

Sequence analysis of the Ras-MAPK pathway genes *SOS1*, *EGFR* & *GRB2* in silver foxes (*Vulpes vulpes*): candidate genes for hereditary hyperplastic gingivitis

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Abstract Hereditary hyperplastic gingivitis (HHG) is an autosomal recessive disease that presents with progressive gingival proliferation in farmed silver foxes. Hereditary gingival fibromatosis (HGF) is an analogous condition in humans that is genetically heterogeneous with several known autosomal dominant loci. For one locus the causative mutation is in the *Son of sevenless homologue 1* (*SOS1*) gene. For the remaining loci, the molecular mechanisms are unknown but Ras pathway involvement is suspected. Here we compare sequences for the *SOS1* gene, and two adjacent genes in the Ras pathway, *growth receptor bound protein 2* (*GRB2*) and *epidermal growth factor receptor* (*EGFR*), between HHG-affected and unaffected foxes. We conclude that the known HGF causative mutation does not cause HHG in foxes, nor do the coding regions or intron–exon boundaries of these three genes contain any candidate mutations for fox gum disease. Patterns of molecular evolution among foxes and other mammals reflect high conservation and strong functional constraints for *SOS1* and *GRB2* but reveal a lineage-specific pattern of variability in *EGFR* consistent with mutational rate differences, relaxed functional constraints, and possibly positive selection.

Keywords Hereditary gingival fibromatosis · *Son of sevenless homologue 1* · *Vulpes vulpes* · Candidate gene sequencing · Molecular evolution

Introduction

Hereditary hyperplastic gingivitis (HHG) is a rare disease of the oral cavity that occurs primarily in captive silver foxes (a coat colour variant of the red fox, *Vulpes vulpes*), and is characterized by progressive proliferation of the gingival tissues starting at 2–3 years of age. The fibrous overgrowths eventually lead to encapsulation of the teeth and inhibition of normal function. HHG is inherited in an autosomal recessive pattern, with sex-biased penetrance favoring males over females (Dyrendahl and Henricson 1960). HHG frequently co-occurs with superior fur quality (length and thickness of guard hairs) suggesting the possibility that a pleiotropic gene is responsible for both phenotypes. Its pathology indicated large epithelial extensions in the keratinized collagen of the gingival tissue. In 2004 HHG was first documented in Newfoundland and Labrador Canada, in a farmed fox population after importation of European silver fox lines (Clark et al. 2014). Gross examination demonstrated proliferative gingival tissue containing a red granular surface. The histology demonstrated thick collagen bundles. These results were in keeping with both the original Dyrendahl and Henricson reports as well as with those reported in by Schulze et al. (2008), who documented the first case of HHG in a wild fox (Clark et al. 2014; Dyrendahl and Henricson 1960; Schulze et al. 2008). However, despite initial documentation, the underlying genetics and cellular mechanisms causing HHG remain unknown.

An analogous condition that affects humans is hereditary gingival fibromatosis (HGF). HGF typically manifests with the onset of permanent dentition and results in slow, progressive, benign fibrous enlargements of the maxillary and mandibular keratinized gingival tissue (Hart et al. 2002; Ye et al. 2005). HGF is both functionally and

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aesthetically problematic. Treatment involves quadrant-by-quadrant gingivectomy, but there is a common recurrence of the overgrowth (Ramer et al. 1996). The majority of HGF cases show autosomal dominant inheritance, with autosomal recessive cases occurring rarely (Goldblatt and Singer 1992; Shashi et al. 1999). HGF is a genetically heterogeneous disease that can occur as an isolated condition, part of an underlying syndrome or chromosomal abnormality, or in a non-hereditary form where it occurs in association with certain pharmacological agents (Shashi et al. 1999). Linkage analysis of isolated cases has mapped HGF to three different chromosomal locations: 2p21–p22, 5q13–q22 and 2p22.3–p23.3 (Hart et al. 2002; Ye et al. 2005). At 2p21–p22, the causative mutation is a single nucleotide insertion in codon 1,083 of the *Son of sevenless homolog 1 (SOS1)* gene (Hart et al. 2002). This causes a frameshift mutation, leading to the premature truncation of the C-terminal domain and resulting in a constitutive activation of its downstream products. Within the 5q13–q22 loci the Ras pathway genes *G protein coupled receptor 113 (GPR113)* and *ethanolaminephosphotransferase 1 (SELI)* have been screened with no reported causative mutations (Ye et al. 2005). The 2p22.3–p23.3 locus has recently been refined to a 6.56 cM region (Pampel et al. 2010), within which no causative mutations have been reported.

HHG and HGF are analogous conditions with very similar manifestations and disease progression. A family has been reported that displays hypertrichosis (excessive hair growth) in addition to the gingival overgrowth (Mangino et al. 2003), which may be reminiscent of the dense fur of HHG-affected silver foxes. Given the strong similarity between the human and fox diseases and the known molecular basis of one form of HGF, our goal was to perform functional candidate gene analysis as a means to establish or eliminate particular gene mutations as causative of HHG in foxes. The premise of this functional candidate gene approach is the implication that a mutation or gene associated with a disease in one organism may be associated with the analogous condition in another (Aguirre-Hernandez and Sargan 2005). Additionally, candidate genes can be identified on the basis that disruption of their predicted gene function may be expected to result in the disease phenotype.

Here we report and compare the DNA sequences of three candidate genes for HHG in silver foxes: *SOS1* (sequences have been submitted to GenBank), where the single known causative mutation of HGF is located, and the closely-associated *Growth factor receptor bound 2 protein (GRB2)* (sequences have been submitted to GenBank) and *Epidermal growth factor receptor (EGFR)* (sequences have been submitted to GenBank) genes. These three genes encode components of the Ras cell signaling pathway. The

pathway is initiated by ligand binding to the epidermal growth factor receptor (EGFR) which results in activation of its tyrosine receptor kinase, which then activates Growth factor receptor bound 2 protein (GRB2) (Qian et al. 2000). GRB2 sequesters the Son of sevenless 1 protein (SOS1) to the cell membrane, which binds to form a GRB2–SOS1 complex. Then SOS1 then activates Ras via its guanine nucleotide exchange factor function. Once initiated, Ras activates numerous pathways involved in the cell cycle, cell migration and cell proliferation. To perform candidate gene analysis, exonic coding regions, exon–intron boundaries, and partial introns of these three genes were sequenced from both affected ranched silver foxes and unaffected wild and farmed red foxes and compared. To further inform interpretation of any candidate mutations identified, we also examine rates and patterns of molecular evolution in *SOS1*, *GRB2* and *EGFR* genes among mammals.

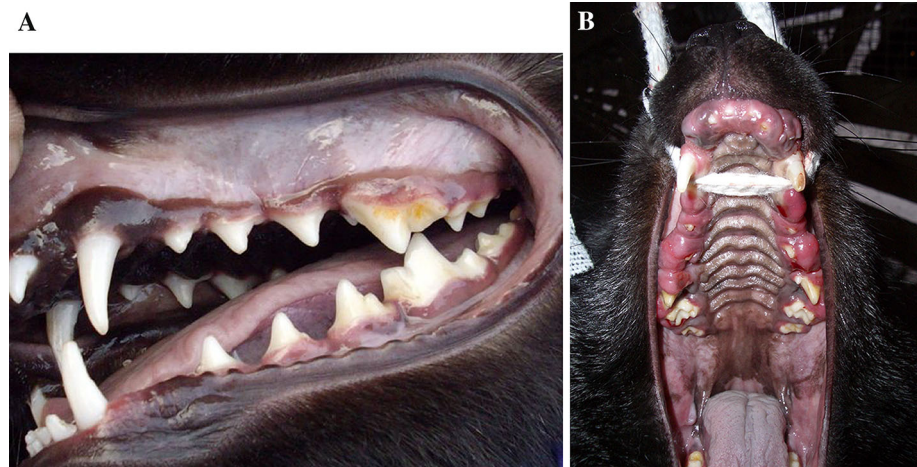
Materials and methods

Sample collection and DNA extraction

In 2004, a provincial government veterinarian on a fox farm in Newfoundland and Labrador, Canada, first documented the presence of HHG within the island of Newfoundland (Fig. 1). It was determined that the affected silver foxes were either the original Finnish silver foxes imported for their superior fur quality or first generation descendants of a cross between the new Finnish silver fox males with the Canadian silver fox females (Clark et al. 2014).

HHG-affected and unaffected silver foxes were obtained from the same Newfoundland fox farm. Provincial government veterinarians examined these animals and after gross manual examination determined the presence or absence of HHG. HHG showed phenotypic variation in severity, with diagnosis being made in the early stages of the disease when there was the presence of a thin layer of gingival tissue at the dental margin with the crown of the tooth that was slightly raised, red and granular. Provincial government veterinarians anesthetized these animals and small gingival sections were collected. Additionally, gingival and skeletal muscle samples were obtained from both HHG-affected and unaffected foxes post-mortem during pelting season. The Newfoundland and Labrador Department of Environment and Conservation Wildlife Division and Department of Natural Resources Animal Health Division provided wild unaffected red fox samples. The wild unaffected foxes from the Newfoundland and Labrador Department of Environment and Conservation Wildlife Division were captured from the area surrounding the St.

Fig. 1 **a** Silver fox with no gingival overgrowth in 2004. **b** Original Finnish silver fox with gingival overgrowth documented in 2004. (Photos provided by Robert Hudson)



John's International Airport and skeletal muscle was sampled. Animal Health Division unaffected foxes were obtained during a rabies eradication program where salivary glands were sampled. All sampling was compliant with Canadian Council on Animal Care regulations, and approved by the Institutional Animal Care Committee.

DNA extractions were performed with the QiaAmp DNA Mini kit (Qiagen Inc., Mississauga, Canada) according to the manufacturer's tissue protocol.

Primer design

Primers were designed using *Canis lupus familiaris* sequences with GenBank Accession Numbers NC_006600.3 (chromosome 18), NC_006591.3 (chromosome 9), and NC_006599.3 (chromosome 17), as references for *EGFR*, *GRB2* and *SOS1*, respectively. Primer pairs were selected from the intronic regions flanking each target region using either Primer3 (Rozen and Skaletsky 2000) or Oligo 4.1 (Molecular Biology Insights, Inc., Cascade, USA). The following criteria were used to choose primer pairs in Primer3: 23–29 bp length; 20–80 % GC content; primer T_m range 57–63 °C; and expected amplicon size 700–800 bp. The following criteria were used to choose primer pairs using Oligo 4.1: 20–22 bp length; 40–60 % GC content; T_m difference for the pair less than 12 °C; expected amplicon size 128–1,500 bp; optimal annealing temperature 50–57 °C. Some primers were tailed with standard M13 forward or reverse sequences for subsequent sequencing. Primers were manufactured by Operon (Huntsville, USA).

Polymerase chain reaction (PCR) and DNA sequencing

Each PCR contained 2.5 μL 10 × buffer, 0.5 μL dNTPs (New England Biolabs Ltd, Whitby, Canada), 1 μL of 10 μM

forward primer, 1 μL of 10 μM reverse primer, 0.2 μL Hot Star Taq Plus (Qiagen Inc., Mississauga, Canada), 19 μL distilled water and 1 μL template DNA. The thermal profile used was 95 °C for 15 min, followed by 40 cycles of 93 °C for 30 s, target-specific annealing temperature for 30 s, and 72 °C for 2 min, followed by 72 °C for 6 min. Amplified PCR products were purified using either Pall Life Sciences Multi-Well Plate Manifolds (Pall Corporation, Port Washington, USA) or the QIAquick PCR purification kit (Qiagen Inc., Mississauga, Canada). Target-specific annealing temperatures were as recommended by the primer selection software.

DNA sequencing reactions were performed with both forward and reverse primers for each PCR amplicon using BigDye Terminator v3.0 chemistry (Applied Biosystems Inc., Foster City, USA) 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, USA). Purified DNA sequencing reactions were electrophoresed on the Applied Biosystems Inc. 3,730 DNA Analyzer, in the GaP Facility of the CREAT Network at Memorial University of Newfoundland.

Data analysis

Raw data was collected using Sequence Analysis v5.2 (Applied Biosystems Inc., Foster City, USA) and imported into Sequencher v4.8 (Gene Codes Corp., Ann Arbor, USA). Contigs were created by assembling reads to the reference sequence using an 85 % minimum gap percentage and a 20 % minimum overlap, followed by manual trimming and editing of sequence each read. Consensus sequences for each individual animal for each gene were constructed and exported.

For each of the three genes examined, HHG-affected farm foxes and wild HHG unaffected foxes were sequenced. Initial sequence comparisons were performed using the HHG-affected and wild HHG-unaffected foxes. For any DNA sequence that demonstrated a trend segregating the HHG-affected farm foxes and HHG unaffected wild foxes, additional samples including HHG-unaffected farm foxes with no direct familial relation to the HHG-affected farm foxes were sequenced to explore any true segregating sequence differences between affected and unaffected groups of foxes.

Analyses of molecular evolution were conducted within MEGA 5.0 (Tamura et al. 2011). These included rates of synonymous and non-synonymous sequence variation (Nei-Gojobori algorithm), Z tests of selection (using Nei-Gojobori proportions), and HyPhy tests of site-specific selection. Three levels of taxonomic hierarchy were considered for each gene: within *V. vulpes*; between *V. vulpes* and *Canis lupus familiaris* (domestic dog); and among mammals using sequences accessed from GenBank (*Bos taurus*, *Mus musculus*, *Rattus norvegicus*, *Pan troglodytes*, *Homo sapiens*). For the *EGFR* gene, a McDonald-Kreitman test of selection (MacDonald and Kreitman 1991) was performed and a test of lineage-specific selection was conducted by comparing a model with a fixed dN/dS (ω) ratio parameter to a model in which this parameter was free to vary, using the log-likelihood ratio test implemented in PAML v4.5 (Yang 2007).

Results

Mutational differences between affected and unaffected foxes

The *EGFR* gene was sequenced for 19 unaffected foxes and 13 HHG-affected foxes; the total coverage for each individual ranged from 2,731 to 9,418 bp. The *SOS1* gene was sequenced for 15 unaffected foxes and 14 HHG-affected foxes. The total coverage for each individual for this gene ranged from 194 to 12,454 bp. The *GRB2* gene was sequenced for 12 unaffected foxes and 10 HHG-affected foxes, and the total coverage for this gene for each individual ranged from 318 to 2,139 bp. For each gene, there was 100 % coverage of the protein-coding region and the intron/exon boundaries for all HHG-affected and wild unaffected foxes. There were no fixed mutations segregating the HHG-affected from the unaffected sets of samples in any coding portions or intron/exon boundaries of the *EGFR*, *SOS1* or *GRB2* genes. There were no heterozygous sites conserved across affected foxes in any of these gene regions, as may be expected for a dominant mutation.

Table 1 Measures of sequence diversity in the coding portions of the *EGFR*, *GRB2*, and *SOS1* genes, within *Vulpes vulpes*, between *Vulpes vulpes* and *Canis familiaris*, and among mammals (pairwise averages)

| Comparison | Gene (nt) | | |
|--|-----------------|---------------|-----------------|
| | EGFR (3,543) | GRB2 (654) | SOS1 (4,032) |
| Within <i>V. vulpes</i> | | | |
| No. variable sites (%) | 12 (0.339) | 2 (0.306) | 4 (0.099) |
| Nonsynonymous | 4 | 0 | 0 |
| Synonymous | 8 | 2 | 4 |
| <i>V. vulpes</i> versus <i>C. familiaris</i> | | | |
| No. variable sites (%) | 41 (1.157) | 5 (0.760) | 19 (0.470) |
| Nonsynonymous (dN) | 20 (0.008) | 0 (0) | 9 (0.003) |
| Synonymous (dS) | 21 (0.031) | 5 (0.036) | 10 (0.011) |
| Among mammals | | | |
| P distance | 0.129 | 0.062 | 0.069 |
| dN | 0.047 | 0.001 | 0.014 |
| dS | 0.401 | 0.288 | 0.257 |
| dN/dS | 0.118 | 0.003 | 0.054 |
| Z test P value | 0 (purifying) | (purifying) | 0 (purifying) |

The 5' regulatory region of the *EGFR* gene was examined. In humans, this region is still not fully characterised but it contains several features within the 500 bp upstream of the 5' start codon. This is a GC rich section (GC content of 88 %) that contains multiple transcriptional start sites and several CCGCCC repeats (Ishii et al. 1985; Liu et al. 2005). Here, 1,775 bp upstream of the 5' start codon of the *EGFR* gene were examined and no CCGCCC repeat was present. In the 500 bp immediately upstream of the *EGFR* start codon in foxes the GC content was 52.4 % (158 Gs and 104 Cs). This region did not contain any fixed differences between the HHG-affected and unaffected foxes. In humans, a polymorphism at position -216 G/T is associated with promoter activity (Liu et al. 2005). This site was an invariant G in foxes.

Molecular evolution of the *EGFR*, *GRB2*, and *SOS1* genes in foxes and other mammals

Across all measures of sequence diversity at all hierarchical levels *EGFR* demonstrated the highest level of variation among the three genes (Table 1). *SOS1* was more variable than *GRB2* at non-synonymous sites, but less so at synonymous sites (Table 1) both between canids and among mammals. Variability of all genes was consistent with purifying selection (Z test dN/dS \ll 1; $P = 0$) with the highest functional constraints associated with *GRB2* and the lowest with *EGFR*. As the Z test is a very conservative test of positive selection requiring dN/dS (ω) >1 to reject

Table 2 Lineage-specific rates of nonsynonymous (dN) and synonymous (dS) substitution in the *EGFR* gene of mammals, and their ratio (ω), determined using phylogenetic analysis by maximum likelihood (PAML) branch-site model

| Species | dN | dS | ω |
|--------------------------|--------|--------|----------|
| <i>Rattus norvegicus</i> | 0.0088 | 0.1353 | 0.0652 |
| <i>Mus musculus</i> | 0.0097 | 0.0987 | 0.0980 |
| <i>Homo sapiens</i> | 0.0075 | 0.0165 | 0.0453 |
| <i>Pan troglodytes</i> | 0.0019 | 0.0122 | 0.0603 |
| <i>Vulpes vulpes</i> | 0.0062 | 0.0241 | 0.2580 |
| <i>Canis familiaris</i> | 0.0019 | 0.0074 | 0.2627 |
| <i>Bos taurus</i> | 0.0370 | 0.2754 | 0.1343 |

neutrality, we used site-specific test of selection (HyPhy) for all genes, and a McDonald-Kreitman test of selection for the *EGFR* gene, where the most variability was observed. HyPhy tests did not reveal evidence of site-specific selection in any gene ($P > 0.15$ for all sites in *EGFR*; $P > 0.29$ for all sites in *SOS1*; $P > 0.67$ for all sites in *GRB2*). The McDonald-Kreitman ratio of non-synonymous-to-synonymous changes was higher between *V. vulpes* and *Canis lupus familiaris* (20/20) than within *V. vulpes* (4/7), suggestive of positive selection, but the difference was not significant (Fisher exact test $P = 0.298$).

A lineage-specific test of selection was performed using phylogenetic analysis by maximum likelihood (PAML) with the branch-site model for the multispecies phylogeny of the *EGFR* gene (Yang 2007). The PAML model allowing ω (dN/dS) ratios to vary was significantly more likely than the model in which they were fixed ($\chi^2 = 32.7$; $P = 0$), to suggest the possibility of positive selection affecting amino acid sequence evolution in some of the mammalian lineages. The branch model indicated a range of lineage-specific ω ratios from 0.0453 for *H. sapiens* to 0.2627 for *Canis familiaris* (Table 2). The highest ω ratios were seen in the *Canis* (0.2627) and *Vulpes* (0.2580) lineages, suggesting the possible presence of positive selection in these lineages.

Discussion

HHG is an autosomal recessive gum disease occurring almost exclusively in ranched silver foxes (Dyrendahl and Henricson 1960). While nothing is known about the underlying genetic mutation(s) leading to its prolific gingival overgrowth phenotype, an analogous condition occurring in humans, HGF, has a known causative genetic mutation (Hart et al. 2002). In the present study, we explore the gene containing this mutation, *SOS1*, and two genes with functions in the same cell signaling pathway,

EGFR and *GRB2*, to determine if these genes carry any potential HHG causative mutations, in addition, to elicit the molecular evolutionary patterns of these genes in foxes and compare to other canids and mammals to assess the likelihood that mutations in these genes could drastically change the phenotype.

Mutational differences between HHG-affected and unaffected foxes

Since research into HGF suggests involvement of the Ras signaling pathway (Xiao et al. 2001), our aim was to start with the known HGF-associated gene *SOS1*, then examine the closely-associated *GRB2* gene and the adjacent upstream *EGFR* gene, to determine whether any mutational differences exist between HHG-affected and unaffected foxes that could affect protein function and potentially be causative of HHG.

The *SOS1* gene in humans spans 138,915 bp, contains 23 exons and encodes a seven domain protein consisting of a histone folding domain, a Dbl homology domain, a Pleckstrin homology domain cassette, a helical linker, a Ras exchange motif, a Cdc25 domain and a proline rich C-terminal which binds to *GRB2* (Findlay and Pawson 2008). The human *GRB2* gene is located at chromosomal position 17q24–q25, spans 87,634 bp, and consists of five exons. The ubiquitously expressed *GRB2* protein contains three domains: a Src homology 2 (SH2) domain flanked on either side by SH3 domains (Dharmawardana et al. 2006). The SH2 domain binds to tyrosine phosphorylated regions while the SH3 domains bind to other proline rich regions on other proteins such as to the C terminal domain of the *SOS1* protein. Duplication of the *GRB2* gene has been associated with leukemia's and tumours (Dharmawardana et al. 2006).

The results of sequence analyses of both the *SOS1* and *GRB2* genes in HHG-affected and unaffected fox demonstrated similar findings. Neither gene showed any fixed differences between these two groups that could represent putative causative mutations of HHG in either the coding regions or at the exon–intron boundaries. There were very few polymorphic sites in these genes at all among foxes, and no non-synonymous changes. These findings do not rule out the possibility that a mutation could exist in the upstream promoter/enhancer regions for either of these genes, or potentially elsewhere in the introns. Nonetheless, they do suggest at least that amino acid differences in the protein products of these genes, frameshift mutations leading to truncation or extension of the protein product, or failure to correctly excise introns, which would radically alter the protein product, are not the cause of the gum disease phenotype in foxes. We can confirm that the known

HGF causative mutation in *SOS1* is not found in HHG-affected foxes.

The 188,307 bp human *EGFR* gene, located on chromosome seven position p12, consists of 34 exons that are expressed as four protein variants (Lurje and Lenz 2009). All EGFR protein variants contain three domains: a variable extracellular receptor domain; a short, hydrophobic, membrane-spanning domain; and a tyrosine kinase intracellular carboxy-terminal domain (Scaltriti and Baselga 2006). Ligand binding results in a conformational change in the extracellular domain leading to receptor dimerization and subsequent autophosphorylation of the intracellular tyrosine residues, enabling the “docking” and activation of the GRB2 protein. Constitutive activity of this gene has been linked to cancer and cancer progression, and EGFR has been researched as a target for drug therapies (Scaltriti and Baselga 2006). Here, no mutational differences separated HHG-affected foxes from unaffected ones, indicating that similar to GRB2 and SOS1, structural differences in the encoded protein are not associated with HHG.

In humans, the 5' regulatory region of the EGFR gene, unlike most eukaryotic 5' regulatory regions, does not contain TATA or CAAT boxes. Instead this region has a GC content of ~88 %, and contains several CCGCCC repeats and multiple RNA initiation sites. A polymorphism at position –216G/T acts independently of other promoter region sequences to bind specificity protein 1 (Sp1), a transcription factor required for promoter activity (Liu et al. 2005); the thymine causes an increase in promoter activity. We found no fixed differences in the region immediately upstream of the *EGFR* gene in the two groups of foxes, including at position –216, hence have no evidence to suggest a promoter difference as causative of HHG. But, the GC content in foxes, similar to *C. familiaris*, was not as high as would be expected if this region functions the way it does in humans, so further exploration of the promoter region will be required before this gene's involvement in HHG can be eliminated. However there are fewer CpG islands in promoter regions in canines than in humans (Auton et al. 2013), which could account for the lower CG content seen in the upstream region of the *EGFR* gene in both *Canis* and *Vulpes*.

Molecular evolution of the SOS1, GRB2, and EGFR genes in mammals

The *SOS1* and *GRB2* genes showed similar molecular evolutionary patterns to each other, in that both genes were highly conserved at the intraspecific, intra-family, and among-genera levels in mammals. Less than 1 % of sites were variable in either gene between the domestic dog and the fox, separated by ~15 million years of evolution, while among mammals ~6 % of sites varied in pairwise

comparisons. Additionally, non-synonymous substitutions and ω ratios were low for both these genes, especially *GRB2*, consistent with strong functional constraints. The GRB2 protein functions as an adapter protein between a receptor and the cytoplasmic kinases (Kraskouskaya et al. 2013), while SOS1 is a nucleotide exchange factor activating both the Ras and Rac cascade pathways (Pierre et al. 2011). Consistent with high conservation, disruptions in both these genes have known disease consequences; the absence of GRB2 protein in mice, for example, is incompatible with life (Cheng et al. 1998), and in humans, mutations in *SOS1* lead to diseases like HGF1 and Noonan syndrome (Pierre et al. 2011).

Unlike *SOS1* and *GRB2*, *EGFR* showed high levels of sequence variation at both synonymous and non-synonymous sites, and *EGFR*'s ω ratio was about 40 times greater than *GRB2*. Elevated synonymous substitution rates may indicate an increase in mutation rate, relaxed selection related to alternative codon usage, or a recombination hotspot (Auton et al. 2013; Comeron and Aguade 1998). Elevated dN rates and ω values suggest either reduced functional constraints or positive Darwinian selection. To determine which, we considered four tests of selection. The Z test could not be rejected, but this test is known to be conservative, requiring ω ratios greater than unity. Nonetheless, site-specific tests also did not indicate positive selection at any codon sites ($P > 0.15$) nor could the McDonald-Kreitman test reject the equality of the non-synonymous-to-synonymous ratios of polymorphisms within *Vulpes* to fixed differences between *Vulpes* and *Canis*. However, the latter was higher, consistent with adaptive evolution; furthermore, of the 20 fixed non-synonymous differences between *Canis* and *Vulpes*, eight (40 %) were located in the third FU (Furin-like repeat) domain while only seven were between domains. The PAML lineage-specific test of selection supported a model allowing the ω ratio to vary among lineages, pointing to the possibility of positive selection in certain lineages (Yang 2007). Notably the *Canis*, *Vulpes*, and *Bos* ω values are the highest among mammals.

Why might there be positive selection in *EGFR* in the *Vulpes*, *Canis*, or *Bos* lineages? One possibility is that selection pressure is diversifying this receptor's ability to respond to different stimuli or participate in multiple pathways. The *EGFR* ω ratios pattern supports the deep evolutionary divergence between the clade of mammals containing the primates and rodents relative to the one containing the Cetartiodactyla 64–104 million years ago (Murphy et al. 2001), so the molecular evolutionary pattern of either relaxed functional constraints or positive selection we observe in *EGFR* may date to this divergence. Nonetheless this pattern suggests that mutations in EGFR may be more readily tolerated and less likely to lead to disease than those in *GRB2* or *SOS1*.

Conclusions

We have determined that HHG-affected silver foxes do not carry the same *SOS1* mutation that causes HGF in humans. We rule out the involvement of coding region or exon–intron boundary mutations in two adjacent Ras pathway genes, *EGFR* and *GRB2* although we cannot eliminate the possibility that promoter, upstream binding, or other regulatory mutations might be changing gene expression patterns of these genes in affected versus unaffected foxes. It is possible that the mutation still lies in a gene somewhere within the Ras pathway. We have also demonstrated high evolutionary conservation of the *SOS1* and *GRB2* genes among mammals. The *EGFR* gene is more highly variable than the other two genes, in a pattern consistent with relaxed functional constraints or possibly even positive Darwinian selection especially in the lineage containing *Canis* and *Vulpes*.

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