Brief Gene Mapping Report

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Physical mapping[1] of the *ATP2A2* gene to equine chromosome 8p14→p12 by FISH and confirmation by linkage and RH mapping

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[1] To our knowledge this is the first time this gene has been mapped in horse.

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Rationale and significance

The ATPase, Ca^{++} transporting, cardiac muscle, slow twitch 2 gene *(ATP2A2)* encodes one of the SERCA Ca^{2+}-ATPases, which are intracellular pumps located in the sarcoplasmic or endoplasmic reticula of muscle cells (MacLennan et al., 1985; Ruiz-Perez et al., 1999). This enzyme catalyzes the hydrolysis of ATP coupled with the translocation of calcium from the cytosol to the sarcoplasmic reticulum lumen, and is involved in regulation of the contraction/relaxation cycle. *ATP2A2* maps to HSA12q23→q24.1 at 109,182,152 to 109,251,615 and consists of 21 exons spanning 69,464 bp (Otsu et al., 1993). Mutations in the human *ATP2A2* gene cause the autosomal dominantly inherited condition keratosis follicularis, also known as Darier-White disease, characterized by loss of adhesion between epidermal cells and abnormal keratinisation (Jacobsen et al., 1999; Sakuntabhai et al., 1999).

Further studies including a null mutation in one copy of the *ATP2A2* gene in mice revealed that serca2 protein levels were reduced by about a third, sarcoplasmic reticulum calcium stores and calcium release in isolated cardiomyocytes were decreased. In addition, these mice had reduced myocyte contractility (Ji et al., 2000). Aged heterozygous mutant *(Atp2a2*$^{+/-}$*)* mice developed squamous cell tumors, which led to the conclusion that *Atp2a2*-haploinsufficiency predisposes murine keratinocytes to neoplasia, and that perturbation of Ca^{2+} homeostasis or signaling can be a primary initiating event in cancer (Liu et al., 2001).

Materials and methods

BAC isolation and characterization of the equine ATP2A2 clone

The equine BAC library CHORI-241 was screened for a BAC clone containing the *ATP2A2* gene. High-density BAC colony filters were probed according to CHORI protocols (http://bacpac.chori.org) with a heterologous ^{32}P-labelled insert of a human *ATP2A2* cDNA clone (IMAGp998K2010072) from the Resource Center/Primary Database of the German Human Genome Project (http://www.rzpd.de/). A clone with an insert of approximately 220 kb was identified and designated CH241-167G5. DNA was isolated from the clone using the Qiagen plasmid midi kit (Qiagen, Hilden, Germany). End sequences from the BAC insert were obtained using the ThermoSequenase kit (AmershamBiosciences, Freiburg, Germany) and a LI-COR 4200 automated sequencer (LI-COR Inc., Lincoln, NE, USA). The CH241-167G5 SP6 and T7 end sequences were deposited in the EMBL nucleotide database under acces-

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sion numbers AJ937347 and AJ937348. A BLASTN sequence comparison of the equine SP6 BAC end sequence with the Build 35.1 of the human genome sequence revealed four significant matches (BLAST E-value of each match $1.4e^{-65}$). The first match was over 101 bp (identity = 89.1 %), which started at 109,369,448 bp of HSA12q24.11 approximately 118 kb downstream of human *ATP2A2.* The further BLASTN matches over 63, 61, and 30 bp started at 109,369,792 bp, 109,369,269 bp and 109,369,371 bp of HSA12q24.11. Two of the three BLASTN matches were located within exon 1 of the HSU79274 (protein predicted by clone 23733) on HSA12q24.11.

The identity of the BAC clone with respect to the presence of the *ATP2A2* gene was further verified by obtaining DNA sequences from a shotgun plasmid library generated from the BAC. For this, DNA from the clone CH241-167G5 was isolated using the Qiagen Large Construct kit (Qiagen, Hilden, Germany), was mechanically sheared to obtain fragments of approximately 2 kb, and subsequently used to construct a shotgun plasmid library. In total 96 plasmid subclones were sequenced as described above. Sequence data were analyzed with Sequencher 4.1.4 (GeneCodes, AnnArbor, MI, USA). Comparison of the sequences with the human genome sequence (Build 35.1) revealed a significant match (BLAST E-value $1e^{-64}$) over 228 bp (87% identity) starting at 109,223,486 with exon 6 and parts of intron 5 and 6 of the human *ATP2A2* gene. A $(GT)_{25}$ dinucleotide repeat was identified in the sequence, and flanking PCR primers ATP2A2_MS5_F: 5′-TAA GAA GAA AGA GAG TGA TG-3′ and ATP2A2_MS5_R: 5′-GCT TTT CGT TTG GAA TAA ACC-3′ were obtained for the microsatellite (AJ937349).

PCR conditions and microsatellite analysis

The PCR amplification (12 μl final volume) was performed on a PTC 200™ thermal cycler (MJ Research, Watertown, MA, USA) using 20 ng of genomic equine DNA, 1.2 μl 10× PCR buffer, 0.24 μl DMSO, 0.2 μl dNTPs (5 mM each), 0.5 μl of each primer (10 pmol/μl) and 0.5 U *Taq* DNA polymerase (Qbiogene, Heidelberg, Germany). One primer of the pair was endlabeled with fluorescent IRD700 to enable fluorescent PCR fragment detection. Reaction started with a denaturing step at 94°C for 4 min followed by 35 cycles using the following protocol: denaturation for 30 s at 94°C, annealing for 30 s at 58°C and extension for 45 s at 72°C. The PCR was completed with a final cooling at 4°C for 10 min. Raw data were genotyped using Gene Profiler 3.55 software (Scanalytics Inc., Fairfax, USA). Marker characteristics were determined by genotyping horses of three different breeds and the two three-generation, full-sib reference families with 67 offspring created at the Animal Health Trust, Newmarket, UK (NRF) (Swinburne et al., 2000) using the above mentioned PCR primer pair.

Chromosome preparation

Equine metaphase spreads for FISH on GTG-banded chromosomes were prepared using phytohemagglutinin stimulated blood lymphocytes from a normal horse. Cells were harvested and slides prepared using standard cytogenetic techniques. Prior to fluorescence in situ hybridization the chromosomes were GTG-banded and well-banded metaphase chromosomes were photographed using a highly sensitive CCD camera and IPLab 2.2.3 software (Scanalytics).

Fluorescence in situ hybridization

The equine BAC clone CH241-167G5 containing the equine *ATP2A2* gene was labelled with digoxygenin by nick translation using a nick-translation mix (Roche Diagnostics, Mannheim, Germany). FISH on GTG-banded horse chromosomes was performed using 500 ng of digoxygenin labelled BAC DNA, with 20 μg sheared total equine DNA and 10 μg salmon sperm DNA as competitors. After overnight hybridization, signal detection was performed using a digoxygenin-FITC detection kit (Qbiogene, Heidelberg, Germany). The chromosomes were counterstained with DAPI (4′,6-diamidino-2-phenylindole) and propidium iodide and embedded in antifade. Previously photographed metaphase spreads were re-examined for hybridization signals using a Zeiss Axioplan 2 microscope (Zeiss, Jena, Germany) equipped for fluorescence. Chromosomes were identified according to the international system for chromosome nomenclature of domestic horses (ISCNH 1997, Bowling et al., 1997).

Probe name: CH241-167G5
Probe type: BAC clone from equine genomic BAC library CHORI-241
Insert size: 220 kb
Vector: pCMV-Sport6
Proof of authenticity: PCR and DNA sequencing
Gene reference for the human ATP2A2 gene: Sakuntabhai et al. (1999)

Radiation hybrid (RH) mapping

The 5,000-rad TAMU equine radiation hybrid (RH) panel was genotyped to map the SP6 BAC end marker located about 118 kb downstream of *ATP2A2*. Primer design for RH-mapping was performed. Primers for PCR amplification of a 975-bp fragment were designed from the CH241-167G5 SP6 BAC end sequence (AJ937347) using the PRIMER3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primers were: ATP2A2_F: 5′-GAA GGA AAT GCT GTG GGA AG-3′ and ATP2A2_R: 5′-CTC CTC CCT AAC CCC TCA AG-3′. PCR was carried out in a 20-μl reaction containing 25 ng RH cell line DNA, 10 pmol of each primer and 0.85 U *Taq* polymerase (Qbiogene, Heidelberg, Germany). The reaction conditions started with a denaturing step at 94°C for 5 min followed by 34 cycles using the following protocol: denaturation for 45 s at 94°C, annealing for 45 s at 60°C and extension for 5 min at 72°C. The PCR was completed with a final cooling at 4°C for 5 min. PCR products were separated on a 1.5% agarose gel. After scoring positive signals, a two-point analysis (Slonim et al., 1997; http://equine.cvm.tamu.edu/cgi-bin/ecarhmapper.cgi) was conducted to find associations between ATP2A2 versus the markers of the first generation whole genome equine RH map.

Linkage mapping

The two full-sib reference families with 67 offspring (NRF) (Swinburne et al., 2000) were genotyped to map the microsatellite marker located within intron 5 of *ATP2A2*. PCR conditions, primer pairs and fragment analysis were as described above. Linkage analysis was performed using CRI-MAP program version 2.4 (Green et al., 1990). Maximum likelihood estimates of recombination fraction (theta) were calculated using the TWO-POINT option with a lod-score threshold >3 to determine linkage group and chromosomal assignment versus the markers of the comprehensive horse linkage map including 626 markers linearly ordered and 140 markers assigned to a chromosomal region (Penedo et al., 2005).

Results and discussion

Polymorphism

Table 1 shows the respective sample sizes, number of alleles, allele sizes, expected heterozygosity and polymorphism information content for the microsatellite ATP2A2_MS5. A total of 8 alleles with sizes ranging between 131 and 177 bp were observed in different horse breeds.

Chromosomal location

The equine genomic BAC clone CH241-167G5 containing the *ATP2A2* gene was mapped to ECA8p14→p12 following examination of 37 metaphases spreads (Fig. 1).

Mapping data
Most precise location: ECA8p14→p12
Number of cells examined: 37
Number of cells with specific signals: 0 (10), 1 (4), 2 (9), 3 (7), 4 (7) chromatids per cell
Location of background signals (site with >2 signals): none observed

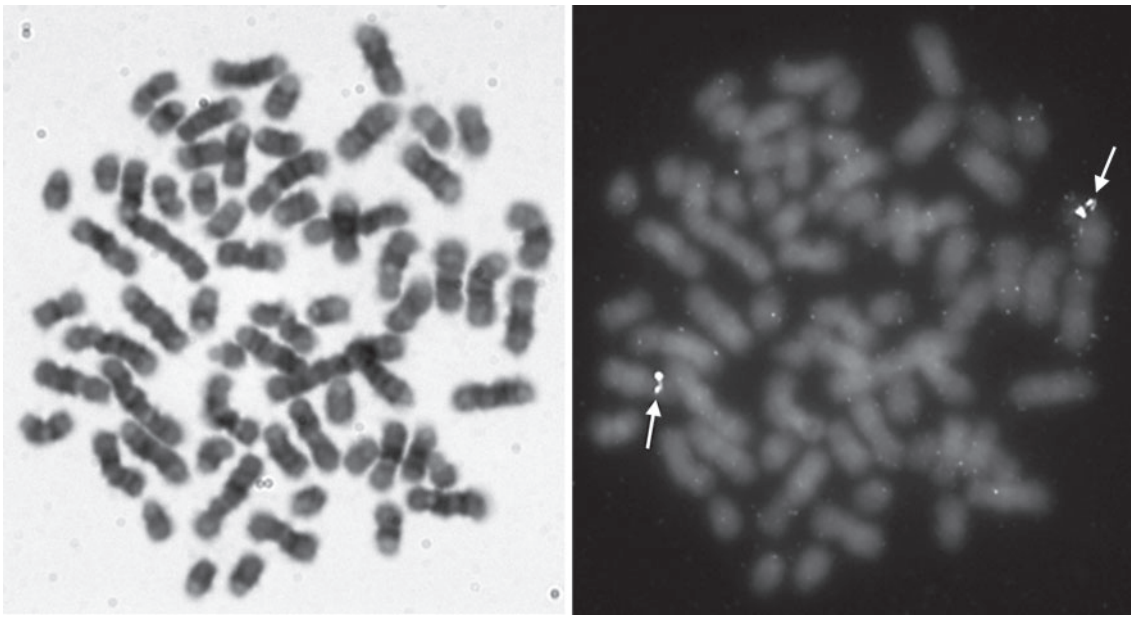

Fig. 1. Chromosomal assignment of the equine BAC CH241-167G5 containing *ATP2A2* by FISH analysis. G-banded metaphase spread before (left) and after (right) hybridization. Double signals indicated by arrows are visible on both ECA8 chromosomes.

Table 1. Characterization of the newly developed *ATP2A2*-associated microsatellite ATP2A2_MS5

Breed/Source	Sample size	No. of alleles	Allele size min-max (bp)	Expected heterozygosity	PIC[a]
South German coldblood	10	8	131–177	0.90	0.76
Saxon Thuringa coldblood	6	5	131–177	0.83	0.68
Hanoverian warmblood	10	7	131–171	0.90	0.76
NRF	67	8	135–171	0.87	0.72

[a] Polymorphism Information Content; Hardy-Weinberg equilibrium in each population genotyped.

RH analysis of the sequence tagged site (STS) marker of the BAC clone CH241-167G5 showed a retention frequency of 21.7%. Two-point linkage analysis revealed close linkage to the microsatellite UM034 (22.06 cR; LOD >3.0). The linked microsatellite marker was previously mapped on ECA8 at 58.7 cM of the horse linkage map (Penedo et al., 2005) and by RH mapping at 17.3 cR of ECARH08b (Chowdhary et al., 2003). The microsatellite ATP2A2_MS5 showed recombination fractions of 0.06 with ECA8 microsatellites LEX023 (Lod score = 23.7) at 71.0 cM and UCDEQ46 (Lod score = 17.8) at 58.7 cM. Thus the FISH, RH and genetic linkage results corroborate each other. The most closely linked equine genes, *SART3* (squamous cell carcinoma antigen recognised by T cells 3) and *FLJ11021* (similar to splicing factor, arginine/serine-rich 4), on ECA8 were also mapped on HSA12q24 and HSA12q24.31 (Chowdhary et al., 2003).

The physical assignment of the equine *ATP2A2* gene on ECA8p14→p12 agrees with the current equine-human comparative ECA8 RH map, which showed conserved synteny to HSA12q24 (Chowdhary et al., 2003).

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