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Andrew Wang April 10, 2017

Overexpression of MAMLD1 in Human and Dogs with Cushing's disease

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An abstract of
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Abstract

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Cushing disease is a secondary hypercortisalism caused by adrenocorticotropic (ACTH) secreting pituitary adenomas. Similar to humans, dogs, also suffer from this disorder. In an effort to map common genomic features Cushing disease between of humans and beagles, we performed whole transcriptome RNA-Seq. Comparing Cushing pituitary adenomas to normal pituitary from humans and dogs, we identified 4 genes (MAMLD1, MNX1, RASEF, TBX19) to be significantly ($P<0.05$) overexpressed in both species. Immunohistochemistry of 31 additional human pituitary adenomas revealed MAMLD1 to be strongly positively expressed in the nucleus of ACTH secreting tumor cells which are normally absent in healthy pituitary tissues. Although our cohort is relatively small, this data presents new insights into the shared genetic profile of human and beagle Cushing's pituitary adenomas and provides a rationale for potential use of a beagle model in development of precision therapeutics. Although other genomic studies have been have been recently conducted in humans with Cushing's, our results provide the first insights into the comparative genomic characterization of Cushing's disease in humans and dogs with respect to MAMLD1 overexpression.

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Introduction

The polyprotein, proopiomelancortin (POMC), packages ACTH within residential corticotropic cells that makes up 20% of cells within the anterior pituitary gland {Slominski, 2000 #166}. When ACTH is secreted through the hypophyseal veins they activate the zona fasciculata cells within the adrenal cortex to release cortisol which exerts systemic effects {Aguilera, 1994 #167}. Cellular corticotropic pituitary adenomas (PA) are rare noncancerous tumors on the pituitary gland that invade surrounding structures and secrete excessive amounts of adrenocorticotropin (ACTH) causing secondary hypercortisalism or Cushing disease (CD) {Lamberts, 1982 #168}. With an incidence five times greater in middle to late aged women, pituitary adenomas are rare (3-5:1,000,000), with approximately 10,000 new cases diagnosed per year worldwide with considerable morbidity {Steffensen, 2010 #169}{Etxabe, 1994 #170}. Phenotypic presentations of chronically elevated ACTH or cortisol include weight gain, moon face, myopathy, excessive hair growth, high blood pressure, increased blood glucose levels, erratic mood swings, poor concentration, and impaired immunological and reproductive functions {Newell-Price, 2006 #171}{Kirk, 2000 #172}{Etxabe, 1994 #173}. Untreated patients are susceptible to inflammatory diseases and cardiovascular complications {Feelders, 2012 #174}.

Treatment for CD often defaults to surgically removing the tumor followed by lifelong hormone supplementation {Mampalam, 1988 #175} {Trainer, 1993 #176} {Magiakou, 1994 #178}. However, despite neurosurgical interventions, Patil et al. demonstrated pituitary adenomas recur in approximately 17.4% of cases {Patil, 2008 #177}. To add to the diseases complication, studies have shown in 30% of identified CD cases, surgery may not be an option as surgeons are unable to physically locate the PA for resection {Guiot, 1976 #179}. Although noninvasive treatment options are available, they often result in generalized systemic effects, prohibiting their use in

individuals who have a history of hyperglycermia, cholelithiasis, cardiovascular complications, and other steroid hormone disorders {Bertagna, 2013 #180} {Feelders, 2010 #181} {Tritos, 2011 #182}. Therefore, a noninvasive treatment through specifically targeted protein inhibition would be of interest to help manage the pathogenesis of CD.

With CD being a rare phenomenon, only a few dysfunctional genes (e.g. *POMC*) have been linked to the condition. However, these genes are not viable options for targeted interventions due to a majority of these genes possessing multifunctional roles within the body {Slominski, 2000 #166}{Fragoso, 2003 #183}{Bilodeau, 2006 #184}{Stratakis, 2010 #185}{Reincke, 2015 #187}{Ma, 2015 #188}. This issue prompted us to investigate a more complete catalogue of genomic aberrations that may lead to dysfunctional protein expression observed in CD that could potentially lead to the development of targeted pharmaceutical interventions.

Dogs possess an 84% genetic overlap with humans, and they naturally develop Cushing's disease with incidence (1500:1000000) and recurrence (8.2%) risks analogous to humans. {de Bruin, 2009 #189}{Lindblad-Toh, 2005 #190}{Karlsson, 2008 #27}{Krzywinski, 2009 #192}. The use of comparing a dog model (beagles) to humans has advantages over transgenic mouse models, particularly the fact that dogs shares similar environmental exposures and diet to humans {Karlsson, 2008 #27}.

Utilizing both human and dog pituitary adenoma and normal pituitary tissues, we chose to comprehensively assess the transcriptomes of the 2 species using RNA-Seq. Whole Exome Sequencing (WES) was also performed on the human CD specimens. Although genetics studies have been previously conducted in humans with CD {Evans, 2001 #186}{Slominski, 2000

#166}{Fragoso, 2003 #183}{Bilodeau, 2006 #184}{Stratakis, 2010 #185}{Reincke, 2015 #187}{Ma, 2015 #188}, our results provide the first direct comparisons of human and dogs.

Methods

Human cohort

This study was approved by the Emory University Institutional Review Board (IRB00045827). Thirty two patients with PAs (avg lateral 1.16 +/-0.44 cm x AP 0.88 +/-0.29 cm x cranial 0.9 +/-0.49 cm) were recruited through the Department of Neurosurgery at Emory University Hospital. Phenotypic presentations of CD were based on symptoms of hypercortisolism (weight gain, moon face, myopathy, excessive hair growth, erratic mood swings, poor concentration, and impaired immunological and reproductive functions) and biochemical testing (elevated level of blood pressure, blood glucose, free cholesterol, and low levels of CRH). Pathology reports indicated immuno positive ACTH, negative TP53, FSH, TSH, LH, GH, and MIB-1 proliferation index <3%. Blood testing revealed elevated ACTH levels (PA avg 72.92 +/-43.91 pg/ml > normal avg 5-25 pg/ml). Additional ACTH stimulation testing, low dose dexamathasone suppression (LDDS) testing, and MRI images confirmed secondary hypercortisolism caused by a PA. Frozen specimens were available for 6 patients, and those were used for nucleic acid isolation and sequencing. Normal human pituitary specimens from one male (ND01199-09) and one female (ND01218-06) were purchased from the Coriell Biorepository (Camden, NJ).

Dog cohort

Beagles with ACTH secreting PAs were recruited through the Department of Clinical Sciences of Companion Animals – General Surgery at the University of Utrecht. Pituitary specimens from 6 healthy beagles and 7 Cushing PAs were studied. Through owner consents, the ethics committee approved the study. Phenotypic presentations of CD manifested as hypercortisolism (hair lose, pot belly, thinning skin, skin infections, dehydration, and inactiveness). Diagnosis of

pituitary dependency confirmed CD through pathology reports of an ACTH secreting tumor, ACTH stimulation testing, LDDS testing, and/or MRI images of PA.

Nucleic acid extraction and sequencing

RNA and DNA were extracted from human and dog frozen tissues using E.Z.N.A.® kits (Omega Bio-tek, Norcross, GA).Specimen quantity and quality were assessed using Agilent Bioanalyzer and Qubit.

Human exome sequence analysis

One hundred basepair paired end fastq files were aligned to the human hg19 reference genome using BWA 0.7.5.a; duplicate reads were removed with Picard tools version 1.111 Li and Durbin, 2009]. Deduplicated bam files were used for copy number estimation using Control-FREEC with recommended setting for exome sequencing. Normalized FREEC output values were segmented with DNACopy. We note that, as only one matched normal sample was available, the same control sample was used for all six Cushing's samples. Mutations in all samples were called using Varscan2 without a matched normal {Koboldt, 2012 #194}. Human Cushings 1, had a matched normal sample and mutations were called using Varscan2 in somatic mode. This sample was also analyzed with Mutect version 1.1.4 using standard parameters following probabilistic indel realignment and base recalibration using GATK version 3.3.0. All predicted mutations were annotated using ANNOVAR.

RNA sequence analysis

Fastq files were aligned to the hg19 human or CanFam3 reference genome using Tophat 2.0.1 unders standard parameters. Human refseq and ensembl transcripts were quantified using Cufflinks 2.0.1. Human refseq transcripts were also quantified using HTSeq 0.6.1. Mutations were called in the dog samples using Varscan2 as above.

Immunohistochemistry

Thirty one paraffin blocks of tissues were sectioned at 5 μm. Immunostaining was performed on formalin fixed paraffin embedded (FFPE) sections. Slides were deparaffinized in xylene and tissue was hydrated by a descending ethanol sequence. After rehydration, slides were incubated with 3% H_2O_2 to inactivate endogenous peroxidases and blocked with 1% BSA for 10 mins. Antibodies against TBX19 (Sigma Aldrich, rabbit 1:125), MAMLD1 (Sigma Aldrich, rabbit 1:250), MNX1 (Sigma Aldrich, rabbit 1:500), RASEF (Sigma Aldrich, mouse 1:200), USP8 (Sigma Aldrich, rabbit 1:100), USP48 (Sigma Aldrich, rabbit 1:100), and POMC (Abcam, rabbit 1:8000) were used. The antibodies were then incubated at 4°C overnight and visualized with diaminobenzidine. Image analysis was performed under a light microscope at x400 magnifications.

Immunohistochemistry scoring

Scoring was complete in concordance to the 0-3+ scoring system. Score of 0 indicated negative immunostaining. Mild positive (1+) showed weak immunostaining of less 30% of tumor cells. Positive (2+) represented complete membranous staining and either uniform or weak in at least

50% of cells. Finally, strongly positive (3+) showed uniform intense nuclear and/or cytoplasmic staining in at least 80% of cells.

ACTH determination

If not stated otherwise, ACTH was determined by immunostaining, blood testings and/or LDDS. ACTH values were normalized and compared to normal healthy ACTH levels and are presented as pg/ml. LDDS testing of either low-dose (1-2 mg) and high-dose (8 mg) variations were administered and the levels of cortisol are measured to obtain the results.

Statistical analysis

RNA seq results are expressed as lower quartile (Q1), median (Q2) and upper quartile (Q3). Statistically significant results between groups were considered when $P < 0.05$ in a one tail T-test.

Results

Whole Exome Sequencing

To identify single nucleotide variants and recurrent mutations that could be associated to CD PAs, we performed whole-exome sequencing on 6 human ACTH secreting PA. Tumor specific variants, such as somatic point mutations, and loss of heterozygosity events, were identified. Ensembl VEP was used to determine the effect of the identified variants on genes, transcripts and protein sequence, as well as regulatory regions. As a result, whole exome sequencing revealed widespread aneuploidy (red: copy number gain, blue: copy number loss) among 6 human CD corticotroph adenomas [Figure 1].

Utilizing VarScan 2, the copy number profile of the human samples are displayed [Figure 1]. We selected copy number variation (CNV) based on at least 1x fold-change in copy number in a given candidate. As expected, since these tissues were acquired from female patients, Cushing's 2-6 showed gains in the X chromosome as compared to Cushing's 1 which was from a male. Cushing's 1 showed gains in 6p, 7, 12p, and 14 with minor losses in 19. For Cushing's 2 gains were in 1q, 5, 7-9, 12, and 14 with loss in 18. A minor loss was observed in Cushing's 3 with no notable gains. Cushing's 4 was characterized as more losses (6, 16, 21, 22) than gains (13). Conversely, Cushing's 5 displayed gains in 3q, 5, 6q, 8, 9p, 12, and 13, as compared to weak loss events in 15q, 16, and 22. Cushing's 6 showed variable minor gains in chromosomes 4-7, and 13, with few losses in 18q and 19p.

As demonstrated, the copy number data of Cushing's PA showed relative conserved interchromsomally changes across chromosomes 10 and 11. Gains were predominantly found in

chromosomes 5, 7, 8, 12, 13, and 14. Majority of losses were found across chromosomes 15, 16, 19 and 22. With the copy number primarily showed more losses in Cushing's 4, few structural abnormalities in Cushing's 3, and minor variable distribution changes in Cushing's 6. Taken together, there was weak concordance of arm-level copy number in patients 3, 4 and 6 as compared to patients 1, 2 and 5. Although a definitive trend was unable to be drawn due to the small sample size, Cushing's PA generally showed more copy number gains.

RNA-Sequencing

To investigate the differential gene expression (DGE) in patients with CD, RNA-seq was performed for transcriptome profiling on 6 human and 7 dog Cushing's samples. Molecular fragment strands were normalized by the number of transcripts and number of base pair of length to each gene using the human and dog reference genome to produce fragments per kilobase of transcripts per million mapped reads (FPKM) values.

Unsupervised hierarchical clustering of RNA-seq FPKM values of Cushing's PAs compared to normal PAs in dog and human demonstrated heterogeneity of widespread aneuploidy among CD PA [Figure 2]. Genes that were generally overexpressed in both Cushing's models were underexpressed in normal. Conversely, genes that were more expressed (red) in normal had lower FPKM values (blue) in Cushing's. In concordance to FPKM expressions across the genes in Humans, Cushing's 1, 4 and 5 were grouped more similarly as compared to Cushing's 2, 3, and 6 [Figure 2A]. In contrast, Cushing's dog samples were categorized into two groups (Cushing's 21 and 23; Cushing's 22, 25 and 24) with a subset Cushing's group (Cushing's 20 and 26) showing a closer DGE profile with the normal healthy group [Figure 2B]. Taking A and

B together, human and dog Cushing's demonstrate similar DGE profiles as compared to the healthy normals.

Comparing 504 highly expressed and 1434 under expressed genes in humans (red) with 123 highly expressed and 800 under expressed genes in dogs (blue), RNA-seq results revealed 14 highly expressed genes and 239 under expressed shared (purple) genes in both human and dog Cushing's samples [Figure 3]. By aligning the mRNA fragments to the respect models reference genomes revealed the identity of the 14 shared highly expressed genes as AVPR1B, CEP55, FAM131B, FZD9, GLDC, MAMLD1, MNX1, POMC, PTGER4, RASEF, TBX19. TK1 and VIPR2.

In order to elucidate if these potentially 14 commonly highly expressed genes in Cushing's tissues (red) are abnormally expressed than to normal (blue), we compared the FPKM of the 14 genes in 6 human cushing (HC) to 2 human normals (HN), and 7 dog cushing (DC) to 6 dog normals (DN) [Figure 4]. Overall, Cushing samples in humans consistently showed greater FPKM values as compared to normal (blue) [Figure 4A] with dogs following a similar trend [Figure 4B]. This demonstrates these 14 genes generally possess a greater transcription fraction percentage. In the majority of H/DC genes, 75% of the samples displayed more transcriptome fragments than H/DN genes. The Q1 and Q3 of H/DC genes are comparatively longer than H/DN genes indicating a high level of variance and disagreement between Cushing samples. Interquartile examination shows H/DC genes possessing a greater range compared to H/DN. Hence, reinforcing the high degree of differential variance and gene expression in Cushing PAs.

However, with uniformly elevated FPKM values in the medians, H/DC genes showed increased transcription in the potentiated mutated genes of interest as listed above.

Statistical analysis in human samples revealed significant ($P<0.05$) results for TBX19, RASEF, MAMLD1 and MNX1. In dogs, genes POMC, TBX19, BIRC5, RASEF, TK1, GLDC, CEP55, FAM131B, MAMLD1 and MNX1 yielded significant results ($P<0.05$). Thus, TBX19, RASEF, MAMLD1 and MNX1 were significant ($P<0.05$) in both models. Our study provides the first direct observation of RNA-seq gene expression of PA in dog and man with CD.

Immunohistochemistry

To determine if significantly differentially overexpressed genes (TBX19, RASEF, MAMLD1, MNX1) are observable at the protein level, IHC was performed on 31 human Cushing's tissue and scored from 0 to 3+. In addition, due to literature suggesting other genes that may be linked to CD, POMC, USP8, and USP48 were stained for {Slominski, 2000 #166} {Reincke, 2015 #187}{Ma, 2015 #188}.

Figure 5 gives a detailed summary of the IHC. Genes TBX19, MAMLD1, RASEF, and USP48 stained nuclear [Figure 4B-D,G,H, K, L). Only POMC stained cytoplasmic [Figure 4A]. Both MNX1 and USP8 stained nuclear and cytoplasmic [Figure 4E,F,I,J]. Panels C, E, G, I, and K scored 1+ whereas the genes direct counterparts D, F, H, F and L scored 3+. Significant genes in both human and dogs from the RNA seq data that scored 2+ were MAMLD1 (2.58+/-0.72) and MNX1 (2.10+/-0.83), while TBX19 (0.06+/-0.35) and RASEF (1.55+/-0.93) displayed weak

staining. Genes of interest POMC (2.77+/-0.56), USP8 (2.52+/-0.85), and USP48 (2.32+/-0.83) consistently scored over 2.3+.

With MAMLD1 demonstrating the highest level of protein expression amongst the significant DGE genes, the gene was further examined. Although 1+ staining occurred in 4 Cushing's tissues [Figure 6A], 5 tissues a scored 2+ and 22 tissues scored a 3+ [Figure 6B] yielding an average of 2.58+/-0.72 nuclear staining. Human Cushing's 1, 3, 6 with FPKM levels 54.55, 53.56 and 66.21 scored 3+, whereas Cushing's 2 and 5 scored 1+ and 2+ with FPKM values of 18.84 and 8.69, respectively [Figure 6]. Moreover, MAMLD1 seems to only exclusively stain tumor tissues as surrounding normal tissues were negative (0) [Figure 6C].

Discussion

Whole Exome Sequencing

Although ACTH secreting PA are sporadic in nature, genetic alterations have been implicated in its tumorigenesis {Evans, 2001 #186} {Slominski, 2000 #166} {Fragoso, 2003 #183}{Bilodeau, 2006 #184}{Stratakis, 2010 #185}{Reincke, 2015 #187}{Ma, 2015 #188}. Our study revealed widespread copy number heterogeneity among our Cushing's samples with a general trend towards copy number gain. The number of somatic mutations per case were low, and no changes were observed in chromosomes 10 and 11. Previous studies, have linked USP8, GNAS, and AIP somatic mutations to PA {Slominski, 2000 #166} {Fragoso, 2003 #183}{Bilodeau, 2006 #184}{Stratakis, 2010 #185}{Reincke, 2015 #187}{Ma, 2015 #188}. In spite of the fact that none of these gene mutations were observed in more than 1 sample, we expect these mutations would be found if we increase our sample size for exome sequencing. Collectively, our study found diverse gene mutations in ACTH secreting PA, suggesting CD may result from genetic alterations, and that each tumor to be pathologically distinct.

Recurrent mutations in deubiquitnating enzymes

In two independent human studies completed by Reike et al. (2015) and Ma et al. (2015), ~54% (n= 12/22) of ubiquitin-specific protease 48 (USP8) mutations were linked to CD by early activation of the EGFR signaling through increasing the genes deubiquitinating enzyme function clustering at the nucleus {Reincke, 2015 #187} {Ma, 2015 #188}. To determine if our cohort shared similar genetic characteristics, we subjected 6 human CD samples to WES in order to reveal altering somatic mutations. In spite of this key piece of information, chromosome 15, 14-3-3 USP8 mutations were not observed perhaps due to our sample size. In addition, our IHC

score revealed USP8 (2.52+/-0.85) localizations in the nucleus and cytoplasm Our results are consistent with Sbiera et al. (2016) which performed WES on 28 Cushing's dogs and found no USP8 mutations with IHC score of ~1.74+/-0.20 within the same cellular compartments {Sbiera, 2016 #195}. Taking these conflicting results together, the mutation driving these cases are still unknown. Nonetheless, although no USP8 mutations were found, interestingly, 2 out of 6 Cushing PA had somatic missense mutations in a similar family gene ubiquitin-specific protease 48 (USP48) was identified and found to localize in the nucleus (2.52+/-0.83). In both tumors single point mutations were found on chromosome 1 located at p.M415I/V. The affected amino acids were found to be highly conserved across a variety of vertebrate species. All mutations were located between amino acid 415, close to the protein's catalytic domain. Whole-exome and targeted sequencing indicated that both the wild-type and mutant alleles were present in tumor tissue, consistent with a heterozygous state of the USP48 mutation. These results suggest, while human and dog cotricotrophic adenomas share similar genetic backgrounds, our results show the mutations in the USP family seems to play a role for the pathogenesis of CD in both species suggesting a possible end point convergence.

RNA-Sequencing

Genetic alterations have been implicated in ACTH secreting PA's tumorigenesis {Evans, 2001 #186} {Slominski, 2000 #166} {Fragoso, 2003 #183}{Bilodeau, 2006 #184}{Stratakis, 2010 #185}{Reincke, 2015 #187}{Ma, 2015 #188}. Our current study revealed that human and dog CD demonstrated similar DGE profiles. Although variations are observed among the Cushing's groups, generally in both species, sites where FPKM were lower in normal were higher in Cushing's, and sites where expression was higher were lower in Cushing's. Amongst the

Cushing's groups, samples may be subdivided based on similar DGE profile. This suggests perhaps there are Cushing's subtypes that may lead to certain patients experiencing similar clinical outcomes. Another plausible explanation would be that DGE may depend on the onset and progression of the presented Cushing's disease, and that patients or dogs in a Cushing's subgroups clustering together represents a similar history or timeline of the illness. To the same merit, Cushing's grouped more similar to normal tissues may suggest either during tissue preparation not enough tumor was collected or these patients were diagnosed with Cushing's disease at a much early stage.

Collectively, human and dog Cushing's PA harbor 14 highly expressed genes. These tumor specific novel and functional variants were analyzed and linked to previous literature CEP55, BIRC5, and TK1 to cellular growth {Fabbro, 2005 #196}{Tracey, 2005 #197}{Schwartz, 2004 #198}; MNX1, MAMLD1, and TBX19, to transcription factors {Dalgin, 2011 #199}{Flanagan, 2014 #200}{Fukami, 2008 #201}{Liu, 2001 #203}; AVPR1, and VIPR2 to Phospholipase C activation{Tamma, 2013 #204}{Assis, 2014 #205}; VIPR2, and PTGER4 to PKA initiation{Åkesson, 2005 #206}{Qu, 2015 #207}; GLDC to mitochondrial Ca 2+ signaling {Boneh, 2005 #210}; RASEF, FAM131B to ERK and IKK complex{Miller, 2016 #211}{Cin, 2011 #212}; FZD9 to WNT pathway{Winn, 2006 #209}; and POMC to ACTH synthesis {Slominski, 2000 #166}. With a high degree of these 14 proteins associated to primarily the ERK, IKK, WNT and Notch signaling pathways, this suggests a dysfunctional expression in any of these genes could lead to downstream cascading effects that may lead to the pathogenesis of an ACTH-secreting coticotrophic adenoma.

After performing statistical analysis on the FPKM values by comparing the CD to normal, genes MAMLD1, MNX1, RASEF, and TBX19 were significantly ($P<0.05$) in both models. In addition, POMC, TK1, GLDC, CEP55, and FAM131B were only found significant ($P<0.05$) in dog CD tissues. We hypothesize this discrepancy was due to perhaps there is a large difference in the number of significant genes in humans (4) as compared to dogs (10) due to the dogs having more normal controls to help adjust for the small sample size. Nonetheless, by elucidating 4 significant commonly overexpressed RNA transcripts on CD, we were interested in examining if differentially expressed mRNA conferred at the protein level to exert a biological functional significance leading to CD.

Proopionmelanocortin

Throughout this experimentation, although POMC was not found to be significant in human Cushing's samples (n = 2) but was significant in the dog the RNA-seq data, POMC was strongly positively expressed in the cytoplasm (2.77 +/-0.56) and served as our positive control due to its role in ACTH secretion. We hypothesize perhaps POMC was not found significant in humans due to a the tissue sample being small and expressing extremely low levels of mRNA transcription to skew the data. Through IHC confirmation of POMC, our results become consistent with the previous studies demonstrating POMCs role in ACTH secretion leading to the production of cortisol in the adrenal cortex.

Immunohistochemistry

Across 32 human Cushing's samples, an immunohistochemistry score rating from 0-3 was assessed and results revealed Mastermind Like Domain Containing 1 (MAMLD1) to the most overexpressed in CD at an average of 2.58 +/-0.72 followed by MNX1 (2.10+/- 0.83), RASEF (1.55+/-0.93) and TBX19 (0.06+/-0.35). Although not much else is known about MAMLD1, studies have found MAMLD1 to be found on the X chromosome with 774 amino acids and weighing ~83kDa {Fukami, 2008 #201}. Acting as a transcriptional co-activator in NOTCH signaling, Shimojo et al. (2011) found MAML family binds to the repressor protein RBPJ that modulates HES expression within the nucleus {Shimojo, 2011 #214} {Nam, 2006 #213}{Monahan, 2009}. MAMLD1 KO studies have been linked to the congenital disorder hypospadias {Chen, 2010 #202}. Moreover, IHC results demonstrate MAMLD1 stained strongly in the nucleus and only on tumor tissues. This result is consistent with MAMLD1's role as a transcriptional co-activator during pituitary neurodevelopment, and would expect grown healthy adults to not have MAMLD1 expressions in their normal corticotropic cells {Zhu, 2007 #215}. Since ACTH secreting corticotropic cells show MAMLD1 expression, this suggests MAMLD1 may be linked to the abnormal corticotropic cell development.

Pathogenesis of CD with MAMLD1 mutations

We propose increased expression of MAMLD1 allows for increased translation HES. Normally, the repressor protein RBPJ inhibits HES expression [Figure 7I]. Since studies have suggested MAMLD1 binds to RBPJ, perhaps MAMLD1 binding releases the RBPJ gene suppressor complex allowing the expression of HES [Figure 7II]. During neuronal development, HES seems to be involved in corticotrophic proliferation and differentiation within the pituitary glands via

turning off cell cycle inhibitors, and provides negative feedback to self inhibits HES1's cell cycle regulatory element suppression {Monahan, 2009 #217}. Knockout studies conducted by Goldberg et al. (2011) have shown HES KO leads underdeveloped hypermorphic pituitary {Goldberg, 2011 #218} {Monahan, 2009 #217}. Thus, perhaps in CD PAs, overexpression of MAMLD1 not only allows for HES expression but also negates the HES1 negative regulatory feedback by preventing upstream repressive protein binding. With Monahen et al.'s (2009) study on HES with MAMLD1 and Zhu et al.'s (2007) study on Notch signaling pathway regulating corticotrophic differentiation, this suggests abnormal ACTH-secreting corticotrophics are produced leading to adenomas {Monahan, 2009 #217} {Shimojo, 2011 #214} {Zhu, 2007 #215}. Interestingly, in Oyama et al. (2001) paper, NEUROD1 activates the transcription of POMC which eventually leads to the production of ACTH {Oyama, 2001 #216}. Thus, another mode of action of the pathogenesis of CD is through the corticotrophic differentiation induced by HES1-MAMLD1 complex, NEUROD1 elements may become overexpressed. Moreover, in relating the Notch proposed mechanism with recent literature in identifying EGFR/ERK signaling causing CD from Reike et al. (2015) and Ma et al. (2015), a study done by Trembley et al. (2013) has shown ERK may promote Notch signaling {Reincke, 2015 #187} {Ma, 2015 #188} {Tremblay, 2014 #219}. Taking these results together, it is plausible perhaps both Notch and ERK pathway act in concert to increase the expression of MAMLD1 to drive unregulated corticotrophic differentiation. For future studies, it would be of great interest to explore these potential mechanistic pathways that may govern the pathogenesis of CD.

Our findings have several important clinical implications. Since CD PAs may recur and may be undetectable, excess morbidity may persist that decreases the quality of life of those suffering

from CD {Feelders, 2012 #174}. Thus, an improved targeted noninvasive therapies are of high interest. Our study suggests perhaps there are overexpressed proteins that are disrupting the cellular cycle leading to abnormal differentiation of corticotrophs. With further future testing behind our proposed pathway, we hypothesize MAMLD1 inhibitor supplement would help decrease and manage ACTH secretion to better manage CD. Moreover, since MAMLD1 seems to only stain for corticotropic tumor cells, MAMLD1 staining may be utilized to diagnose CD in neuropathology departments in hospitals. Furthermore, with the results from the human and dog models demonstrating similar genetic profiles for CD, our investigation strongly supports the usage of dogs as a feasible model for human CD for continued Cushing's profiling and clinical trials studies. Taken together, the findings from our study suggests genetic alteration in PAs contributes to the differentiate gene expression of MAMLD1 leading to corticotrophic ACTH secreting tumorgenesis.

Figures and Graphs

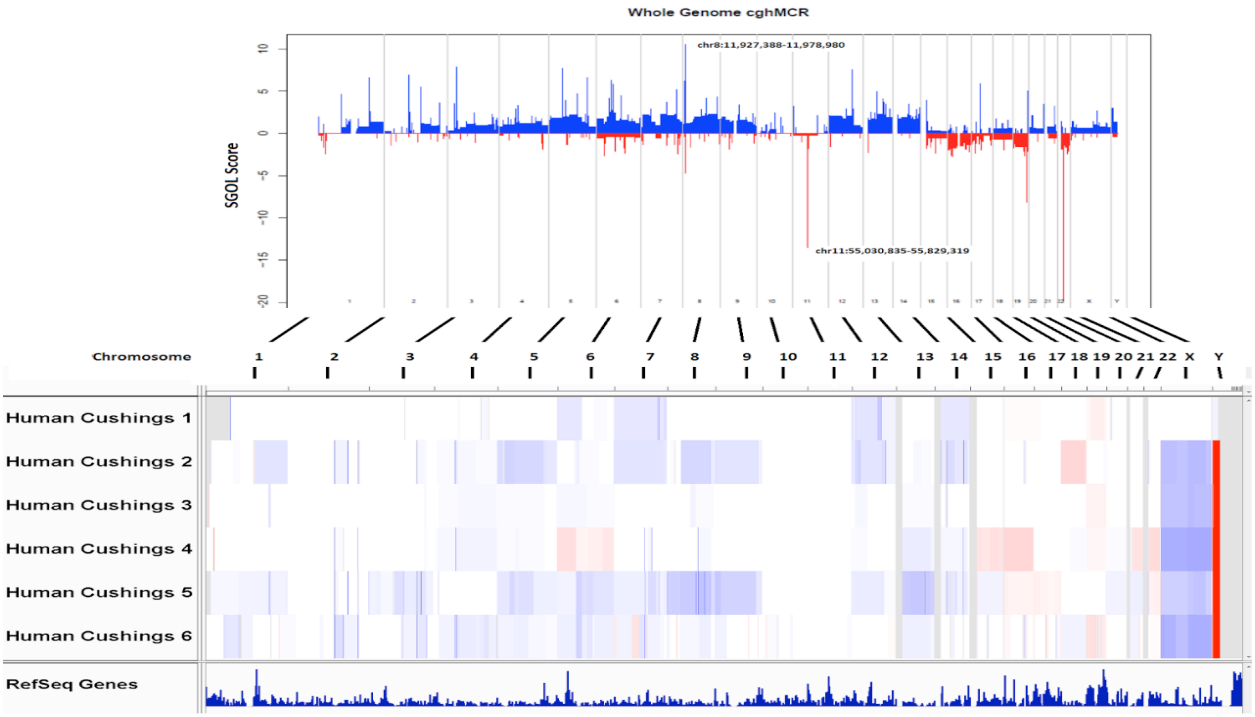


Figure 1: Copy number variation of somatic mutations in corticotroph adenomas. Whole Exome Sequencing of 6 human Cushing pituitary adenoma specimens. Copy number gains (blue) and losses (red) are shown as a GISTIC profile (top) and for individual specimens. Prominent regions of gain (chr8:11,927,388-11,978,980_hg19) and loss (chr11:55,030,835-55,829,319_hg19) highlighted in the GISTIC profile represent regions of benign copy number variation. We were unable to identify any focal gains or losses associated with pituitary adenoma using these 6 Cushing specimens, but gain of chromosomes 5, 7, 8, 9, 12, 13 and 14 and loss of chromosome 19 were recurrent copy number abnormalities.

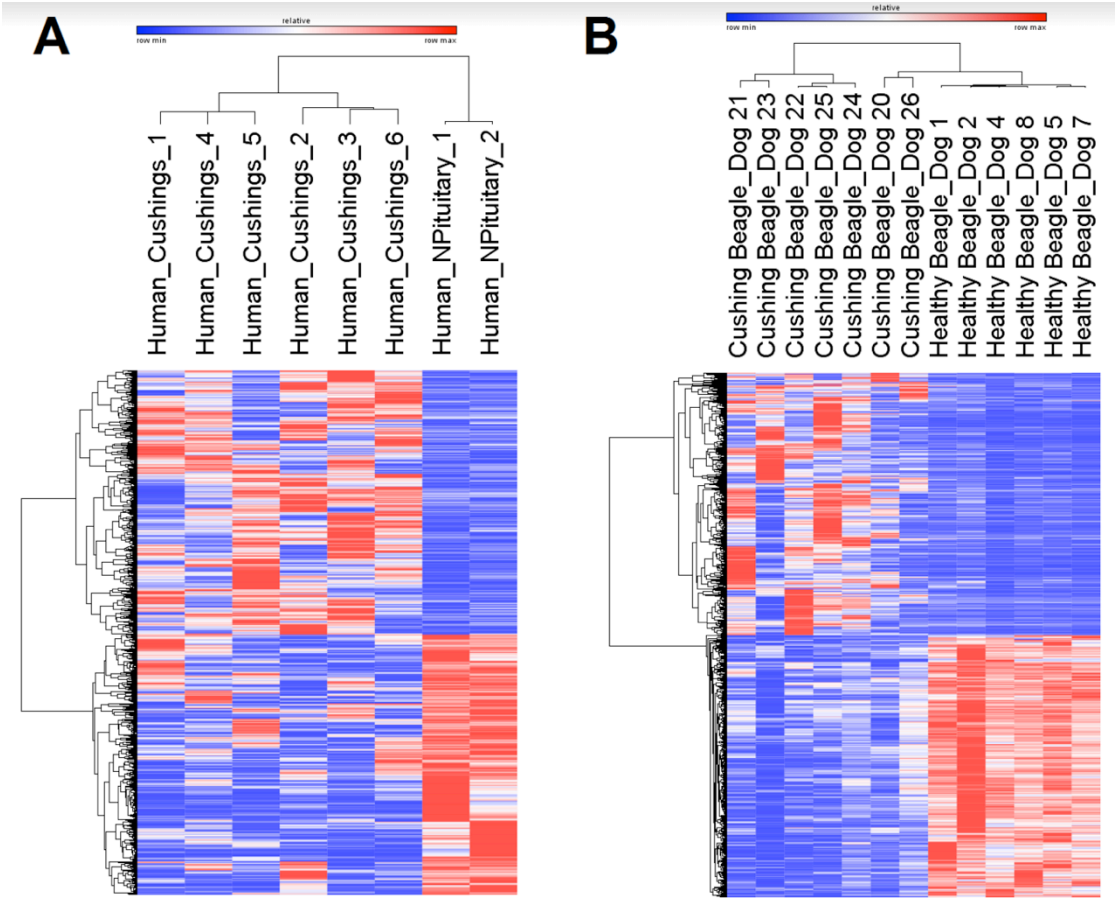


Figure 2: Unsupervised hierarchical clustering of differentially expressed genes in Cushing pituitary adenoma vs normal pituitary in dog and man. Normalized RNA-Seq FPKM values of genes were subject to unsupervised hierarchical clustering comparing 6 human Cushing adenomas to 2 normal human pituitary specimens (A) and 7 dog Cushing adenomas to 6 normal dog pituitary samples (B). Differential gene expression (DGE) profiles clearly separated the Cushing from normal pituitary for dog and man specimens.

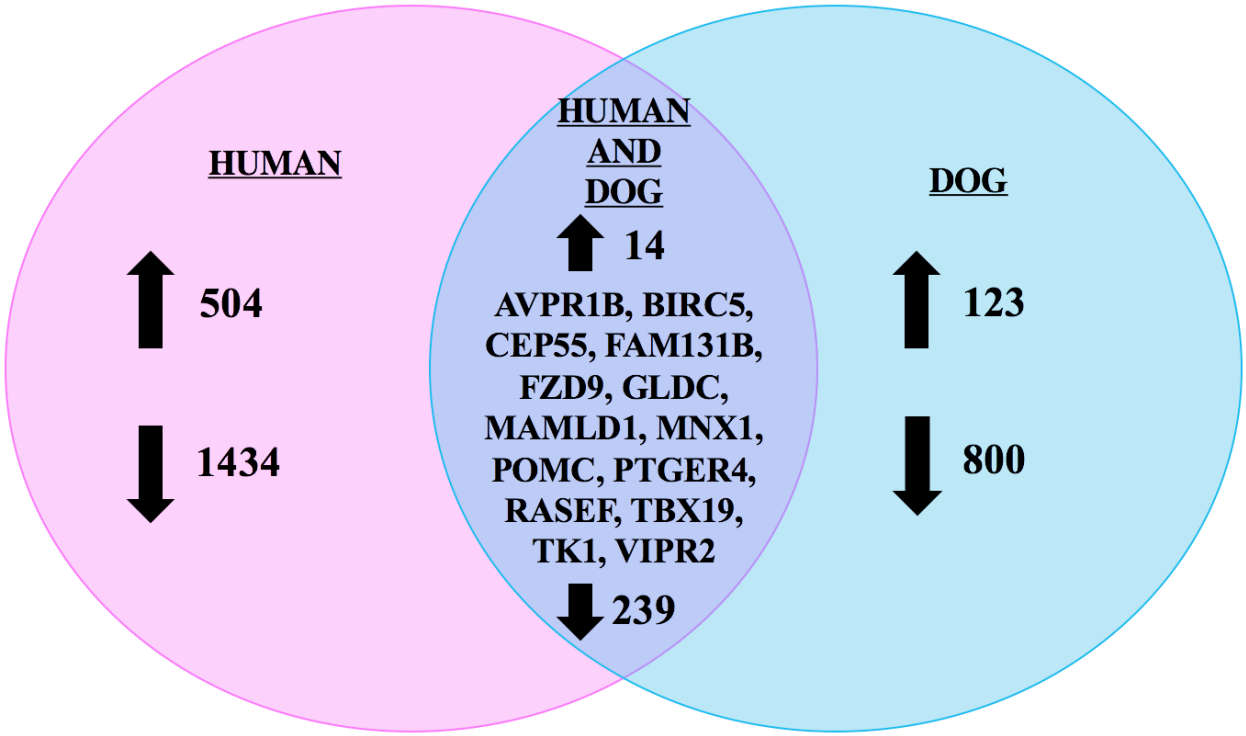


Figure 3: Shared gene expression changes in humans and dogs with Cushing disease. Genes were grouped by overexpression (>2-fold compared to normal) and underexpression (<2-fold compared to normal) of normalized RNA-Seq FPKM levels of human (pink) and dog (blue) specimens. We identified 504 and 1434 human genes that were overexpressed and underexpressed, respectively, and 123 and 800 dog genes that were overexpressed and underexpressed. Overlap of the 2 datasets demonstrated 14 genes that were overexpressed and 239 that were underexpressed in dog and man. The 14 commonly overexpressed genes were: *AVPR1B, BIRC5, CEP55, FAM131B, FZD9, GLDC, MAMLD1, MNX1, POMC, PTGER4, RASEF, TBX19, TK1,* and *VIPR2*.

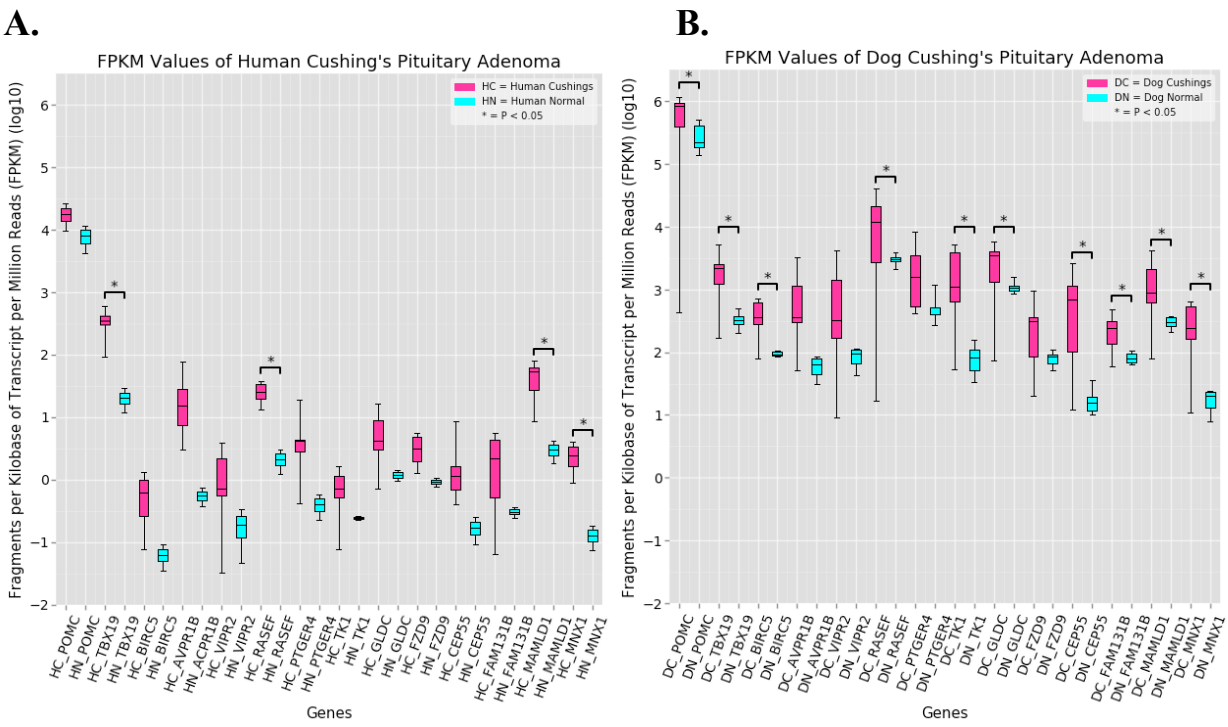


Figure 4: Boxplot comparison of overexpressed genes in humans and dogs. Comparisons are shown for human Cushing (HC, n=6), normal human pituitary (HN, n=2), dog Cushing (DC, n=7), and normal dog pituitary (DN, n=6). Normalized FPKM levels for 14 overexpressed genes Cushing (pink) and normal pituitary (blue) are shown for humans (A), and dogs (B). MAMLD1, MNX1, RASEF, and TBX19 were significantly overexpressed (*) in Cushing as compared to normal pituitary ($P<0.05$) in both human and dog specimens. POMC, TK1, GLDC, CEP55, and FAM131B were significantly ($P<0.05$) overexpressed in only dog tissues.

Panels	Protein	IHC Scoring H_n=31	Cellular Staining Site
A	POMC	2.77	Cytoplasmic
B	TBX19	0.06	Nuclear
C,D	MAMLD1	2.58	Nuclear
E,F	MNX1	2.1	Nuclear + Cytoplasmic
G,H	RASEF	1.55	Nuclear
I,J	USP8	2.52	Nuclear + Cytoplasmic
K,L	USP48	2.32	Nuclear

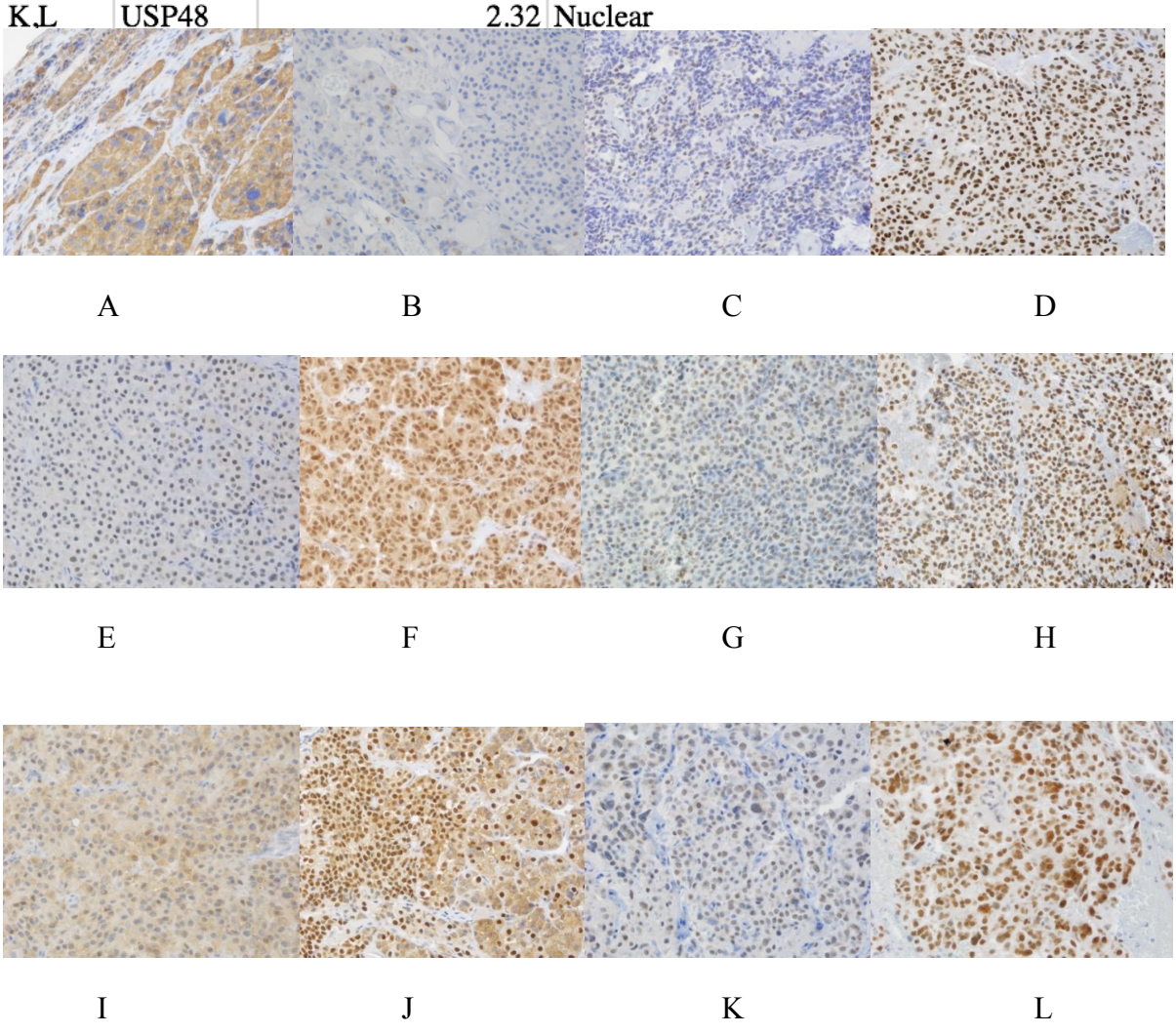
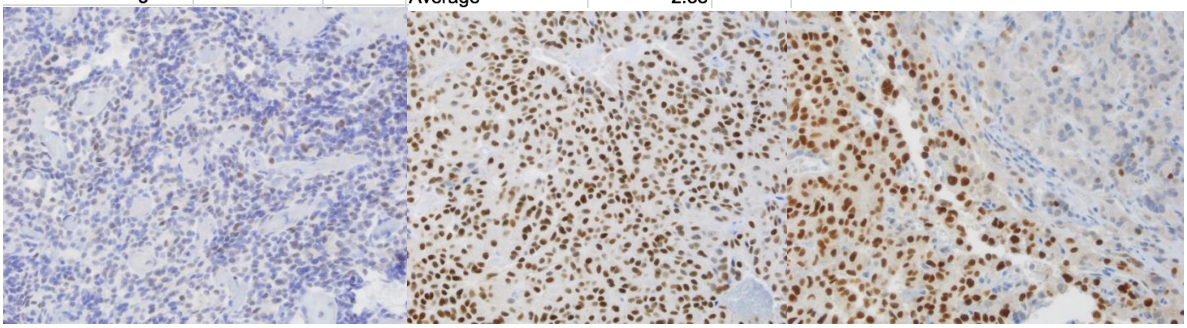


Figure 5: Immunohistochemistry for 7 proteins suspected to overexpressed by RNA-Seq. Thirty one human Cushing adenomas were sectioned, stained, and scored (0-3) for POMC, TBX19, MAMLD1, MNX1, RASEF, USP8, and USP48. Panels B, C, E, G, I, and K scored 1+ whereas A, D, F, H, F and L scored 3+. Significant genes in both human and dogs from the RNA seq data that scored 2+ were MAMLD1 and MNX1, while TBX19 and RASEF displayed weak staining. Genes of interest POMC, USP8, and USP48 consistently scored over 2.3+.

ID	IHC MAMLD1	FPKM
Human Cushings 1	3	54.5552
Human Cushings 2	1	18.8391
Human Cushings 3	3	53.5637
Human Cushings 4	NA	80.6422
Human Cushings 5	2	8.68483
Human Cushings 6	3	66.2096
Human Cushings 7	3	NA
Human Cushings 8	1	NA
Human Cushings 9	3	NA
Human Cushings 10	2	NA
Human Cushings 11	3	NA
Human Cushings 12	1	NA
Human Cushings 13	2	NA
Human Cushings 14	3	NA
Human Cushings 15	3	NA
Human Cushings 17	3	NA
Human Cushings 18	3	NA
Human Cushings 19	2	NA
Human Cushings 20	3	NA
Human Cushings 21	3	NA
Human Cushings 22	3	NA
Human Cushings 23	3	NA
Human Cushings 24	3	NA
Human Cushings 25	3	NA
Human Cushings 26	2	NA
Human Cushings 27	3	NA
Human Cushings 28	3	NA
Human Cushings 29	3	NA
Human Cushings 30	1	NA
Human Cushings 31	3	NA
Human Cushings 32	3	NA
Average	2.58	



A B C

Figure 6: Elevated FPKM transcripts of MAMLD1 were shown in immunohistochemistry. Five out of the six originally sequenced CD PA tissues with the addition of 26 human Cushing's tissues were sectioned, stained, and scored (0-3) for MAMLD1. A.) showed 1+ weak nuclear staining, B.) showed 3+ strong nuclear staining, and C.) compares 3+ tumor to the surrounding normal tissue. Four of the sequenced tissues (Cushing's 1, 2, 3, 6) FPKM levels correlated with the IHC scoring while Cushing 5 did not. The average IHC score for MAMLD1 in 31 human CD PA tissues was 2.58 +/- 0.72.

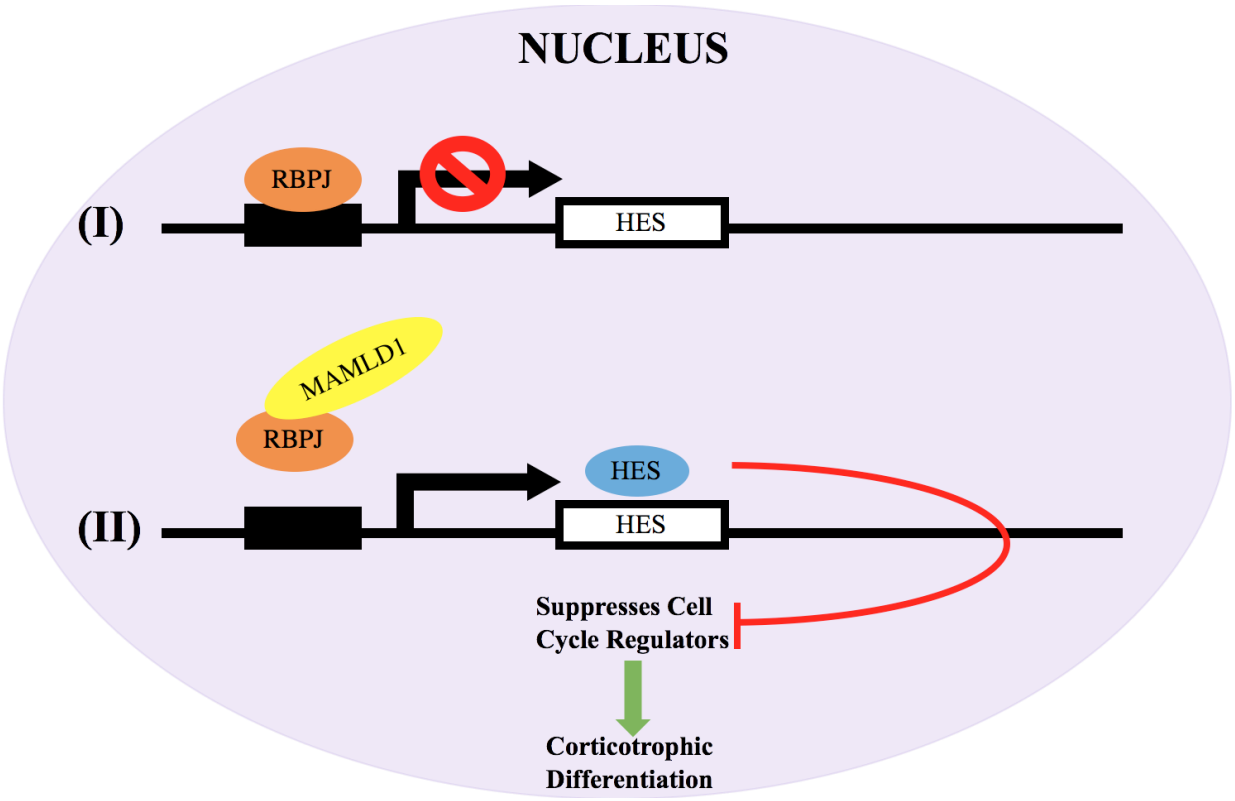


Figure 7: Proposed MAMLD1 interaction pathway leading to unregulated ACTH secreting cell proliferation. I.) RBPJ binds to the promoter of HES to inhibit translation. II.) Transcriptional coactivator MAMLD1 binds to remove repressor protein RBPJ allowing HES translation. HES acts to suppress cell cycle regulator elements allowing corticotrophic cell differentiation.

References

1 Aguilera, G. Regulation of pituitary ACTH secretion during chronic stress. Frontiers in neuroendocrinology 15, 321-350 (1994).

2 Åkesson, L., Ahrén, B., Edgren, G. & Degerman, E. VPAC2-R mediates the lipolytic effects of pituitary adenylate cyclase-activating polypeptide/vasoactive intestinal polypeptide in primary rat adipocytes. Endocrinology 146, 744-750 (2005).

3 Assis, P. A. et al. Mycobacterium tuberculosis expressing phospholipase C subverts PGE synthesis and induces necrosis in alveolar macrophages. BMC microbiology 14, 128 (2014).

4 Bertagna, X. et al. LCI699, a potent 11β-hydroxylase inhibitor, normalizes urinary cortisol in patients with Cushing's disease: results from a multicenter, proof-of-concept study. The Journal of Clinical Endocrinology & Metabolism 99, 1375-1383 (2013).

5 Bilodeau, S. et al. Role of Brg1 and HDAC2 in GR trans-repression of the pituitary POMC gene and misexpression in Cushing disease. Genes & development 20, 2871-2886 (2006).

6 Boneh, A. et al. A single nucleotide substitution that abolishes the initiator methionine codon of the GLDC gene is prevalent among patients with glycine encephalopathy in Jerusalem. Journal of human genetics 50, 230-234 (2005).

7 Chen, Y. et al. Mutational study of the MAMLD1-gene in hypospadias. European journal of medical genetics 53, 122-126 (2010).

8 Cin, H. et al. Oncogenic FAM131B–BRAF fusion resulting from 7q34 deletion comprises an alternative mechanism of MAPK pathway activation in pilocytic astrocytoma. Acta neuropathologica 121, 763-774 (2011).

9 Dalgin, G. et al. Zebrafish mnx1 controls cell fate choice in the developing endocrine pancreas. Development 138, 4597-4608 (2011).

10 de Bruin, C. M., BP; Kooistra, HS; Hanson, JM; Lamberts, SW; Hofland, LJ. Cushing's disease in dogs and humans. Horm Res. 1, 140-143 (2009).

11 Etxabe, J. & Vazquez, J. Morbidity and mortality in Cushing's disease: an epidemiological approach. Clinical endocrinology 40, 479-484 (1994).

12 Etxabe, J. & Vazquez, J. Morbidity and mortality in Cushing's disease: an epidemiological approach. Clinical endocrinology 40, 479-484 (1994).

13 Evans, C.-O. et al. Novel Patterns of Gene Expression in Pituitary Adenomas Identified by Complementary Deoxyribonucleic Acid Microarrays and Quantitative Reverse Transcription-Polymerase Chain Reaction 1. The Journal of Clinical Endocrinology & Metabolism 86, 3097-3107 (2001).

14 Fabbro, M. et al. Cdk1/Erk2-and Plk1-dependent phosphorylation of a centrosome protein, Cep55, is required for its recruitment to midbody and cytokinesis. Developmental cell 9, 477-488 (2005).

15 Feelders, R. A. et al. Pasireotide alone or with cabergoline and ketoconazole in Cushing's disease. New England Journal of Medicine 362, 1846-1848 (2010).

16 Feelders, R. A., Pulgar, S. J., Kempel, A. & Pereira, A. M. MANAGEMENT OF ENDOCRINE DISEASE: The burden of Cushing's disease: clinical and health-related quality of life aspects. European journal of endocrinology 167, 311-326 (2012).

17 Flanagan, S. E. et al. Analysis of transcription factors key for mouse pancreatic development establishes NKX2-2 and MNX1 mutations as causes of neonatal diabetes in man. Cell metabolism 19, 146-154 (2014).

18 Fragoso, M. C. B. V. et al. Cushing’s syndrome secondary to adrenocorticotropin-independent macronodular adrenocortical hyperplasia due to activating mutations of GNAS1 gene. The Journal of Clinical Endocrinology & Metabolism 88, 2147-2151 (2003).

19 Fukami, M. et al. Mastermind-like domain-containing 1 (MAMLD1 or CXorf6) transactivates the Hes3 promoter, augments testosterone production, and contains the SF1 target sequence. Journal of Biological Chemistry 283, 5525-5532 (2008).

20 Goldberg, L. B., Aujla, P. K. & Raetzman, L. T. Persistent Expression of Activated Notch Inhibits Corticotrope and Melanotrope Differentiation and Results in Dysfunction of the HPA Axis. Developmental biology 358, 23-32, doi:10.1016/j.ydbio.2011.07.004 (2011).

21 Guiot, G. & Derome, P. in Advances and technical standards in neurosurgery 3-33 (Springer, 1976).

22 Karlsson, E. K. & Lindblad-Toh, K. Leader of the pack: gene mapping in dogs and other model organisms. Nature Reviews Genetics 9, 713-725 (2008).

23 Kirk, L., Hash, R. B., Katner, H. P. & Jones, T. Cushing's disease: clinical manifestations and diagnostic evaluation. American family physician 62, 1119-1127 (2000).

24 Koboldt, D. C. et al. VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. Genome research 22, 568-576 (2012).

25 Krzywinski, M. et al. Circos: an information aesthetic for comparative genomics. Genome research 19, 1639-1645 (2009).

26 LAMBERTS, S. W., LANGE, S. A. D. & STEFANKO, S. Z. Adrenocorticotropin-secreting pituitary adenomas originate from the anterior or the intermediate lobe in Cushing's disease: differences in the regulation of hormone secretion. The Journal of Clinical Endocrinology & Metabolism 54, 286-291 (1982).

27 Lässer, C., Eldh, M. & Lötvall, J. Isolation and characterization of RNA-containing exosomes. JoVE (Journal of Visualized Experiments), e3037-e3037 (2012).

28 Lindblad-Toh, K. et al. Genome sequence, comparative analysis and haplotype structure of the domestic dog. Nature 438, 803-819 (2005).

29 Liu, J. et al. Tbx19, a tissue-selective regulator of POMC gene expression. Proceedings of the National Academy of Sciences 98, 8674-8679 (2001).

30 Ma, Z.-Y. et al. Recurrent gain-of-function USP8 mutations in Cushing's disease. Cell research 25, 306-317 (2015).

31 Magiakou, M. A., Mastorakos, G., Gomez, M. T., Rose, S. R. & Chrousos, G. P. Suppressed spontaneous and stimulated growth hormone secretion in patients with Cushing's disease before and after surgical cure. The Journal of Clinical Endocrinology & Metabolism 78, 131-137 (1994).

32 Mampalam, T. J., Tyrrell, J. B. & Wilson, C. B. Transsphenoidal Microsurgery for Cushing DiseaseA Report of 216 Cases. Annals of Internal Medicine 109, 487-493 (1988).

33 Miller, J. R. et al. RNA-Seq of Huntington’s disease patient myeloid cells reveals innate transcriptional dysregulation associated with proinflammatory pathway activation. Human molecular genetics 25, 2893-2904 (2016).

34 Monahan, P., Rybak, S. & Raetzman, L. T. The Notch Target Gene Hes1 Regulates Cell Cycle Inhibitor Expression in the Developing Pituitary. Endocrinology 150, 4386-4394, doi:10.1210/en.2009-0206 (2009).

35 Nam, Y., Sliz, P., Song, L., Aster, J. C. & Blacklow, S. C. Structural basis for cooperativity in recruitment of MAML coactivators to Notch transcription complexes. Cell 124, 973-983 (2006).

36 Newell-Price, J., Bertagna, X., Grossman, A. B. & Nieman, L. K. Cushing's syndrome. The Lancet 367, 1605-1617 (2006).

37 Oyama, K., Sanno, N., Teramoto, A. & Osamura, R. Y. Expression of neuro D1 in human normal pituitaries and pituitary adenomas. Mod Pathol 14, 892-899, doi:10.1038/modpathol.3880408 (2001).

38 Patil, C. G. et al. Late recurrences of Cushing's disease after initial successful transsphenoidal surgery. The Journal of Clinical Endocrinology & Metabolism 93, 358-362 (2008).

39 Qu, F. et al. Prostaglandin E receptor 4 (PTGER4) involved in host protection against immune challenge in oyster, Crassostrea hongkongensis. Fish & shellfish immunology 42, 316-324 (2015).

40 Reincke, M. et al. Mutations in the deubiquitinase gene USP8 cause Cushing's disease. Nature genetics 47, 31-38 (2015).

41 Sbiera, S. et al. Lack of Ubiquitin Specific Protease 8 (USP8) Mutations in Canine Corticotroph Pituitary Adenomas. PloS one 11, e0169009 (2016).

42 Schwartz, J. L., Tamura, Y., Jordan, R., Grierson, J. R. & Krohn, K. A. Effect of p53 activation on cell growth, thymidine kinase-1 activity, and 3′-deoxy-3′ fluorothymidine uptake. Nuclear medicine and biology 31, 419-423 (2004).

43 Shimojo, H., Ohtsuka, T. & Kageyama, R. Dynamic expression of notch signaling genes in neural stem/progenitor cells. Frontiers in neuroscience 5, 78 (2011).

44 Slominski, A., Wortsman, J., Luger, T., Paus, R. & Solomon, S. Corticotropin releasing hormone and proopiomelanocortin involvement in the cutaneous response to stress. Physiological reviews 80, 979-1020 (2000).

45 Steffensen, C., Bak, A. M., Zøylner Rubeck, K. & Jørgensen, J. O. L. Epidemiology of Cushing's syndrome. Neuroendocrinology 92, 1-5 (2010).

46 Stratakis, C. A. et al. The role of germline AIP, MEN1, PRKAR1A, CDKN1B and CDKN2C mutations in causing pituitary adenomas in a large cohort of children, adolescents, and patients with genetic syndromes. Clinical genetics 78, 457-463 (2010).

47 Tamma, R. et al. Regulation of bone remodeling by vasopressin explains the bone loss in hyponatremia. Proceedings of the National Academy of Sciences 110, 18644-18649 (2013).

48 Tracey, L. et al. Expression of the NF-ϰB targets BCL2 and BIRC5/Survivin characterizes small B-cell and aggressive B-cell lymphomas, respectively. The Journal of pathology 206, 123-134 (2005).

49 Trainer, P. et al. Transsphenoidal resection in Cushing's disease: undetectable serum cortisol as the definition of successfuI treatment. Clinical endocrinology 38, 73-78 (1993).

50 Tremblay, I., Paré, E., Arsenault, D., Douziech, M. & Boucher, M.-J. The MEK/ERK Pathway Promotes NOTCH Signalling in Pancreatic Cancer Cells. PLOS ONE 8, e85502, doi:10.1371/journal.pone.0085502 (2014).

51 Tritos, N. A., Biller, B. M. & Swearingen, B. Management of Cushing disease. Nature Reviews Endocrinology 7, 279-289 (2011).

52 Winn, R. A. et al. Antitumorigenic effect of Wnt 7a and Fzd 9 in non-small cell lung cancer cells is mediated through ERK-5-dependent activation of peroxisome proliferator-activated receptor γ. Journal of Biological Chemistry 281, 26943-26950 (2006).

53 Winn, R. A. et al. Antitumorigenic effect of Wnt 7a and Fzd 9 in non-small cell lung cancer cells is mediated through ERK-5-dependent activation of peroxisome proliferator-activated receptor γ. Journal of Biological Chemistry 281, 26943-26950 (2006).

54 Zhu, X., Gleiberman, A. S. & Rosenfeld, M. G. Molecular physiology of pituitary development: signaling and transcriptional networks. Physiol Rev 87, 933-963, doi:10.1152/physrev.00006.2006 (2007).