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Insulin-Like Growth Factor—Phosphatidylinositol 3 Kinase Signaling in Canine Cortisol-Secreting Adrenocortical Tumors

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Background: Hypercortisolism is a common endocrine disorder in dogs, caused by a cortisol-secreting adrenocortical tumor (AT) in approximately 15% of cases. In adrenocortical carcinomas of humans, activation of the phosphatidylinositol 3 kinase (PI3K) signaling pathway by insulin-like growth factor (IGF) signaling represents a promising therapeutic target.

Objectives: To investigate the involvement of PI3K signaling in the pathogenesis of ATs in dogs and to identify pathway components that may hold promise as future therapeutic targets or as prognostic markers.

Animals: Analyses were performed on 36 canine cortisol-secreting ATs (11 adenomas and 25 carcinomas) and 15 normal adrenal glands of dogs.

Methods: mRNA expression analysis was performed for PI3K target genes, PI3K inhibitor phosphatase and tensin homolog (*PTEN*), IGFs, IGF receptors, IGF binding proteins and epidermal growth factor receptors. Mutation analysis was performed on genes encoding *PTEN* and PI3K catalytic subunit (*PIK3CA*).

Results: Target gene expression indicated PI3K activation in carcinomas, but not in adenomas. No amino acid-changing mutations were detected in *PTEN* or *PIK3CA* and no significant alterations in IGF-II or IGFR1 expression were detected. In carcinomas, *ERBB2* expression tended to be higher than in normal adrenal glands, and higher expression of inhibitor of differentiation 1 and 2 (*ID1* and *ID2*) was detected in carcinomas with recurrence within 2.5 years after adrenalectomy.

Conclusions and Clinical Importance: Based on these results, ERBB2 might be a promising therapeutic target in ATs in dogs, whereas ID1 and 2 might be valuable as prognostic markers and therapeutic targets.

Key words: Adrenal; Dog; Hypercortisolism; Insulin-like growth factor; Phosphatidylinositol 3 kinase.

Hypercortisolism is 1 of the most common endocrine disorders in dogs.[1] Approximately, 15% of spontaneous cases of hypercortisolism in dogs are due to cortisol-secreting adrenocortical adenomas or carcinomas.[1] Therapeutic options for dogs with adrenocortical tumors (ATs) are limited: complete adrenalectomy of the affected adrenal gland is the treatment of choice, provided no metastases are present at the time of presentation.[2,3] However, surgery is not possible or successful in all cases, and tumor recurrence and metastasis occur regularly.[4] Options for medical management are limited to mitotane, a chemotherapeutic agent, or trilostane, which can only alleviate the clinical signs of hypercortisolism. The lack of reliable prognostic markers further complicates treatment.

A pathway with the potential to provide both therapeutic targets and prognostic markers is the phosphatidylinositol-3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling pathway (Fig 1). The PI3K pathway is 1 of the most frequently activated signal transduction pathways in cancers of humans, the activation of which also has been documented in ATs in humans.[5–7] Pathway activation is initiated by receptor tyrosine kinases, such as the type 1 insulin-like growth factor (IGF) receptor (IGFR1) or dimers of the

Abbreviations:

ACC	adrenocortical carcinoma
AT	adrenocortical tumor
BCL2L1	B-cell lymphoma 2 related protein
CCND1	cyclin D1
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERBB	erythroblastic leukemia viral oncogene homolog
GUSB	beta-glucuronidase
HPRT	hypoxantine phosphoribosiltransferase
ID	inhibitor of differentiation
IGFBP	insulin-like growth factor binding protein
IGF	insulin-like growth factor
IGFR	insulin-like growth factor receptor
INSR	insulin receptor
mTOR	mammalian target of rapamycin
p-AKT	phosphorylated AKT
PI3K	phosphatidylinositol 3 kinase
PIK3CA	phosphatidylinositol 3 kinase catalytic subunit
PTEN	phosphatase and tensin homolog
qPCR	quantitative RT-PCR
RPS19	ribosomal protein S19
RPS5	ribosomal protein S5
SGK1	serum glucocorticoid regulated kinase 1
SNAI1	snail
SNAI2	slug
SPRP	small proline rich protein
TRAIL	tumor necrosis factor superfamily member 10
UTR	untranslated region
XIAP	X-linked inhibitor of apoptosis

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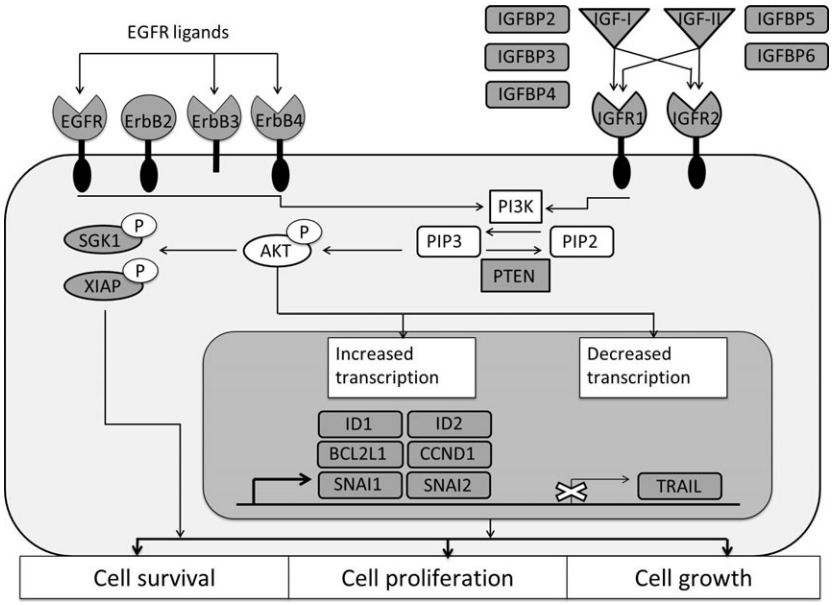

Fig 1. Schematic representation of the phosphatidylinositol 3 kinase (PI3K) signaling pathway. Upon ligand binding, the epidermal growth factor receptors (EGFR and ERBB 2–4) form homo- and heterodimers, activating their tyrosine kinase domain. The IGF receptors (IGFR1 and IGFR2) are activated by the binding of insulin-like growth factor (IGF) I or II. IGF actions are modulated by high affinity IGF binding proteins (IGFBP 2–6). IGFR1 and the EGF receptors relay their intracellular signal to PI3K, which converts the inactive PIP2 to the active PIP3. Phosphatase and tensin homolog (PTEN) counters PI3K action by converting PIP3 back to PIP2. PIP3 production leads to the phosphorylation of AKT to phospho-AKT (P-AKT), which in turn activates SGK1 and XIAP through phosphorylation, while it induces mRNA expression of target genes *ID1*, *ID2*, *SNAI1*, *SNAI2*, *BCL2L1* and *CCND1*, and inhibits mRNA expression of *TRAIL*. Through its target genes, activation of the PI3K pathway results in increased cell survival, proliferation and growth. Target genes for qPCR analysis in this study are indicated in dark gray. SGK1, serum glucocorticoid regulated kinase 1; XIAP, X-linked inhibitor of apoptosis; *ID1,* inhibitor of differentiation 1; *ID2*, inhibitor of differentiation 2; *SNAI1*, snail; *SNAI2*, slug; *BCL2L1*, B-cell lymphoma 2 related protein; *CCND1*, cyclin D1; *TRAIL*, tumor necrosis factor superfamily member 10.

epidermal growth factor (EGF) receptor family (EGFR, ERBB2–4), and counteracted by the competitive PI3K inhibitor phosphatase and tensin homolog (PTEN). Upon activation of the PI3K pathway, phosphorylated AKT (p-AKT) and its downstream effectors stimulate cell proliferation, survival and growth by transcriptional and posttranslational mechanisms.[5]

The PI3K pathway contains multiple targets for therapeutic intervention, the choice of which depends on the mode of activation. In adrenocortical carcinomas (ACC) of humans, frequent overexpression of *IGF-II* and *IGFR1* indicates IGF-signaling as a likely mode for PI3K activation.[8–10] Selective IGFR1 kinase inhibitors thus could be of benefit, and indeed have shown antitumor effects both in cell culture studies and preclinical and early phase clinical trials in humans with ACC.[6,10] For EGFR-induced PI3K pathway activation, several specific inhibitors already have been approved for clinical use in humans.[11] Activation of the PI3K pathway also may occur downstream of the receptors, for instance as a result of mutations in the genes encoding *PTEN* or the PI3K catalytic subunit (*PIK3CA*) or because of decreased expression of *PTEN*.[12,13] In these cases, single or dual inhibitors of PIK3CA and mTOR could be employed.[14] In a human ACC cell line, use of these compounds has resulted in decreased cell proliferation and cortisol secretion.[15,16]

In ACC of humans, *IGF-II* is the most frequently and strongly overexpressed gene,[17,18] whereas in adenomas overexpression occurs only rarely.[18,19] Additionally, high *IGF-II* expression in ACC of humans is associated with aggressive tumor behavior and increased risk of metastasis.[20,21] Therefore, in humans IGF-II is a diagnostic and prognostic marker for ATs.

The aim of this study was to investigate involvement of the PI3K signaling pathway in the pathogenesis of cortisol-secreting ATs in dogs, to identify pathway components that may hold promise as future therapeutic targets or may serve as prognostic markers. Pathway activation was evaluated by means of target gene expression analysis, whereas mRNA expression analysis and mutation analysis were used to indicate mode of activation.

Materials and Methods

Patient Material

Patient material used in this study consisted of 36 cortisol-secreting ATs from dogs and 15 whole tissue explants of normal canine adrenal glands. All normal adrenal glands from healthy dogs were available as archived tissue for comparison with AT tissue obtained from patients. The tumor group consisted of histologically confirmed ATs from patients with clinical signs of hypercortisolism, referred to the Department of Clinical Sciences of Companion Animals of the Faculty of Veterinary Medicine in Utrecht between 2001 and 2012. The diagnosis of ACTH-independent hypercortisolism because of a cortisol-secreting AT was based upon (1) increased urinary corticoid-to-creatinine ratios, that were

not suppressible with high doses of dexamethasone, (2) suppressed or undetectable basal plasma ACTH concentrations[1] and (3) demonstration of an AT by ultrasonography, computed tomography or both.[22] All dogs subsequently underwent unilateral adrenalectomy. The dogs' ages at the time of surgery ranged from 2 to 12 years (mean, 9 years). Seven dogs were mongrels and the other dogs were of 22 different breeds. Eighteen of the dogs were male (8 castrated) and 18 female (12 spayed).

After resection, all ATs and normal adrenal glands were immediately put on ice for inspection, and material was saved for quantitative RT-PCR (qPCR) analysis and histopathology. Fragments for RNA isolation were snap frozen in liquid nitrogen within 10 minutes after resection and stored at −80°C until further use. The remaining part of the tissue was immersed in formalin for fixation and embedded in paraffin after 24–48 hours. Permission to use the 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 was performed on formalin-fixed and paraffin-embedded tissue slides of all samples and used to confirm the diagnosis and classify the tumors. All histological evaluations were performed by a single pathologist. Classification was performed based on criteria described previously.[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 11 adenomas and 25 carcinomas.

Follow-up

Of the dogs in the tumor group, follow-up information was available for 15 dogs with histologically confirmed carcinomas: 7 of these dogs developed signs of hypercortisolism within 2.5 years after surgical removal of the tumor. Recurrence of hypercortisolism was confirmed by endocrine testing, and was caused by metastases in 6 of these dogs, and by regrowth of the AT in 1 dog. The remaining 8 dogs were in remission for at least 2.5 years after adrenalectomy.

Total RNA Extraction and Reverse Transcription

Total RNA for quantitative RT-PCR analysis was isolated from the adrenal tissue using the RNeasy mini kit,[a] according to manufacturer's protocols. An additional DNAse step was performed to avoid genomic DNA contamination. RNA concentrations were measured on the NanoDrop ND-1000.[b] Synthesis of cDNA 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.

Quantitative RT-PCR

Primers for qPCR were designed to detect the mRNA expression levels of PI3K pathway target genes (Table 1), PI3K pathway inhibitor PTEN and major components of the IGF and EGF axis (Table 2). Primer design was performed using Perl-primer v1.1.14 according to the parameters in the Bio-Rad iCycler manual, and primers were ordered from Eurogentec.[d] For all primer pairs, a temperature gradient was performed to determine the optimal annealing temperature. Formation of the proper PCR products was confirmed by a sequencing reaction, using the ABI3130XL Genetic analyzer[e] according to the manufacturer's protocol.

For mRNA expression analysis of the EGF receptors, PI3K target genes and PTEN, a 10× diluted pool of cDNA samples was used to create a 4-fold standard dilution series. The remaining cDNA was diluted 5 times with milliQ water, to achieve a working stock. Reactions were performed on a CFX384 real-time PCR detection system.[c] The following genes were measured as follows: EGF receptor (*EGFR*), erythroblastic leukemia viral oncogene homolog 2–4 (*ERBB2–4*), snail (*SNAI1*), slug (*SNAI2*), B-cell lymphoma 2 related protein (*BCL2L1*), cyclinD1 (*CCND1*), inhibitor of differentiation 1 and 2 (*ID1* and *ID2*), tumor necrosis factor superfamily member 10 (*TNFSF10* or *TRAIL*), serum glucocorticoid regulated kinase 1 (*SGK1*), X-linked inhibitor of apoptosis (*XIAP*) and *PTEN*. To correct for differences in cDNA concentration, ribosomal protein S5 (*RPS5*), *RPS19*, small proline rich protein (*SPRP*) and hypoxantine phosphoribosiltransferase (*HPRT*) were used as reference genes.[24,25]

For mRNA expression analysis of *IGF-I*, *IGF-II*, *IGFR1*, *IGFR2*, IGF binding proteins (*IGFBP 2–6*) and the insulin receptor (*INSR*), an undiluted pool of cDNA samples was used to create a 4-fold standard dilution series. The remainder of the cDNA was diluted 10 times with milliQ water, to achieve a working stock. Reactions were performed on a MyIQ single color real-time PCR detection system.[c] To correct for differences in cDNA concentration, *RPS5*, *RPS19*, *SPRP* and beta-glucuronidase (*GUSB*) were used as reference genes.[24,25]

Detection was performed using SYBRgreen supermix[c] and data were analyzed using CFX Manager 3.0[c] for the CFX384 real-time PCR data and using iQ5 software[c] for the MyIQ single color real-time PCR data. The raw data were used to calculate the reaction efficiency. Reaction efficiencies between 90% and 110% were accepted. Analysis of the relative expression levels of the reference genes disclosed no significant differences among groups and reference gene expression was shown to be stable using GeNorm software,[21] justifying the use as reference genes. Calculation of normalized relative expression levels for each of the target genes was performed using the $2^{-\Delta\Delta CT}$ method.[26]

Mutation Analysis

Mutation analysis was performed on *PTEN* and *PIK3CA*. Primers for PCR amplification (Table 3) and sequence primers (Table 4) were designed using Perl-primer v1.1.14 according to BioRad iCycler parameters, and ordered from Eurogentec.[d] Sequence primers were located along the entire transcript, at a distance of 300–500 bp apart, or closer together when needed for complete coverage. Amplification primers were located in the 3′ and 5′untranslated regions (UTRs) of the gene to ensure amplification of the complete coding region. For *PTEN*, the canine 3′ UTR is not annotated and was deduced from the human 3′ UTR and the canine genomic sequence. If a gene could not be amplified in 1 stretch, overlapping primer pairs were used. All amplification primers were tested on a pool of adrenal samples, to determine the optimal annealing temperature. Correct product formation was evaluated by means of gel electrophoresis and sequencing.

After PCR optimization, target genes in all samples were amplified on a C1000 Touch thermal cycler[c] using Phusion Hot Start Flex DNA Polymerase.[f] The 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 sequences obtained were aligned to the NCBI consensus mRNA sequence using DNAstar Lasergene core suite 9.1 SeqMan

Table 1. Quantitative RT-PCR primer pairs for the detection of PI3K target genes: *SNAI1*, *SNAI2*, *BCL2L1*, *ID1* and *ID2*, *CCND1*, *SGK11*, *TNFSF10* or *TRAIL*, and *XIAP*. All positions are based on the mRNA sequence, as published on the NCBI website.

qPCR Primers (Position)	Sequence (5′–3′)	Annealing Temperature (°C)	Product Length (bp)
cf_SNAI1			
Fw	CAA GAT GCA CAT CCG AAG C	61.6	133
Rv	CAG TGG GAG CAG GAA AAC		
cf_SNAI2			
Fw	CTT CAC TCC GAC TCC AAA CG	60	148
Rv	TGG ATT TTG TGC TCT TGC AG		
cf_BCL2L1			
Fw	GGG GTG GTG AGG TAC AAA AA	61.6	112
Rv	CTG GGT CTA GCG TCC AAA AG		
cf_ID1			
Fw	CTC AAC GGC GAG ATC AG	59.5	135
Rv	GAG CAC GGG TTC TTC TC		
cf_ID2			
Fw	GCT GAA TAA ATG GTG TTC GTG	60.5	114
Rv	GTT GTT CTC CTT GTG AAA TGG		
cf_CCND1			
Fw	GCC TCG AAG ATG AAG GAG AC	60	117
Rv	CAG TTT GTT CAC CAG GAG CA		
cf_TNFSF10			
Fw	GCT GAT CCT CAT CTT CAC TG	62	90
Rv	TCC TGC ATC TGC TTC AG		
cf_SGK11			
Fw	TGG GCC TGA ACG ACT TTA TT	62	124
Rv	GAG GGG TTG GCA TTC ATA AG		
cf_XIAP			
Fw	ACT ATG TAT CAC TTG AGG CTC TGG TTT C	54	80
Rv	AGT CTG GCT TGA TTC ATC TTG TGT TAT G		

Accession numbers used: *SNAI1*: XM_543048.1; *SNAI2*: XM_543048.1; *CDH1*: XM_536807.3; *BCL2L1*: NM_001003072.1; *ID1*: XM_847117.2; *ID2*: XR_134413.1; *CCND1*: NM_001005757.1; *SGK11*: XM_003432525.1; *TNFSF10*: NM_001130836; *XIAP*: XM_003435664.1.

PI3K, phosphatidylinositol 3 kinase; *SNAI1*, snail; *SNAI2*, slug; *BCL2L1*, B-cell lymphoma 2 related protein; *ID1* and *ID2*, inhibitor of DNA binding 1 and 2; *CCND1*, CyclinD1; *SGK11*, serum/glucocorticoid regulated kinase 1; *TNFSF10* or *TRAIL*, tumor necrosis factor superfamily member 10; *XIAP*, X-linked inhibitor of apoptosis; Fw, forward primer; Rv, reverse primer.

software. Mutations altering the amino acid sequence were confirmed by repeat RNA extraction and sequencing in both sense and antisense directions.

Statistical Analyses

Statistical analyses were performed using SPSS20.[i] Because of the non-normal distribution of most of the variables, the non-parametric Mann-Whitney *U*-test was used to compare mRNA expression levels among groups. The relative mRNA expression levels were compared between ATs (adenomas and carcinomas) and normal adrenals and between ATs with and without recurrent disease. For the first comparison, a Bonferroni correction was applied and $P < .025$ was considered significant, whereas for the latter comparison, a $P < .05$ was considered significant.

Results

Relative mRNA Expression of PI3K Target Genes

To evaluate whether activation of the PI3K pathway was present in cortisol-secreting ATs of dogs, mRNA expression analysis was performed on a selection of 10 known target genes of the pathway. Based on the literature, PI3K activation would increase mRNA expression of *ID1*,[27,28] *ID2*,[29] *SNAI1* and *SNAI2*,[30] *CCND1* and *BCL2L1*.[31] In contrast, lower mRNA expression of *TRAIL*[31,32] would be expected (Fig 1). The activation of SGK1 and XIAP occurs by means of phosphorylation; so for these genes, no effect on mRNA expression would be expected[33,34] (Fig 1). In ACC of dogs, the relative mRNA expression levels when compared to those in normal adrenal glands (Fig 2A) were significantly higher for *ID1* (2.1-fold, $P = .021$) and *SNAI1* (1.8-fold, $P = .024$), and significantly lower for *SGK1* (0.5-fold, $P = .009$). The relative mRNA expression of *TRAIL* (0.6-fold, $P = .028$) tended to be lower. Thus, in carcinomas 3 of the 7 transcriptionally regulated target genes showed a change in accordance with pathway activation, whereas the mRNA expression of the other genes remained unchanged.

In adenomas, the mRNA expression of none of the genes showed a significant change consistent with PI3K pathway activation (Fig 2A). The only significant finding was a lower relative expression of *CCND1* (0.42-fold, $P = .004$) when compared to that of normal adrenal glands.

Table 2. qPCR primer pairs for the detection of genes encoding components of the IGF- and EGF axis, and *PTEN*: *EGFR*, *ERBB2–4*, *PTEN*, *IGF-I* and *IGF-II*, *IGFR1* and *IGFR2*, *IGFBP 2–6*, and *INSR*. All positions are based on the mRNA sequence, as published on the NCBI website.

qPCR Primers (Position)	Sequence (5′–3′)	Annealing Temperature (°C)	Product Length (bp)
cf_EGFR			
Fw	CTG GAG CAT TCG GCA	53	107
Rv	TGG CTT TGG GAG ACG		
cf_ERBB2			
Fw	CGT GCT GGA CAA TGG AGA CC	64	126
Rv	CCG CTG AAT CAA GAC CCC TC		
cf_ERBB3			
Fw	TAG TGG TGA AGG ACA ACG GCA G	64	103
Rv	GGT CTT GGT CAA TGT CTG GCA G		
cf_ERBB4			
Fw	CAG TTC TTG TGT GCG TGC CTG	70	121
Rv	ATG ATC CTG TGC CGA TGC C		
cf_PTEN			
Fw	AGA TGT TAG TGA CAA TGA ACC T	62	101
Rv	GTG ATT TGT GTG TGC TGA TC		
cf_IGF-I			
Fw	TGT CCT CCT CGC ATC TCT T	58	125
Rv	GTC TCC GCA CAC GAA CTG		
cf_IGF-II			
Fw	CTT CTG GAG ACC TAC TGT GC	61	128
Rv	CTG CTT CCA GGT GTC GTA TTG		
cf_IGFR1			
Fw	CAT GCC TTG GTC TCC CTG T	60	129
Rv	GGT GGT CCC AAT CCC AAA G		
cf_IGFR2			
Fw	GAG TTC AGC CACG AGA C	54	94
Rv	GCA TTG TCA CCA TCA AGG		
cf_IGFBP2			
Fw	GAT CTC CAC CAT GCA CCT TC	60	127
Rv	GCT GCC CGT TCA GAG ACA TCT TG		
cf_IGFBP3			
Fw	CTG CAC ACG AAG ATG GAT GT	61	127
Rv	TAT TCC GTC TCC CGC TTG TA		
cf_IGFBP4			
Fw	AGC CTG CAG CCC TCT GAC A	59	120
Rv	TGG TGC TGC GGT CTC GAA T		
cf_IGFBP5			
Fw	TCG CAG AAA GAA GCT GAC C	60	131
Rv	GAA GCC TCC ATG TGT CTG C		
cf_IGFBP6			
Fw	CAA TCC TGG TGG TGT CC	54	136
Rv	AGA AGC CCT TAT GGT CAC		
cf_INSR			
Fw	GTG ACA GAC TAT TTA GAT GTC CC	60	166
Rv	ACT CAG GGT TTG AAG AAG C		

Accession numbers used: *EGFR*: XM_533073.3; *ERBB2*: NM_001003217.1; *ERBB3*: XM_538226.4; *ERBB4*: XM_003640190.2; *PTEN*: NM_001003192.1; *IGF-I*: XM_848024.1; *IGF-II*: XM_858107.1; *IGFR1*: XM_853622.1; *IGF-IIR*: NM_001122602; *IGFBP2*: XM_545637.2; *IGFBP3*: XM_548740.2; *IGFBP4*: XM_845091.1; *IGFBP5*: XM_847792.1; *IGFBP6*: XM_844250; *INSR*: XM_542108.

EGFR, epidermal growth factor receptor; *ERBB2–4*, erythroblastic leukemia viral oncogene homolog 2–4; *PTEN*, phosphatase and tensin homolog; *IGF-I* and *IGF-II*, insulin-like growth factor 1 and 2; *IGFR1* and *IGFR2*, IFG receptor type 1 and 2; *IGFBP 2–6*, IGF binding protein 2–6; *INSR,* insulin receptor; Fw, forward primer; Rv, reverse primer.

When comparing dogs with recurrent disease within 2.5 years to dogs remaining in remission (Fig 2B), a significantly higher expression of both *ID1* (2.0-fold, $P = .033$) and *ID2* (2.4-fold, $P = .019$) was detected in dogs with recurrent disease. No significant changes were detected in the expression of other target genes.

Relative mRNA Expression of PTEN and the Components of IGF and EGF Signaling

To evaluate for a potential cause of PI3K activation, mRNA expression levels of the components of the IGF axis, the EGF receptors and PI3K-inhibitor *PTEN* were

Table 3. PCR primers pairs for amplification of phosphatase and tensin homolog (*PTEN*) and phosphatidylinositol 3 kinase, catalytic subunit alpha (*PIK3CA*). All positions are based on the mRNA sequence, as published on the NCBI website.

PCR Primers (Position)	Sequence (5′–3′)	Annealing Temperature (°C)	Product Length (bp)
cf_PTEN			
Fw75	TCC TCC TTC CTC TCC AG	55	748
Rv822	TGA ACT TGT CTT CCC GTC		
Fw718	CAA TGT TCA GTG GCG GA	55	743
Rv1460	CGA GAT TGG TCA GGA AGA G		
cf_PIK3CA			
F1	TTT CTG CTT TGG GAC AGC	55	1536
R1537	CTG GGA ACT TTA CCA CAC TG		
Fw1438	TGC TGA ACC CTA TTG GTG	55	449
Rv1887	TAC AGT CCA GAA GCT CCA		
Fw1516	GCA GTG TGG TAA AGT TCC	55	1843
Rv3359	CAG TCT TTG CCT GTT GAC		

Accession numbers used: *PTEN*: NM_001003192.1; *PIK3CA*: XM_545208.3.
Fw, forward primer; Rv, reverse primer.

Table 4. Sequencing primers for the mutation analysis of phosphatase and tensin homolog (*PTEN*) and phosphatidylinositol 3 kinase, catalytic subunit alpha (*PIK3CA*). All positions are based on the mRNA sequence, as published on the NCBI website.

Sequence Primers (Position)	Sequence (5′–3′)
cf_PTEN	
Fw474	CAC TGT AAA GCT GGA AAG GG
Fw1157	TGT AGA GGA GCC ATC AAA CC
Rv249	CAA AGG GTT CAT TCT CTG GG
Rv595	TGT CTC TGG TCC TTA CTT CC
Rv1285	CCT GTA TAC GCC TTC AAG TC
cf_PIK3CA	
Fw375	GTA ATT GAG CCA GTA GGC
Fw707	CAA CCA TGA CTG TGT TCC
Fw1153	TCT ATC ATG GAG GAG AAC CC
Fw1483	CTC CAT GCT TAG AGT TGG AG
Fw1767	CTA CCC AAA CTG CTT CTG
Fw2183	TAG GCA AGT TGA GGC TAT GG
Fw2582	TGG TTG TCT GTC AAT CGG
Fw2806	TGG GAA TTG GAG ATC GTC
Fw2963	GAT TAG TAA AGG AGC CCA GG
Rv358	AAA GCC GAA GGT CAC AAA GC
Rv730	GTT CTG GAA CAC AGT CAT GG
Rv1236	TAG CCA TTC ATT CCA CCT GG
Rv1750	CAC AAT AGT GTC TGT GGC TC
Rv2205	TTC CAT AGC CTC AAC TTG CC
Rv2642	GTG TGA GAA TTT CGC ACC
Rv2993	TTT GTG CAT TCC TGG GCT
Rv3271	CAT GCT GCT TAA TGG TGT GG

Accession numbers used: *PTEN*: NM_001003192.1; *PIK3CA*: XM_545208.3.
Fw, forward primer; Rv, reverse primer.

evaluated. Analysis of the components of the IGF axis in carcinomas (Fig 3A) disclosed a significantly higher expression of *IGFBP2* (5.8-fold, $P = .001$) and a significantly lower expression of *IGFBP5* (0.5-fold, $P = .001$). Likewise, in adenomas, *IGFBP2* expression was significantly higher (7.2-fold, $P = .013$) and *IGFBP5* expression was significantly lower (0.4-fold, $P < .001$). No significant differences in the expression levels of *IGF-II* or the *IGFR1* were detected.

Analysis of the EGF receptors in carcinomas identified a tendency to higher *ERBB2* expression (1.7-fold, $P = .027$) and significantly lower expression of *ERBB3* (0.4-fold, $P = .003$). In adenomas, the only significant change was lower *ERBB3* expression (0.2-fold, $P = .001$).

When comparing dogs with recurrent disease within 2.5 years to dogs remaining in remission (Fig 3B), significantly higher expression of *IGF-I* (2.7-fold, $P = .042$), *IGFBP5* (6.8-fold, $P = .042$) and *INSR* (2.3-fold, $P = .040$) was detected.

Mutation Analysis of PTEN and PIK3CA

To determine whether inactivating mutations of *PTEN* or activating mutations of *PIK3CA* might be responsible for activation of the PI3K pathway, mutation analysis was performed. Mutation analysis of *PTEN* identified the presence of 1 silent mutation in codon 325 (CTC>CTT), which was present in 6 ATs and occurred in both homo- and hetero-zygous form. No amino acid-changing mutations were detected in of any of the ATs.

Mutation analysis of *PIK3CA* identified the presence of 3 different heterozygous silent mutations (codon 149 CCA>CCC, codon 438 TCT>TCA and codon 842 GTG>GTT) in 3 different ATs. No amino acid-changing mutations were detected in any of the ATs.

Discussion

In this study, we aimed to investigate activation of the PI3K pathway in cortisol-secreting adrenocortical adenomas and carcinomas of dogs, to identify both potential therapeutic targets and prognostic markers for use in dogs with ATs. The presence of PI3K activation

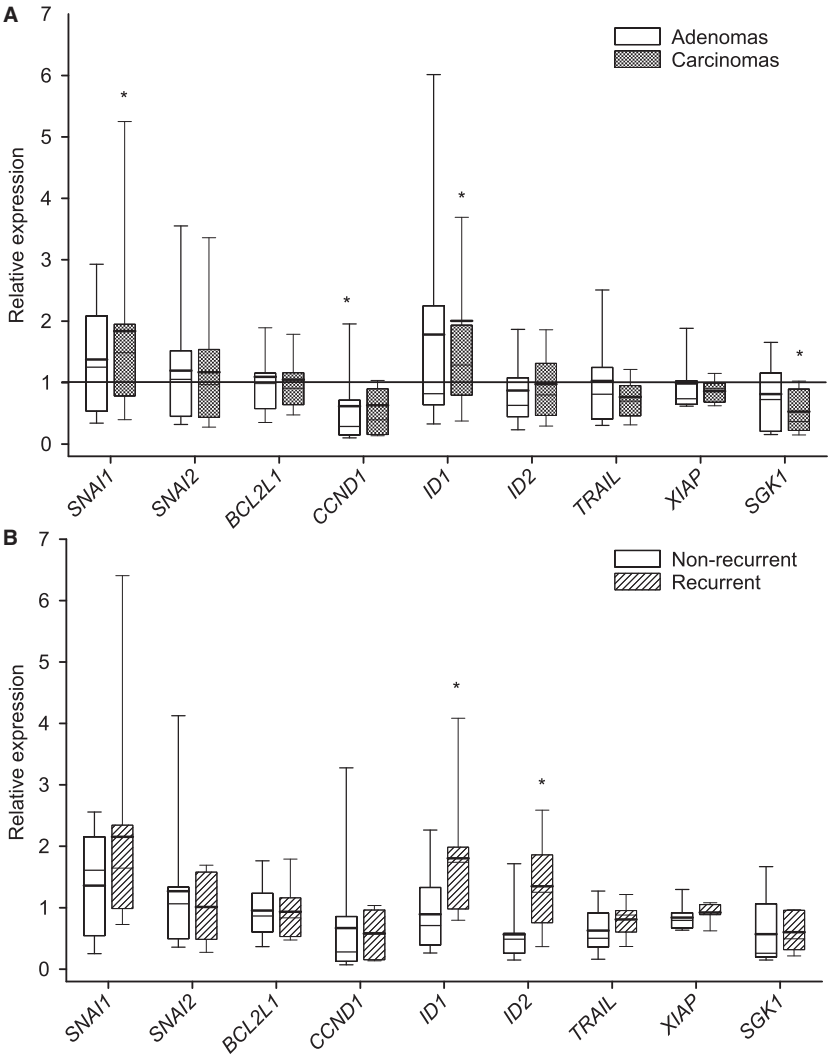

Fig 2. Box-and-whisker plot describing the relative mRNA expression of phosphatidylinositol 3 kinase target genes in 37 canine adrenocortical tumors (ATs) and 15 normal adrenal glands, as measured by qPCR and calculated using the $2^{-\Delta\Delta CT}$ method. Relative expression in adenomas and carcinomas when compared to normal adrenal glands **(A)**. Relative expression in ATs with recurrence within 2.5 years after adrenalectomy, when compared with ATs remaining in remission for at least 2.5 years **(B)**. Significant changes ($P < .05$ for A and $P < .025$ for B) are marked with an asterisk. N, normal adrenal gland; C, adrenocortical carcinoma; A, adrenocortical adenoma; *SNAI1*, snail; *SNAI2*, slug; *BCL2L1*, B-cell lymphoma 2 related protein; *ID1* and *ID2*, inhibitor of DNA binding 1 and 2; *CCND1*, CyclinD1; *SGK11*, serum/glucocorticoid regulated kinase 1; *TNFSF1*, tumor necrosis factor superfamily member 10; *XIAP*, X-linked inhibitor of apoptosis.

was assessed by means of target gene expression analysis. In adenomas, none of the target genes showed a significant change consistent with PI3K pathway activation, and there was even a significantly lower expression of *CCND1*. This may be explained by the fact that adrenocortical adenomas, based on histological characteristics and tumor behavior, are a highly differentiated tumor type, and repression of *CCND1* expression is thought to be a hallmark of cell differentiation.[35] In line with this reasoning, lower *CCND1* expression was detected in adrenocortical adenomas of humans when compared to carcinomas.[36] In contrast, in ACC of dogs all target genes that showed significant alteration in mRNA expression were altered in accordance with PI3K activation.

The main modes of activation of the PI3K pathway are by intracellular alterations of signaling pathway components or through receptor tyrosine kinase signaling. With regard to intracellular pathway alterations, activating mutations of *PIK3CA* and inactivating mutations of *PTEN* are well-documented.[14] In this study, no amino acid-changing mutations were detected in either *PIK3CA* or *PTEN* and no overall decreased expression of *PTEN* was detected in ATs.

With regard to receptor tyrosine kinase induced activation, 1 of the most strongly documented changes that can activate the PI3K pathway in ACC of humans, is activation of the IGF axis. Several studies have demonstrated that in ACC of humans, *IGF-II* is the most overexpressed gene.[19,37] In the healthy individual, 1 of the alleles of the

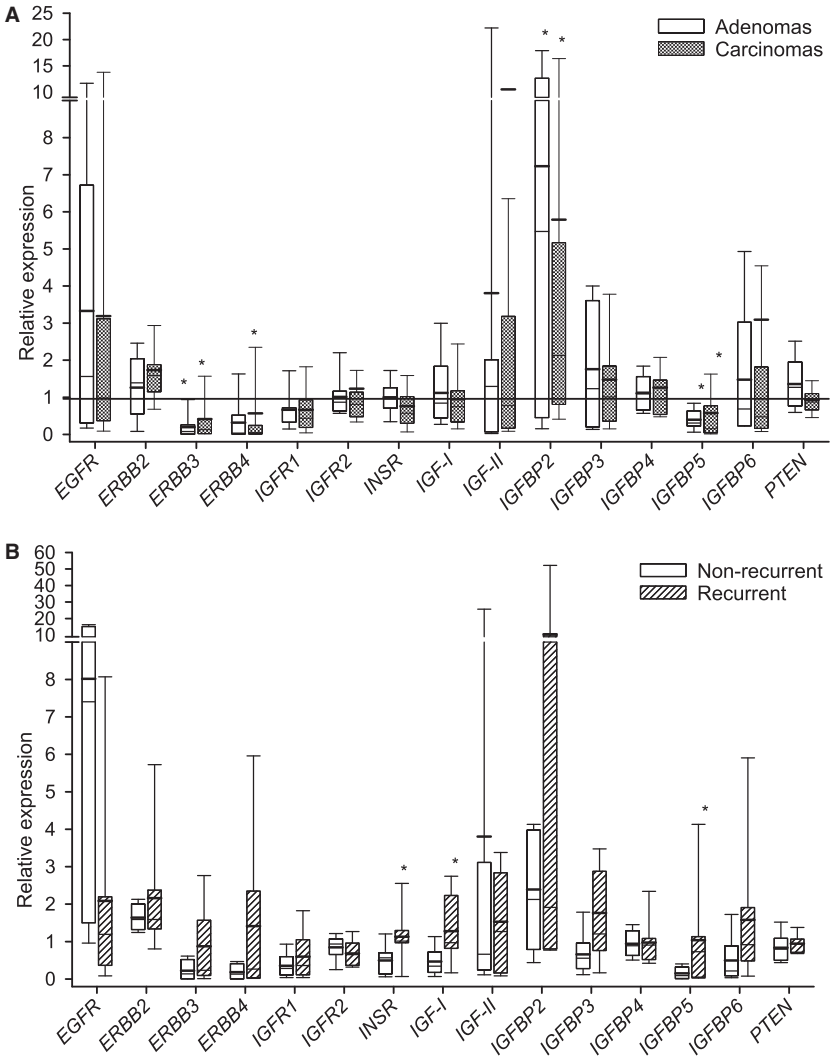

Fig 3. Box-and-whisker plot describing the relative mRNA expression of genes encoding components of the IGF- and EGF axis, and *PTEN* in 37 canine ATs and 15 normal adrenal glands, as measured by qPCR and calculated using the $2^{-\Delta\Delta CT}$ method. Relative expression in adenomas and carcinomas when compared to normal adrenal glands **(A)**. Relative expression in ATs with recurrence within 2.5 years after adrenalectomy, when compared with ATs remaining in remission for at least 2.5 years **(B)**. Significant changes ($P < .05$ for A and $P < .025$ for B) are marked with an asterisk. N, normal adrenal gland; C, adrenocortical carcinoma; A, adrenocortical adenoma; *PTEN*, phosphatase and tensin homolog; *IGF-I* and 2, insulin like growth factor 1 and 2; *IGFR1*, IFG receptor type 1; *IGF-IIR*, IGF receptor type 2; *IGFBP2–6*, IGF binding protein 1–6; *INSR*, insulin receptor; *EGFR*, epidermal growth factor receptor; *ERBB2–4*, erythroblastic leukemia viral oncogene homolog 2–4.

IGF-II locus is epigenetically silenced postnatally, but in ACC genetic and epigenetic alterations in the 11p15 locus cause both alleles to be active, resulting in *IGF-II* overexpression.[17] Likewise, high IGF-II protein expression is characteristic of ACC in humans.[17,18] The mechanism by which high IGF-II expression leads to activation of the PI3K pathway is by binding IGFR1, which like IGF-II frequently shows increased expression in ACC of humans.[8,9] Taken together, these data suggest that in ACC of humans changes in the IGF axis could be responsible for PI3K activation. This notion is supported by studies in H295R human adrenocortical carcinoma cells, in which IGF-II – IGFR1 signaling results in an increase in p-AKT.[6,7,38] Remarkably, this study did not identify a higher expression of either *IGF-II* or *IGFR1* in ATs of dogs.

For *IGFBP2,* higher mRNA expression was detected in adenomas and carcinomas of dogs. The IGFBPs function as regulatory components of IGF signaling,[39] and some studies have indicated that high IGFBP2 expression may contribute to ACC pathogenesis. In murine Y1 AT cells, long-term increased *IGFBP2* expression enhanced the malignant phenotype[40] and in *IGF-II*-overexpressing ACC of humans increased expression of IGFBP2 has been reported.[20] However, the significance of high *IGFBP2* expression in the absence of *IGF-II* overexpression is unknown. Theoretically, high IGFBP2 expression could lead to higher IGF-II availability and thus increase IGFR1/PI3K signaling. However, the fact that *IGFBP2* overexpression also was detected in adenomas in the absence

of increased target gene expression does not support a functional role of *IGFBP2* overexpression in PI3K activation in dogs.

In contrast, *IGFBP5* showed an overall lower expression in ATs, but a 6.8-fold higher expression in those carcinomas showing recurrence within 2.5 years after adrenalectomy. In different types of cancer in humans, *IGFBP5* overexpression has been noted as a prognostic marker. In particular, in breast cancer, high *IGFBP5* expression is associated with a shorter recurrence-free, metastasis-free and overall survival and *IGFBP5* overexpression in breast cancer cell lines conferred resistance to IGFR1 inhibition.[41] Our results therefore indicate the mRNA expression of *IGFBP5* as a relevant prognostic factor in ACC in dogs.

An alternative mechanism for receptor tyrosine kinase-induced PI3K pathway activation is EGFR signaling. In this respect, the tendency toward higher *ERBB2* mRNA expression in ACC of dogs is interesting. ERBB2 (also known as HER2) is a receptor tyrosine kinase that lacks a ligand-binding domain, and functions by heterodimerization with the other EGF receptors. Aberrant ERBB2 activation, for instance because of receptor overexpression, results in activation of the PI3K pathway, growth stimulation and tumorigenesis in different tumor types, of which ERBB2-positive breast cancer is the most prominent example.[11,42] Different drugs targeting ERBB2 have been approved for clinical use in cancer treatment, including antibodies that inhibit heterodimerization (trastuzumab and pertuzumab[43,44]) and tyrosine kinase inhibitors that affect both EGFR (ie, ERBB1) and ERBB2 activity (lapatinib and afatinib[45,46]). Recent studies suggest that simultaneously targeting EGFR and ERBB2 further increases antitumor activity.[11] Based on the higher expression of *ERBB2*, this receptor might be a promising new therapeutic target in dogs with ACC, either singly or in combination with EGFR inhibition.

Aside from therapeutic targets, we also aimed to investigate the pathway components for potential new prognostic markers. In this regard, aside from *IGFBP5*, *SGK1*, *ID1* and *ID2* also are worth mentioning. The relative mRNA expression of *SGK1* was significantly lower in carcinomas. At first glance, this seems surprising, because *SGK1* is activated by PI3K signaling only at the protein level, by phosphorylation.[33] Recently, however, *SGK1* microdeletions and low *SGK1* mRNA expression have been reported in cortisol-secreting ATs of humans, but not in nonsecreting or aldosterone-secreting ATs.[47–49] Low SGK1 protein expression was found to be an independent prognostic factor for shorter overall survival.[48] Although this study did not identify significant differences in *SGK1* mRNA expression between dogs with and without recurrent disease, low *SGK1* expression in cortisol-secreting carcinomas does suggest a functional role for *SGK1*. Additional studies involving SGK1 protein expression and overall survival analyses are needed to determine whether SGK1 expression might prove to be a prognostic marker in these tumors.

In the group of dogs with recurrent disease, we did find an increase in both *ID1* and *ID2* expression. This observation is in accordance with studies reporting an association between high *ID1* and *ID2* expression and poor prognosis in several different tumor types in humans.[50] Inhibitor of differentiation proteins are thought to keep cells in a poorly differentiated, proliferative state, and therefore a role for ID1 and 2 in the pathogenesis of malignant ATs in dogs appears likely. Based on our results, ID1 and 2 show promise as new prognostic markers for ACC in dogs. Future studies are needed to determine whether targeting IDs might also be feasible as a new therapeutic option.[51,52]

In conclusion, our results suggest the presence of PI3K activation in cortisol-secreting ACC in dogs, but not in adenomas. In contrast to 1 of the most prominent features of ACC in humans, no significant alterations in *IGF-II* or *IGFR1* expression were detected. Therefore, our results suggest that inhibition of IGF signaling is not likely to prove successful in dogs with ATs. However, we did find higher expression of *ERBB2*, providing a preclinical rationale for studying the potential of ERBB2 inhibition, as used in ERBB2-positive breast cancer, in dogs with ACC. Finally, the lower expression of *SGK1* in carcinomas and the higher expression of *IGFBP5*, *ID1*, and *ID2* in ATs with early recurrence may represent an important step in the search for prognostic markers for cortisol-secreting ACC in dogs.

Footnotes

[a] Quiagen, 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, Foster City, CA
[h] Amersham, Buckinghamshire, UK
[i] IBM, Armonk, NY

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Conflict of Interest Declaration: The authors disclose no conflict of interest.

Off-label Antimicrobial Declaration: The authors declare no off-label use of antimicrobials.

References

1. Galac S, Reusch CE, Kooistra HS, Rijnberk A. Adrenals. In: Rijnberk A, Kooistra HS, eds. Clinical Endocrinology of Dogs and Cats. Second, revised and extended edition ed. Hannover: Schlütersche; 2010:93–154.

2. Schwartz P, Kovak JR, Koprowski A, et al. Evaluation of prognostic factors in the surgical treatment of adrenal gland

tumors in dogs: 41 Cases (1999–2005). J Am Vet Med Assoc 2008;232:77–84.

3. van Sluijs FJ, Sjollema BE, Voorhout G, et al. Results of adrenalectomy in 36 dogs with hyperadrenocorticism caused by adreno-cortical tumour. Vet Q 1995;17:113–116.

4. Barrera JS, Bernard F, Ehrhart EJ, et al. Evaluation of risk factors for outcome associated with adrenal gland tumors with or without invasion of the caudal vena aava and treated via adrenalectomy in dogs: 86 Cases (1993–2009). J Am Vet Med Assoc 2013;242:1715–1721.

5. Martini M, De Santis MC, Braccini L, et al. PI3K/AKT Signaling pathway and cancer: An updated review. Ann Med 2014;46:372–383.

6. Barlaskar FM, Spalding AC, Heaton JH, et al. Preclinical targeting of the type I insulin-like growth factor receptor in adrenocortical carcinoma. J Clin Endocrinol Metab 2009;94: 204–212.

7. Fassnacht M, Weismann D, Ebert S, et al. AKT is highly phosphorylated in pheochromocytomas but not in benign adrenocortical tumors. J Clin Endocrinol Metab 2005;90:4366–4370.

8. Kamio T, Shigematsu K, Kawai K, Tsuchiyama H. Immunoreactivity and receptor expression of insulinlike growth factor I and insulin in human adrenal tumors. An immunohistochemical study of 94 cases. Am J Pathol 1991;138:83–91.

9. Weber MM, Auernhammer CJ, Kiess W, Engelhardt D. Insulin-like growth factor receptors in normal and tumorous adult human adrenocortical glands. Eur J Endocrinol 1997;136:296–303.

10. Almeida MQ, Fragoso MC, Lotfi CF, et al. Expression of insulin-like growth factor-II and its receptor in pediatric and adult adrenocortical tumors. J Clin Endocrinol Metab 2008;93: 3524–3531.

11. Tebbutt N, Pedersen MW, Johns TG. Targeting the ERBB family in cancer: Couples therapy. Nat Rev Cancer 2013;13:663–673.

12. Martini M, Ciraolo E, Gulluni F, Hirsch E. Targeting PI3K in cancer: Any good news? Front Oncol 2013;3:108.

13. Hopkins BD, Hodakoski C, Barrows D, et al. PTEN function: The long and the short of it. Trends Biochem Sci 2014;39:183–190.

14. Willems L, Tamburini J, Chapuis N, et al. PI3K and mTOR signaling pathways in cancer: New data on targeted therapies. Curr Oncol Rep 2012;14:129–138.

15. De Martino MC, van Koetsveld PM, Feelders RA, et al. The role of mTOR inhibitors in the inhibition of growth and cortisol secretion in human adrenocortical carcinoma cells. Endocr Relat Cancer 2012;19:351–364.

16. Doghman M, Lalli E. Efficacy of the novel dual PI3-kinase/mTOR inhibitor NVP-BEZ235 in a preclinical model of adrenocortical carcinoma. Mol Cell Endocrinol 2012;364:101–104.

17. Gicquel C, Raffin-Sanson ML, Gaston V, et al. Structural and functional abnormalities at 11p15 are associated with the malignant phenotype in sporadic adrenocortical tumors: Study on a series of 82 tumors. J Clin Endocrinol Metab 1997;82:2559–2565.

18. Schmitt A, Saremaslani P, Schmid S, et al. IGFII and MIB1 immunohistochemistry is helpful for the differentiation of benign from malignant adrenocortical tumours. Histopathology 2006;49:298–307.

19. Giordano TJ, Thomas DG, Kuick R, et al. Distinct transcriptional profiles of adrenocortical tumors uncovered by DNA microarray analysis. Am J Pathol 2003;162:521–531.

20. Boulle N, Logie A, Gicquel C, et al. Increased levels of insulin-like growth factor II (IGF-II) and IGF-binding protein-2 are associated with malignancy in sporadic adrenocortical tumors. J Clin Endocrinol Metab 1998;83:1713–1720.

21. Gicquel C, Boulle N, Logie A, et al. Involvement of the IGF system in the pathogenesis of adrenocortical tumors. Ann Endocrinol (Paris) 2001;62:189–192.

22. Galac S, Kool MM, Naan EC, et al. Expression of the ACTH Receptor, steroidogenic acute regulatory protein, and steroidogenic enzymes in canine cortisol-secreting adrenocortical tumors. Domest Anim Endocrinol 2010;39:259–267.

23. Labelle P, Kyles AE, Farver TB, De Cock HE. Indicators of malignancy of canine adrenocortical tumors: Histopathology and proliferation index. Vet Pathol 2004;41:490–497.

24. Brinkhof B, Spee B, Rothuizen J, Penning LC. Development and evaluation of canine reference genes for accurate quantification of gene expression. Anal Biochem 2006;356:36–43.

25. Schlotter YM, Veenhof EZ, Brinkhof B, et al. A GeNorm algorithm-based selection of reference genes for quantitative real-time PCR in skin biopsies of healthy dogs and dogs with atopic dermatitis. Vet Immunol Immunopathol 2009;129:115–118.

26. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001;25:402–408.

27. Perk J, Iavarone A, Benezra R. Id family of helix-loop-helix proteins in cancer. Nat Rev Cancer 2005;5:603–614.

28. Birkenkamp KU, Essafi A, van der Vos KE, et al. FOXO3a induces differentiation of Bcr-Abl-transformed cells through transcriptional down-regulation of Id1. J Biol Chem 2007;282:2211–2220.

29. Belletti B, Prisco M, Morrione A, et al. Regulation of Id2 gene expression by the insulin-like growth factor I receptor requires signaling by phosphatidylinositol 3-kinase. J Biol Chem 2001;276:13867–13874.

30. Lau MT, Leung PC. The PI3K/Akt/mTOR signaling pathway mediates insulin-like growth factor 1-induced E-cadherin down-regulation and cell proliferation in ovarian cancer cells. Cancer Lett 2012;326:191–198.

31. Lam EW, Francis RE, Petkovic M. FOXO transcription factors: Key regulators of cell fate. Biochem Soc Trans 2006;34:722–726.

32. Allen JE, El-Deiry WS. Regulation of the human TRAIL gene. Cancer Biol Ther 2012;13:1143–1151.

33. Firestone GL, Giampaolo JR, O'Keeffe BA. Stimulus-dependent regulation of serum and glucocorticoid inducible protein kinase (SGK) transcription, subcellular localization and enzymatic activity. Cell Physiol Biochem 2003;13:1–12.

34. Dan HC, Sun M, Kaneko S, et al. Akt phosphorylation and stabilization of X-linked inhibitor of apoptosis protein (XIAP). J Biol Chem 2004;279:5405–5412.

35. Klein EA, Assoian RK. Transcriptional regulation of the cyclin D1 gene at a glance. J Cell Sci 2008;121:3853–3857.

36. Pereira SS, Morais T, Costa MM, et al. The emerging role of the molecular marker p27 in the differential diagnosis of adrenocortical tumors. Endocr Connect 2013;2:137–145.

37. de Fraipont F, El Atifi M, Cherradi N, et al. Gene expression profiling of human adrenocortical tumors using complementary deoxyribonucleic acid microarrays identifies several candidate genes as markers of malignancy. J Clin Endocrinol Metab 2005;90:1819–1829.

38. Cantini G, Lombardi A, Piscitelli E, et al. Rosiglitazone inhibits adrenocortical cancer cell proliferation by interfering with the IGF-IR intracellular signaling. PPAR Res 2008;2008:904041.

39. Ribeiro TC, Latronico AC. Insulin-like growth factor system on adrenocortical tumorigenesis. Mol Cell Endocrinol 2011;351:96–100.

40. Hoeflich A, Fettscher O, Lahm H, et al. Overexpression of insulin-like growth factor-binding protein-2 results in increased tumorigenic potential in Y-1 adrenocortical tumor cells. Cancer Res 2000;60:834–838.

41. Becker MA, Hou X, Harrington SC, et al. IGFBP ratio confers resistance to IGF targeting and correlates with increased invasion and poor outcome in breast tumors. Clin Cancer Res 2012;18:1808–1817.

42. Hynes NE, MacDonald G. ErbB receptors and signaling pathways in cancer. Curr Opin Cell Biol 2009;21:177–184.

43. Hudis CA. Trastuzumab—Mechanism of action and use in clinical practice. N Engl J Med 2007;357:39–51.

44. Barthelemy P, Leblanc J, Goldbarg V, et al. Pertuzumab: Development beyond breast cancer. Anticancer Res 2014;34:1483–1491.

45. Reid A, Vidal L, Shaw H, de Bono J. Dual inhibition of ErbB1 (EGFR/HER1) and ErbB2 (HER2/neu). Eur J Cancer 2007; 43:481–489.

46. Spicer JF, Rudman SM. EGFR inhibitors in non-small cell lung cancer (NSCLC): The emerging role of the dual irreversible EGFR/HER2 inhibitor BIBW 2992. Target Oncol 2010;5:245–255.

47. Ronchi CL, Leich E, Sbiera S, et al. Single nucleotide polymorphism microarray analysis in cortisol-secreting adrenocortical adenomas identifies new candidate genes and pathways. Neoplasia 2012;14:206–218.

48. Ronchi CL, Sbiera S, Leich E, et al. Low SGK1 expression in human adrenocortical tumors is associated with ACTH-independent glucocorticoid secretion and poor prognosis. J Clin Endocrinol Metab 2012;97:E2251–2260.

49. Ronchi CL, Sbiera S, Leich E, et al. Single nucleotide polymorphism array profiling of adrenocortical tumors—Evidence for an adenoma carcinoma sequence? PLoS One 2013;8:e73959.

50. Nair R, Teo WS, Mittal V, Swarbrick A. ID proteins regulate diverse aspects of cancer progression and provide novel therapeutic opportunities. Mol Ther 2014;000:000–000.

51. Sumida T, Murase R, Onishi-Ishikawa A, et al. Targeting Id1 reduces proliferation and invasion in aggressive human salivary gland cancer cells. BMC Cancer 2013;13:141.

52. Fong S, Debs RJ, Desprez PY. Id genes and proteins as promising targets in cancer therapy. Trends Mol Med 2004;10:387–392.