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Activating Mutations of *GNAS* in Canine Cortisol-Secreting Adrenocortical Tumors

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Background: Cushing's syndrome or hypercortisolism is a common endocrinopathy in dogs. In approximately 15% of cases, the disorder is caused by adrenocorticotropin (ACTH)-independent hypersecretion of cortisol by an adrenocortical tumor (AT). Without other explanation, the cortisol hypersecretion has been referred to as autonomous.

Objectives: To investigate whether ACTH-independent hypersecretion of cortisol may be associated with aberrant activation of the melanocortin 2 receptor (MC2R)-cyclic AMP (cAMP)-protein kinase A (PKA) pathway.

Animals: All analyses were performed on 44 cortisol-secreting ATs (14 adenomas and 30 carcinomas) derived from dogs diagnosed with ACTH-independent hypercortisolism.

Methods: Mutation analysis was performed of genes encoding the stimulatory G protein alpha subunit (*GNAS*), *MC2R*, and PKA regulatory subunit 1A (*PRKAR1A*) in all ATs.

Results: Approximately one-third of all ATs harbored an activating mutation of *GNAS*. Missense mutations, known to result in constitutive activation, were present in codon 201 in 11 ATs, in codon 203 (1 AT), and in codon 227 (3 ATs). No functional mutations were found in *MC2R* and *PRKAR1A*.

Conclusions and Clinical Importance: Activation of cAMP signaling is a frequent event in canine cortisol-secreting ATs and may play a crucial role in both ACTH-independent cortisol production and tumor formation. To the best of our knowledge, this is the first report of potentially causative mutations in canine cortisol-secreting ATs.

Key words: Adrenal; Cushing's syndrome; Dog; Gs alpha.

Cushing's syndrome or hypercortisolism is relatively common in dogs, with an estimated incidence of approximately 1–2 cases per 1000 dogs per year. In approximately 15% of cases, this disorder is due to a cortisol-secreting adrenocortical tumor (AT).[1,2] Clinical signs of such a tumor include centripetal obesity, atrophy of muscles and skin, exercise intolerance, polyphagia, polyuria, and polydipsia[3,4] and are a consequence of ACTH-independent hypersecretion of cortisol.

In the healthy adrenal cortex, cell proliferation and steroidogenesis are regulated by melanocortin 2 receptor (MC2R) signaling. Upon ACTH binding to the MC2R, the stimulatory G protein alpha subunit (Gsalpha) activates adenylyl cyclase, producing cAMP. This, in turn, induces protein kinase A (PKA) activity, which results in activation of transcription factors such as cAMP response elements (CREB) that mediate ACTH effects and induce target gene transcription (Fig 1).[5] Aberrant activation of the MC2R-cAMP-PKA pathway therefore may be a cause of ACTH-independent hypersecretion of cortisol by ATs.

Despite extensive search, no activating mutations of the *MC2R* have ever been described.[6–8] Mutations that constitutively activate cAMP production mimic MC2R activation in their effects. The best known example is the gsp-oncogene, which arises from a mutation in the stimulatory G protein alpha subunit gene (*GNAS*), and leads to activation of Gsalpha.[9,10] Activating *GNAS* mutations cause McCune-Albright syndrome in humans[11] and also occur in various endocrine tumors, for instance growth hormone-secreting pituitary tumors in humans and thyroid tumors in humans and cats.[12–1413,14] However, only a few cases of activating *GNAS* mutations have been described in adrenocortical adenomas of humans,[9,15,16] and no activating *GNAS* mutations have been described in dogs.

Abbreviations:

AC	adenylate cyclase
AMP	adenosine monophosphate
AT	adrenocortical tumor
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
CREB	cAMP response elements
FAK	focal adhesion kinase
GDP	guanidine diphosphate
GNAS	stimulatory G protein alpha subunit gene
GNASL	stimulatory G protein alpha subunit, long variant
GNASS	stimulatory G protein alpha subunit, short variant
Gsα	stimulatory G protein alpha subunit
MAPK	mitogen-activated protein kinase
MAS	McCune Albright syndrome
MC2R	melanocortin 2 receptor
NFKB	nuclear factor kappa-B
PDE	phosphodiesterase
PKA	protein kinase A
PPNAD	primary pigmented nodular adrenocortical disease
PRKAR1A	PKA regulatory subunit 1A
SNP	single nucleotide polymorphism
TF	transcription factor
UTR	untranslated region

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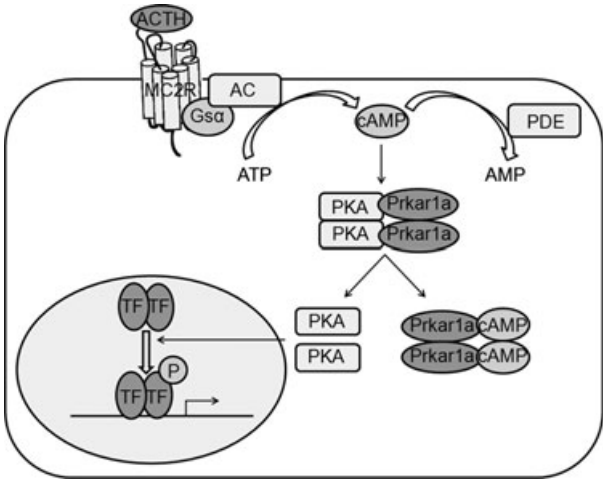

Fig 1. Schematic overview of the MC2R-cAMP-PKA pathway. Binding of ACTH to the MC2R activates adenylate cyclase (AC) through Gsα. AC converts ATP to cAMP, which separates protein kinase A (PKA) from its regulatory subunit PRKAR1A, enabling it to phosphorylate (P) and subsequently activate transcription factors (TF). Phosphodiesterase (PDE) inhibits the pathway by converting the active cAMP to inactive AMP. MC2R, melanocortin 2 receptor; Gsα, stimulatory G protein alpha subunit; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; AMP, adenosine monophosphate; PRKAR1A, protein kinase A regulatory subunit 1A.

Inactivating mutations of the gene encoding PKA regulatory 1 alpha (*PRKAR1A*) subunit cause increased basal and cAMP-stimulated PKA activity.[17,18] Inactivating germ line mutations of this gene are found in approximately two-thirds of people with Carney complex,[19] in whom endocrine tumors are common. The most common endocrine gland manifestation in affected people is ACTH-independent hypercortisolism because of primary pigmented nodular adrenocortical disease (PPNAD).[19] Inactivating *PRKAR1A* mutations also are a relatively common finding in sporadic cortisol-secreting adenomas of humans.[20] In dogs, 1 case report describes a syndrome similar to human Carney complex, but no mutations in PRKAR1A have ever been detected in dogs.[21]

Although the knowledge of canine ATs has expanded considerably in recent years, the molecular origin of these adrenocortical neoplasms and the mechanism behind their autonomous cortisol production still are largely unknown, and the role of the MC2R-cAMP-PKA signaling pathway has never been addressed. Therefore, we report here the results of mutation analysis of the full cDNA sequences of *MC2R*, *GNAS* and *PRKAR1A* in 44 canine cortisol-secreting ATs.

Materials and Methods

Animals and Tests

The study included 44 canine cortisol-secreting ATs and 2 normal adrenal glands (whole tissue explants). Normal adrenal glands were obtained from healthy Beagle dogs, euthanized for reasons unrelated to the present study and for which approval was obtained from the Ethical Committee of Utrecht University.

All ATs were derived from patients referred to the Department of Clinical Sciences of Companion Animals of the Faculty of Veterinary Medicine in Utrecht between 2001 and 2012. Suspicion of hypercortisolism was based on the history, physical examination findings, and routine laboratory findings. The diagnosis of ACTH-independent hypercortisolism due to an AT was based upon (i) increased urinary cortisol secretion, which was not suppressible with a high dose of dexamethasone; (ii) suppressed or undetectable basal plasma ACTH concentrations[2]; and (iii) demonstration of an AT by ultrasonography, computed tomography, or both.[22] All ATs were removed by unilateral adrenalectomy. The dogs' ages at the time of surgery ranged from 2 to 13 years (mean, 9 years). Six dogs were mongrels and the others were of 26 different breeds. Twenty-two of the dogs were male (10 castrated) and 22 female (15 spayed). Permission to use AT tissue for this study was obtained from all patient owners, and the study was approved by the Ethical Committee of Utrecht University.

Histopathology

Histopathological evaluation of ATs was performed on formalin-fixed and paraffin-embedded tissue slides of all samples and used to confirm the diagnosis and to classify the tumors. All histological evaluations were performed by a single pathologist. Classification was based on the criteria described by Labelle et al.[23] Classification as a carcinoma was based on histological evidence of vascular invasion, peripheral fibrosis, capsular invasion, trabecular growth, hemorrhage, necrosis, and single cell necrosis. Typical histological characteristics of adenomas were hematopoiesis, fibrin thrombi, and cytoplasmic vacuolization. Based on these criteria, the tumor group consisted of 14 adenomas and 30 carcinomas.

Total RNA Extraction and Reverse Transcription

Tissue fragments for RNA isolation were snap frozen in liquid nitrogen within 10–20 minutes after surgical removal. Total RNA was isolated from the samples using the RNeasy mini kit,[a] according to the manufacturer's protocols. A DNAse step was performed to avoid DNA contamination. RNA concentrations were measured on the NanoDrop ND-1000.[b] cDNA synthesis was performed using the iScript cDNA synthesis kit,[c] according to the manufacturer's protocols. For all samples, 1 cDNA reaction was performed without Reverse Transcriptase (RT−), to check for contamination with genomic DNA.

PCR

Primers for PCR were designed using Perl-primer v1.1.14 according to the parameters in the Bio-Rad iCycler manual, and ordered from Eurogentech.[d] Forward primers were located in the 5′ untranslated region (UTR) of the genes of interest, whereas reverse primers were located in the 3′UTR. For the *MC2R*, the canine UTR sequences were not available, and were predicted based on the human UTR sequences and the canine genomic sequence. Overlapping primer pairs were used when a gene could not be amplified in 1 stretch. For all primer pairs, a PCR temperature gradient was performed to determine the optimal annealing temperature.

Formation of the proper PCR products was evaluated by gel electrophoresis, to check for the correct product length. In case of correct product lengths, a sequencing reaction was performed to confirm the identity of the transcript, using the ABI3130XL

Genetic analyzer[e] according to the manufacturer's protocol. After optimization of the protocol, the complete cDNA of all target genes was amplified in all ATs. PCR reactions were performed using Phusion Hot Start Flex DNA Polymerase[f] on a Dyad Disciple Peltier Thermal Cycler (BioRad[c]) for *PRKAR1A* and on a C1000 Touch thermal cycler (BioRad[c]) for *MC2R* and *GNAS*. All PCR primers and their characteristics are listed in Table 1.

Sequencing

All sequence primers were designed using Perl-primer v1.1.14, and ordered from Eurogentech. Primers were located every 300-500 base pairs along the entire transcript, or closer together when additional primers were needed for complete coverage. All PCR primers also were used as sequence primers. PCR products were amplified for sequencing using the BigDye Terminator version 3.1 Cycle Sequencing Kit[g] and filtrated using Sephadex G-50 Superfine.[h] Sequencing reactions were performed on an ABI3130XL Genetic analyzer, according to the manufacturer's instructions. The obtained sequences were compared to the consensus mRNA sequence using DNAstar Lasergene core suite 9.1 SeqMan software (DNASTAR, Madison, WI). All mutations affecting the amino acid sequence were confirmed by repeat RNA extraction, and sequenced in both sense and antisense directions. All sequence primers and their characteristics are listed in Table 2.

Results

Mutation analysis of *MC2R* identified 3 different silent point mutations and 1 amino-acid changing (missense) point mutation. The silent mutations or single nucleotide polymorphisms (SNPs) found in codon 38 (GGG>GGA), codon 237 (GCG>GCC), and codon 286 (GCG>GCA) were present in 8, 21, and 21 ATs, respectively, and occurred both in hetero- and homozygous form. The missense mutation, a V291I substitution, was present in 3 of the 44 ATs (2 carcinomas, 1 adenoma) and was present only in heterozygous form (Fig 2A).

Mutation analysis of *GNAS* showed the presence of a splice variant, 1 silent point mutation, and 7 different missense mutations. The splice variant of *GNAS*, in which exon 3 is missing, is analogous to the human *GNAS* transcript variant 3 (GenBank: NM_080426.2) or *GNAS*-short (*GNASS*). It was present in all ATs and normal adrenal glands, alongside the full length transcript. The silent mutation was found in codon 201 (CGT>CGC) and was present in 8 ATs in both hetero- and homozygous form. Missense mutations were present in 14 of the 44 ATs, including 4 of the 14 adenomas and 10 of the 30 carcinomas. All missense mutations were heterozygous. Eleven of the 14 missense mutations were located in codon 201 (Fig 2B). They were present in 8 carcinomas and 3 adenomas and comprised the following substitutions: R201C (5×), R201H (4×), R201S (1×), and R201L (1×). A missense mutation in codon 203 (L203P) was present in 1 adenoma (Fig 2C). Missense mutations in codon 227 (Fig 2D) were present in 2 carcinomas (Q227H and Q227R). An overview of the different missense mutations is presented in Table 3.

Mutation analysis of *PRKAR1A* showed the presence of 2 different silent mutations. A silent mutation in codon 317 (AGA>CGA) was present in 4 carcinomas and a silent mutation in codon 311 (GAG>GAA) was present in 1 adenoma. Mutations that changed the amino acid sequence were not found in any of the ATs.

Discussion

In this study, *GNAS* mutations were detected in 14 of the 44 cortisol-secreting ATs of dogs, whereas no functional mutations were found in *MC2R* and *PRKAR1A*. All *GNAS* mutations detected in the ATs of these dogs previously have been described in the human literature, and have been found to cause constitutive activation of cAMP signaling.[14,24–26] Although additional in vitro assays would be necessary to establish a causal relationship, our results strongly suggest the involvement of increased cAMP signaling, caused by activating *GNAS* mutations, in the pathogenesis of a subset of cortisol-secreting ATs in dogs. This finding even may provide an explanation for autonomous, ACTH-independent, cortisol secretion in the affected subset of ATs.

In cortisol-secreting ATs of humans, activation of the cAMP signaling pathway is a well-known phenomenon; however, activating *GNAS* mutations in these tumors are extremely rare, and only have been

Table 1. PCR primers for the amplification of canine *MC2R*, *GNAS*, and *PRKAR1A*. All positions are based on the mRNA sequence, as published on the NCBI website.

PCR Primers	Sequence (5′-3′)	Location	Annealing Temperature	Product Length
MC2R Fw 69	CGAGGCAGAGTAACACCT	−41/−24	55°C	674
MC2R Rv 743	GGAAGCGTCAAGATCTTCC	614/632		
MC2R Fw 410	CACAGCGGATGACATTATGG	300/319	55°C	781
MC2R Fw 1190	AAGCATGAGCATTTGTTGGT	1061/1080		
GNAS Fw 352	CCATGGGCTGCCTCGGAAACA	352/372	56°C	1357
GNAS Rv 1708	TTAAGCAAGCGGAAGGGAAGAAA	1686/1708		
PRKAR1A Fw 20	GCTATCGCGGAGTAGAG	20/36	59°C	1336
PRKAR1A Rv 1355	AGAGGAAGAGAAAGCAGTC	1337/1355		

Accession numbers used were as follows: *MC2R*: XM_003638756.1, *GNAS*: NM_001003263.1, *PRKAR1A*: XM_537577.3. *MC2R*, melanocortin 2 receptor; *GNAS*, stimulatory G protein alpha subunit; *PRKAR1A*, protein kanise A regulatory subunit 1A; Fw, Forward primer; Rv, Reverse primer.

Table 2. Sequencing primers for the mutation analysis of canine *MC2R*, *GNAS*, and *PRKAR1A*. All positions are based on the mRNA sequence, as published on the NCBI website.

Sequence Primers	Sequence (5′-3′)	Location
MC2R Fw 810	CCCTTTGTCCTTCATGTTCTG	700/720
MC2R Rv 340	TATACAAGCTGCCCATCATATCAG	206/229
GNAS Fw 777	TCCCTCCTGAGTTCTATGAG	778/797
GNAS Fw 1226	AACAAGCAAGACCTGCTC	1127/1244
GNAS Rv 845	CTCATAGCAGGCACGCACTCC	825/845
GNAS Rv 951	CAGCGAAGCAGATCCTG	936/952
GNAS Rv 1504	CTGAATGATGTCACGGCA	1488/1505
PRKAR1A Fw 174	GGGAATGTGAGCTCTATGTC	175/194
PRKAR1A Fw 329	CTGCAGAAAGCAAGCTCC	330/347
PRKAR1A Fw 575	TTTGATGCCATGTTTCCAG	576/594
PRKAR1A Fw 750	GAACACCTAGAGCAGCCA	751/768
PRKAR1A Fw 975	TGGTACAGGGAGAACCAG	976/993
PRKAR1A Rv 452	CTCCTCCGTGTAGACTTCG	434/452
PRKAR1A Rv 699	CCCATTCGTTGTTGAC	684/699
PRKAR1A Rv 855	GCTTTCTCAGAGTGCTTCC	837/855
PRKAR1A Rv 975	CTGGTTCTCCCTGTACCA	975/993
PRKAR1A Rv 1255	AAACTGTTGTACTGCTGGA	1237/1255

Accession numbers used: *MC2R*: XM_003638756, *GNAS*: NM_001003263, *PRKAR1A*: XM_537577.3. *MC2R*, melanocortin 2 receptor; *GNAS*, stimulatory G protein alpha subunit; *PRKAR1A*, protein kinase A regulatory subunit 1A; Fw, Forward primer; Rv, Reverse primer.

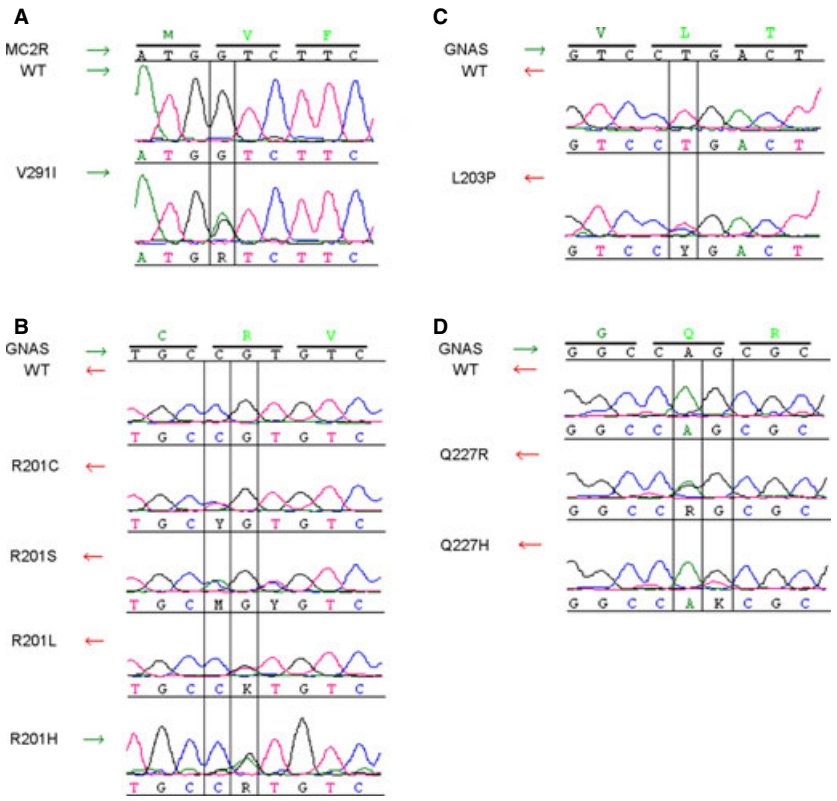

Fig 2. Representative examples of the mutations found in codon 291 of the *MC2R* (**A**) and in codons 201 (**B**), 203 (**C**), and 227 (**D**) of *GNAS* in canine cortisol secreting ATs. Reference sequences are based on XM_003638756.1 (*MC2R*) and NM_001003263.1 (*GNAS*). WT, wild type; *MC2R*, melanocortin 2 receptor; *GNAS*, stimulatory G protein alpha subunit.

described in benign lesions.[9,15,16] Activating *GNAS* mutations in humans are associated with McCune Albright syndrome, in which they result in macronodular hyperplasia of the adrenal glands and hypercortisolism.[11] *GNAS* mutations also have been detected in pituitary and pancreatic tumors of humans and in

Table 3. Overview of all missense mutations of *GNAS* in 44 canine cortisol secreting ATs. All nucleotide positions are based on the mRNA sequence (NM_001003263.1), as published on the NCBI website. All amino acid positions are based on the protein sequence (NP_001003263.1), as published on the NCBI website.

Mutation	Nucleotide	Codon	Basepair Change	Amino Acid Change	Number of ATs
Arg201Cys	954	201	**C**GT>**T**GT	Arg>Cys	5
Arg201His	955	201	C**G**T>C**A**T	Arg>His	4
Arg201Ser	954	201	**C**GT>**A**GT	Arg>Ser	1
Arg201Leu	955	201	C**G**T>C**T**T	Arg>Leu	1
Gln227His	1034	227	CA**G**>CA**T**	Gln>His	1
Gln227Arg	1033	227	C**A**G>C**G**G	Gln>Arg	1
Leu203Pro	961	203	C**T**G>C**C**G	Leu>Pro	1

GNAS, stimulatory G protein alpha subunit. Bold text denotes the basepair change within the codon.

thyroid tumors of humans and cats.[12–14] In humans, substitutions of Arg^{201} are most common, followed by Gln^{227} substitutions[11,13,14] whereas Arg^{201} and Gln^{227} also were the affected codons in thyroid tumors of cats.[12] Likewise, in our canine cohort most of the mutations were substitutions of Arg^{201} and $Gln.^{227}$

Four possible substitutions of Arg^{201} in *GNAS* have been described in humans, in decreasing occurrence: R201C, R201H, R201S, and R201L.[11,24,26] Of these mutations, only R201C and R201H previously have been reported in the adrenal cortex, including cortisol-secreting ATs.[10,15,27] In cats, R201C is the only known mutation affecting Arg^{201}.[12] In our canine AT cohort, all 4 known Arg^{201} mutations were identified, with a higher frequency of R201C and R201H mutations. Five different substitutions have been described at the 2nd hotspot for human *GNAS* mutations (ie, Gln^{227}) of which Q227H, Q227L and Q227R are most common.[14,24] A single report of a Q227H substitution in a cortisol-secreting adrenocortical adenoma in a human has been published.[9] In thyroid tumors of cats, both Q227R and Q227L have been reported.[12] In our canine AT cohort, both Q227H and Q227R substitutions were detected. The L203P substitution at Leu^{203} found in 1 AT in a dog has only been described in a thyroid tumor of a human.[25]

The *GNAS* splice variant that was present in all samples was analogous to human transcript variant 3.[28] This transcript variant corresponds to a shorter Gsalpha protein (GNASS), which was found to be co-expressed with the long variant (GNASL) in nearly all cell types, although the relative amounts vary depending on the tissue type. In the adrenal cortex of humans, GNASL was found to be the predominant isoform.[29] Both variants induce cAMP production, and some investigations have indicated differences in their activity, affinity of GDP binding and receptor interaction.[30–32] However, whether these differences result in clinically relevant biological effects still is unclear.[33] Moreover, the presence of both variants in all ATs and normal adrenal glands in our canine cohort makes a causal role in canine adrenocortical tumorigenesis unlikely.

cAMP is the main cellular signal for inducing cortisol secretion.[5] Therefore, the activating *GNAS* mutations in ATs of dogs, resulting in constitutive cAMP production, may explain the ACTH-independent cortisol production for this subgroup. Apart from cortisol production, increased cAMP signaling also is known to play a role in adrenal tumorigenesis.[34] Activating mutations in *GNAS* induce tumor formation in cAMP-sensitive tissue types by increasing cell proliferation. The mutated *GNAS* thus is referred to as the gsp oncogene.[35] *GNAS* activating mutations have been shown to result in induction of mitogen-activated protein kinase (MAPK or ERK) and p53 signaling, focal adhesion kinase (FAK) pathways, and nuclear factor kappa-B (NFκB) expression.[27] Both P53 and Ras-Raf-MAPK pathways are well known for their roles in carcinogenesis and have been implicated as factors in adrenal tumorigenesis.[36,37] The FAK pathway and NFKB also have been implicated as factors in the pathogenesis of various tumor types.[38,39] Therefore, it is likely that the activating *GNAS* mutations found in ATs of dogs play a role in tumorigenesis.

In contrast to the high prevalence of activating *GNAS* mutations in cortisol-secreting ATs of dogs, no mutations affecting the amino acid sequence were found in *PRKAR1A*. This contrasts with the important role of *PRKAR1A* mutations in adrenal pathology in humans and mice. Mutations in *PRKAR1A* cause Carney complex in humans, with PPNAD as one of the main consequences[19] When cAMP-PKA activation is present in ATs of humans, it nearly always originates from *PRKAR1A* mutations or other PKA signaling abnormalities.[34] Likewise, in the mouse, *PRKAR1A* inactivation and AT formation are closely linked. Transgenic mice lacking PRKAR1A activity in the adrenal cortex develop ACTH-independent Cushing's syndrome[40] and activated PKA signaling because of PRKAR1A loss of function results in a tumor formation syndrome similar to human Carney complex.[41,42]

In dogs, a single case report describes a syndrome similar to human Carney complex, but in this dog *PRKAR1A* was not altered.[21] The absence of missense *PRKAR1A* mutations in these canine AT cohort, combined with the fact that no mutations in PRKAR1A have ever been detected in dogs, appear to indicate a difference in the molecular origin of cAMP-PKA activation between adrenal glands of dogs and their human and murine counterparts. The pathways affected by cAMP-PKA activation have been shown to differ

depending on the molecular origin of the activation. cAMP-PKA activation attributable to *PRKAR1A* mutations stimulates a different set of cellular pathways and target genes than activation caused by *GNAS* mutations.[27] Both *PRKAR1A* and *GNAS* mutations activate MAPK and P53 signaling, whereas the FAK pathway and NFKB specifically are induced by *GNAS* mutations, and *PRKAR1A* mutations induce activation of the Wnt-pathway, one of the main cellular pathways implicated in AT pathogenesis in humans.[27,43] However, although differences exist between germ line *PRKAR1A* defects and somatic *GNAS* mutations, dogs and humans still share common MAPK and p53 signaling pathway activations that might be important targets for treatment in both species.

Mutation analysis of the *MC2R* identified the presence of a V291R missense mutation in 3 ATs, which has not been described previously in the literature. However, this substitution is not likely to have a functional effect on the receptor, because valine and isoleucine are alike in polarity and charge. Moreover, in the human MC2R, isoleucine and not valine is the consensus amino acid. Otherwise, no mutations in the *MC2R* were found, corresponding to the situation in humans, where activating *MC2R* mutations have never been identified.

In conclusion, this study demonstrates the presence of activating *GNAS* mutations in a large portion of both benign and malignant cortisol-secreting ATs in dogs. These results strongly suggest increased cAMP signaling as a factor in the pathogenesis of these tumors and may explain the autonomous secretion of cortisol in the affected subset of ATs. This study thus is the first to identify potentially causal mutations in cortisol-secreting ATs of dogs.

Footnotes

[a] Qiagen, Hilden, Germany
[b] NanoDrop Technologies, Wilmington, DE
[c] Bio-Rad, Hercules, CA
[d] Eurogentec, Maastricht, the Netherlands
[e] AB Applied Biosystems, Carlsbad, CA
[f] New England BioLabs Inc, Ipswich, MA
[g] Applied Biosystems
[h] Amersham, Buckinghamshire, UK

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