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Research Article

Localization of CO4107 Marker, CTR1 and Wilson (ATP7B) BAC Clones on Dog Chromosomes

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Abstract: Copper toxicosis (CT) is a common autosomal recessive disorder in dogs. Recently, the CT locus was closely linked to microsatellite marker CO4107. Currently, the gene underlying CT is unknown. In contrast to that of many other animals, knowledge regarding the canine karyotype is quite sparse. Except for the X and Y, all the chromosomes are acrocentric. Peripheral blood was used in this study and high-resolution G-banding and FISH were performed on dog chromosomes. In order to evaluate the chromosomal locus of the ATP7B gene (Wilson F4 and Wilson B1), CTR1 and the CO4107 marker were isolated from canine BAC clones. Dual-color FISH results showed that BAC clones containing Wilson F4, Wilson B1, CO4107 marker and CTR1 were localized on chromosomes 4q23, 22q11, 10q26 and 11q22.2-22.5, respectively.

Key Words: Wilson F4, Wilson B1, CO4107 marker, CTR1, dog chromosome

CO4107, CTR1 ve Wilson (ATP7B) BAC Markırlarının Köpek Kromozomları Üzerinde Lokalizasyonu

Özet: Bakır zehirlenmesi (CT) köpeklerde yaygın gözlenen otozomal resesif bir hastalıktır. CT lokusunun son zamanlarda mikrosatellit markır CO4107 ile sıkı bağlantılı olduğu belirlenmiştir. Şu anda CT geni tanımlanmamıştır. Bir çok hayvan karyotipine göre, köpek karyotipi hakkındaki bilgiler oldukça azdır. Bunun nedeni X ve Y kromozomu hariç diğer tüm kromozomların akrosentrik olmasıdır. Bu çalışmada periferal köpek kanından kromozom elde edilerek yüksek rezolusyonlu G bantlama yapılmış ve bantlanmış kromozomları üzerinde FISH yöntemi uygulanmıştır FISH probları (ATP7B geni Wilson F4 ve Wilson B1, CTR1 ve CO4107) köpek BAC klonlarından elde edilmiştir. İki-renkli FISH yönteminin sonucuna göre, BAC klonları Wilson F4, WilsonB1, CO4107 markırı ve CTR1 sırası ile 4q23, 22q11, 10q26 ve 11q22.2-22.5 kromozomlar üzerinde lokalize olduğu belirlenmiştir.

Anahtar Sözcükler: Wilson F4, Wilson B1, CO4107 markır, CTR1, Köpek kromozomları

Introduction

Copper (Cu) is required for many biological processes, but it is toxic at high cellular concentrations. Therefore, the Cu levels must be strictly controlled in the cells. Cu is transported across the plasma membrane by CTR1 and then transferred to small, soluble cytoplasmic Cu transporters such as Atx 1, Lys7 and Cox17 (1,2).

Wilson disease (WD) is an autosomal recessive disorder of the Cu transport system. Cu is stored in the liver and subsequently in the brain and other organs (3). The underlying defect in WD is an ATPase. Recently, mutation in the ATP7B cDNA has been shown in rats and toxic milk mutation in mice (4,5).

Canine copper toxicosis (CT) is also an autosomal recessive disorder that results in the accumulation of Cu in the liver. This leads to chronic hepatitis and cirrhosis. Although canine CT resembles WD in many ways, there are marked differences in the normal ceruloplasmin levels observed in CT. However, patients with WD typically show greatly reduced serum ceruloplasmin levels (6).

Recently, a polymorphic microsatellite marker (CO4107) has been identified that is closely linked to the CT locus in dogs (7,8). The aim of this study was to determine the localization of the CO4107 marker, ATP7B, and CTR1 in dog chromosomes. For this purpose, peripheral dog blood was cultured and FISH was used on GTG-banded dog chromosomes. The probes used for the FISH experiment were isolated in bacterial artificial chromosome (BAC) clones.

Materials and Methods

Canine Peripheral Blood Cultures

Peripheral blood is the most frequently used tissue for postnatal chromosome studies, because it is easy to obtain and simple to culture. The method used in this study of the canine chromosome was described by Fischer et al (9). We also made some minor modifications. Five milliliters of heparinized peripheral dog blood was diluted 1:1 with PBS and layered over 2 different tubes, each containing 3 ml of Ficoll/Histopaque 1077. The tubes were centrifuged for 10 min at 3000 rpm, and the middle phase was transferred to a new tube and washed twice with a 1:1 volume of RPMI 1640.

Growth Medium

The growth medium contained 80 ml of RPMI 1640 with Glutamax and 25 mM HEPES (Gibco) supplemented with 3 ml of phytohemagglutinin (PHA), 20 ml of fetal calf serum and 1 ml of penicillin/streptomycin.

Culturing Procedure

Using an aseptic technique, a 0.1 ml lymphocytes suspension (5.10^5 cell/ml) was added to 6 ml of culture medium. After mixing the contents of each culture tube by gently inverting it a few times, the culture tubes were incubated in a slanted position at 37 °C for 72 h. The slanted position creates more surface area between the liquid and gaseous phases and allows cells to settle over a larger surface area of the culture tube, which provides optimal culture conditions for cell growth. Twenty-four hours before collecting the cells, 0.1 ml of MTX (5 µg/ml) was added to the tubes.

Harvesting

Solution

Colcemid solution (10 µg/ml), hypotonic solution (0.075 M KCl) and fixative solution (3 parts absolute methanol to 1 part glacial acetic acid) were used.

Procedure

After the 72 h culture period, 0.1 ml of colcemid solution was added to each culture tube and mixed with gentle shaking. The tubes were incubated at 37 °C for an additional 25 min. The tubes were centrifugated at 1200 rpm for 8 min and the supernatant was discarded. The pellet was resuspended using 10 ml of hypotonic solution (pre-warmed at 37 °C) and the tubes were incubated at 37 °C for a further 7 min. After the tubes had been centrifugated at 1200 rpm for 8 min and the supernatant discarded, the pellet was resuspended using 10 ml of fresh fixative. The tubes were centrifugated at 1200 rpm for 8 min and the supernatant was discarded. This procedure was repeated 3 times. The pellet was resuspended and 0.5 to 1 ml of fresh, cold fixative solution was added to the tubes. Then 3 or 4 drops of the cell suspension were dropped onto a cold, wet glass slide and placed on a hotplate to dry.

GTG-Banding

The Giemsa bands (G-bands) were obtained by digesting the chromosomes with the trypsin, the most widely used material in clinical laboratories for routine chromosome analysis. This technique is known as GTG (G-bands by trypsin using Giemsa).

Solutions

Trypsin solution (Dissolve 0.1 g of Difco trypsin (1:250) in 100 ml of Gurr Buffer) and staining solution (5 % Giemsa with Gurr Buffer) were used.

Procedure

After aging the slides by placing them in hydrogen peroxide in water 1:1 (v/v) at room temperature for 10 to 30 s, they were rinsed in distilled water. The slides were immersed in the trypsin solution for 5 to 10 s. Before staining with Giemsa dye for 6 min, the slides were rinsed in distilled water and dried at room temperature

Fluorescent In Situ Hybridization

The BAC probes were labeled with DIG-11-dUTP or Biotin-11-dUTP using nick translation (BioNick Labelling System, Boehringen, Germany). Fluorescent in situ hybridization (FISH) using the BAC probes was performed after GTG-banded chromosomes on destained slides according to the protocol of Fischer et al. (9). We made some minor modifications. Briefly, the labeled probes were dissolved in 100 µl of TE. The competition of 100 ng probes was performed with 100x excess sheared genomic canine DNA and precipitated with a 0.1 volume of 3M NaAc and a 2.5 volume of absolute ethanol. The precipitate was washed with 70% ethanol and dissolved in 11 µl of hybridization mix per slide. G-banded slides were treated with xylol for 1 min, xylol/ethanol (v:v) for 1 min, and methanol/acetic acid(3/1) for 5 min and were then air-dried. After the slides were treated for 55 s with 70% acetic acid, they were washed 3 times

directly in PBS for 2 min. The slides were dehydrated through an ethanol series and air-dried.

The probes were denatured for 5 min at 80 °C and denaturation was stopped on ice for 3 min. The denatured probes were incubated for 30 min at 37 °C to permit competition in the repeats in the probes with the sheared genome DNA. The slides were denatured by treating them with 100 ml of 70% formamide in 2 x SSC in pH 5.0 and covered with a cover slide and incubated for 3 min at 70 °C. The slides were directly incubated in 70% ethanol for 2 min at -20 °C, in 96% and 100% ethanol for 2 min each at room temperature and air-dried. The hybridization was performed with an 11 µl probe, and the material was dissolved in hybridization mix on the slides. The slides were covered with a 24 x 24 mm cover slide and incubated in a humidified room for 1 or 2 days at 37 °C. After hybridization, slides were washed in 50% formamide, 2 x SSC at pH 5, 2 x SSC at 42 °C for 3 x 2 min each, in 4 x SSC and 0.05% Tween-20 for 2 min at room temperature, followed by preincubation in 4 x SSC and 0.05% Tween-20 and 5% nonfat dry milk for 10 min at 37 °C. To visualize the biotinylated probe in red (Cy3) and the disoxygenated probe in green (fluorescein isothiocyanate FITC), the slides were incubated with Avidin-Cy3 (1:50) and or mouse anti-dioxygenin (1:100) for 30 min at 37 °C. The slides were washed for 3 x 5 min in SSC. The results were evaluated using a Zeiss fluorescence microscope coupled to a CCD camera.

Results

After the dog blood culture had been obtained and the slides prepared, high-resolution G-banding was applied to the chromosomes (Figure 1). FISH was performed using hybridization with BAC clones on G-banded slides. The FISH signal was seen on 4 different canine chromosomes. The probes and their localization on chromosomes were based on the idiogram described by Reimann et al. (10). After FISH, the signals were checked to determine where the signals were situated on the same G-banded metaphase (Figures 2A and 3A). The results showed that while BAC CO4107 maps to CFA10q26 and BAC Wilson F4 (ATP7B) maps to CFA4q23 (Figure 2B), BAC Wilson B1 (ATP7B) maps to CPA22q11 and CTRI maps to CFA11q22.2-22.5 (Figure 3B). In some metaphases BAC clone (Wilson F4) also has a second, weaker signal at CFA4q31. However, during FISH on G-banded slides, some metaphases were lost.

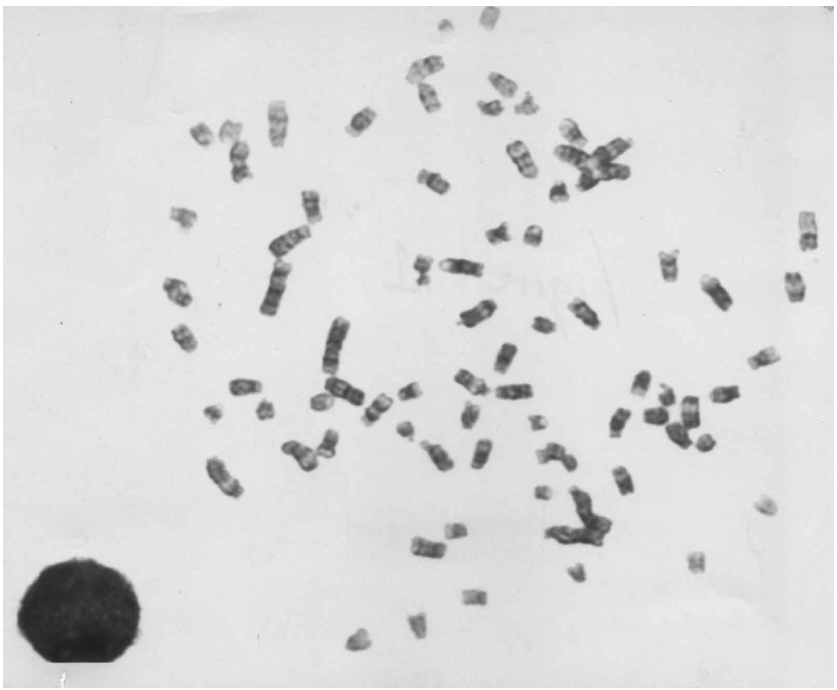
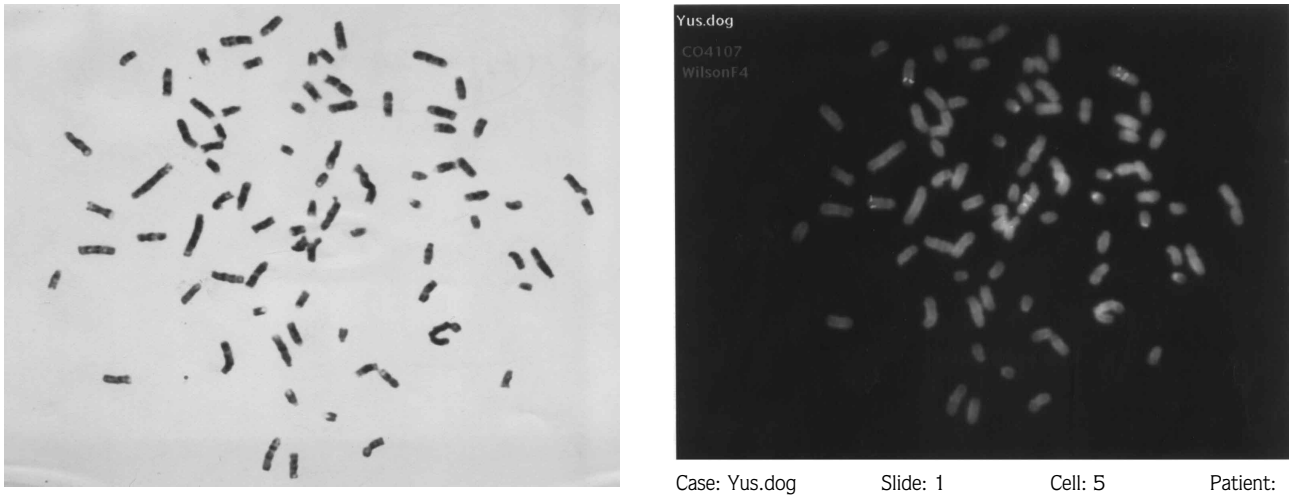


Figure 1. GTG-banding dog chromosome.

(A) (B)



Figures 2A and 2B. GTG-banded/FISH approach with CO4107 (green) and Wilson F4 (red) probes on the same metaphase.

(A) (B)

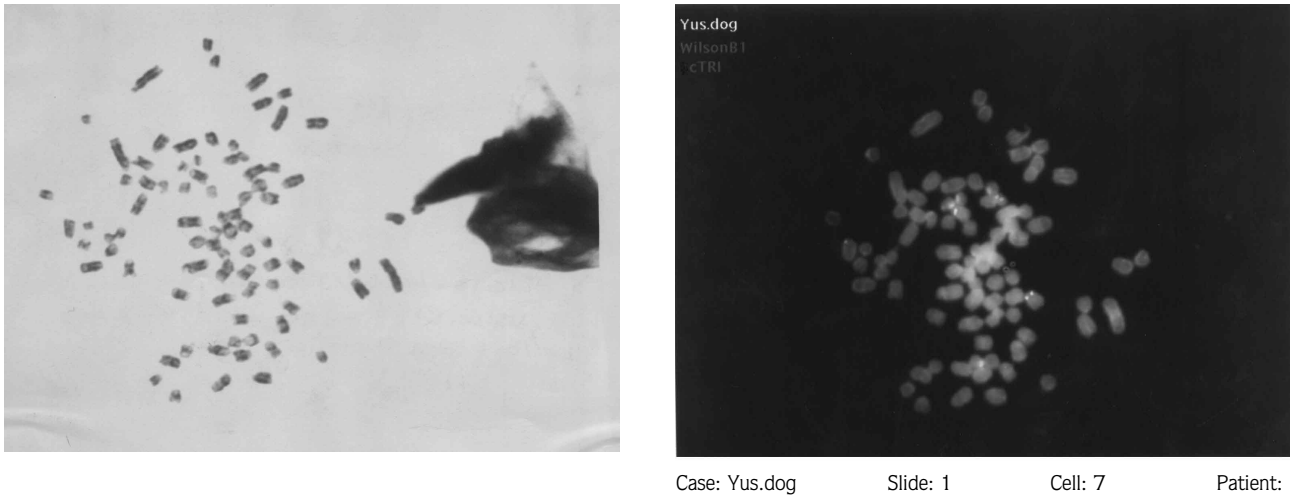


Figure 3A and 3B. GTG-banded/FISH approach with Wilson B1 (green) and CTR1 (red) probes on the same metaphase.

Discussion

There is increasing interest in dog chromosomes. Many projects are underway aimed at producing a high-quality genetic map for the dog genome (Dog Genome Project). Canine peripheral blood culture gives a very high quality dog chromosome for high resolution banding. However, cytogenetic investigations in the dog are difficult to compare with human chromosomes because all dog chromosomes are quite small and acrocentric except for X and Y, and different nomenclatures have also been used (10).

The DNA microsatellite marker CO4107, linked to the Bedlington terrier copper toxicosis locus CT, is used diagnostically in the USA to detect the disease allele. This marker has been typed in Bedlington terriers of known disease status in the United Kingdom (7,8).

In this study we were able to obtain the map of the marker CO4107 on canine chromosome 10q26. So far, no recombination events between CO4107 and CT have been reported in the literature. This suggests that the CO4107 marker could be extremely close to the CT locus. Therefore, the most likely location for the CT locus is also on canine chromosome 10q.

CTR1 was mapped to dog chromosome 11q22. In addition, the canine has the gene, CTR2, mapped to chromosome 11q22.5. The 2 genes were closely linked.

The ATP7B gene and CT locus map onto different chromosomes. Therefore, the ATP7B gene can be excluded as the primary candidate gene for canine CT. However, 2 different ATP7B loci were identified on the same chromosome. Nanji et al. unpublished data have shown that the ATP7B gene has a pseudogene (11). Our result could indicate that the second FISH signal may represent this pseudogene.

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