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Microsatellites within the feline androgen receptor are suitable for X chromosome-linked clonality testing in archival material

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Abstract

Objectives A hallmark of neoplasms is their origin from a single cell; that is, clonality. Many techniques have been developed in human medicine to utilise this feature of tumours for diagnostic purposes. One approach is X chromosome-linked clonality testing using polymorphisms of genes encoded by genes on the X chromosome. The aim of this study was to determine if the feline androgen receptor gene was suitable for X chromosome-linked clonality testing.

Methods The feline androgen receptor gene, was characterised and used to test clonality of feline lymphomas by PCR and polyacrylamide gel electrophoresis, using archival formalin-fixed, paraffin-embedded material.

Results Clonality of the feline lymphomas under study was confirmed and the gene locus was shown to represent a suitable target in clonality testing.

Conclusions and relevance Because there are some pitfalls using X chromosome-linked clonality testing, further studies are necessary to establish this technique in the cat.

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Introduction

Most tumours arise from a single cell of origin and are therefore considered clonal proliferations.[1–3] Clonality testing has developed into a significant accessory technique in human cancer diagnostics.[4] Clonality testing is especially important for the diagnosis of haematological malignancies such as lymphoma and leukaemia because lymphatic neoplasia and hyperplasia are not always easily differentiated histologically.[5,6]

To improve lymphoma diagnostics in veterinary medicine several techniques have been applied to analyse clonal antigen receptor rearrangement, including Southern blotting [7–10] and PCR.[7,9–16] Further techniques have been used for clonality testing in veterinary medicine and targeted clonally integrated feline leukaemia virus (FeLV) provirus within the host's genome,[17] mutations within *c-kit*,[18] clonal polymorphisms within mitochondrial DNA,[19] and polymorphisms of microsatellites and mitochondrial DNA in the transmissible venereal tumour.[20] Clonality testing involving X-linked genes has also been used in dogs and cats,[21,22] both employing the androgen receptor gene.

To date, there is no routinely implementable clonality test for non-lymphoid neoplasms in cats.[23] The analysis of clonal inactivation of genes located on the X chromosome in females (X-linked clonality testing) has nevertheless been applied to many human tumours.[24,25] This approach is based on the fact that females carry two copies of the genes located on the X chromosome, of which one is inactivated to avoid an 'overdose' of gene expression. The inactivated allele of X-chromosomal genes is randomly selected during early embryogenesis and inactivation is based on methylation of cytosine residues

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within CpG-rich islands.[26] To test for clonality, genomic DNA is digested by methylation-sensitive endonucleases, such as *Hpa*II or *Hha*I. In the following PCR only the inactivated allele of the corresponding gene can be amplified.[27] One gene with such a polymorphism located next to a CpG island that is heterozygous in a large fraction of individuals is the human androgen receptor. This gene features a polymorphic short tandem repeat of CAG,[27] which has been used to demonstrate clonality of tumours in women.[28] This test has been termed the HUMARA assay.[28]

The androgen receptor gene of cats has already been sequenced.[29] Similar to its canine counterpart it includes two stretches of CAG repeats. However, possible polymorphic microsatellites within exon 1 have not yet been reported. Therefore, we investigated exon 1 of the feline androgen receptor for potentially useful microsatellites and applied them in clonality tests. We then designed the assay such that archival formalin-fixed and paraffin embedded material could be tested.

Materials and methods

Case selection

To assess the presence of microsatellites within the feline androgen receptor we used the DNA of 42 cats from a previous study.[30] The newly developed assay was then applied to 50 feline lymphomas and lymph nodes with reactive lymphocytic hyperplasia that had already been thoroughly investigated.[15,31]

Nucleic acid extraction, PCR amplification of the feline androgen receptor exon 1 and polyacrylamide gel electrophoresis

Genomic DNA was extracted from macro-dissected, formalin-fixed and paraffin-embedded tissue samples with the Gentra Puregene Tissue Kit (Qiagen), including RNase treatment as described.[30]

Primers spanning both CAG repeats and primers for the first CAG region within exon 1 of the feline androgen receptor were newly designed according to a publicly available sequence (Table 1; GenBank Accession: AJ893545) using GeneFisher.[33] Primers for the second CAG stretch of the feline androgen receptor were adapted from primers designed for the canine androgen receptor.[32]

PCR was performed as described elsewhere.[21] In short, the master mix consisted of 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each primer and 0.024 U/µl Taq DNA polymerase (GoTaq; Promega). Cycling conditions encompassed an initial denaturing at 94°C for 5 mins followed by 40 cycles of DNA melting at 92°C for 30 s, annealing for 15 s, amplification at 72°C for 80 s in the case of the long amplicons (>600 base pairs) and 20 s in the case of the short ones. This was followed by a final amplification at 72°C for 5 mins. Annealing temperatures for specific primer combinations are summarised in Table 1.

Using a semi-nested PCR protocol, products of the first round of PCR were diluted 1:500 with 5 mM Tris buffer. Cycling conditions were modified in the second round of PCR by using only 30 cycles, 59°C as the primer annealing temperature and 15 s elongation time. After amplification, PCR products were screened by agarose gel electrophoresis using 2% gels impregnated with ethidium bromide.

To detect deactivated alleles of the canine androgen receptor genomic DNA was digested prior to PCR amplification with the methylation-sensitive endonuclease *Hpa*II (restriction site: CCGG) (Fermentas). Genomic DNA (1 µg) was digested with 10 U endonuclease over night at 37°C in a final volume of 20 µl.

Table 1 Primer sequences, melting temperatures, amplicon sizes and positions

No	Identification	Primer sequence	Temperature (°C)	Amplicon size (bp) in AJ893545
	Outer primers			
1	FeARof1*	5'-CAAGACCTATCGAGGAGCTTTC-3'	60–55†	645
2	FeARor1*	5'-GTCGAACTGCCACCTAGGTAAC-3'		
	First polymorphic CAG repeat – FELARA1			
3	FELARAf1	5'- TATTCCAGAGCGTGCGCGAAG -3'	57	245
4	FELARAr1	5'- GCTGTTGTGAAGGCTGCTGTTC -3'		
	Second polymorphic CAG repeat – FELARA2[32]			
5	FELARAf2*	5'-GACTCAGCTGCCCCATCCAC-3'	57	250
6	FELARAr2*	5'-GGTAACTGTCCTTGGAGGAGG-3'		
	Additional combinations used			
3/6	FELARAf1 and FELARAr2		59	606
1/4	FeARof1 and FELARAr1		57	269
5/2	FELARAf2 and FeARor1		57	265

*Designed for the canine androgen receptor but also works with the feline counterpart
†A touch-down protocol was applied starting at 60°C, reducing the temperature by 0.5°C per cycle for 10 cycles followed by 30 cycles at 55°C
bp = base pairs

To test complete digestion, a male sample in which only one active (ie, digestion-sensitive) allele is present was also digested and later amplified. Polyacrylamide gel electrophoresis (PAGE) was carried out as described before.[21]

Sequence analysis

To analyse the sequence and exact repeat number of the feline androgen receptor gene PCR products were purified as described and submitted to a commercial laboratory (SeqLab, Göttingen, Germany) for sequencing.[15] Sequence analysis was carried out using Blast Search (NCBI) and ClustalW (EMBL-EBI). GeneDoc 2.6.003 was used to create the multiple sequence alignments.

Results

First, microsatellites within the feline androgen receptor of 56 male cats ('myocardium' and 'lymphoma' groups) were studied because they only feature one allele. Analysis of sequences obtained from PCR products of the first potential microsatellite within the feline androgen receptor revealed that, in contrast to the situation in dogs,[21] no length polymorphism was present in the cats investigated. Additionally, within the feline gene three alternative triplets (CAA, CAA and GAG) were identified when compared with the canine gene (see Figure 1a). However, the GAG triplet is also included in the canine gene, but it follows one position downstream. Both carnivores featured a stretch of different triplets – three in the cat and four in the dog – within this microsatellite when compared with the human sequence.

In contrast, sequences of the second microsatellite revealed distinct length polymorphisms in cats. While dogs had 10–13 consecutive tandem repeats within this region, cats had 16–20 CAG repeats (see Figures 1b and 2, as well as Figure 3 for a schematic; see also Table 2). In dogs the consecutive tandem repeats of 'CAG' are interrupted by 'CAA' triplets; the feline sequences lacked interspersed 'CAA' triplets (see Figure 1b). Higher numbers of tandem repeats (especially 18 and 19) were more frequent in our data sets than the lower numbers (Table 2).

The first microsatellite that is highly polymorphic in humans is shorter in dogs and cats (Figure 1a). In contrast, the second microsatellite, which is not polymorphic in humans, is much longer in cats and especially in dogs (Figure 1b).

Of all 32 female animals ('myocardium' and 'lymphoma' groups) tested in this study, 12 were heterozygous (37.5%) (Table 2, Figure 4). Similarly, 10/22 (45.4%) lymphomas from female animals were heterozygous. These animals featured different combinations of alleles regarding the number of tandem repeats, with 18 and 19 repeats being the most frequent (Table 2).

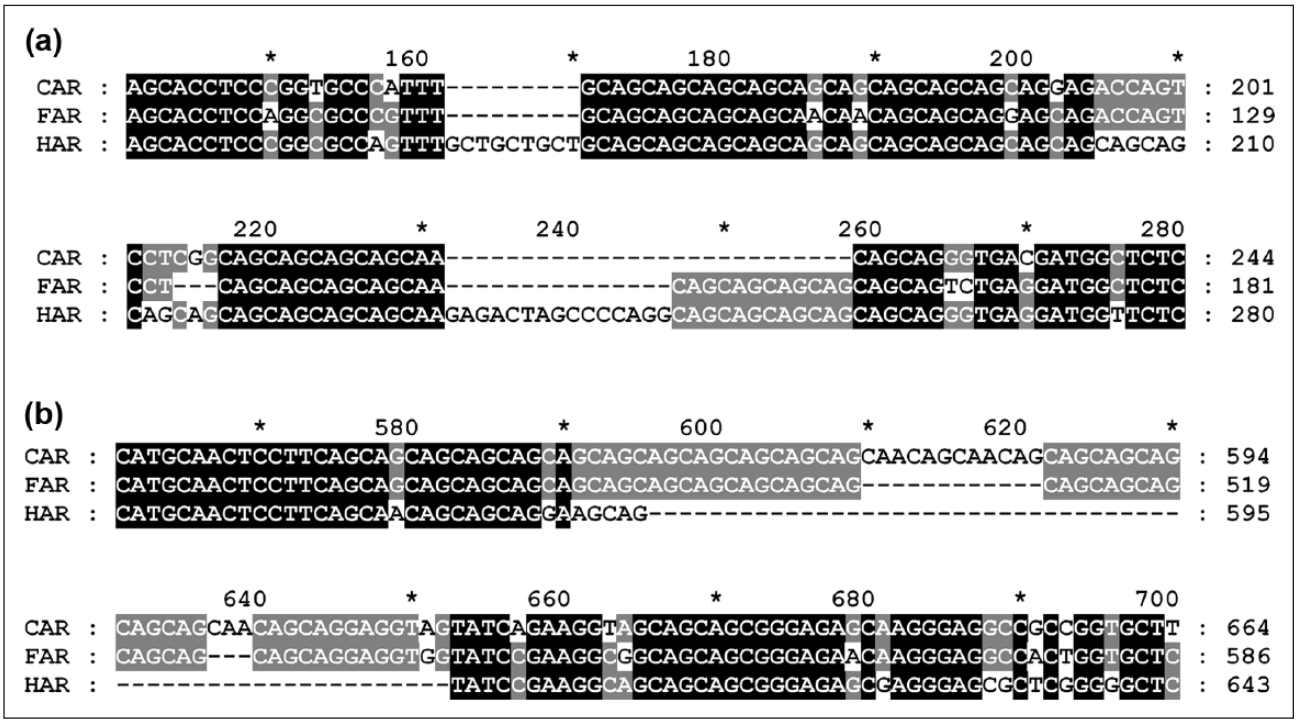

Figure 1 Alignment of exon 1 of the canine (CAR; GenBank Accession: L11357), feline (FAR; GenBank Accession: AJ893545) and human (HAR; GenBank Accession: NM_000044) androgen receptor. (a) Encompasses the first tandem repeat. It is polymorphic in humans and rarely in dogs. (b) Encompasses the second tandem repeat that is polymorphic in dogs and cats but not humans

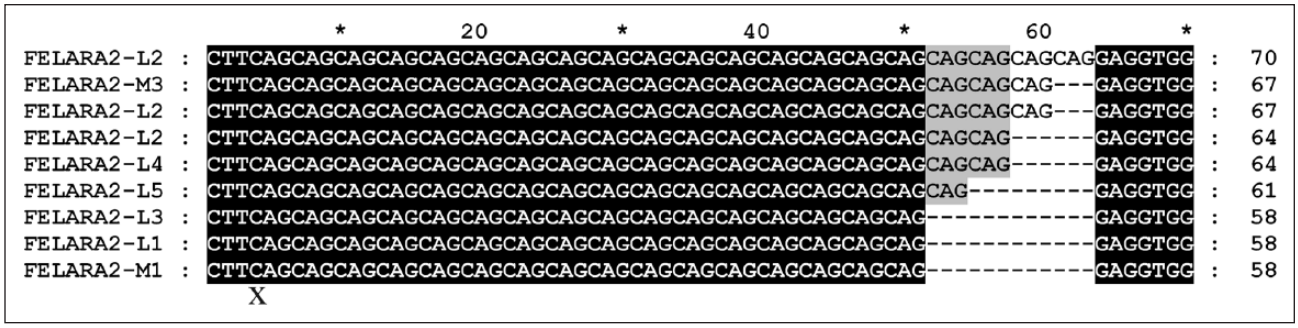

Figure 2 Alignment of different variants of the polymorphic CAG tandem repeat in cats. This microsatellite includes 16–20 repetitions of CAG triplets

Of nine lymphomas with a heterozygous polymorphism in the feline androgen receptor gene, eight revealed only one band after digestion with *Hpa*II and PCR amplification (see Figure 4). Therefore, they displayed non-random X chromosome inactivation. One lymphoma displayed a markedly weakened second band (see Figure 4, sample 3). In contrast, two lymphatic hyperplasias retained two bands. Seven of the nine lymphomas were FeLV provirus positive and none were FeLV antigen positive. Both cases of lymphatic hyperplasia contained FeLV provirus.[31] Three of the lymphomas had been previously diagnosed as clonal entities and the two lymphatic hyperplasias as polyclonal.[15]

As was the case in dogs, digestion of genomic DNA prior to amplification was most effective using the long PCR fragments. In the case of the shorter fragments, especially when using semi-nested PCR, digestion had to be extended. Using the long fragments, however, some DNA isolated from archival material may not be amplifiable by PCR, as was the case in two cases of lymphoma and myocardial tissue, respectively.

PAGE of the PCR products revealed a phenomenon we previously observed in dogs.[21] Additional larger DNA fragments were visualised after PAGE that could not be observed after agarose gel electrophoresis. Sequencing of this material only resulted in originally targeted sequences. Therefore, these bands represent probably duplicated PCR fragments.

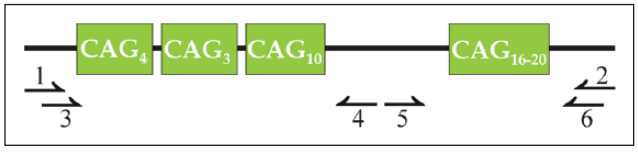

Figure 3 Schematic of exon 1 of the feline androgen receptor gene. It includes both tandem repeats and the location's primer annealing sites. 1 = FeARof1; 2 = FeARor1; 3 = FELARAf1; 4 = FELARAr1; 5 = FELARAf2; 6 = FELARAr2

Discussion

To characterise the microsatellites within the feline androgen receptor, PCR products of the androgen receptor gene of male cats were sequenced and studied because they only feature one allele. Therefore, sequencing of this locus was possible without the necessity of differentiating two possibly different alleles. Sequencing results, especially regarding frequency and length of polymorphisms of the feline androgen receptor, were in accordance with results of a recent study using fresh material.[22] However, in the previous study far more heterozygous female animals were identified (68.0%) than in the present study (37.5%). These findings are supported by our

Table 2 Numerical distribution of the different alleles discovered in the cases studied

FELARA2					
	Homozygous animals (n)				
	CAG_{16}	CAG_{17}	CAG_{18}	CAG_{19}	CAG_{20}
Myocardium* ♀/♂	0/1	0/3	3/10	5/14	0/2
Lymphoma† ♀/♂	1/2	0/3	1/9	10/11	0/1
	Heterozygous animals (n)				
	CAG_{16+18}	CAG_{17+18}	CAG_{18+19}	CAG_{18+20}	
Myocardium	1	0	1	0	
Lymphoma	0	3	6	1	

*DNA of two animals could no longer be amplified
†DNA of two animals could no longer be amplified

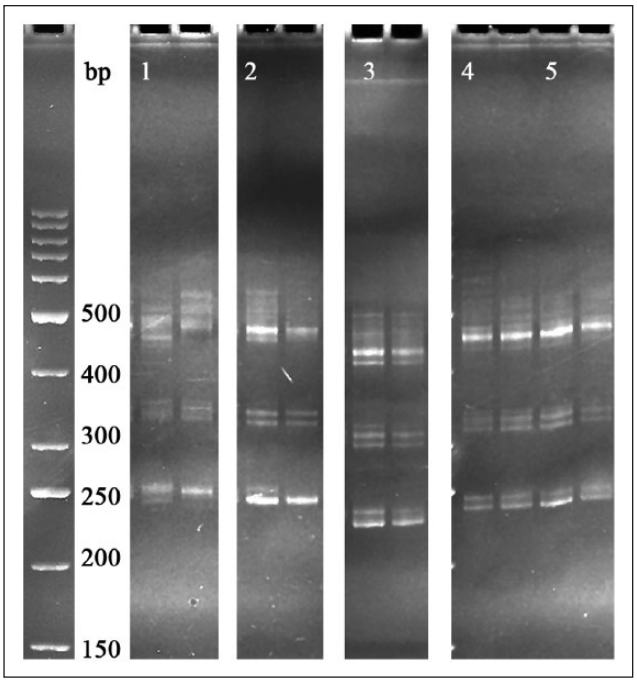

Figure 4 Clonality analysis of feline lymphomas (1–3) and lymphatic hyperplasias. Each sample comprises two lanes from two different PCRs. The left includes native DNA the right *Hpa*II-digested DNA. Samples 1 and 2 display non-random deactivation of one androgen receptor gene; that is, they are clonal (one band after *Hpa*II digestion). Sample 3 displays a visibly weakened second band, probably due to a strong non-neoplastic lymphatic infiltration of the clonal, neoplastic cell population. Samples 4 and 5 display random inactivation of the androgen receptor gene (two bands in both lanes), resulting in amplification of both alleles, indicating a polyclonal cell population. bp = base pairs

recent findings in the analysis of the heterozygosity of dogs, where we identified 19% of animals as heterozygous,[21] while older reports identified 43% of animals as heterozygous.[34]

The lack of interspersed 'CAA' triplets within the second microsatellite in cats when compared with dogs (see Figure 1b), results in a higher rate of heterozygous cats, leading to better applicability of this test in cats than in dogs.

Thorough digestion of the non-deactivated X chromosome prior to PCR by a methylation-sensitive endonuclease has been identified as a crucial aspect for the assay presented here. In this study, as in a previous one,[21] digestion was most effective using the long fragments for PCR amplification. When using shorter fragments, digestion had to be extended or higher concentrations of endonuclease had to be used. In this case, digestion controls from male animals have to be used. This phenomenon is a result of the different number of restrictions sites covered by the PCR fragments. The long fragment includes three restriction sites, whereas the short fragments FELARA1 and FELARA2 each contain only one restriction site. As we used archival material we had to cope with limited amounts of starting material and therefore had to use nested PCR to produce enough material for the PAGE. However, digestion should be no problem using fresh or freshly frozen material and the long PCR fragment. Another alternative could be direct amplification of the short fragments.

All lymphomas from heterozygous animals that were tested revealed a non-random deactivation of one allele of the feline androgen receptor and, therefore, are of clonal origin. In contrast, the two lymphatic hyperplasias analysed revealed a random deactivation and therefore, as was expected, are of polyclonal origin.

One case of lymphoma in this study revealed only a weakened band upon digestion; this could be due to a reactive infiltration by lymphocytes. Incomplete digestion of the active allele could be a further reason for this result.

It is absolutely vital with this technique to first check whether informative pattern, that is, heterozygous alleles, are present in the animal to be tested. Both samples should be directly compared upon PAGE to visualise clearly any changes after digestion by a methylation-sensitive endonuclease.

This study demonstrates the usefulness of the polymorphic tandem repeat within exon 1 of the feline androgen receptor for clonality analysis, and it can be expected that the assay can also be applied to other tumour types. Because of contrary results regarding the rate of heterozygosity, further studies are needed to establish the true extent to which this test can be utilised in routine diagnostics.

However, there are further caveats that need to be addressed. In humans, a skewing of the X chromosome inactivation pattern in haematopoietic cells, especially granulocytes, of some ageing women has been identified.[35] It needs to be determined if such a phenomenon exists in cats. This should be visible in a PAGE of PCR products obtained from non-digested DNA. To be certain only to identify truly clonal populations, total loss of a second band could be regarded as hallmark of a clonal cell population.[35]

Furthermore, the two polymorphic tandem repeats within the androgen receptor gene are approximately 1 kb downstream from the promoter region of the gene. This region is vital in gene regulation; that is, activation and inactivation. In some humans methylation patterns of the promoter region of the androgen receptor gene and within exon 1 are not completely concordant.[36] In the future it should be determined whether this rather remote location exhibits the same methylation pattern as the direct vicinity of the promoter region in the cat. Additionally, more than one polymorphic locus should be studied to determine truly monoclonal cell populations.

Conclusions

Until now there have been no routine assays to evaluate clonality in feline neoplasms with the exception of lymphomas;[22] therefore, it is necessary to further establish X chromosome-linked clonality testing for basic research of carcinogenesis in this species.

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