# Mutation analysis of the NR5A1 and P53 tumor suppressor gene of normal adrenals and cortisol-secreting adrenocortical tumors in dogs

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## Abstract

The steroidogenic factor 1 (SF-1) plays an important role in adrenal development in humans and mice and there are also indications that the regulation of the SF-1 protein level is critical for the cortisol-secreting adrenocortical tumor (AT) development. The NR5A1 gene encodes for SF-1. Also the P53 tumor suppressor gene plays a role in the development of childhood ATs. In this study we searched for activating mutations in the P53 tumor suppressor gene and the NR5A1 gene. No activating mutations were detected in the NR5A1 gene, only 3 silent mutations in the NR5A1 gene of 3 carcinomas. In the P53 tumor suppressor gene one activating mutation was detected in a carcinoma and one silent mutation in an adenoma. The results of this study suggests that the activation of SF-1 in canine ATs is not a result of mutation and that P53 mutations also do not play an important role.

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## Introduction

Hypercortisolism or Cushing's syndrome is a common condition in dogs caused by prolonged exposure to abnormal high plasma concentrations of cortisol. The estimated incidence of Cushing's syndrome is about 1-2 cases per 1000 dogs a year.[1] The production of cortisol is regulated by the hypothalamus – pituitary – adrenal axis.[2] The hypothalamus produces CRH, corticotrophin release hormone, as response to stress or episodic signals. CRH stimulates the anterior pituitary gland to produce ACTH, adrenocorticotrophic hormone, which will stimulate the production of cortisol.[2] The production of CRH is inhibited by high cortisol blood concentrations (negative feedback).

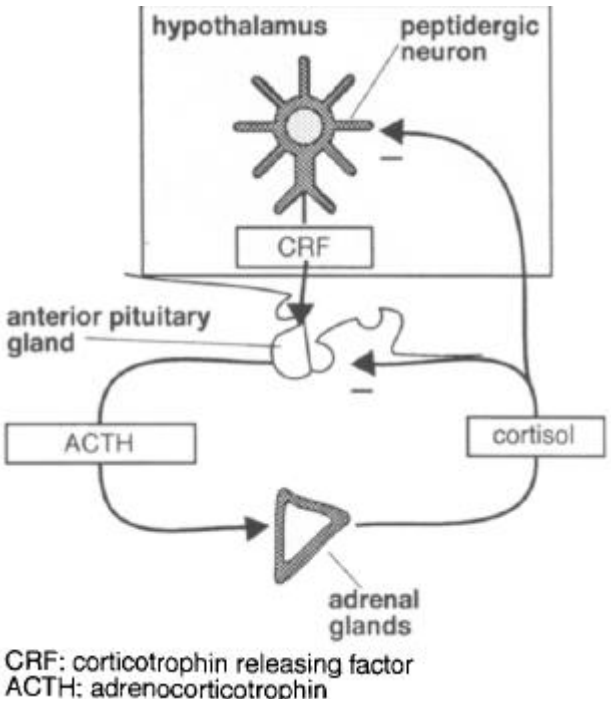


*Figure 1: Hypothalamus - pituitary - adrenal axis [2]*

Clinical signs associated with hypercortisolism are polyuria, polydipsia, lethargy, polyphagia, enlargement of the abdomen, muscle weakness, obesity and a thin coat (figure 2).[3,4] These clinical signs are related to the function of corticosteroids in metabolism; Corticosteroids stimulate gluconeogenesis and lipogenesis at the expense of proteins. A thin coat is caused by a reduced proliferation of keratinocytes and fibroblasts and a disturbed metabolism of the extracellular matrix proteins. Polyuria and polydipsia is caused by the interference with the action of vasopressin and a reduced osmoregulation of vasopressin release.[5]

*Figure 2: Two dogs with Cushing's syndrome*

Cushing's syndrome is classified as spontaneous or iatrogenic.[3,4] Spontaneous hypercortisolism is a disease which occurs in middle-aged and old dogs and is in 80 to 85% of the cases ACTH-dependent and for 15-20% ACTH-independent.[3] ACTH-dependent hypercortisolism arises mostly from hyper secretion of ACTH by a pituitary adenoma, which stimulates the adrenal gland to produce more glucocorticoids. In rare cases the cause could be an ectopic ACTH-secretion syndrome. ACTH-independent hypercortisolism is mostly a result of hyper secretion of glucocorticoids by a benign or malignant AT. ACTH-independent hypercortisolism can also be caused by a primary adrenocortical hyperplasia. The prevalence of adenomas and carcinomas is equally.[5] To differentiate between adenomas and carcinomas, in absence of metastases, is often difficult.[6] Labelle et all (2004) showed that there are 10 histopathologic criteria which are diagnostically useful for differentiating between adenomas and carcinomas. Carcinomas are significant larger than adenomas and show a trabecular growth pattern. Adenoma's showed hematopoiesis in 83% of the cases and fibrin thrombi in dilated veins in contrast with carcinomas.[6] Cushing's syndrome can also be caused by an excessive or prolonged administration of corticosteroids, also called iatrogenic hypercortisolism.[3,4] The appearance of clinical signs by administration of corticosteroids is dependent on the duration and severity of the exposure.

For diagnosing hypercortisolism and differentiating between ACTH-dependent and ACTH-independent hypercortisolism, several tests are used to demonstrate two characteristics of all forms; an increased production of cortisol and a variable sensitivity to glucocorticoid feedback. These tests measure the amount of cortisol in the urine or plasma. Determining the urinary cortisol to creatinine ratio (UCCR) is the most used test.[4] This test provides information about the secretion of cortisol over a period of time. It also adjusts for fluctuations because of the pulsatile release of cortisol.[5] This ratio will be measured in 2 samples of morning urine. The pituitary-adrenal system can be tested by using dexamethasone, which is a synthetic glucocorticoid.[4] This test is called the high dose dexamethasone suppression test (HDDST) and starts after determining the UCCR. If the next morning urine sample has more than 50 % reduction in the UCCR than last two days, the diagnosis of ACTH-dependent hypercortisolism is made. When the UCCR is less than 50% reduced, a CT-scan is needed to differentiate between ACTH-dependent and ACTH-independent hypercortisolism. Further differentiation is also possible by measuring the plasma ACTH concentrations. In dogs with cortisol-secreting ATs, basal ACTH concentration is normally suppressed.

The treatment of ACTH-independent hypercortisolism caused by an adrenocortical tumor is surgical removal. Cortisol-secreting ATs can be malignant and spread to other organs. In this case surgical removal of all the cancer is not an option and Lysodren can be used for destruction of the tumor.

Little is known about what triggers the ACTH-independent cortisol secretion and unrestricted growth of canine ATs. In normal adrenal glands the zona fasciculata secretes

cortisol, which is mainly controlled by ACTH.[7] ACTH binds to its melanocortin 2 receptor (MC2R) and leads to an activation adenyl cyclase A for producing cAMP. This results in the activation of protein kinase A (PKA), which can be activated by various ligands, and is the main mediator of cAMP signaling in cells.[8] PKA activation results in the activation of various transcription factors. One of these factors is the steroidogenic factor 1 (SF-1). A common finding in ATs is the abnormal activation of the protein kinase A (PKA) signaling pathway.[8] This abnormal activation can be caused by mutations in different genes which are part of the signaling cascade. To date, there have never been found mutations in the MC2R gene, but overexpression of SF-1 is a common finding in adrenocortical tumors.[1, 9, 10]

SF-1, also called NR5A1 or AD4BP, is an orphan nuclear receptor which regulates the expression of cytochrome P-450 steroid hydroxylases and steroidogenic acute regulatory protein (StAR), which are enzymes important for adrenal steroidogenesis.[11] SF-1 binding sites are present in the promotors of almost every gene involved with steroidogenesis. The NR5A1 gene encodes for SF-1. SF-1 is a transcription factor which not only regulates the expression of steroidogenic enzymes, but also plays an important role in the normal development of the adrenal glands and steroid-producing organs. Ingraham et all (1994) demonstrated that mice with homozygous disruption of the NR5A1-gene lacked adrenal glands and gonads, which proves the developmental role of SF-1.[12] SF-1 was also shown to be important for residual adrenal gland growth of a contralateral adrenal gland after unilateral adrenalectomy. [13] The regulation of SF-1 activity is achieved by several pathways; positive and negative cofactors, phospholipid ligand availability, epigenetic and tissue-specific gene expression regulation and gene dosage and post-translational modifications (figure 3).[14]

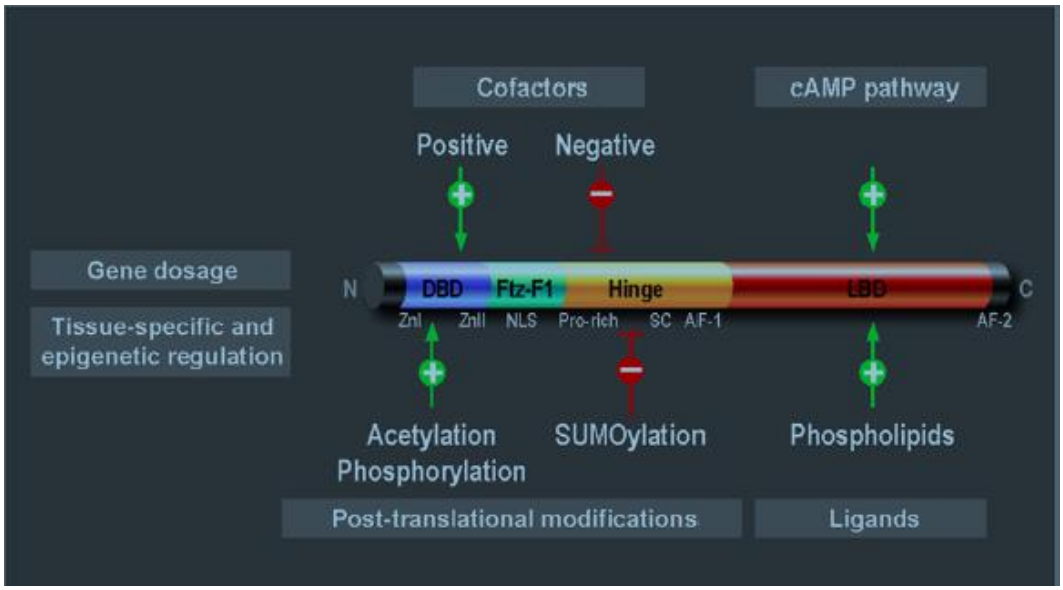


*Figure 3: Mechanisms of control of the transcriptional activity of the SF-1 protein* [14]

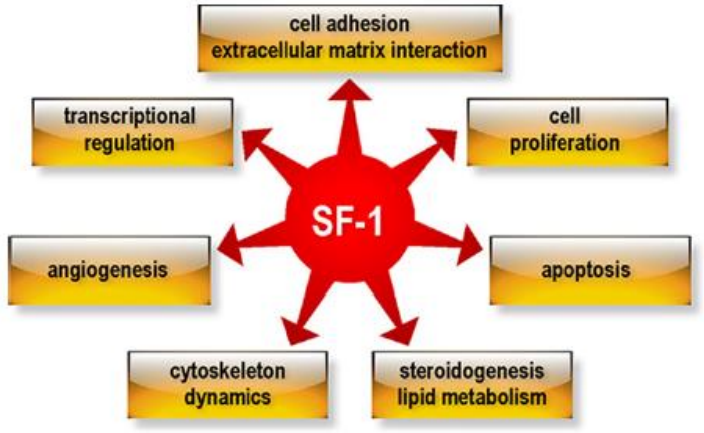


*Figure 4: Gene categories regulated by SF-1 in adrenocortical tumor cells* [14]

There are several studies in which SF-1 has been shown to play a role in adrenocortical tumor development (figure 4).[14] Dogmann et all (2007) showed that an increased dosage of SF-1 increases proliferation of human adrenocortical cells, modulates the pattern of steroid secretion and triggers the formation of ATs in mice.[15] There also seems to be a direct correlation between the amount of SF-1 copy numbers and the adrenal proliferative potential. In another study was showed that SF-1 is a high valuable marker for the diagnosis of ATs.[16, 17] SF-1 protein staining determines the adrenocortical origin of an adrenal mass. In this study it was also proved that a high expression of SF-1 is associated with poor survival. Another important finding is that SF-1 inverse agonists inhibited the cell proliferation of SF-1 stimulated adrenocortical carcinoma cells.[18]

ATs are also seen in children. In Southern Brazil ATs in childhood occurs 10-15 times more than worldwide estimates.[9] In these ATs it has been shown that the NR5A1 gene is amplified and that the SF-1 protein is overexpressed compared to normal adrenal glands.[9,10] Another characteristic of childhood ATs in Southern Brazil is the high incidence of a P53 tumor suppressor gene mutation. 35 of 36 patients had an identical point mutation of P53, which causes an amino acid substitution of arginine to histidine.[19] The prevalence of P53 mutations in children with ATs varies from 50 to 80%. This in contrast with adults where the prevalence is low, 3 to 6%.[20] Acquired mutations in the P53 gene are common and are identified in most major cancer types in human and dogs.[21]

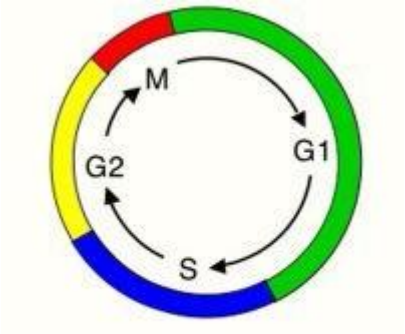


*Figure 5: Somatic cell cycle* [17]

The P53 tumor suppressor gene encodes for a protein which is involved in the control of the cellular proliferation.[22]

The normal cell division consists of 4 phases (figure 5). Two important phases are the S phase, in which DNA replication occurs, and the M phase, in which mitosis occurs. Between these phases there are two gaps, the G1 and G2 phase. During G1 the cell prepares for DNA

synthesis and during G2 the cell prepares for mitosis. Cells in G1 can temporarily or permanently leave the cell cycle and enter the arrested phase Go.

Just before the S-phase starts there is a checkpoint, called the G1 checkpoint. P53 controls the G1-checkpoint. When a cell suffers DNA damage, P53 will be activated. P53 will stimulate the transcription of Cdk inhibitor P21, which will arrest the cell-cycle.[22] This will initiate DNA repair mechanisms or apoptosis, so P53 act as a tumor suppressor gene. Mutations in the P53 gene are in most cases loss-of-function mutations, so tumorigenesis can occur.[22] For example, the Li-Fraumeni syndrome, which is an autosomal dominant syndrome characterized by the development of several mesenchymal and epithelial neoplasms at various sites, is caused by a germline mutations in the P53 gene.[23]

Up to now, the screening for the mutations in the SF-1 and P53 gene in canine cortisol-secreting ATs has not been done yet.

## Research goal

The goal of this research is to search for activating mutation(s) in the NR5A1 gene that encodes for SF-1 and loss-of-function mutations in the P53 tumor suppressor gene in dogs with cortisol-secreting ATs.

# Materials & Methods

## Material

The materials used for this study were already obtained for a previous study.[1] Normal adrenal glands and 54 canine cortisol-secreting ATs. The normal adrenal glands were obtained from healthy beagle dogs, which were euthanized for other reasons than this study. Approval for euthanization was obtained by the Ethical Committee of Utrecht University. The cortisol secreting ATs were obtained from patients of the faculty of Veterinary medicine in Utrecht between 2001 and 2014. The age of the dogs at time of surgery ranged from 2 to 13 years, with an average of 9 years. Six patients were mongrels and the others were of 26 different breeds. From the 54 patients, half were females and the other half males. The diagnosis of ACTH-independent hypercortisolism due to a cortisol-secreting ATs was based on increased cortisol secretion in urine, a decreased sensitivity to dexamethasone, low or non-detectable blood concentrations of ACTH and the detection of an AT by computer tomography or ultrasonography. Permission to use the cortisol-secreting ATs for this study was obtained from all patient owners.

Histopathology evaluation was performed in a previous study on all cortisol-secreting ATs and was performed on formalin-fixed and paraffin-embedded tissue slides. The classification of the tumors was based on criteria described by Labelle.[6] Carcinomas showed vascular invasion, trabecular growth, necrosis, peripheral fibrosis, capsular invasion and hemorrhage. Adenomas showed fibrin thrombi, cytoplasmic vacuolization and hematopoiesis. In this study there were 14 were adenomas and 40 carcinomas.

## Methods

- **RNA isolation**

For this study cDNA of the normal adrenal glands and the cortisol-secreting ATs was used. After surgical removal of the tumor, tissue fragments were snap frozen in liquid nitrogen within 10-20 minutes. For most of the tumors RNA isolation was already performed in a previous study.[1] For 9 new tumors RNA had to be isolated. Total RNA was isolated of these tumors using the RNeasy mini kit, according to the manufacturer's protocol. To avoid DNA contamination a DNAse step was included. The outcoming RNA concentrations were measured by the NanoDrop ND-1000. The results are listed in attachment 1. RNA concentrations below 80 ng/µl will not be used.

- **cDNA production**

cDNA was obtained from these 9 samples using iScript cDNA synthesis kit, according to the manufacturer's protocol. For the other tumors, cDNA was already obtained in a previous study. The reaction set up is showed in table 1.

| Component | Volume per reaction |
|---|---|
| 5x iScript Reaction Mix | 4 µl |
| iScript Reverse Transcriptase | 1 µl |
| Nuclease-free water | x µl |
| RNA template (1 µg Total RNA) | x µl |
| | |
| Total volume | 20 µl |
| **Reaction protocol** | |
| **Incubate complete reaction mix:**<br>- 5 minutes at 25 °C<br>- 30 minutes at 42 °C<br>- 5 minutes at 85 °C<br>- Hold at 4°C (optional) | |

*Table 1: Reaction set up cDNA synthesis*

The volume of RNA template of each sample used for this reaction set up is listed in attachment 2 for the cortisol-secreting ATs.

- **Polymerase chain reaction**

Most of the primers for PCR and sequencing of SF-1 and P53 were already designed in a previous study and were ordered from Eurogentech.[1] 2 new primers were designed for the PCR of SF-1 using Perl-Primer v1.1.14, according to the parameters in the manual, and ordered from Eurogentech. 1 forward primers was located in the 5' untranslated region and 1 reverse primers was located in the 3' untranslated region of SF-1. The (new) PCR primers for SF-1 and P53 are listed in table 2.

| PCR primers | Sequence (5' – 3') | Annealing temperature | Product length |
|---|---|---|---|
| **SF-1, part 1** | | | 1150 base pairs |
| - **Fw 381** | CCTGAAGCAGCAGAAGAAGG | 63,9 | |
| - **Rv 1531** | ATCAAACATCTGGAGGGCC | | |
| **SF-1, part 2** | | | 439 base pairs |
| - **Fw99 (new)** | CAAGATCGACAAGACGCAGCGCA | 63,9 | |
| - **Rv539(new)** | CATAGGGCTCTGGGTACT | | |
| **P53** | | | |
| - **Fw-122** | AAGTCCAGAGCCACCATCC | 64,5 | 1503 base pairs |
| - **Rv 1381** | CCTTTATCTCCCGACCTACC | | |

*Table 2: PCR primers SF1 and P53*

For optimization of the primers a PCR temperature gradient was performed to determine the optimal annealing temperature. Only normal adrenal glands were used for determining the PCR temperature gradient. The PCR reactions were performed using the Phusion Hot

Start DNA polymerase on a C1000 touch thermal cycler for SF-1. The reaction set up for SF-1 and P53 is showed in table 3. The cycling instructions for SF-1 and P53 are showed in table 4 and 5.

| Component | Volume reaction (20 µl) | Final concentration |
|---|---|---|
| **H2O** | Add to 20 µl | |
| **GC buffer** | 4 µl | 1x |
| **10 mM dNTPs** | 0,4 µl | 200 µM each |
| **PrimerA (Forward)** | 1 µl | 0,5 µM |
| **PrimerB (reverse)** | 1 µl | 0,5 µM |
| **Template DNA** | 1 µl | |
| **DMSO** | 0,6 µl | (3%) |
| **Phusion Hot Start DNA Polymerase** | 0,2 µl | 0,02 U/µl |

*Table 3: Components PCR SF-1 & P53*

| Cycle step | Temperature | Time | Number of cycles |
|---|---|---|---|
| **Initial duration** | 98 °C | 30 s | 1 |
| **Denaturation**<br>**Annealing**<br>**Extension** | 98 °C<br>55-65 °C<br>72 °C | 5-10 s<br>10-30 s<br>15-30 s/1 kb | 25 - 35 |
| **Final extension** | 72 °C<br>4°C | 5-10 min<br>hold | 1 |

*Table 4: Cycling instructions PCR SF-1*

| Cycle step | Temperature | Time | Number of cycles |
|---|---|---|---|
| **Initial duration** | 98 °C | 30 s | 1 |
| **Denaturation**<br>**Annealing**<br>**Extension** | 98 °C<br>55-65 °C<br>72 °C | 10 s<br>20 s<br>60 s | 40x |
| **Final extension** | 75 °C<br>20°C | 5 min<br>hold | 1 |

*Table 5: Cycling instructions PCR P53*

The PCR products were evaluated by gel electrophoresis to check for the correct length of the product of interest. The agarose gel consists of:

- 50 ml 0,5 TBE
- 0,5 g 1% agarose
- 2 µl ethidiumbromid

A 100-bp ladder and a 1 kb-ladder were used to identify the approximate size of the PCR products. The agarose gel runs for 40 minutes at 80 Volt. After 40 minutes the gel was evaluated by the Bio-Rad Gel Doc 2000. In case of a correct product length, a sequencing reaction was done to confirm the identity of the transcript, using ABI3130XL Genetic Analyzer. With the program Lasergene 11 SeqmanPro the outcoming sequences were compared to the consensus mRNA of SF-1 and P53. After optimization, the cDNA in all cortisol-secreting ATs was amplified for SF-1 and P53.

- **Sequencing**

The primers used for sequencing were partly already known.[1] Four new primers for sequencing were designed for SF-1 using Perl-Primer v1.1.1.14, according to the parameters in the manual, and ordered from Eurogentech. The primers are located every 300-500 base pairs along the transcript of SF-1. The sequencing primers are listed in table 6.

| Sequence primers | Sequence (5' – 3') |
|---|---|
| **SF-1 , part 1** | |
| - **Fw 381** | CCTGAAGCAGCAGAAGAAGG |
| - **Fw 798** | CATTGTGGGCTGTCTGCA |
| - **Fw 1164** | CCAGGAGTTTGTCTGCCT |
| - **Rv 674** | GCATAGGGCTCTGGGTACTC |
| - **Rv 1073** | GTGCTCATCTCCACCTCCT |
| - **Rv 1531** | ATCAAACATCTGGAGGGCC |
| **SF-1, part 2** | |
| - **Fw 99 (new)** | CAAGATCGACAAGACGCAGCGCA |
| - **Fw 862 (new)** | CACATCTACCGCCAGATCC |
| - **Fw 142 (new)** | CTTCCAGAAGTGCCTGAC |
| - **Rv 284 (new)** | CTCCAGCTTGAAGCCATTG |
| - **Rv 539 (new)** | CATAGGGCTCTGGGTACT |
| - **Rv 1477 (new)** | ACCCACCTTCCCAAACAC |
| **P53** | |
| - **Fw -122** | AAGTCCAGAGCCACCATCC |
| - **Fw 29** | CAGTCAGAGCTCAATATCGACCCCC |
| - **Fw 461** | ATCTATAAGAAGTCGGAGTTCG |
| - **Fw 777** | GACGCAACAGCTTTGAGG |
| - **Fw 1010** | TGAATGAAGCCTTGGAGCTG |
| - **Rv 193** | ATCATCTGAGTCTTCGTCTAGCC |
| - **Rv 574** | TTCCACTCGGATGAGATGC |
| - **Rv 908** | AGGCAGTGCTCGCTTGGTAC |
| - **Rv 1123** | TTTATGGCGAGAGGTAGATTGC |
| - **Rv 1381** | CCTTTATCTCCCGACCTACC |

*Table 6: Sequence primers of SF-1 and P53*

For sequencing the PCR products the BigDye Terminator cycle sequencing was used. First the products were amplified using BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit and the C1000 touch thermal cycler. The reaction set up for cycle sequencing is listed in table 7.

| **Cycle sequencing** | |
|---|---|
| 1. | For each reaction, add the following reagents to a separate tube: |
| - | Terminator Ready Reaction Mix 1 µl |
| - | Diluted PCR product 2 µl |
| - | Primer, 3.2 pmol 1 µl |
| - | 5x Sequence buffer 2 µl |
| - | MilliQ 4 µl |
| - | Total volume 10µl |
| 2. | Mix well and spin briefly. |
| 3. | Place the tubes in a thermal cycler: with heated lid. |
| 4. | Repeat the following for 25 cycles: |
| - | rapid thermal ramp to 96 °C |
| - | 96 °C for 30 sec. |
| - | rapid thermal ramp to 50 °C |
| - | 50 °C or 55 °C for 15 sec. (depends on the annealing temp. in pcr) |
| - | rapid thermal ramp to 60 °C |
| - | 60 °C for 30 sec (+/- 1 min/ kb) |
| 5. | Rapid thermal ramp to 4 °C and hold until ready to purify. |
| 6. | Spin down the contents of the tubes in a micro centrifuge. |
| 7. | Proceed to “Sephadex purification” |

*Table 7: Reaction set up cycle sequencing*

After amplifying, the PCR product were filtrated using Sephadex G-50 superfine. The reaction set up for sephadex purification is listed in table 8.

| **Sephadex purification** | |
|---|---|
| 1. | Load dry Sephadex into all 96-wells of a Multiscreen MAHV plate using the column loader as follows: |
| - | Add Sephadex G-50 to the Column Loader. |
| - | Remove excess resin off the top of the column loader with the scraper. |
| - | Place Multiscreen MAHV plate upside-down on the top of the Column Loader. |
| - | Invert both Multiscreen MAHV plate and the Column Loader. |
| - | Tap on top or side of the Column Loader to release the resin. |
| 2. | Using a multi-channel pipettor, add 300 µl milli-Q water to each well to swell resin. Incubate at room temperature for 3 hr. |
| - | Once the mini-columns are swollen in Multiscreen plates, they can be stored in the refrigerator at 4 °C for up to two weeks, by tightly sealing the plates with parafilm. |

| |
|---|
| 3. Place a Centrifuge Alignment Frame on top of a standard 96-well microplate, then place the MAHV plate on the assembly, without lid. Centrifuge at 1900 rpm for 5 min. to pack the mini-columns. |
| 4. Carefully add 10-20 µl milliQ water to the sequencing reactions (10 µl) and pipet everything to the center of the columns. |
| 5. Tape off the unused mini-columns. |
| 6. Place the MAHV plate (without lid) on top of a sequencing plate (an MicroAmp Optical 96-Well Reaction Plate) and centrifuge at 1900 rpm for 5 min (The position of the samples must correspond with empty wells in the sequencing plate). |

*Table 8: Reaction set up sephadex purification*

After the sephadex purification, sequencing was performed using the ABI3130XL, according to manufacturer's instructions. The outcoming sequences were compared to the consensus mRNA using DNAstar Lasergene 11 SeqmanPro software.

The PCR and sequencing primers are also listed in attachment 3 & 4 with the cDNA sequence of SF-1 and P53. The reference sequences were based on AB537893.1 (P53, NCBI) and ENSCAFG00000023086 (SF-1, Ensembl). Only Ensembl has the correct cDNA sequence of SF-1.

## Results

The results of the RNA-isolation of 9 new cortisol-secreting ATs are showed in attachment 1. The last result of one carcinoma (patient Verhage) was used to produce cDNA. One sample was excluded from the study (patient Oehlert), as there was too less nanogram of RNA per microliter to produce cDNA. The amount of RNA needed for producing cDNA was measured by the amount of RNA in each sample (ng/µl). The amounts are listed in attachment 2.

### Steroidogenic factor 1

The optimal annealing temperature for the PCR primers of SF-1 (F381 & R1531) was between 64,5 °C and 63,3 °C, so 63,9 °C was taken as optimal temperature for the PCR. At this temperature no aspecific products were formed, only the product of interest. The PCR was repeated at 63,9 °C for 3 normal adrenal glands. The PCR products showed by gel electrophoresis to contain a product of the correct length (near 1200 basepairs). These products where sequenced using ABI3130XL and where confirmed to be the cDNA of SF-1.

The optimal annealing temperature for the new primers of SF-1 (F99 & R538) was also 63,9 °C. The PCR was repeated for 3 normal glands and showed by gel electrophoresis to contain a product of the correct length (near 500 basepairs) and when sequenced, they were confirmed to be the first part of the cDNA of SF-1.

One carcinoma was excluded from this study, because no PCR product could be formed.

No activating mutations in the coding region of SF-1 were found in normal adrenal glands, adenomas and carcinomas. In 3 carcinomas a silent point mutation was found. In 2 carcinomas in codon 69 basepair 373 was changed, GCG > GCA (figure 5A en 5B). In remaining a silent point mutation in codon 143 was detected, basepair 595 was changed, GGG > GGT (figure 5C). All silent point mutations occurred in heterozygous form.

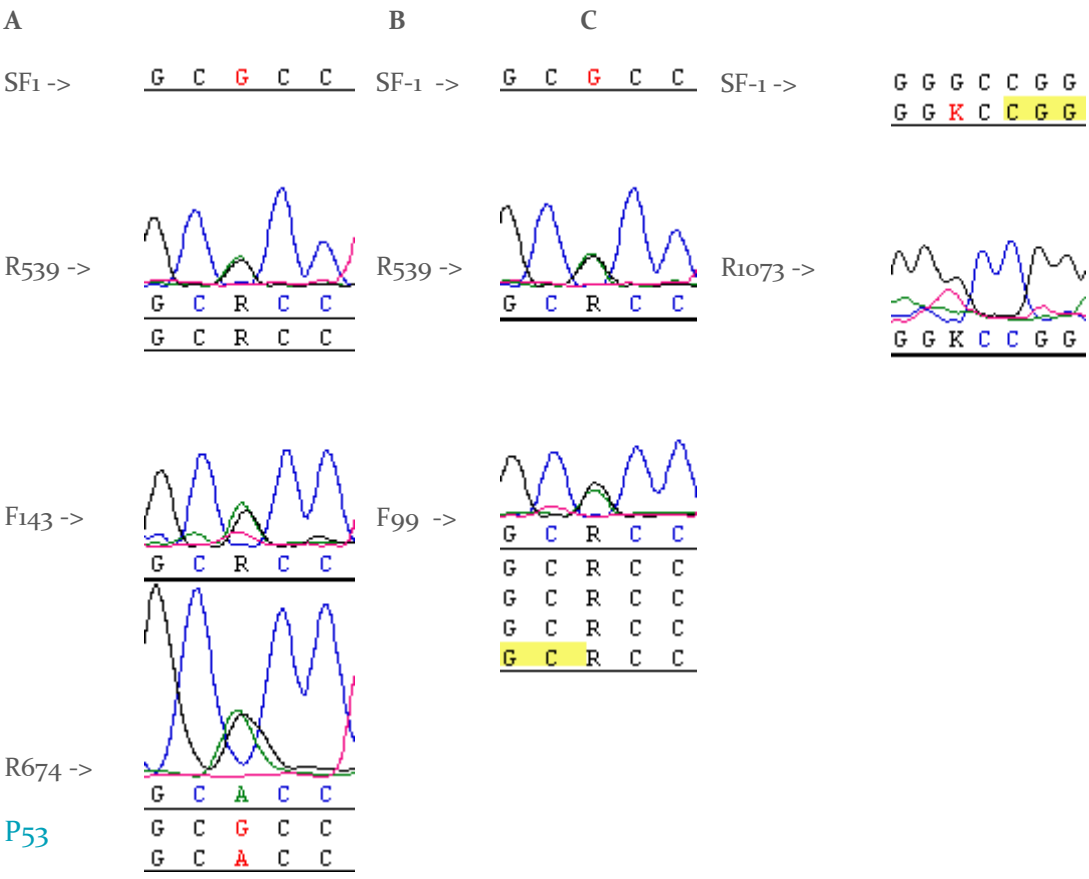


*Figure 6: SF-1 Silent mutations found in 3 carcinomas. (A) codon 69 (B) codon 69 (C) codon 143*

The optimal annealing temperature for the primers of P53 (F-122 & R1381) was between 65°C and 63,3 °C, so 64,5°C was taken as optimal temperature for the PCR. At this temperature no aspecific products were formed, only the product of interest. The PCR was repeated for 3 normal adrenal glands at 64,5 °C. The PCR products showed by gel electrophoresis a product of the correct length (near 1500 bp). These products where sequenced using ABI3130XL and where confirmed to be the cDNA of P53.

The normal adrenal glands showed no (activating) mutations in the coding region of P53.

The adenomas showed no activating mutations. In 1 adenoma a silent point mutation was found in codon 214. Basepair 798 was changed, GGC > GGT (figure 6A). One carcinoma showed an activating point mutation in codon 177 (figure 6B). Basepair 686 was changed, GCC -> GAC, which causes an amino acid change of alanine to aspartic acid. All point mutations occurred in heterozygous form.

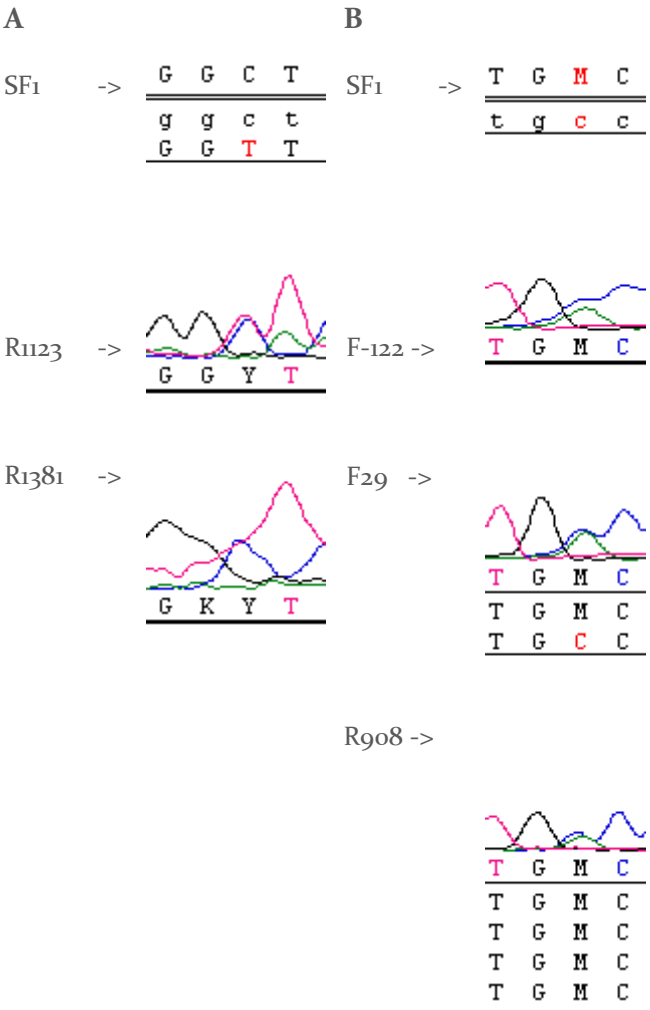


*Figure 7: P53 Silent mutation found in 1 adenoma (A) codon 214, and 1 carcinoma (B) codon 177*

## Discussion

In this study we searched for mutations in the NR5A1 gene and the P53 tumor suppressor gene of cortisol-secreting ATs. In the NR5A1 gene no functional mutations were found, so the activation of SF-1 in canine cortisol-secreting ATs is not a result of mutation. One functional mutation was found in the P53 tumor suppressor gene in 1 of 40 carcinomas.

In a previous study, mice with a NR5A1 disruption lacked adrenal glands.[12] It was also shown that an increased dosage of SF-1 increases the proliferation of adrenocortical cells, induces tumor formation in mice and decreases apoptosis of human adrenocortical cells.[15] In children with adrenocortical tumors from Southern Brazil it has been shown that the NR5A1 gene is amplified and the SF-1 protein is overexpressed.[9,10] Sbiera et all (2010) showed that a high SF-1 expression is associated with poor clinical outcome.[17] In addition it was demonstrated that SF-1 inverse agonists inhibited adrenocortical carcinoma cell proliferation.[18] These findings suggests that SF-1 possesses an important role for tumor development in men. However, recent reports indicate that SF-1 expression possesses a role in canine-cortisol secreting ATs as well.[25] The results of our study demonstrate that mutations in the NR5A1 gene do not play a role in the activation of SF-1.

In future, research into mechanisms involved in the regulation of SF-1 could be considered. Among candidates, DAX-1 and Wnt-signaling pathway could be interesting, as well as post-translational modifications of SF-1 and influence of ligands (cAMP and phospholipids). So far, no functional mutations were detected, the receptor of ACTH.[1] In about one third of the canine cortisol-secreting ATs, activating mutations in the stimulatory G protein alpha subunit gene (GNAS), which activates adenylcyclase to produce cAMP, were demonstrated and the link with SF-1 expression still needs to be evaluated.[1]

DAX-1 (dosage-sensitive sex reversal adrenal hypoplasia congenital (AHC) critical region on the X-chromosome, NR0B1) is a nuclear transcription factor and is a co-repressor of other nuclear receptors, including SF-1.[24] DAX-1 is also essential for the development and differentiation of the adrenal cortex. In humans mutations in DAX1 causes AHC. DAX-1 has a suppressive effect on SF-1 and when DAX-1 is present in high concentrations, SF-1 concentrations should be low and vice versa. A deregulation of the relationship between DAX-1 and SF-1 may also be associated with adrenocortical tumorigenesis.

The Wnt-signaling pathway also influences the SF-1 regulation.[24] The Wnt family has crucial roles in development, cell growth and differentiation. A homozygous missense mutation in Wnt4 is associated with adrenal hypoplasia in humans. Activation of the Wnt pathway leads to accumulation of β-catenin in the nucleus, which binds to SF-1 and synergistically regulates the expression of several genes, including DAX-1. SF-1 and β-catenin will stimulate DAX-1 expression which, in turn, will lead to a suppression of SF-1. An inactivation of B-catenin in SF-1 expressing cells leads to adrenal agenesis.[26] Tissier et all (2005) showed an

activation of the Wnt pathway in 21 of 39 adrenocortical tumors in human.[27, 28] 11 of these 21 tumors showed an activating mutation in the β-catenin gene. It is also seen in more cancers.

Post-translational modifications of SF-1, like acetylation and phosphorylation positively modulate SF-1 transcriptional activity, and deserve further research. Finally, increase of transcriptional activity with phospholipids, should be investigated as well.

More research needs to be done to determine whether the activation of the Wnt pathway, DAX-1 or adenylcyclase could influence the SF-1 expression in canine cortisol-secreting ATs.

Acquired mutations in the P53 gene are common findings in most major cancer types in humans and dogs. [21] In childhood ATs there's also found a P53 mutation in these tumors.[19] 35 of 36 patients had an identical point mutation of P53. The prevalence of P53 mutations in children with ATs is about 50 to 80 percent. In this study we found one functional mutation in codon 177. Codon 177 corresponds to codon 189 on the human gene and is not a hotspot codon.[29] There have been found P53 mutations in canine osteosarcoma and lymphoma.[30, 31]

## Conclusion

In this study we showed no activating mutations in the NR5A1 gene of cortisol-secreting ATs, only 3 silent point mutations. In the p53 tumor suppressor gene 1 activating point mutation was found in 1 of 54 adrenocortical tumors and 1 silent point mutation. The findings of this study suggest that mutations in these genes are not associated with adrenocortical tumor development.

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# Attachements

## 1. Results RNA isolation

Plots | Report

Test type: Nucleic Acid

2/27/2014 2:50 PM

Exit

Report Name

Max Buffer Size 200

Buffer Mode Save Report & Clear

| Sample ID | User ID | Date | Time | ng/ul | A260 | A280 | 260/280 | 260/230 | Constant | Cursor Pos. | Cursor abs. | 340 raw |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| harms | Default | 2/27/2014 | 2:35 PM | 179.28 | 4.482 | 2.159 | 2.08 | 0.49 | 40.00 | 230 | 9.089 | 0.015 |
| piet | Default | 2/27/2014 | 2:36 PM | 186.71 | 4.668 | 2.457 | 1.90 | 2.25 | 40.00 | 230 | 2.070 | 36.831 |
| piet | Default | 2/27/2014 | 2:36 PM | 128.71 | 3.218 | 1.541 | 2.09 | 1.85 | 40.00 | 230 | 1.742 | 2.893 |
| verhage | Default | 2/27/2014 | 2:37 PM | NaN | NaN | 9.749 | NaN | NaN | 40.00 | 230 | -0.085 | 46.103 |
| verhage | Default | 2/27/2014 | 2:40 PM | 374.66 | 9.366 | 6.045 | 1.55 | NaN | 40.00 | 230 | NaN | 37.343 |
| verhage | Default | 2/27/2014 | 2:42 PM | 0.38 | 0.009 | 0.009 | 1.02 | 0.61 | 40.00 | 230 | 0.016 | 0.007 |
| verhage | Default | 2/27/2014 | 2:43 PM | 241.75 | 6.044 | 2.910 | 2.08 | 0.62 | 40.00 | 230 | 9.764 | 0.010 |
| speulstra | Default | 2/27/2014 | 2:44 PM | 219.00 | 5.475 | 2.661 | 2.06 | 1.21 | 40.00 | 230 | 4.532 | 0.032 |
| molenaar | Default | 2/27/2014 | 2:44 PM | 412.88 | 10.322 | 5.154 | 2.00 | 2.11 | 40.00 | 230 | 4.883 | 0.009 |
| oehlert | Default | 2/27/2014 | 2:45 PM | 23.70 | 0.592 | 0.313 | 1.89 | 0.89 | 40.00 | 230 | 0.667 | 0.040 |
| iersel | Default | 2/27/2014 | 2:46 PM | 188.71 | 4.718 | 2.289 | 2.06 | 0.68 | 40.00 | 230 | 6.900 | 0.015 |
| raber | Default | 2/27/2014 | 2:46 PM | 201.33 | 5.033 | 2.444 | 2.06 | 0.47 | 40.00 | 230 | 10.608 | 0.031 |
| mathot | Default | 2/27/2014 | 2:46 PM | 206.06 | 5.152 | 2.515 | 2.05 | 2.03 | 40.00 | 230 | 2.537 | 0.021 |

## 2. cDNA

Component Volume per reaction, sample Mathot: 206,1 ng/microliter

| | | |
|---|---|---|
| • 5x iScript Reaction Mix | 4 | µl |
| • iScript Reverse Transcriptase | 1 | µl |
| • Nuclease-free water | 10,15 | µl |
| • RNA template(100fg to 1µg Total RNA)* | 4,85 | µl |
| • Total Volume | 20 | µl |

Component Volume per reaction, sample Raber: 201,3 ng/microliter

| | | |
|---|---|---|
| • 5x iScript Reaction Mix | 4 | µl |
| • iScript Reverse Transcriptase | 1 | µl |
| • Nuclease-free water | 10,03 | µl |
| • RNA template(100fg to 1µg Total RNA)* | 4,97 | µl |
| • Total Volume | 20 | µl |

Component Volume per reaction, sample Verhage: 241,8 ng/microliter

| | | |
|---|---|---|
| • 5x iScript Reaction Mix | 4 | µl |
| • iScript Reverse Transcriptase | 1 | µl |
| • Nuclease-free water | 10,86 | µl |
| • RNA template(100fg to 1µg Total RNA)* | 4,14 | µl |
| • Total Volume | 20 | µl |

Component Volume per reaction, sample Iersel: 188,7 ng/microliter

| | | |
|---|---|---|
| • 5x iScript Reaction Mix | 4 | µl |
| • iScript Reverse Transcriptase | 1 | µl |
| • Nuclease-free water | 9,70 | µl |
| • RNA template(100fg to 1µg Total RNA)* | 5,30 | µl |
| • Total Volume | 20 | µl |

Component Volume per reaction, sample Speulstra: 219 ng/microliter

| | | |
|---|---|---|
| • 5x iScript Reaction Mix | 4 | µl |
| • iScript Reverse Transcriptase | 1 | µl |
| • Nuclease-free water | 10,43 | µl |
| • RNA template(100fg to 1µg Total RNA)* | 4,57 | µl |
| • Total Volume | 20 | µl |

Component Volume per reaction, sample Molenaar: 412,9 ng/microliter

| | | |
|---|---|---|
| • 5x iScript Reaction Mix | 4 | µl |
| • iScript Reverse Transcriptase | 1 | µl |
| • Nuclease-free water | 12,58 | µl |
| • RNA template(100fg to 1µg Total RNA)* | 2,42 | µl |
| • Total Volume | 20 | µl |

Component Volume per reaction, sample Pieterman: 128,7 ng/microliter

- 5x iScript Reaction Mix 4 µl
- iScript Reverse Transcriptase 1 µl
- Nuclease-free water 7,30 µl
- RNA template(100fg to 1µg Total RNA)* 7,70 µl
- Total Volume 20 µl

Component Volume per reaction, sample Harmsworth: 179,3 ng/microliter

- 5x iScript Reaction Mix 4 µl
- iScript Reverse Transcriptase 1 µl
- Nuclease-free water 9,42 µl
- RNA template(100fg to 1µg Total RNA)* 5,58 µl
- Total Volume 20 µl

## 3. Sequence cDNA SF-1 Canis lupus familiaris and associated primers

**Sequentie cDNA SF-1 Ensemble**
**Coding region: 167 - 1321**

CATCCTCGGACCGGGCTCTCTCGCATTTTGCCGTCAGGCCTTTGGCAGGCCCCTGGCGCA
GAACTGCGAGCCCTACACGTGCACGGAGAGCCAGAGCTGCAAGATCGACAAGACGCAGCG
CAAGCGCTGTCCCTTCTGCCGCTTCCAGAAGTGCCTGACAGTGGGGATGCGCCTGGAAGC
TGTGCGTGCTGACCGCATGCGGGGTGGCCGGAACAAATTCGGGCCCATGTACAAACGGGA
CCGTGCCCTGAAGCAGCAGAAGAAGGCACAGATTCGAGCCAATGGCTTCAAGCTGGAGAC
GGGTCCTCCGATGGGGGTGCCTCCCCCACCGCCTCCCCCGCCGGACTACATGCTGCCCCC
CGGCCTGCACGCGCCTGAGCCCAAGGGCCTGGCCTCTGGTCCACCTGCTGGGCCGCTGGG
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TTACCTCTATCCCAGCTTCCCTGGCCGTGCCATCAAGTCTGAGTACCCAGAGCCCTATGC
CAGTCCCCCGCAGCCGGGGCCGCCCTATGGCTACCCAGAGCCCTTCTCCGGAGGGCCGGG
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CAGCCTCCTGTGCCGGATGGCTGACCAGACCTTCATCTCCATCGTGGACTGGGCACGCAG
GTGCATGGTCTTCAAAGAGCTAGAGGTGGCTGACCAGATGACACTGCTGCAGAACTGCTG
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CATCCTGCTGGTCACCGGGCAGGAGGTGGAGATGAGCACGGTGGCGGCCCAGGCGGGCTC
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GCAGAGAGTCCTGGTGGGGGCCCTGGAAGGGACTGGAGCTGAAGCTGGGGAGCCCTGGGG
GTGGGGGCTGTGGCCCGGGGCCCTAGGCCGGCTCCCCCGTCCCACTGTCTCCCTCCAGGA
ACAGCGGGGGGCGCCCCGCGGACTTCCCGGGCTCCCAGGGAGGGCGGCGCCGCCTCTCGA
CGGAGGGACTCCTCGCCTGCGCGCTGCGTGGGCCTGGGGAGGCCTGGTGGGGGGTCA

| | | | |
|---|---|---|---|
| Coding region | | | |
| Forward primer 99: | 5' CAAGATCGACAAGACGCAG 3' | (nieuw) | PCR |
| Forward primer 143: | 5' TCCAGAAGTGCCTGACAG 3' | (nieuw) | PCR |
| Forward primer 381 (247): | 5' CCTGAAGCAGCAGAAGAAGG 3' | | PCR |
| Forward primer 798 (664): | 5'CATTGTGGGCTGTCTGCA 3' | | Seq |
| Forward primer F862: | 5' CACATCTACCGCCAGATCC 3' | (nieuw) | Seq |
| Forward primer F1164 (1030): | 5' CCAGGAGTTTGTCTGCCT 3' | | Seq |
| Reverse primer 284: | 5' CCATTGGCTCGAATCTGTG 3' | (nieuw) | Seq |
| Reverse primer 538: | 5' CATAGGGCTCTGGGTACTC 3' | (nieuw) | PCR |
| Reverse primer 674 (540): | 5' GCATAGGGCTCTGGGTACTC 3' | | Seq |
| Reverse primer 1073 (939): | 5' GTGCTCATCTCCACCTCCT 3' | | Seq |
| Reverse primer 1477: | 5' ACCCACCTTCCCAAACAC 3' | (nieuw) | PCR |
| Reverse primer 1531 (1397): | 5' ATCAAACATCTGGAGGGCC 3' | | PCR |

## 4. Sequence mRNA P53 Canis lupus familiaris and associated primers

**gene** 1..2110 **CDS** 157..1302

ORIGIN

```
1    agttttgagc ttctcaaaag tccagagcca ccatcctggg cgcaggtagc cgccgggctc
61   cgggatcctc tggcgtccgg cctcggagcg tgcttcccag gacggtgaca cgctcccctg
121  aggttcggca cccacactcc cttcccagga gctgcgatgc aagagccaca gtcagagctc
181  aatatcgacc ccctctgag ccaggagaca ttttcagaat tgtggaacct gcttcctgaa
241  aacaatgttc tgtcttcgga gctgtgccca gcagtggatg agctgctgct cccagagagc
301  gtcgtgaact ggctagacga agactcagat gatgctccca ggatgccagc cacttctgcc
361  cccacagccc ctggaccggc cccctcctgg cccctatcat cctctgtccc ttccccgaag
421  acctaccctg gcacctatgg gttccgtttg gggttcctgc attccgggac agccaagtct
481  gttacttgga cgtactcccc tctcctcaac aagttgtttt gccagctggc gaagacctgc
541  cccgtgcagc tgtgggtcag ctccccaccc ccacccaata cctgcgtccg cgctatggcc
601  atctataaga agtcggagtt cgtgaccgag gttgtgcggc gctgccccca ccatgaacgc
661  tgctctgaca gtagtgacgg tcttgccccct cctcagcatc tcatccgagt ggaaggaaat
721  ttgcgggcca agtacctgga cgacagaaac acttttcgac acagtgtggt ggtgccttat
781  gagccacccg aggttggctc tgactatacc accatccact acaactacat gtgtaacagt
841  tcctgcatgg gaggcatgaa ccggcggccc atcctcacta tcatcaccct ggaagactcc
901  agtggaaacg tgctgggacg caacagcttt gaggtacgcg tttgtgcctg tcccgggaga
961  gaccgccgga ctgaggagga gaatttccac aagaaggggg agccttgtcc tgagccaccc
1021 cccgggagta ccaagcgagc actgcctccc agcaccagct cctctccccc gcaaaagaag
1081 aagccactag atggagaata tttcaccctt cagatccgtg ggcgtgaacg ctatgagatg
1141 ttcaggaatc tgaatgaagc cttggagctg aaggatgccc agagtggaaa ggagccaggg
1201 ggaagcaggg ctcactccag ccacctgaag gcaaagaagg ggcaatctac ctctcgccat
1261 aaaaaactga tgttcaagag agaagggccc gactcagact gacttccccct gcttcctgtc
1321 ctcccccaac acgcacaccc tcactctcgt cctccttccc tggcattttt ggaattggga
1381 gcttgagcac acctattccg gtgtgttact ttgcatggta cagatgtgcc tcagaaacac
1441 cctagattct tccatttgcc tttcccgtaa gtctctacta aagaagttgg cctgcacagt
1501 tggtaggtcg ggagataaag gacgttccag cctggttttt aagtttttta ctgtgtagaa
1561 ccatgggaga ggtaggaaaa tgttcctgaa tgttgggagc attttatagc ccctaagaca
1621 ctgagtcatg agcccagttc tctatcattg tagccagtgg ctgaattttt cctaatttca
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1801 ctgtgtgggt gctcattccc tgccctggcc aaatcctgtc cagccttttt gggagacccc
1861 tgcacctaag atgaaatctc actcccatct cacaccctgc agaatcccat ccctggtgta
1921 ggatctttgt ctaccaaggg cttttactgc ttccccccc ccccgccccc aattgctcag
1981 gatcaatttc ttttgcactc tgtgaggcac atctgcattc atgcattgat caccccacc
2041 cctcctcctc ttccctttt atatcccgtt tttatatcaa tctcttattt tacaataaaa
2101 ctttactacc
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| | | | |
|---|---|---|---|
| | Coding region | | |
| | Forward primer -122: | 5’ AAGTCCAGAGCCACCATCC 3’ | PCR |
| | Forward primer 29: | 5’ CAGTCAGAGCTCAATATCGACCCCC 3’ | Seq |
| | Forward primer 461: | 5’ ATCTATAAGAAGTCGGAGTTCG 3’ | Seq |
| | Forward primer 777: | 5’ GACGCAACAGCTTTGAGG 3’ | Seq |
| | Forward primer 1010: | 5’ TGAATGAAGCCTTGGAGCTG 3’ | Seq |
| | Reverse primer 193: | 5’ ATCATCTGAGTCTTCGTCTAGCC 3’ | Seq |
| | Reverse primer 574: | 5’ TTCCACTCGGATGAGATGC 3’ | Seq |
| | Reverse primer 908 | 5’ AGGCAGTGCTCGCTTGGTAC 3’ | Seq |
| | Reverse primer 1123: | 5’ TTTATGGCGAGAGGTAGATTGC 3’ | Seq |
| | Reverse primer 1381: | 5’ CCTTTATCTCCCGACCTACC 3’ | PCR |