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Genomic analysis of gum disease and hypertrichosis in foxes

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ABSTRACT. Since the 1940s, a proliferative gingival disease called hereditary hyperplastic gingivitis (HHG) has been described in the farmed silver fox, *Vulpes vulpes* (Dyrendahl and Henricson 1960). HHG displays an autosomal recessive transmission and has a pleiotropic relationship with superior fur quality in terms of length and thickness of guard hairs. An analogous human disease, hereditary gingival fibromatosis (HGF), is characterized by a predominantly autosomal dominant transmission and a complex etiology, occurring either as an isolated condition or as a part of a syndrome. Similar to HHG, the symptom most commonly associated with syndromic HGF is hypertrichosis. Here we explore potential mechanisms involved in HHG by comparison to known genetic information about hypertrichosis co-occurring with HGF, using an Affymetrix canine genome microarray platform, quantitative PCR, and candidate gene sequencing. We conclude that the mitogen-activated protein kinase pathway is involved in HHG, however despite involvement of the *mitogen-activated protein kinase kinase 6* gene in congenital hypertrichosis with gingival fibromatosis in humans, this gene did not contain any fixed mutations in exons or exon-intron boundaries in HHG-affected foxes, suggesting that it is not causative of HHG in the farmed silver fox population.

Differential up-regulation of *MAP2K6* gene in HHG-affected foxes does implicate this gene in the HHG phenotype.

Key words: Hereditary hyperplastic gingivitis; Hypertrichosis; Mitogen activated protein kinase pathway; MAP2K6; Hereditary gingival fibromatosis

INTRODUCTION

Hereditary hyperplastic gingivitis (HHG) presents as tumour-like growths of the gingival tissue on the mandible in farmed silver foxes, a coat colour variant of the red fox (*Vulpes vulpes*) (Dyrendahl and Henricson, 1960). HHG is characterized by progressive proliferation of the gingival tissues starting at approximately two to three years of age leading to the encapsulation of the teeth and inhibition of normal function. The HHG inheritance pattern is autosomal recessive with sex-biased penetrance displaying an increased occurrence in males over females (Dyrendahl and Henricson, 1960). HHG frequently co-occurs with superior fur quality, described as length and thickness of guard hairs, suggesting that a pleiotropic relationship is responsible for both phenotypes. Despite its discovery in Sweden in the 1940s (Dyrendahl and Henricson 1960) this disease was only documented in Newfoundland and Labrador, Canada, in 2004, when Finnish foxes selected for their denser, longer fur quality were introduced into the breeding population of farmed Canadian silver fox lines (Figure 1) (Clark et al., 2015). The molecular etiology of HHG and its association with fur quality remain unknown.

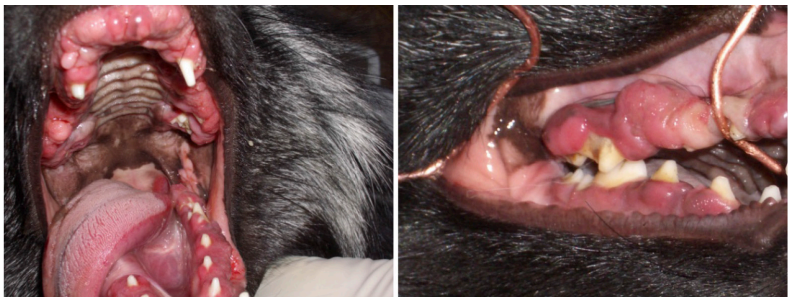

Figure 1. Initial hereditary hyperplastic gingivitis presentation in original Finnish silver foxes in 2004 (Photos provided by Robert Hudson).

Human hereditary gingival fibromatosis (HGF), an analogous condition to HHG, is a rare disease of the oral cavity with progressive benign fibrous enlargements of the maxillary and mandibular keratinized gingival tissue (Hart et al., 2002). HGF affects both sexes equally and average disease onset occurs during permanent dentition, with progression worsening through adolescence (Fletcher, 1966; Coletta and Graner, 2006; Breen et al., 2009). While its underlying origin and cellular mechanisms are still mostly unknown, HGF can be broadly classified as primary or secondary HGF (Shashi et al., 1999). Primary HGF can be further divided into genetic aetiologies and HGF-associated syndromes, while secondary HGF involves an acquired drug-induced HGF phenotype. The focus to date has been on the understanding the genetic aetiologies of HGF with little focus on the HGF-associated syndromes.

Syndrome-associated HGF presents with high phenotypic variability (Haytac and Ozcelik, 2007). The most commonly associated presentation with HGF is hypertrichosis (Coletta and Graner, 2006). This association can occur with or without mental retardation and displays a dominant inheritance pattern (Coletta and Graner, 2006). Haplotype analysis and linkage studies have demonstrated that when HGF with hypertrichosis occurs, it is not linked to two other known, genetic forms of HGF (HGF1 and HGF2) suggesting a distinct genetic form and etiology of HGF (Mangino et al., 2003).

Hypertrichosis is defined as an abnormal excess of hair growth that is not primarily androgen dependent nor influenced by race, sex or age (Beighton 1970). The incidence estimates of this rare condition have been about one in a billion but as these do not account for hypertrichosis in association with numerous syndromes they are probably underestimates (Beighton 1970; Garcia-Cruz et al. 2002). Hypertrichosis can be broadly split into congenital or acquired categories, and the congenital category is subdivided based on a general or localized hair distribution. The generalized distribution is considered a syndrome and is further subdivided according to whether the hypertrichosis is the primary or secondary syndrome characteristic. The subtypes with gingival hyperplasia are classified as syndromes with hypertrichosis as the primary symptom, and include congenital hypertrichosis lanuginose (CHL) and gingival fibromatosis with hypertrichosis. In addition, there is a group of syndromes with hypertrichosis and acromegaloid facial features with or without gingival fibromatosis, for example Cantu syndrome, that are thought to be phenotypic variations of the same disease (Czeschik et al., 2013).

HHG and HGF are diseases with similar manifestations and presentations; in particular the HGF-associated syndrome with hypertrichosis is similar in presentation to HHG-affected foxes with longer denser fur qualities. The goal of this study was to combine the current knowledge of HGF-associated syndromes with hypertrichosis with the genome-enabling capacity of the dog genome to examine potential molecular and cellular pathways involved in silver fox HHG. Utilization of a cross-species platform is supported by karyotyping, cytogenetic genome markers and gene sequence comparisons between *Vulpes vulpes* and *Canis lupus familiaris* that provide strong evidence supporting the close relationship between the two species (Switonski et al. 2009). Specifically, the Affymetrix GeneChip Canine Genome 2.0 Array was employed to compare the global gene expression patterns of HHG-affected farmed silver foxes and HHG-unaffected farmed silver foxes. We conducted an extensive review of the congenital forms of hypertrichosis with gingival fibromatosis in humans and examined their known chromosomal regions and in the context of the HHG microarray expression patterns. In addition we report candidate gene sequencing based mutational analysis of the *mitogen activated protein kinase kinase 6* (*MAP2K6*) gene in the silver fox, for which a role in hypertrichosis has been debated (Sun et al., 2009).

MATERIAL AND METHODS

Sample collection

Samples of HHG-affected and HHG-unaffected gum tissues were taken from a total of thirty foxes from a Newfoundland and Labrador fox farm. Eight of the samples were used for the microarray experiment and the remainder for quantitative polymerase chain reaction (qPCR) validation experiments. Within the microarray samples, there were two pairs of related

foxes and four individuals sharing no direct familial connections. Each fox used was assessed for the presence or absence of HHG and then graded on a severity scale (Clark et al., 2015).

Gingival samples were collected in two manners at different times, either during pelting season or in the late spring after whelping season. During pelting season samples were collected immediately after the animal was euthanized. Samples collected after whelping season were taken from live anesthetised foxes. Provincial government veterinarians performed all sampling and the Institutional Animal Care Committee at Memorial University approved use of samples for this research in accordance with Canadian Council on Animal Care Guidelines. Gingival sections (<500 mg) were collected and immediately placed into 5 mL of RNAlater (Qiagen Inc., Mississauga, ON, Canada) for 4 h followed by storage at -20°C.

RNA preparation

RNA was extracted using RNeasy Fibrous Tissue Mini Kit, according to protocol specifications (Qiagen Inc.). An additional off-column DNase procedure was performed to ensure the quality of the RNA. RNA quality was assessed using 1.5% agarose gel electrophoresis and spectrophotometry using the NanoDrop ND1000 (NanoDrop Technologies, Inc., Wilmington, DE, USA).

Microarray experiments and analysis

RNA samples were sent to the Microarray Facility at the Toronto Centre for Applied Genomics, Hospital for Sick Children, in Toronto, Canada, where RNA integrity was assessed using the Agilent BioAnalyzer (Affymetrix Inc. and Agilent Technologies Inc., Santa Clara, CA, USA). RNA was labeled using the Affymetrix 3'IVT Express kit (Affymetrix Inc.) according to the manufacturer's protocol. Each sample was hybridized to an Affymetrix GeneChip Canine Genome 2.0 Array and scanned using an Affymetrix GeneChip Scanner 3000 (Affymetrix Inc.).

Microarray '.CEL' files were analyzed using Bioconductor (Gentleman et al. 2004). Raw data was converted to expression measures using a GCRMA normalization and summarization algorithm. Non-parametric Mann-Whitney *t*-tests were used to test for statistical significance of differential expression values.

Using the NCBI human genome build 36.3 gene lists were created based on the genes found within the chromosomal loci 17q24.2-q24.3 and 8q22, which are known to be associated with hypertrichosis with gingival fibromatosis (http://www.ncbi.nlm.nih.gov/projects/mapview/map_search.cgi?taxid=9606&query=human&SITE=HumanGuide&SUBMIT=y&submit=Go). These gene lists were cross-referenced to the microarray expression results and annotated with gene names and functions using NetAffx Analysis Centre (Affymetrix Inc., Santa Clara, USA). Additionally, genes were placed in Wikipathways to investigate involvement in known canine signaling pathways (Kelder et al. 2012).

qPCR

cDNA was transcribed from total RNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems Inc., Foster City, CA, USA). Initial qPCR assays were conducted in house with subsequent assays performed at the Microarray Facility at the Toronto Centre for

Applied Genomics, Hospital for Sick Children in Toronto, Canada. For each assay, cDNA was combined with 10 μL Taqman Master Mix (2X) and 1 μL Taqman Gene Expression Assay (20X; 900 nM primer and 250 nM probe) (Applied Biosystems Inc.). Reaction mixtures were placed into MicroAmp Fast Optical 96-Well Reaction Plates (Applied Biosystems Inc.) and subjected to thermal cycling in the StepOnePlus Real Time PCR System (Applied Biosystems Inc.) according to the following profile: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s, 60°C for 1 min. A selection of MAPK signaling pathway genes were selected for qPCR testing including transforming growth factor β 3 (*TGFb3*) (Assay ID Cf02625275_m1), mitogen-activated protein kinase kinase kinase 6 (*MAP2K6*) (Assay ID Cf02683089_m1) and mitogen-activated kinase kinase kinase 1 (*MAP3K1*) (Assay ID Cf02635276_m1). Elongation factor, RNA polymerase II (*ELL2*) (Cf02707156_m1) for the in house qPCR and the breakpoint cluster region (*BCR)* (Cf02664178_m1) genes for the remaining qPCR samples were selected as the endogenous control genes.

Relative quantification of each gene was calculated using the ΔΔCT method. For the in house qPCR the unaffected sample with the lowest standard error was selected as the reference sample while for the remaining qPCR samples the unaffected sample with the lowest expression was selected as the reference sample. Computed fold changes were tested for significance with two-tailed *t*-tests. All samples were tested in triplicate and samples with a standard deviation greater than 0.5 were removed from the dataset. Any samples that were identified as outliers within biological groups according to Data Assist v.3.0 were also discarded (Life Technologies, Grand Island, NE, USA).

Primer design for candidate gene sequencing

Primers were designed to amplify the *MAP2K6* gene in foxes using the *Canis lupus familiaris* sequence (GenBank Accession No. NC_006591.3). Primer pairs were selected from the intronic regions flanking each target region using NCBI Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). All parameters were set to default except for the "Primer Melting Temperatures" which were as follows: maximum melting temperature: 65°C; minimum melting temperature: 50°C; and optimum melting temperature: 57°C. Primers were manufactured by Operon Inc. as PCReady primers (Huntsville, AL, USA).

DNA preparations and PCR

DNA extractions were performed with the QiaAmp DNA Mini kit (Qiagen Inc.) according to the manufacturer's tissue protocol. Each PCR contained 2.5 μL 10X buffer, 0.5 μL dNTPs (New England Biolabs Ltd., Whitby, Canada), 1 μL 10 μM forward primer, 1 μL 10μM reverse primer, 0.2 μL 5 U/μL Qiagen Hot Star Taq Plus (Qiagen Inc., Mississauga, Canada), 15 μL distilled nuclease free water and 1 μL template DNA. The thermal profile used was 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, target-specific annealing temperature for 30 s, and 72°C for 1 min, followed by 72°C for 10 min. Amplified PCR products were purified using either Pall Life Sciences Multi-Well Plate Manifolds (Pall Corporation, Port Washington, WI, USA) or the QIAquick PCR purification kit (Qiagen Inc.). Target-specific annealing temperatures were as recommended by the primer selection software. PCR products were analyzed using 1.5% agarose gel electrophoresis.

DNA sequencing and analysis

DNA sequencing reactions were performed with both forward and reverse primers for each PCR amplicon using BigDye Terminator v3.0 chemistry (Applied Biosystems Inc.) utilizing the following thermal profile: 96°C for 6 min, then 25 cycles of 96°C for 10 s, 50°C for 5 s, 60°C for 4 min. Sequencing reaction purification was carried out using either ethanol precipitation or an Agencourt CleanSeq method (Beckman Coulter Inc., Danvers, MA, USA). Purified DNA sequencing reactions were electrophoresed on the Applied Biosystems Inc. 3730 DNA Analyzer, in the GaP Facility of the CREAIT Network at Memorial University of Newfoundland.

Raw data was collected using Sequence Analysis v5.2 (Applied Biosystems Inc.) and imported into Sequencher v4.8 (Gene Codes Corp., Ann Arbor, MI, USA). Contigs were created by assembling reads to the reference sequence, Accession Number NC_006591.3 (chromosome 9) using an 85% minimum gap percentage and a 20% minimum overlap, followed by manual trimming and editing of sequence each read.

RESULTS

Differential gene expression in HHG-affected compared to HHG-unaffected silver foxes based on the Affymetrix GeneChip Canine Genome 2.0 Array

Bioconductor analyzed 43,035 gene ID probes in both the HHG-affected and HHG-unaffected groups. Of these, 1154 probesets demonstrated a statistically significant differential gene ID probe expression when comparing the HHG-affected to HHG-unaffected samples as determined by a Mann-Whitney *t*-test ($P < 0.05$). 508 probesets demonstrated at least a two-fold differential up-regulation when comparing HHG-affected foxes to HHG-unaffected foxes and 446 Probe IDs demonstrated a least a two-fold differential down-regulation when making the same comparison.

Figure 2 demonstrates the general expression trend comparison between the HHG-affected and unaffected samples for a selection of RT-qPCR genes involved in the MAPK signaling cascade. The *TGFb3* gene showed a statistically significant fold change ($P < 0.05$) (Table 1). Table one demonstrates the differential expression of genes in hereditary hyperplastic gingivitis-affected foxes compared to unaffected foxes from RT-qPCR validation assays, compared with fold-change found by microarray analysis.

Comparison of gene expression patterns from microarrays to chromosomal loci associated with hypertrichosis with gingival fibromatosis

There are a number of congenital hypertrichosis syndromes that occur with gingival overgrowth, including CHL, Cantu syndrome and hypertrichosis with gingival fibromatosis (Garcia-Cruz et al., 2002). Genes within the loci for each of these syndromes were examined and compared with the differential expression HHG-affected versus HHG-unaffected microarray data in the silver fox samples.

In humans, CHL is associated with chromosomal region 8q22-24 (Tadin et al., 2001). NCBI human genome build 36.3 contained 215 genes in this chromosomal region (accessed July 2013). Eighty-two of those genes correspond to equivalent known canine genes. On the Affymetrix GeneChip Canine Genome 2.0 Array those 82 genes were represented by 170

probesets. Five of those probe IDs demonstrated statistically significant expression differences (Mann Whitney $P < 0.05$) and an additional nine when the requirement for statistical significance was relaxed to $P < 0.10$ to account for the high biological diversity of the samples (Table 2). These 14 probe IDs represented 11 different genes with three that were differentially up-regulated in the HHG-affected versus HHG-unaffected fox samples and eight that were differentially down-regulated in the same comparison.

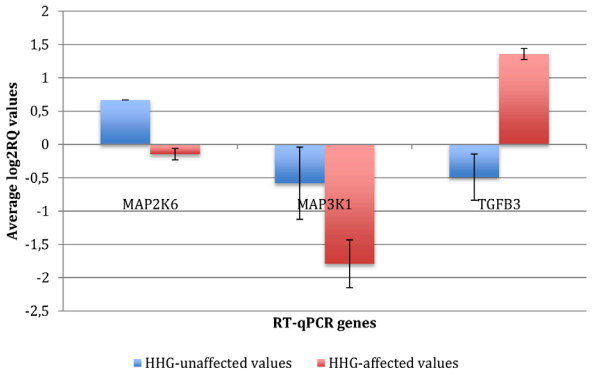

Figure 2. Mean $\log_2$ relative expression ($\log_2$RQ) values for RT-qPCR genes in hereditary hyperplastic gingivitis (HHG)-affected and HHG-unaffected fox samples. Standard errors are indicated by the bars.

Table 1. Differential expression of genes in hereditary hyperplastic gingivitis-affected foxes compared to unaffected foxes from quantitative polymerase chain reaction (qPCR) validation assays, compared with fold-change found by microarray analysis.

Gene symbol	Gene name	qPCR		Microarray		
		Fold change	P value	Affymetrix probe ID	Fold change	P value
MAP2K6	Mitogen-activated protein kinase kinase 6	-1.106	NA*	CfaAffx.16824.1.S1_at	-1.203	0.250
MAP3K1	Mitogen-activated protein kinase kinase kinase	-2.313	0.112	Cfa.19524.1.S1_at	-1.959	0.071
TGFβ3	Transforming growth factor beta 3	3.603	0.026	CfaAffx.26185.1.S1_at	1.084	0.788

*NA: Insufficient samples present in triplicate to perform a *t*-test.

Table 2. Microarray-identified differential expression in hereditary hyperplasticgingivitis (HHG)-affected foxes compared to unaffected foxes for genes found in the human chromosome loci associated with hypertrichosis occurring with gingival overgrowth.

Gene symbol	Gene name	Human chromosome region	Fold change	P value
ABCA5	ATP-binding cassette, sub-family A, member 5	17q24.2-q24.3	1.022	0.393
ABCA6	ATP-binding cassette, sub-family A, member 6	17q24.2-q24.3	1.014	0.571
MAP2K6	Mitogen-activated protein kinase kinase 6	17q24.2-q24.3	-1.203	0.250
PITPNC1	Phosphatidylinositol transfer protein, cytoplasmic 1	17q24.2-q24.3	1.358	0.036
OXR1	Oxidation resistance 1	8q22	-1.288	0.036
NCALD	Neurocalcin delta	8q22	-1.232	0.036
MED30	Mediator complex subunit 30	8q22	-1.789	0.036
COL14A1	Collagen, type XIV, alpha 1	8q22	1.242	0.036
AZIN1	Antizyme inhibitor 1	8q22	-1.228	0.036
TAF2	TATA box binding protein associated factor	8q22	-1.258	0.071
RPL30	Ribosomal protein L30	8q22	-1.303	0.071
RAD21	RAD21 homolog	8q22	-1.431	0.071
NUDCD1	NudC domain containing 1	8q22	-1.551	0.071
KIAA1429	KIAA1429 ortholog	8q22	1.124	0.071
CPQ	Carboxypeptidase Q	8q22	1.122	0.071

Cantu syndrome in humans has a putative causative mutation in the ATP-binding cassette, sub-family C member 9 (*ABCC9*) gene (Czeschik et al., 2013). There is a homologous gene in the canine microarray represented by two probe IDs. Both probe IDs demonstrated an up-regulation in the HHG-affected compared to the HHG-unaffected microarray fox samples but not significantly so ($P > 0.10$). The ABCC9 protein is part of an ATP potassium-sensitive channel composed of four pore-forming subunits of either KCNJ8 or KCNJ11 and four regulatory subunits of the ABCC9 protein (Harakalova et al., 2012). KCNJ8 and KCNJ11 microarray gene expression levels were examined and neither showed any statistically significant differential expression ($P < 0.10$). Hypertrichosis with gingival fibromatosis is associated with the chromosome region 17q24.2-q24.3 in humans (Sun et al., 2009) and this region contains the following genes: *ABCA5*, *ABCA6*, *ABCA10* and *MAP2K6*. ABCA10 was not on the Affymetrix GeneChip Canine Genome 2.0 Array, nor was a description of the gene found in NCBI for canines. The remaining genes were compared to the HHG microarray data but none yielded a statistically significant differential expression with a Mann Whitney *t* test at $P < 0.05$ (Table 2). The *MAP2K6* gene did show statistically significant ($P < 0.05$) differential expression in the RT-qPCR assay with a down-regulation of the gene in the HHG-affected foxes compared to the unaffected foxes (Table 1). NCBI human genome build 36.3 listed 36 genes within that chromosome region (accessed July 2013). Of these there were 20 canine equivalents that were represented on the Affymetrix GeneChip Canine Genome 2.0 Array by 46 probe IDs. Of those probe IDs, the Phosphatidylinositol Transfer Protein, Cytoplasmic 1 (*PITPNC1*) gene was significantly ($P < 0.05$) differentially up-regulated in the HHG-affected versus HHG-unaffected microarray fox samples.

Mutational differences between affected and unaffected foxes in the MAP2K6 gene

The *MAP2K6* gene was sequenced for six HHG-unaffected foxes and six HHG-affected foxes. There was 100% coverage of the protein-coding region and the intron/exon boundaries for several HHG-affected and several HHG-unaffected animals. There were no fixed mutations segregating the HHG-affected from the HHG-unaffected sets of samples in any of coding portions or intron/exon boundaries of the *MAP2K6* gene, nor were there any heterozygous sites that segregated differently in the two groups.

Comparison of HHG microarray gene lists to MAPK pathway genes

The canine c-Jun N-terminal kinase (JNK), and p38 portions of mitogen-activated protein kinase (MAPK) signaling cascade were examined using Wikipathways (Kelder et al., 2012) to determine if any significantly differentially expressed genes within the fox HHG-affected vs. HHG-unaffected microarray comparison could be identified. Three genes showed statistically significant differential expression (Mann-Whitney *t* test $P < 0.05$) and four in total with a relaxed Mann Whitney criterion ($P < 0.10$) (Table 3). Of these four genes, one showed up-regulation in the HHG-affected samples and three showed down-regulation in the HHG-affected samples compared to the HHG-unaffected samples.

Table 3. Microarray analysis of differential expression of hereditary hyperplastic gingivitis (HHG)-affected foxes compared to unaffected foxes for genes found in the c-Jun N-terminal kinase (JNK) and p38 portions of the mitogen-activated protein kinase (MAPK) signaling cascade.

Gene symbol	Gene title	Fold change	Mann-Whitney P value
CASP6	Caspase 6, apoptosis-related cysteine peptidase	-1.213	0.036
IL1B	Interleukin 1, beta	1.189	0.036
MAP3K1	Mitogen-activated protein kinase kinase kinase 1	-1.959	0.071
MAP4K1	Mitogen-activated protein kinase kinase kinase kinase 1	-1.270	0.036

DISCUSSION

HHG is a condition that occurs predominantly in the farmed silver fox population, where HHG appears to have been selected when choosing to breed animals with longer, denser fur (Dyrendahl and Henricson, 1960). HGF is an analogous condition in humans with a complex etiology. The genetic basis of syndromic HGF associated with hypertrichosis is distinct from the isolated genetic forms of HGF (Mangino et al., 2003; Coletta and Graner, 2006). While often viewed as separate but co-occurring, fibromatosis and hypertrichosis may be different phenotypic expressions of a common ectodermal or mesodermal congenital abnormality (Lee et al. 1993). Herein, we use a canine microarray platform to investigate the etiology of hypertrichosis with HHG in the context of what is known about the genetic basis of the human syndrome.

Comparison of HHG microarray gene lists to chromosomal loci of gingival fibromatosis associated with hypertrichosis

CHL is often referred to as Ambras syndrome after paintings that were discovered in the Ambras castle in Austria depicting the child Peter Gonzales, who had this form of hypertrichosis (Freire-Maia et al., 1976). The main features of CHL are the excessive growth of lanugo hair and dental abnormalities (Garcia-Cruz et al., 2002). Typically the latter involve the delayed presentation of primary and secondary teeth as well as the absence of some teeth (Tadin et al., 2001). However, there has been a report of a sporadic case of CHL with mild gingival hyperplasia (Lee et al., 1993). CHL has autosomal dominant inheritance with the occurrence of sporadic cases (Tadin et al., 2001). The CHL mutation has been mapped to the 8q22-q24 chromosomal region (Tadin et al., 2001). The HHG microarray demonstrated 11 genes with a statistically significant ($P < 0.10$) differential expression between the HHG-affected and HHG-unaffected fox samples. While none of these genes is thought to contain the causative mutation, their expression levels and possible contributions to disease phenotype are of note. Of particular interest was the *COL14A1* gene which was up-regulated in the HHG-affected foxes compared to the HHG-unaffected foxes. This gene encodes collagen 14 protein produced by fibroblasts, myofibroblasts and hepatic stellate cells during late embryogenesis (Ruehl et al., 2005). It plays a role in cellular quiescence and differentiation, such as during wound healing, and has been known to induce fibroblast differentiation (Ruehl et al., 2005). As HGF is associated with a more friable tissue (Clark et al., 2015) it is plausible that the increased need for wound healing has resulted in the up-regulation of this gene.

Cantu syndrome is classified primarily with hypertrichosis, cardiomegaly and bone abnormalities and can result from autosomal dominant or recessive inheritance (Kurban et

al., 2011). While typically not associated with gingival fibromatosis it is thought that Cantu syndrome along with the phenotypes of acromegaloid with or without hypertrichosis and gingival fibromatosis are variations along a spectrum of the same disease (Czeschik et al., 2013). The putative causative mutation of this condition is in the ABCC9 gene, located at 12p12.1. In the silver fox microarray study the gene is up-regulated in HHG-affected compared to the HHG-unaffected fox samples but not significantly so. Although the HHG-affected foxes have both gingival and hair overgrowth, they do not show the bone or cardiac abnormalities associated with Cantu syndrome. Hence it is unlikely that ABCC9 is involved in HHG, although RT-qPCR validation of the up-regulation and mutational analysis may challenge this.

Gingival fibromatosis with hypertrichosis is an autosomal dominant condition with terminal hair covering the face, trunk and eyebrows, with progressive gingival hyperplasia starting in childhood (Wendelin et al., 2003). It is associated with chromosomal region 17q24.2-24.3 (Sun et al., 2009). Of interest, this region contains several members of the ATP binding cassette transporter family A (ABCA) family of genes, *MAP2K6*, and the *PITPNC1* gene. The ABCA is a large family of proteins (Albrecht and Viturro, 2007). ABCA5 has been found in lysosomes and endosomes and is thought to be involved in intracellular trafficking (Kubo et al., 2005). In rats, mRNA expression the *ABCA5* transcript has been identified in Leydig cells where testosterone production occurs (Petry et al., 2006). ABCA5 was down-regulated in both male and female HHG-affected fox samples when compared to the unaffected samples so the gingival fibromatosis phenotype is unlikely to be associated with Leydig cell biological function. However, in dogs, the gingival tissue is androgen sensitive, such that testosterone exacerbates the phenotypic presentation of gingival hyperplasia (Pariser and Berdoulay, 2011). If the fox gingival tissues are similar to the canine counterpart then the down-regulation of the *ABCA5* gene may be in response to the already hypertrophied androgen-sensitive gingival tissue. ABCA6 is responsive to cholesterol concentrations and is up-regulated during macrophage differentiation (Kaminski et al., 2001). The up-regulation of ABCA6 in the HHG-affected foxes could be related to disease etiology, although it is plausible that it may be up-regulated as part of an inflammatory response in the gingival tissue leading to an increase in macrophage differentiation.

In the human genome version 36.3, the *PITPNC1* gene is identified in the 17q24.2-24.3 chromosomal region and this gene was up-regulated in the HHG-affected foxes when compared to the HHG-unaffected foxes. The *PITPNC1* gene encodes the RdgBβ protein, a Class IIB PIP protein subfamily (Cockcroft, 2012). RdgBβ expression is tightly regulated, with a substantial level of expression in cardiac and renal tissue (Garner et al., 2011). This protein has been implicated in a variety of processes and the full scope of it biological roles is unknown (Trivedi and Padinjat 2007). One of its roles is during wound healing (Gu and Iyer, 2006). As previously mentioned HGF is a delicate tissue prone to injury (Clark et al., 2015) contributing to an increased need for wound healing, potentially explaining the up-regulation of this gene.

Candidate gene, microarray, and RT-qPCR analysis of the MAP2K6 gene in HHG-affected foxes

The original interest in the 17q24.2-24.3 chromosomal region was in the *MAP2K6* gene (Sun et al., 2009). While other MAPK pathway proteins have been implicated in both hair growth and gingival overgrowth conditions, there was some debate about the *MAP2K6* gene involvement in the combined phenotype (Hart et al., 2002; Sun et al., 2009). In 2008,

a human case was reported with a 2.3Mb deletion encompassing this gene that did not result in either hypertrichosis or gingival overgrowth (Blyth et al., 2008). The microarray analysis of this gene showed a differential down-regulation in the HHG-affected foxes compared to the HHG-unaffected foxes but this difference was not statistically significant. RT-qPCR confirmed the differential down-regulation in the HHG-affected fox samples but again without statistical significance. Depending on the nature of the mutation, however, and due to the inherent biological variability this does not definitely rule it out as a putative HHG gene.

Mutational analysis of the coding regions and exon-intron splice sites of the *MAP2K6* gene did not show any putative candidate mutations within HHG-affected foxes in this gene. The *MAP2K6* gene did demonstrate variability between the fox sequences and the *Canis* reference sequence. In particular, exon 3 carried a nonsynonymous sequence difference between the dog and the foxes, which was variable within foxes. In dogs, the sequence is CGT (codon for histidine), while in the fox samples the variant form of this same allele was CAT (arginine). Although some foxes were heterozygous for these two alleles, there were no fixed differences between affected and unaffected individuals. While *MAP2K6* does not appear to be the causative gene for HHG as least with respect to coding portion of the gene, it is possible that allelic variations contribute to phenotype severity, for example this amino acid change could potentially result in a greater propensity for foxes to develop HGF. The absence of a putative mutation does not completely exclude the possibility of the *MAP2K6* gene involvement in the phenotype of HHG with hypertrichosis, as it is still possible that there is a mutation in the promoter region or within the introns that leads an enhanced expression of the gene. The expression pattern we observe supports the potential involvement of this pathway in the HHG presentation.

Mitogen activated protein kinase pathway

As the MAPK signaling pathway is directly linked to several known isolated genetic forms of HGF and its role in hypertrichosis with gingival fibromatosis (Hart et al., 1998; Xiao et al., 2001; Sun et al., 2009). MAPK signaling starts when a ligand binds to a receptor and activates a multi-tier cascade of mitogen-activate protein kinase phosphorylations ultimately resulting in a change in gene expression in the nucleus (Yang et al., 2013) (Figure 3). MAP2K6 signaling occurs in the c-Jun N-terminal kinase (JNK), and p38 sub-pathways in the MAPK signaling schematic (Wang and Tournier, 2006; Krishna and Narang, 2008). The p38 and JNK pathways are activated by signals such as interleukin B (ILB), transforming growth factor beta (TGFβ) and stress and end with in a common pathway of cell proliferation, differentiation or cellular apoptosis (Kim and Choi, 2010). Based on both the microarray and RT-qPCR data there are several genes in both the JNK and p38 pathways that demonstrate differential expression between the HHG-affected and unaffected samples.

The JNK pathway cellular signal interleukin 1, beta (*IL1B*) is up-regulated in the HHG-affected foxes, and, downstream of the receptor, the caspase 6, apoptosis-related cysteine peptidase (*CASP6*) adapter protein is down-regulated in HHG-affected foxes both demonstrating statistical significance (Mann-Whitney *t* test $P < 0.05$). Subsequent MAPK proteins mitogen-activated protein kinase kinase kinase kinase 1 (*MAP4K1*) and mitogen-activated protein kinase kinase kinase 1 (*MAP3K1*) also display significant ($P < 0.100$) down-regulation in HHG-affected foxes. While the end of the pathway did not contain any genes that displayed statistically significant expression differences the general trend appears to be a

down-regulation of this part of the MAPK pathway. The p38 pathway shows the inverse trend. In this pathway there were two up-regulated cellular signals including *TGFb3* and *IL1*, which acts as a signal for both JNK and p38 sub-pathways (Kelder et al., 2012). Downstream in the pathway is the *MAP2K6* gene, which was down-regulated in the HHG-affected foxes. As the end of this and the JNK sub-pathway are the same we cannot comment on the overall effect of the up-regulation of the upstream members of the p38 sub-pathway. One hypothesis for the combined trend is that the JNK portion of the pathway is down-regulated in the HHG-affected foxes as a compensatory mechanism for the up-regulation of the p38 portion of the pathway in the HHG-affected foxes. Another hypothesis is that the portions of the pathway that are associated with apoptosis, like CASP6, are down-regulated to prevent cell death, adding to the lifespan of the hypertrophied cells. While DNA sequencing has shown no mutation in the coding regions of the *MAP2K6* gene, the overall pattern of differential gene expression in the MAPK pathway still calls the involvement of this pathway into question.

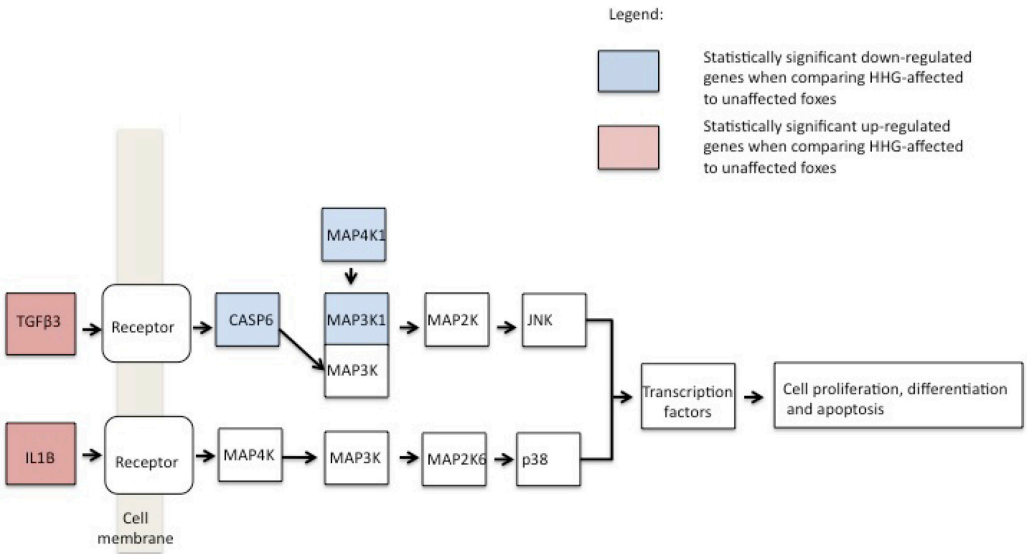

Figure 3. Diagram of several steps in the c-Jun N-terminal kinase (JNK) and p38 sub-pathways of the mitogen-activated protein kinase (MAPK) pathway in canines, highlighting genes differentially expressed on the microarray or in the RT-qPCR results in foxes affected with hereditary hyperplastic gingivitis (HHG). Adapted from Kelder et al. (2012).

Hypertrichosis and gingival hyperplasia are co-occurring phenotypes that could result from a shared underlying etiology. Using a microarray platform the expression of genes in HHG-affected versus HHG-unaffected foxes was explored with respect to genome regions associated with hypertrichosis and gingival hyperplasia in humans. Differential expression of various genes helps to uncover potential contributing mechanisms such as the MAPK pathway involvement. Of note is the statistically significant up regulation of the p38 sub-pathway with the concurrent down regulation of the JNK sub-pathway of the MAPK signaling. While there was no putative mutation found in the MAP2K6 gene in the coding region, the overall differential gene expression trend still suggests involvement of the MAPK pathway.

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