Based on these results, ERBB2 might be a promising therapeutic target in ATs in approximately 15% of cases. 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 receptor 1 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 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 (IGFRI) or dimers of the

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**Abbreviations:**

- ACC: adenocortical 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 phosphoribosyltransferase
- ID: inhibitor of differentiation
- IGFBP: insulin-like growth factor binding protein
- IGFR: insulin-like growth factor receptor
- INS: 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
- SPRR: small proline rich protein
- TRAIL: tumor necrosis factor superfamily member 10
- UTR: untranslated region
- XIAP: X-linked inhibitor of apoptosis

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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 mutation in the gene 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 and (3) demon-
stration of an AT by ultrasonography, computed tomography or both. 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 immedi-
ately put on ice for inspection, and material was saved for quanti-
tative 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 fix-
ation 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 paraaffin-embedded tissue slides of all samples and used to con-
firm the diagnosis and classify the tumors. All histological evalua-
tions were performed by a single pathologist. Classification was performed based on criteria described previously. 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 hematoxylineosin, 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 metasteses 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 according to manufacturer’s protocols. An additional DNaseI step was performed to avoid genomic DNA contamination. RNA concentrations were measured on the NanoDrop ND-1000. Synthesis of cDNA was performed using the iScript cDNA synthesis kit 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. 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 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. The following genes were measured as follows: EGF receptor (EGFR), erythroblastoid leukemia viral oncogene homolog 2–4 (ERBB2–4), snail (SNAIL), slug (SNAI2), B-cell lymph-
oma 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 concentra-
tion, ribosomal protein S5 (RPS5), RPS19, small proline rich protein (SPRP) and hypoxanthine phosphoribosyltransferase (HPRT) were used as reference genes.

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 cre-
ate 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. To correct for differences in cDNA concentration, RPS5, RPS19, SPRP and beta-glucuronidase (GUSB) were used as reference genes.

Detection was performed using SYBRGreen supermix and data were analyzed using CFX Manager 3.0 for the CFX384 real-time PCR data and using iQ5 software 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 soft-
ware, justifying the use as reference genes. Calculation of normalized relative expression levels for each of the target genes was performed using the 2−ΔΔCT method.

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. 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 using Phusion Hot Start Flex DNA Polymerase. The PCR products were amplified for sequencing using the BigDye Terminator version 3.1 Cycle Sequencing Kit and filtered using Sephadex G-50 Superfine. 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.
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. 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 \( ID1, ID2, SNAI1, SNAI2, CCND1, SGK1, TNFSF10 \) 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 | CAA GAT GCA CAT CCG AAG C | 61.6 | 133 |
| Rw | CAG TGG GAG CAG GAA AAC |
| cf_SNAI2 | CTT CAC TCC GAC TCC AAA CG | 60 | 148 |
| Rw | TGG ATT TTG TGC TCT TGC AG |
| cf_BCL2L1 | GGG GTG GTG AGG TAC AAA AA | 61.6 | 112 |
| Rw | CTG GGT CTA GGG TCC AAA AG |
| cf_ID1 | CTC AAC GGC GAG ATC AG | 59.5 | 135 |
| Rw | GAG CAC GGG TTC TTC TC |
| cf_ID2 | GCT GAA TAA ATG GTG TCC GTG | 60.5 | 114 |
| Rw | GTT GTT CTC TTT GTG AAA TGG |
| cf_CCND1 | GCC TCG AAG ATG AAG GAG AC | 60 | 117 |
| Rw | CAG TTT GTC CAC CAG GAG CA |
| cf_TNFSF10 | GCT GAT CCT CAT CTT CAC TG | 62 | 90 |
| Rw | TCC TGC ATC TGC TTC AG |
| cf_SGK1 | TGG GCC TGA ACG ACT TTA TT | 62 | 124 |
| Rw | GAG GGG TTG GCA TCC ATA AG |
| cf_XIAP | ACT ATG TAT CAC TTG AGG CTC TGG TTT C | 54 | 80 |
| Rw | AGT CTG GCT TGA TTG TTC TGT TAC G |

Accession numbers used: \( SNAI1 \): XM_543048.1; \( SNAI2 \): XM_543048.1; \( CDH1 \): XM_536807.3; \( BCL2L1 \): NM_001003072.1; \( ID1 \): XM_847171.2; \( ID2 \): XR_134413.1; \( CCND1 \): NM_001005757.1; \( SGK1 \): XM_003435255.1; \( TNFSF10 \): 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; \( SGK1 \), serum/glucocorticoid regulated kinase 1; \( TNFSF10 \) or \( TRAIL \), tumor necrosis factor superfamily member 10; \( XIAP \), X-linked inhibitor of apoptosis; \( Fw \), forward primer; \( Rw \), 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. 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 \( ID1, ID2, SNAI1 \) and \( SNAI2, CCND1 \) and \( BCL2L1 \). In contrast, lower mRNA expression of \( TRAIL \) would be expected. The activation of \( SGK1 \) and \( XIAP \) occurs by means of phosphorylation; so for these genes, no effect on mRNA expression would be expected. In ACC of dogs, the relative mRNA expression levels when compared to those in normal adrenal glands 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 \( TRAIL \) 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. The only significant finding was a lower relative expression of \( CCND1 \) (0.42-fold, \( P = .004 \)) when compared to that of normal adrenal glands.
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.

### 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, IGFRI and IGFRII, and 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                      | GGG CTT GGT CAA TGT CTG CCA 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 TGT |                             |                     |
| cf_IGFR1 Fw              | CAT GCC TTG GTG 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 TGG TCA CCA TCA AGG |                             |                     |
| cf_IGFBP2 Fw             | GAT CTC CAC CAT GCA CCT TC | 60                          | 127                 |
| Rv                      | GCT GCC GGT TCA GAG ACA TCT TG |                             |                     |
| cf_IGFBP3 Fw             | CTG CAC ACG AAG ATG GAT GT | 61                          | 127                 |
| Rv                      | TAT TCC GTG TCC CGC TGT 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 GGG AAG GAG 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; IGFRI: XM_853622.1; IGFRII: NM_001122602; IGFBP2: XM_545637.2; IGFBP3: XM_548740.2; IGFBP4: XM_845091.1; IGFBP5: XM_847792.1; IGFBP6: XM_842450; 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; **IGFRI** and **IGFRII**, IFG receptor type 1 and 2; **IGFBP 2–6**, IGF binding protein 2–6; **INSR**, insulin receptor; Fw, forward primer; Rv, reverse primer.

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

Analysis of the EGF receptors in carcinomas identified a tendency to higher \textit{ERBB2} expression (1.7-fold, \( P = .027 \)) and significantly lower expression of \textit{ERBB3} (0.4-fold, \( P = .003 \)). In adenomas, the only significant change was lower \textit{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 \textit{IGF-I} (2.7-fold, \( P = .042 \)), \textit{IGFBP5} (6.8-fold, \( P = .042 \)) and \textit{INSR} (2.3-fold, \( P = .040 \)) was detected.

Mutation Analysis of PTEN and PIK3CA

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

Mutation analysis of \textit{PIK3CA} identified the presence of 3 different heterozygous silent mutations (codon 149 CCA \( \rightarrow \) CCC, codon 438 TCT \( \rightarrow \) TCA and codon 842 GTG \( \rightarrow \) 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...
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 \textit{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 \textit{CCND1} expression is thought to be a hallmark of cell differentiation. In line with this reasoning, lower \textit{CCND1} expression was detected in adrenocortical adenomas of humans when compared to carcinomas. 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 \textit{PIK3CA} and inactivating mutations of \textit{PTEN} are well-documented. In this study, no amino acid-changing mutations were detected in either \textit{PIK3CA} or \textit{PTEN} and no overall decreased expression of \textit{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, \textit{IGF-II} is the most overexpressed gene. In the healthy individual, 1 of the alleles of the

![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; \textit{SNAI1}, snail; \textit{SNAI2}, slug; \textit{BCL2L1}, B-cell lymphoma 2 related protein; \textit{ID1} and \textit{ID2}, inhibitor of DNA binding 1 and 2; \textit{CCND1}, CyclinD1; \textit{SGK1}, serum/glucocorticoid regulated kinase 1; \textit{TNFSF1}, tumor necrosis factor superfamily member 10; \textit{XIAP}, X-linked inhibitor of apoptosis.](image-url)
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. Likewise, high IGF-II protein expression is characteristic of ACC in humans. 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. 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. 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, 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 and in IGF-II-overexpressing ACC of humans increased expression of IGFBP2 has been reported. 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 IGF-II overexpression suggests that the role of IGFBP2 in ACC pathogenesis may be more complex.
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 and overall survival and IGFBP5 overexpression in breast cancer cell lines conferred resistance to IGFR1 inhibition. 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 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. Different drugs targeting ERBB2 have been approved for clinical use in cancer treatment, including antibodies that inhibit heterodimerization (trastuzumab and pertuzumab) and tyrosine kinase inhibitors that affect both EGFR (ie, ERBB1) and ERBB2 activity (lapatinib and afatinib). Recent studies suggest that simultaneously targeting EGFR and ERBB2 further increases antitumor activity. 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, 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. 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. Low SGK1 protein expression was found to be an independent prognostic factor for shorter overall survival. 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. 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.

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 IGFR-I or IGFR-I 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

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References

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

2. Schwartz P, Krovak JR, Koprowski A, et al. Evaluation of prognostic factors in the surgical treatment of adrenal gland
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tumors in dogs: 41 Cases (1999-2005). J Am Vet Med Assoc 2008;232:77–84.

3. van Sluijs FJ, Spjollema 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 cava and treated via adrenal-ectomy 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 adreno-cortical tumors. J Clin Endocrinol Metab 2005;90:4366–4370.

8. Kamio T, Shigematsu K, Kawai K, Tsujiyama H. Immunoreactivity and receptor expression of insulin-like 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, Gullini 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 therapi-es. 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 corti-sol secretion in human adrenocortical carcinoma cells. Endocr Relat Cancer 2012;19:351–364.

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

17. Giqcel 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, Saremnsani P, Schmid S, et al. IGFII and MSH6 immunohistochemistry is helpful for the differentiation of benign from malignant adrenocortical tumors. 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, Giqcel 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. Giqcel 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. Gulac S, Kool MM, Naan EC, et al. Expression of the ACTH Receptor, steroidogenic acute regulatory protein, and ste-roidogenic 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 quanti-fication 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 CT(T)) method. Methods 2001;25:402–408.

27. Perk J, Iavaronne 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 path-way 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, Giampiolo 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, Assion 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 adreno-cortical tumors. Endocr Connect 2013;2:137–145.

37. de Frainpont 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;2008:90401.

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

40. Hoeflich A, Fetscher 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, Goldberg 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.