a high risk of mortality during gestation, and those mice that survive to birth usually die before they are 2 weeks old. When these mice transgenically are given noncardiac actin, they survive longer but develop a dilated heart with systolic dysfunction, resembling idiopathic DCM.[21] Second, 2 actin mutations have been described in humans with DCM, including a G-to-A substitution at codon 312 in exon 5 and an A-to-G substitution at codon 361 in exon 6. Both mutations are observed in highly conserved amino acids located in important functional regions of the actin molecule.[11]

The PCR-based SSCP analysis for single base-pair mutations is an accepted technique that assesses electrophoretic mobility shifts that are evident when there is a change in base pairs.[11,22-24] Sensitivity of the technique is slightly dependent on the length of the fragment evaluated and is believed to be at least 90% effective for detecting single base-pair changes within regions < 400 base pairs in length.[24] Actin mutations reported in humans with familial DCM were detected by PCR-based SSCP analysis and were single base-pair changes in exons 5 and 6, which have fragment lengths of 182 and 325 base pairs, respectively.[11] Size of the 5 exons evaluated in the study reported here ranged from 161 to 324 base pairs. Therefore, PCR-SSCP analysis would have been expected to detect a base-pair change within these regions. However, because we did not detect base-pair changes between the affected Doberman Pinschers and control dogs with this technique, and because results of PCR-SSCP analysis some-

times can be affected by electrophoretic conditions, we elected to evaluate a selection of samples from both groups of dogs for each exon, using DNA sequencing, a more specific technique.[22-24]

One limitation of the study reported here was the inability to evaluate the promoter region or the first exon of the actin gene. To our knowledge, sequence information for these areas is not available and will only be obtained when the canine cardiac actin gene is cloned and sequenced, which was beyond the scope of this study. A causative mutation for familial DCM has been identified within the promoter region of another cytoskeletal protein, dystrophin.[9] A thorough evaluation of this area should be performed before the importance of the actin gene and its association with DCM in Doberman Pinschers can be completely understood; however, analysis of exons 2 to 6 of the cardiac actin gene in this group of Doberman Pinschers with DCM did not identify a causative mutation. Although the mutations reported in humans have been observed only in exons 5 and 6, we evaluated exons 2 through 6 by use of PCR-SSCP analysis as well as by use of PCR-based sequencing. The additional exonic regions were evaluated, because abnormalities in actin may arise from mutations in sites other than the ones previously described, and it is likely that mutations within other areas of the actin gene may lead to the development of a similar phenotype.

We concluded that mutations in exons 5 and 6 of the cardiac actin gene that have been reported in humans with DCM do not appear to be involved in the development of familial DCM in Doberman Pinschers. Additionally, evaluation of exons 2 to 6 for causative mutations did not reveal molecular alterations in these dogs. It is possible that cardiac actin mutations are responsible for DCM in some affected Doberman Pinschers or in other breeds of dogs; however, it was not observed in the Doberman Pinschers that we evaluated from 16 unrelated families. Given the strong breed-specific characteristics of this condition in this breed, it seems unlikely that there would be numerous mechanisms responsible for the development of DCM in Doberman Pinschers. We believe that evaluation of genes encoding additional cytoskeletal proteins in Doberman Pinschers with DCM is indicated.

Appendix

Polymerase chain reaction primers for exons 2 to 6 of the canine cardiac actin gene

Exon	Primer sequence	Product size (bp)
2	F 5′ GGAGTTATGGTGGGTATGGGTCAG 3′ R 5′ CTGTGGTACGGCCAGAAGCATACAG 3′	324
3	F 5′ GCATTGTTCTGGACTCTGGGGATG 3′ R 5′ CAGTGGTGACAAAGGAGTAGCCAC 3′	161
4	F 5′ CTGAACGTGAAATTGTCCGTGACA 3′ R 5′ CAATGAAGGAGGGCTGGAAGAGTG 3′	171
5	F 5′ GTATGGAATCTGCTGGCATCCATG 3′ R 5′ CTTAATCTTCATGGTGCTAGGAGC 3′	181
6	F 5′ ATTATTGCTCCCCCTGAGCGTAAATAC 3′ R 5′ AGGTTGCAAGTCCTAGTCTGGTTTATT 3′	324

F = Forward primer. R = Reverse primer. bp = Base pairs. Genbank accession numbers were as follows: exons 2, 3, and 4 = J00071; exon 5 = J00072; exon 6 = J00073.

[a] Hammer TA, Venta PJ, Eyster GE. The genetic basis of dilated cardiomyopathy in Doberman pinschers (abstr). *Anim Genet* 1996;27(suppl 2):109.

[b] Eppendorf centrifuge 5402, Eppendorf Scientific Incorporated, Westbury, NY.

[c] Proteinase K, Boehringer Mannheim Corp, Indianapolis, Ind.

[d] *Taq* polymerase, Amersham Life Science Inc, Arlington Heights, Ill.

[e] MDE nondenaturing polyacrylamide gel, SMC Bioproducts, Rockland, Me.

[f] Qiaquick, Qiagen Inc, Valencia, Calif.

References

1. Kasper EK, Agema WRP, Hutchins GM, et al. The causes of dilated cardiomyopathy: a clinicopathologic review of 673 consecutive patients. *J Am Coll Cardiol* 1994;23:586–590.
2. Towbin JA. The role of cytoskeletal proteins in cardiomyopathies. *Curr Opin Cell Biol* 1998;10:131–139.
3. Keeling PJ, Gang Y, Smith G, et al. Familial dilated cardiomyopathy in the United Kingdom. *Br Heart J* 1995;73:417–421.
4. Mestroni L, Krajinovic M, Severini GM, et al. Molecular genetics of dilated cardiomyopathy. *Herz* 1994;19:97–104.
5. Towbin JA, Hejtmancik F, Brink P, et al. X-linked dilated cardiomyopathy. Molecular genetic evidence of linkage to the Duchenne muscular dystrophy (dystrophin) gene at the Xp21 locus. *Circulation* 1993;87:1854–1865.
6. Fadic R, Sunada Y, Waclawik AJ, et al. Brief report: deficiency of a dystrophin-associated glycoprotein (adhalin) in a patient with muscular dystrophy and cardiomyopathy. *N Engl J Med* 1996; 334:362–366.
7. Bies RD, Maeda M, Roberds SL, et al. A 5' dystrophin duplication mutation causes membrane deficiency of α-dystroglycan in a family with X-linked cardiomyopathy. *J Mol Cell Cardiol* 1997;29:3175–3188.
8. Maeda M, Holder E, Lowes B, et al. Dilated cardiomyopathy associated with deficiency of the cytoskeletal protein metavinculin. *Circulation* 1997;5:17–20.
9. Muntoni F, Cau M, Ganau A, et al. Brief report: deletion of the dystrophin muscle-promoter region associated with X-linked dilated cardiomyopathy. *N Engl J Med* 1993;329:921–925.
10. Li D, Tapscoft T, Gonzalez O, et al. Desmin mutation responsible for idiopathic dilated cardiomyopathy. *Circulation* 1999;100:461–464.
11. Olson TM, Michels VV, Thibodeau SN, et al. Actin mutations in dilated cardiomyopathy, a heritable form of heart failure. *Science* 1998;280:750–752.
12. Smucker ML, Kaul S, Woodfield JA, et al. Naturally occurring cardiomyopathy in the Doberman Pinscher: a possible large animal model of human cardiomyopathy? *J Am Coll Cardiol* 1990;16: 200–206.
13. Monnet E, Orton EC, Salman M, et al. Idiopathic dilated cardiomyopathy in dogs: survival and prognostic indicators. *J Vet Intern Med* 1995;9:12–17.
14. Harpster NK. Boxer cardiomyopathy. A review of the long-term benefits of antiarrythmic therapy. *Vet Clin North Am Small Anim Pract* 1991;21:989–1004.
15. Kittleson MD, Keene B, Pion PD, et al. Results of the multicenter spaniel trial (MUST): taurine- and carnitine-responsive dilated cardiomyopathy in American cocker spaniels with decreased plasma taurine concentration. *J Vet Intern Med* 1997;11:204–211.
16. Gooding JP, Robinson WF, Wyburn RS, et al. A cardiomyopathy in the English cocker spaniel: a clinico-pathological investigation. *J Small Anim Pract* 1982;23:133–149.
17. Calvert CA. Dilated congestive cardiomyopathy in Doberman Pinschers. *Compend Contin Educ Prac Vet* 1986;8:417–427.
18. Calvert CA, Pickus CW, Jacobs GJ, et al. Signalment, survival, and prognostic factors in Doberman pinschers with end-stage cardiomyopathy. *J Vet Intern Med* 1997;11:323–326.
19. Bonagura JD, O'Grady MR, Herring DS. Echocardiography. Principles of interpretation. *Vet Clin North Am Small Anim Pract* 1985;15:1177–1194.
20. Kittleson MD, Eyster GE, Knowlen GG, et al. Myocardial function in small dogs with chronic mitral regurgitation and severe congestive heart failure. *J Am Vet Med Assoc* 1984;184:455–459.

21. Kumar A, Crawford K, Close L, et al. Rescue of cardiac α-actin-deficient mice by enteric smooth muscle (-actin. *Proc Natl Acad Sci USA* 1997;94:4406–4411.

22. Sheffield VC, Beck JS, Kwitek AE, et al. The sensitivity of single-strand conformation polymorphism analysis for the detection of single base substitutions. *Genomics* 1993;16:325–332.

23. Orita M, Suzuki Y, Sekiya T, et al. Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics* 1989;5:874–879.

24. Vidal-Puig A, Moller DE. Comparative sensitivity of alternative single-strand conformation polymorphism (SSCP) methods. *Biotechniques* 1994;17:490–496.