Genetic mapping of a naturally occurring hereditary renal cancer syndrome in dogs

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Canine hereditary multifocal renal cystadenocarcinoma and nodular dermatofibrosis (RCND) is a rare, naturally occurring inherited cancer syndrome observed in dogs. Genetic linkage analysis of an RCND-informative pedigree has identified a linkage group flanking *RCND* (CHP14-C05.377-C05.414-FH2383-C05.771-[*RCND*-CPH18]-C02608-*GLUT4*-*TP53*-ZuBeCa6-AHT141-FH2140-FH2594) thus localizing the disease to a small region of canine chromosome 5. The closest marker, C02608, is linked to *RCND* with a recombination fraction (*θ*) of 0.016, supported by a logarithm of odds score of 16.7. C02608 and the adjacent linked markers map to a region of the canine genome corresponding to portions of human chromosomes 1p and 17p. A combination of linkage analysis and direct sequencing eliminate several likely candidate genes, including tuberous sclerosis 1 and 2 genes (*TSC1* and *TSC2*) and the tumor suppressor gene *TP53*. These data suggest that RCND may be caused by a previously unidentified tumor suppressor gene and highlight the potential for canine genetics in the study of human disease predisposition.

The identification of cancer-susceptibility genes has been facilitated by the study of high-risk families characterized by multiple generations of affected individuals (1–4). The general assumption is that identification of genetic mutations causing the highly penetrant phenotypes studied in these rare families will provide a foundation on which to understand cancer susceptibility in the general population.

Informative high-risk cancer families, however, are uncommon in human populations, making it difficult to identify all but a few highly penetrant cancer-susceptibility genes. In addition, inherent limitations in the structure of human families, such as small size and long generation time, mean that genes that are weakly penetrant give rise to variable phenotypes and that genes that act in a recessive fashion are difficult to study. Finally, the high frequency of phenocopies for some cancers confounds linkage results and further complicates analysis of common cancers.

We have proposed that cancer-susceptibility genes could be mapped more easily in animals, where large families can be generated, directed matings are possible, and multiple generations are easily collected. In addition, because of the shorter life span, clinical symptoms often manifest in relatively short periods of time (5). In a search for appropriate animal models in which to study the genetics of cancer susceptibility, we have elected to focus on the domestic dog, which we believe offers two specific advantages over rodents. First, many types of spontaneous canine cancers are more similar to human tumors in histopathological appearance, biological behavior, and response to therapy than the corresponding rodent tumors (6). Second, purebred dogs have a relatively high frequency of autosomal recessive and genetically complex disorders, many of which, including cancer, tend to be breed specific (7). Indeed, distinct dog breeds often differ significantly in the type and frequency of specific tumors (8, 9).

Although there are many animal models for sporadic cancer, currently, there are few well described examples of naturally occurring inherited cancers in nonhuman mammals. The single exception is the well characterized renal carcinoma syndrome found in the Eker rat, which seems to be caused by germ-line mutations in the *tsc2* gene (10–13).

Recently, we described a second hereditary mammalian cancer syndrome called hereditary multifocal renal cystadenocarcinoma and nodular dermatofibrosis (RCND; refs. 14 and 15), a rare, inherited, naturally occurring cancer syndrome in German Shepherd Dogs. The syndrome is characterized by bilateral, multifocal tumors in kidneys and numerous firm nodules, consisting of dense collagen fibers in the skin and subcutis. Most females develop uterine leiomyomas. Pedigree analysis of a canine family with RCND strongly indicates an autosomal dominant pattern of inheritance (16). There is no precisely corresponding human disease known, but the clinical patterns are reminiscent of diseases associated with mutations in tumor suppressor genes, such as tuberous sclerosis (17), which is associated with inherited mutations in the *TSC1* and *TSC2* genes, and Li–Fraumeni syndrome, which is associated with germ-line mutations in *TP53* (18, 19).

Rapid progress on the development of a canine genome map containing both type I (gene) and II (microsatellite) markers (20–23) has been highlighted by the recent mapping of several canine disease genes, including those associated with two forms of hereditary blindness, progressive rod-cone degeneration (*prcd*), and early retinal degeneration (*erd*) (refs. 24 and 25), as well as genes for copper toxicosis (26) and neuronal ceroid lipofuscinosis (27). The results reported herein represent, to our knowledge, the first localization of a mammalian cancer syndrome to be mapped in a species other than human and rat and localize *RCND* to canine chromosome 5 (CFA5). A combination of linkage analysis and direct mutation screening eliminate several likely candidate genes, including *TSC1*, *TSC2*, and *TP53*, suggesting that RCND may be caused by either a previously unidentified tumor suppressor gene or a previously identified gene with no known association with inherited cancer syndromes.

Methods

Canine Pedigree Development and Phenotypic Assessment. A canine colony segregating RCND was established by breeding one affected male (German Shepherd/Flat Coated Retriever cross)

Abbreviations: CFA*n*, *Canis familiaris* chromosome *n*; HSA*n*, *Homo sapiens* chromosome *n*; RCND, hereditary multifocal renal cystadenocarcinoma and nodular dermatofibrosis; cM, centimorgan; RH, radiation hybrid; lod, logarithm of odds; TSC, tuberous sclerosis complex.

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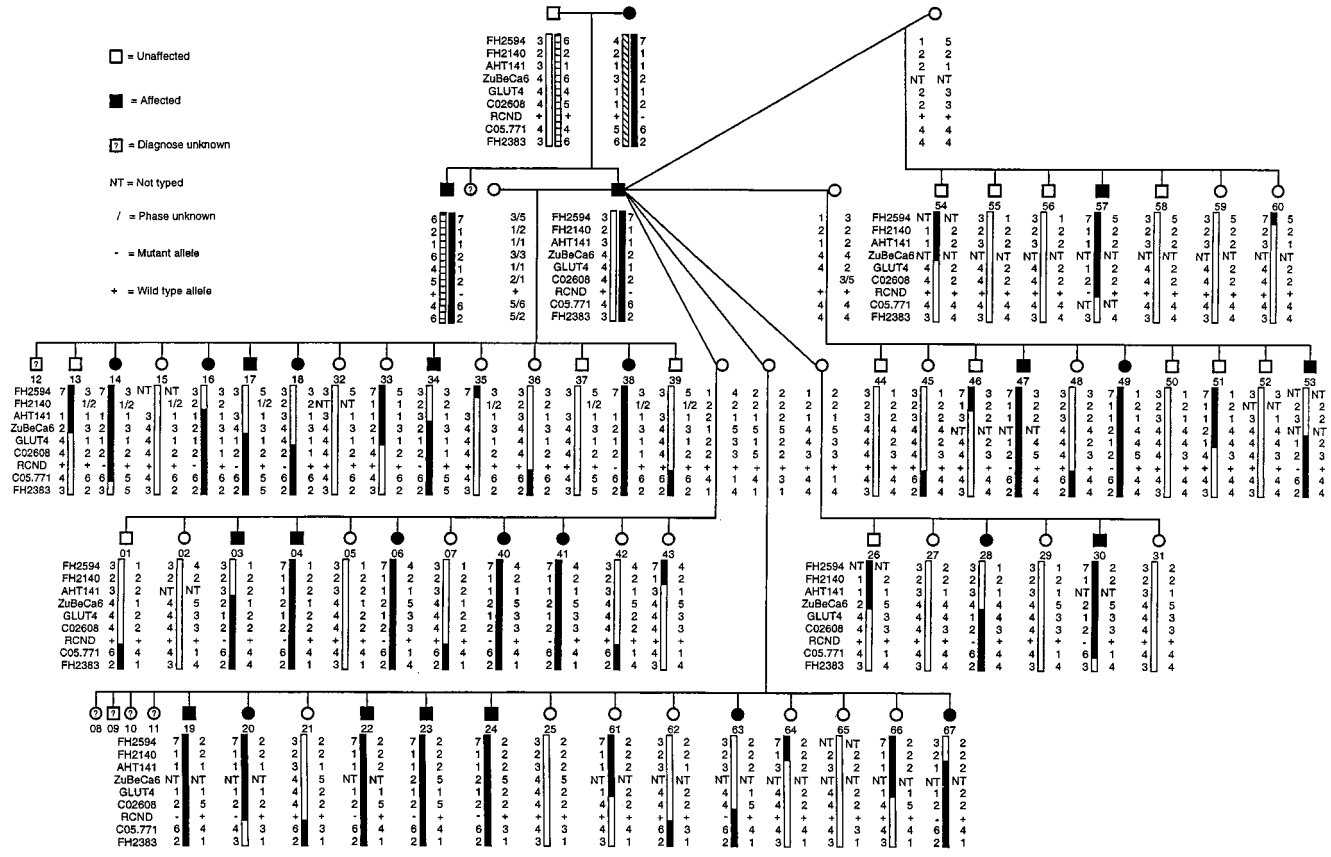

Fig. 1. The canine pedigrees segregating RCND. Affected dogs are represented by black; unaffected are represented by white; and those with an unknown diagnosis are indicated with a question mark. Genotypes of all typed markers are shown, but the bar indicating crossovers is shown only for the affected father's side. The *RCND* locus is shown as + for the wild-type allele and − for the mutant.

with six unaffected females (one German Shepherd and five English Setters) to yield 67 F_2 offspring from nine litters (Fig. 1). All of the dams were unrelated to the proband; the five English Setter females were related. The male used to produce the litters was diagnosed at 1 year of age, and the offspring were diagnosed at 8–12 weeks of age.

Dogs were examined for the presence of multiple microscopic renal cysts by exploratory laparotomy, kidney biopsy or necropsy, and histologic examination as described (28). Microscopic renal cysts are considered to be a precursor of the renal cystadenocarcinomas that are typically observed in mature dogs with RCND and are therefore used as early diagnostic criteria (29). Other diagnostic criteria and their application to early diagnosis have been reported (28).

The Norwegian Animal Research Authority approved colony development, maintenance, and sample collection. Canine DNA samples were handled as specified by the Fred Hutchinson Cancer Research Center Institutional Animal Care and Use Committee.

DNA Extraction and Microsatellite Typing. Genomic DNA was extracted from aliquots of EDTA-anticoagulated whole blood or from samples of muscle tissue by using standard protocols (30). Microsatellites spanning the canine genome at approximately 10-centimorgan (cM) resolution were selected for an initial low-resolution genome screen (31). The majority of markers selected were based on tetranucleotide repeats, which are highly polymorphic in dogs and easy to genotype (32). The primer sequences, amplification conditions, and product sizes are available on our web site: http://www.fhcrc.org/science/dog_genome/markers/all.html. PCR was carried out by using published conditions (33). PCR products were separated by 4–6% PAGE run at 55°C, visualized by autoradiography, and scored manually.

Linkage Analysis. Each marker was checked for Mendelian segregation by using the PREPARE option of the MULTIMAP program (34). The disease trait was assumed to be fully penetrant and was coded as autosomal dominant. Two-point linkage analysis was carried out between *RCND* and each marker and between each pair of markers. The most likely order and spacing of markers within the linkage group was calculated by using multipoint analysis and the GET-LIKELIHOODS function of MULTIMAP.

Radiation Hybrid (RH) Analysis. Markers were typed on DNA isolated from a canine/hamster RH panel (Research Genetics, Huntsville, AL). Canine-specific *TSC1* primers were typed on the RH panel described by Vignaux *et al.* (35) by using previously described protocols (22). The most likely order and spacing of markers within each RH linkage group was calculated by multipoint analysis with the program MULTIMAP (34).

Sequencing of Canine *TP53*. mRNA was isolated from blood of three affected and three unaffected dogs by using a Quickprep Micro mRNA Purification Kit (Amersham Pharmacia). First-strand cDNA was synthesized with a Ready To-Go T Primed

Table 1. Recombination fractions and lod scores for markers flanking the *RCND* locus on CFA5

		FH2383	C05.771	RCND	C02608	GLUT4	ZuBeCa6	AHT141	FH2140	FH2594
FH2383	θ	—								
	lod									
C05.771	θ	0.034	—							
	lod	20.207								
RCND	θ	0.205	0.160	—						
	lod	4.964	6.707							
C02608	θ	0.273	0.218	0.016	—					
	lod	4.140	6.770	16.728						
GLUT4	θ	0.312	0.250	0.097	0.087	—				
	lod	2.504	4.926	10.103	17.707					
ZuBeCa6	θ	0.369	0.300	0.161	0.142	0.085	—			
	lod	0.754	2.062	4.587	6.539	10.325				
AHT141	θ	0.387	0.379	0.233	0.257	0.168	0.039	—		
	lod	0.843	0.919	3.905	5.227	10.525	11.672			
FH2140	θ	0.500	0.500	0.322	0.273	0.212	0.119	0.100	—	
	lod	0.00	0.00	1.432	2.187	4.001	4.593	7.992		
FH2594	θ	0.480	0.459	0.311	0.307	0.258	0.118	0.157	0.014	—
	lod	0.026	0.106	1.777	2.828	4.706	8.088	9.054	16.081	

First-Strand Kit (Amersham Pharmacia). Five pairs of primers were designed from a published canine TP53 cDNA sequence (GenBank accession no. AF060514) to amplify overlapping fragments of canine TP53. The positions of the amplified nucleotides were as follows: nucleotides 4–335, 236–529, 441–781, 677–990, and 893–1,145. Fragments of cDNA were amplified for direct sequencing from 3.5 μg of cDNA. All PCR products were bidirectionally sequenced with the Thermo Sequenase Dye Terminator Cycle Sequencing PreMix Kit (Amersham Pharmacia) according to the manufacturer's instructions and analyzed on an Applied Biosystems 373 fluorescence DNA sequencer.

Sequencing of Canine *TSC1*. Consensus *TSC1* primers were designed from conserved regions of human and rat *TSC1* cDNA (GenBank accession nos. AF013168 and AB011821, respectively). The 5′–3′ sequences of the forward and reverse consensus primers are as follows: forward, CATCGCCTTTATGGAATGTA, and reverse, GAGGGTCCAGTTCATGGTC. The consensus primers were used to amplify canine and hamster genomic DNA, and the resulting PCR products were bidirectionally sequenced by using the same primers. Both canine and hamster sequences were confirmed by BLAST searches as *TSC1*. The canine and hamster sequences were aligned, and canine-specific primers were designed in regions of mismatch between the canine and hamster sequences: forward, GTGCACAGGCTACACTTGGGT, and reverse, GAACCCTGAAAAATTCCACCA.

Results

A set of mapped canine-specific microsatellite markers, distributed at ≈10-cM intervals throughout the genome, was selected for typing on a set of canine pedigrees segregating RCND. The set of pedigrees used is shown in Fig. 1. Two-point linkage was detected initially between the *RCND* locus and ZuBeCa6, a microsatellite marker that has been localized previously to CFA5q12-q13 (36), at a recombination fraction (θ) of 0.161, supported by a logarithm of odds (lod) score of 4.6. Several other polymorphic markers known to be on CFA5 (23, 37, 38) were then genotyped on the same set of pedigrees. Two-point analyses showed the disease locus to be linked to five additional markers on CFA5 with odds of at least 1,000:1 (Table 1). A maximum lod score of 16.7 was observed with C02608 at a θ of 0.016. Thus, we concluded that the *RCND* gene is located on CFA5. Multipoint analyses indicate that *RCND* maps to the interval between C05.771 and C02608 (Fig. 2), ≈2 cM from C02608.

The canine homolog of the tumor suppressor gene *TP53* is located on CFA5 (39). Germ-line mutations in *TP53* are associated with Li–Fraumeni syndrome (18, 19), and RCND has several clinical features in common with Li–Fraumeni syndrome (15, 40, 41) making *TP53* a potential candidate for RCND. To date, no polymorphisms within canine *TP53* have been reported; thus, to determine the position of *TP53* relative to *RCND* and the other markers on CFA5, we constructed an RH map of CFA5. Whole-genome RH maps of the canine genome have been published (22, 31), which include markers on CFA5. The RH panel developed for those studies, however, lacked the region of the genome containing *TP53* (22, 42). A distinct RH panel (Research Genetics), therefore, was used for this analysis. DNA was amplified from the RH panel with the canine-specific *TP53* primer sequence (GenBank accession no. AF060514), as well as with primers for all available CFA5 markers. Multipoint analysis indicated that *TP53* is located between ZuBeCa6 and *GLUT4* (odds of at least 1,000:1), on the opposite side of *GLUT4* from *RCND* (Fig. 2). The linkage map of CFA5, constructed with linkage data from the RCND pedigree, shows the same order of markers as the third generation linkage map (23) and the RH map of CFA5 (Fig. 2).

In parallel with linkage analysis, direct sequencing of *TP53* was carried out. cDNA from the whole coding sequence of *TP53* was sequenced in three unaffected and three affected dogs with primers that amplified the entire coding sequence of the gene. No differences were observed between affected and unaffected individuals.

Other strong candidate genes for RCND include *TSC1* and *TSC2*. Germ-line mutations in both genes have been associated with tuberous sclerosis in humans, another inherited renal cancer syndrome with clinical similarities to RCND (43, 44). To investigate the potential role of *TSC1* in RCND, canine-specific primers were designed that amplified a region of the canine but not the hamster *TSC1* gene. Primers were typed on the canine/hamster RH panel used to construct published whole-genome RH maps of the canine genome (22, 31, 35). *TSC1* was linked significantly to seven markers, all of which localize to CFA9 (results not shown), allowing us to exclude *TSC1* as a potential candidate for RCND. Similar, previously reported experiments localize *TSC2* to CFA6 and additionally allow us to exclude it as a candidate for RCND (33).

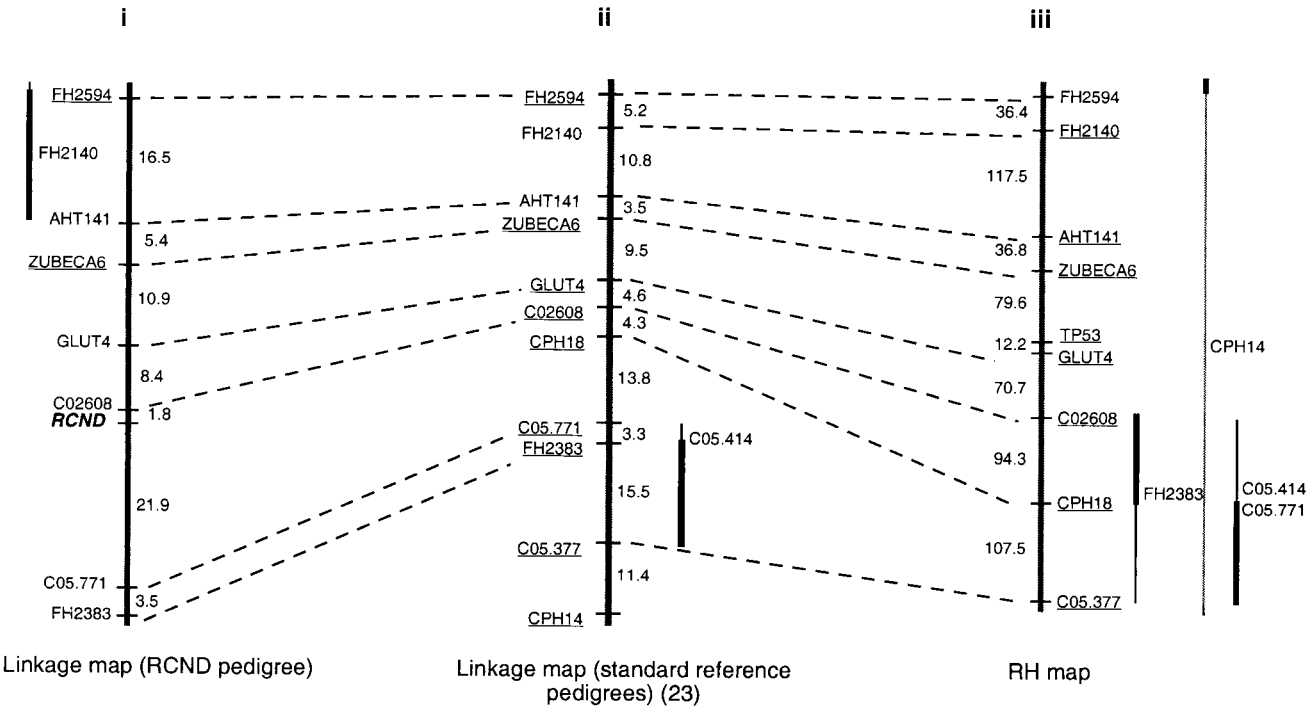

Fig. 2. Comparison of maps of CFA5 constructed with (*i*) linkage data from the *RCND* pedigrees, (*ii*) the standard reference pedigrees (23), and (*iii*) an RH panel. The distances on the linkage map are shown in centimorgans, and the distances on the RH map are shown in centiRays. All markers were assigned to CFA5 with odds of at least 100,000:1 and ordered with odds of at least 10:1. The most likely position or positions of markers that could not be positioned with odds of at least 10:1 are indicated with vertical bars to the sides of the maps. Thickened portions of bars indicate an interval that is favored over other intervals with odds of at least 10:1. Markers that can be ordered with odds of at least 1,000:1 are underlined.

GENETICS

Discussion

Few naturally occurring nonhuman mammalian models for inherited cancer syndromes have been well defined, and the underlying locus has been identified only for the Eker rat (12, 13, 45, 46). The linkage and RH data presented herein firmly establish that the canine cancer syndrome RCND maps to a linkage group containing 13 markers on CFA5. Linkage and RH data suggest that the most likely order of markers is CHP14-C05.377-C05.414-FH2383-C05.771-[*RCND*-CPH18]-C02608-*GLUT4*-*TP53*-ZuBeCa6-AHT141-FH2140-FH2594. We are unable to determine the orientation of *RCND* and CPH18 with respect to the rest of the chromosome, because CPH18 was not informative in the RCND pedigree.

CFA5 contains homologs of genes located on at least four different human chromosomes (HSA; ref. 31). However, the *RCND* locus is located in a region most likely homologous to either HSA17p or HSA1p. The breakpoint between these two chromosomes seems to be very close to the *RCND* locus and thus cannot be mapped precisely until more genes from each HSA have been positioned on the canine map.

The possibility of synteny between *RCND* and either HSA1 or HSA17 suggests several provocative candidate genes, including the tumor suppressor gene *TP53*, which is associated with germ-line mutations in Li–Fraumeni syndrome (18, 19). Li–Fraumeni disease is characterized by a wider array of early onset tumors than is observed in RCND, including sarcomas, leukemias, and later in life, breast cancer (40, 41). *TP53* has not been associated with any polymorphisms in dogs and thus can be placed only on the RH map, whereas *RCND* is localized on the linkage map. The position of these two loci, relative to one another, was made by comparing their positions relative to other markers that have been placed on both maps. The RH map places *TP53* about 12.2 centiRays (equivalent to about 2 cM) from *GLUT4*. The third generation linkage map of CFA5 positions *GLUT4* 4.6 cM from C02608, which is 1.8 cM from *RCND*, on the opposite side of *GLUT4* from *TP53*. Thus, because there are no discrepancies between the order of markers on the two maps and because we find no mutations in the coding region of *TP53* in affected dogs, *TP53* is unlikely to be the relevant disease gene. An exception could occur if significant undetected microrearrangements were in this region of the map and if *TP53* mutations were located outside the coding region of the gene within promoters, enhancers, or downstream regulatory elements.

Linkage analysis places the *RCND* locus close to marker CPH18, which Mellersh *et al.* (31) have placed 21.2 centiRays or about 3.5 cM from the canine homolog of *DIO1*, which maps to HSA1p32. The tumor suppressor gene *p73* maps to HSA1p36 (47) and thus is also a good candidate gene for RCND that will be worth further investigation.

Phenotypic similarities between RCND and some human disorders have suggested several other candidate genes. Tuberous sclerosis complex (TSC) is a syndrome characterized by a high incidence of cutaneous neoplastic nodes and multiple renal neoplasms, with a complex and sometimes inconsistent phenotype. The syndrome has an autosomal dominant mode of inheritance and is caused by mutations in the *TSC1* and *TSC2* genes (43, 44). Although RCND bears some similarities to TSC, the renal carcinomas associated with TSC are typically hamartomas or angiomyolipomas, whereas those associated with RCND are cystadenocarcinomas. Interestingly, the clinical syndrome presented by the Eker rat also bears some similarity to RCND, with kidney tumors and reproductive tract leiomyomas being common in both diseases (17, 48). RCND, however, is distinct in that

it includes skin tumors and lacks vascular neoplasms. Both TSC genes are excluded by our linkage studies. The present study localizes canine *TSC1* to CFA9, and we have previously localized *TSC2* to CFA6 (33).

One additional candidate we have considered is *KRT9*, mutations of which are associated with epidermolytic palmoplantar keratoderma (49). This rare syndrome is usually characterized by skin tumors, but few families have been described recently that also have different malignancies (50). Canine *KRT9* has been cloned (51) and mapped to CFA9 (22); therefore, it is excluded as the *RCND* gene.

It is noteworthy that there are several other genes associated with inherited renal cancers. These include the Wilms' tumor gene (*WT1*; ref. 52), the von Hippel Lindau gene (*VHL*; refs. 53–56), the neurofibromatosis gene (*NF1*; ref. 57), and polycystic kidney disease genes 1 and 2 (*PKD1* and *PKD2*; refs. 58 and 59). The canine homologs of *WT1* and *NF1* have been mapped to CFA9 (22, 38), and *PKD1*, which is involved in 85% of autosomal dominant polycystic kidney disease in humans, has been mapped to CFA6 (33). These can therefore be excluded from further consideration as candidates. Von Hippel Lindau syndrome also shares several clinical features with RCND, commonly results in bilateral renal cysts and tumors, and may therefore represent an additional candidate worthy of study. Patients with Von Hippel Lindau syndrome, however, also experience tumors in the central nervous system and cysts in the pancreas and central nervous system, which are not observed in RCND. Neither of the canine homologs *PDK2* or *VHL* has been cloned or mapped, and thus, neither can be excluded formally at this time.

The mapping of a unique cancer syndrome to CFA5 shows the potential of the dog to map genes for intractable human genetic disorders. Canine lineages provide a tool with which to overcome problems of locus heterogeneity that often confound human studies. In addition, large pedigrees enhance the statistical power of linkage analysis studies. Placement of additional genes and informative markers on the canine map will be necessary before identification of a disease gene by means other then candidate gene analysis can begin in earnest; however, results published herein indicate that investigation of other canine inherited disease syndromes could make a unique contribution to our understanding of mammalian biology and cancer susceptibility.

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