Veterinary and Comparative Oncology

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Journal:	*Veterinary and Comparative Oncology*
Manuscript ID	Draft
Manuscript Type:	Short Communication
Keywords:	ameloblastoma, animal model, canine acanthomatous ameloblastoma, HRAS, oncogenic mutation, odontogenic tumor

Ultra-frequent *HRAS* p.Q61R somatic mutation in canine acanthomatous ameloblastoma reveals pathogenic similarities with human ameloblastoma

Abstract

Ameloblastoma is a locally aggressive odontogenic tumor that occurs in humans and dogs. Most ameloblastomas in humans (AM) harbor mutually-exclusive driving mutations in *BRAF*, *HRAS*, *KRAS*, *NRAS* or *FGFR2* that activate MAPK signaling. The remarkable clinical and histological similarities between canine acanthomatous ameloblastoma (CAA) and AM suggest they may harbor similar driving mutations, yet no studies have investigated these genes in CAA. Here we demonstrate that 94% of CAA harbor a somatic *HRAS* p.Q61R mutation, suggesting that the molecular mechanisms of tumorigenesis are conserved, and thus qualifying the dog as a potentially useful model of disease. Given the relevance of *RAS* mutations in the pathogenesis of odontogenic tumors and other types of cancer, the results of this study are of comparative, translational, and veterinary value.

Keywords: ameloblastoma; animal model; canine acanthomatous ameloblastoma; *HRAS*; oncogenic mutation; odontogenic tumor.

Ameloblastoma is an odontogenic tumor that occurs in humans and dogs.[1-3] As in humans, ameloblastomas in dogs are more common in the mandible than in the maxilla, and are typically locally aggressive and destructive (**Figure 1**).[3] The standard of care for ameloblastomas has traditionally been wide surgical excision of the affected area of the mandible or maxilla.[4,5] However, surgery is highly invasive and may result in significant disfigurement and dysfunction.[6,7] Novel therapeutic approaches based on molecular oncogenic mechanisms are expected to replace or complement current surgical solutions and thus help minimize patient morbidity while improving possible outcomes.[8]

Human ameloblastomas (AM) express early dental epithelial transcription factors such as *PITX2,* pointing out to the origin from a dental laminal cell.[9] Recent discoveries have shown that the majority of AM are driven by mutually-exclusive *BRAF*, *HRAS*, *NRAS*, *KRAS* or *FGFR2* mutations that activate the MAPK pathway regulating cell survival.[7,10-14] Co-occurring *SMO* mutations that alter the Hedgehog signaling pathway to regulate cell fate have also been detected, especially in maxillary tumors.[11,12] Both pathways are mutated in various cancers and have been targeted for therapy, making them of particular interest in modern oncology.[7]

Tooth development is similar in humans and dogs, and AM and canine acanthomatous ameloblastomas (CAA) show remarkable clinical and histological and similarities.[3,13,15] Therefore, it is likely that both tumors harbor similar oncogenic mutations. The purpose

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of this study was to determine whether CAAs harbor any of the oncogenic mutations frequently found in AM.

Our study consisted of the molecular characterization of candidate oncogenic mutations in 16 CAA cases from client-owned dogs (**Table 1**). Eight gingival oral squamous cell carcinoma (OSCC) tumor samples, as well as 4 healthy gingival samples, all from different dogs, were used as controls. Dog owners signed an informed consent for use of tissues and blood for research purposes prior to collection. Sample collection and experimental procedures were in accordance with a protocol (#2005-0151) approved by Cornell University's Institutional Animal Care and Use Committee. All tumors were confirmed by histological assessment of routine H&E stained formalin-fixed and paraffin-embedded tumor sample by a board-certified veterinary pathologist (GED) (**Figure 2**).

Frozen study and control tissues were homogenized in Trizol (Thermo Fisher) using a bead mill without being allowed to thaw. RNA was isolated following the manufacturer's protocol with the following modifications: an extra chloroform extraction was added prior to precipitation, 1 µl glycoblue (Thermo Fisher) was added immediately prior to precipitation, and the RNA pellet was washed twice with 75% ethanol. RNA concentration was measured with a Nanodrop and integrity determined with a Fragment Analyzer (AATI).

RNAseq libraries were generated with the NEBNext Ultra II Directional library prep kit (NEB) using 250ng input total RNA. Single-end 85nt reads were generated on a NextSeq500 instrument (Illumina). Reads were trimmed with cutadapt[16] and mapped to the CanFam3 reference genome with Tophat[17] v2.1.1 to investigate polymorphisms in candidate oncogenic driver genes.

For Sanger sequencing and restriction fragment length polymorphisms (RFLP) validation assays, each RNA sample was used to generate cDNA using MMLV reverse transcriptase (Sigma) according to the manufacturer's instructions in a final 20µl reaction containing 500ng total RNA, 33uM random nonamer primers, and 25uM oligo-dT20 primers. The reaction was incubated at 37°C for 1 hour followed by 85°C for 10 minutes to inactivate the reverse transcriptase. Genomic DNA was isolated from EDTA blood samples by the Cornell Veterinary Biobank using a standard salt precipitation technique, as previously reported.[18]

PCR primers were designed within *HRAS* exon 2 flanking the p.Q61R mutation using Primer-BLAST11 at NCBI: forward primer ATCGGAAGCAAGTGGTCATCG, reverse primer ACTGGTGGATGTCCTCAAAGG. Each PCR reaction contained 1x Q5 Hot Start High Fidelity Master (New England Biolabs), 25uM each PCR primer (IDT), and 1µl cDNA (from tumor RNA) or 500ng genomic DNA (from blood) in a 50µl reaction volume. PCR reactions were cycled as follows: denaturation at 98°C/3min; 35 cycles of 98°C/10sec - 62°C/10sec - 72°C/15sec; final extension 72°C/5min. Reactions were cleaned up with a PCR purification kit (Qiagen buffers, Omega HiBind columns), eluted in 50μl EB, and quantified with a Nanodrop. PCR amplicons were sequenced at the Cornell BRC Genomics Facility using 8 μl PCR product (120-400ng) combined with 25pmol reverse primer to generate antisense reads across the variant position. KB Basecaller software was used to compensate for mobility differences between dyes. Restriction digest reactions containing 45ng PCR product, 1x buffer, 1μl restriction enzyme (New England Biolabs) in total 20μl reaction volume and were incubated at the recommended temperature for 20 minutes. Digested samples were run on a native 6% PAGE gel at 100V for 2 hours and stained with GelStar (Lonza).

Analysis of RNA sequencing data identified reads that indicated a possible *HRAS* missense mutation (A → G) that would alter the protein sequence from glutamine (cAg) to arginine (cGg) at position 61 (p.Q61R mutation) in 11 of 12 CAA samples, while no deleterious sequence variants were observed in *BRAF*, *NRAS*, *KRAS*, *FGFR2* or *SMO* (see **Supporting Information**). To validate the occurrence of the *HRAS* p.Q61R mutation in CAA, we designed a PCR assay for canine *HRAS* exon 2 flanking the variant site (amplicon size 168bp) that can be used on either RNA (cDNA) or genomic DNA samples. The presence of the variant allele was assessed by two different approaches: Sanger sequencing and RFLP. The presence of the variant allele was detected by Sanger sequencing in cDNA from 15 of 16 (94%) CAA samples (inclusive of the 12 samples used for RNA sequencing plus 4 additional independent samples), as evidenced by a T nucleotide (antisense strand) as well as the wild-type C nucleotide (**Figure 3; Supporting Information**). To determine whether the mutant allele was present in other (non-tumor) tissues in some of the same dog or arose within the tumor,

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we also sequenced genomic DNA isolated from paired blood samples from 7 dogs. All 7 had only the wild-type allele, indicating a somatic mutation occurred in the tumor in each case.

We further investigated these results using RFLP analysis on all 16 CAA samples available for analysis. We developed RFLP assays for both the mutant and wild-type alleles: HpaII cuts only the mutant allele (CCGG) to generate bands of 122bp and 46bp;d BstNI cuts the wild-type allele as well as an adjacent invariant site, so the wild-type allele yields 46bp and 107bp bands, whereas samples harboring the mutant allele have a band at 122bp (the 15bp fragment cut from the wild-type product is not visible on the gel). In all cases, the RFLP assays were internally consistent and agreed with the Sanger sequencing, confirming that all but one CAA sample had the mutant allele. RFLP analysis for the mutant allele (HpaII assay) in genomic DNA from 7 paired blood samples again confirmed the *HRAS* p.Q61R mutation is somatic and not inherited.

To confirm that the *HRAS* p.Q61R mutation was specific to CAA, we performed Sanger sequencing on 8 gingival OSCC and 4 healthy gingival samples from 12 different dogs. Oral squamous cell carcinoma was chosen as a control tissue because this tumor type represents the most common oral malignancy of epithelial origin in dogs, and shares some histological features with CAA.[6,19,20] Sanger sequencing showed that only wild-type *HRAS* alleles were present in healthy gingival samples (see **Supporting Information**). All OSCC samples had wild-type *HRAS* alleles except case 22 which

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showed an *HRAS* p.Q61L mutation, consistent with a previous report showing low frequency of this mutation in this tumor type.[21]

The presence of a somatic *HRAS* p.Q61R mutation in 94% of the CAA tumor samples analyzed in this study demonstrates that the mutational basis of CAA and AM is comparable (**Figure 4**), and strongly implicates conserved oncogenic molecular mechanisms. The known clinical and histological similarities between CAA and AM,[15] and now the observation of common oncogenic mutations in both species, invokes the opportunity to leverage dogs as a natural model of this disease. Moreover, given that *RAS* mutations are also well known drivers of several types of cancer in people,[22] our findings suggest that dogs may potentially be used as translational models for targeted treatments of MAPK pathway mutated neoplasms. This is a significant finding considering that dog models offer many advantages over small and (other) large animal models (i.e. primates), and can be particularly valuable for drug discovery and development purposes.[23]

The apparent prominent role of *RAS* mutations in tumorigenesis of odontogenic neoplasms is not unprecedented.[14] Indeed, *RAS* mutant mice have been shown to develop odontogenic neoplasms.[24] Additionally, most adenomatoid odontogenic tumors harbor *RAS* mutations.[25] Nevertheless, some interesting comparisons should be made between CAA and AM, which emphasize the need for additional studies. One notable difference lies in the frequency of the *HRAS* p.Q61R mutation. Contrasting with our findings, only 20% of AM harbor *RAS* mutations, with *HRAS* p.Q61R occurring with a frequency of 6% or less.[10-13]

Similarly, while two thirds or more of AM harbor a *BRAF* V600E mutation,[10-13] synonymous mutations have not been identified in CAA,[26] underscoring potentially significant differences in the mutational profiles. Nevertheless, given that both *BRAF* and *RAS* mutations activate MAPK signaling,[14] this difference does not necessarily translate in differences in the biologic mechanisms involved. As well, our findings suggest that MAPK signaling is involved in both maxillary and mandibular CAA. This would be consistent with the hypothesis that MAPK activating mutations dominate oncogenesis of maxillary and mandibular AM, and that *SMO* and other simultaneous mutations act as secondary drivers.[12]

It should be noted that the scope of this pilot study focused on candidate genes known to be mutated in AM,[10-13] and that it is possible that other genes could be mutated in CAA tumors, including non-coding RNAs.[27] Furthermore, whether the *HRAS* p.Q61R allele found in the tumors analyzed in this study acts as a primary or secondary oncogenic driver cannot be ascertained. Genome-scale mutational analysis as well as molecular phenotyping will help elucidate ways in which the dog may be a suitable natural model of AM, or may uncover subtypes of this disease.

The veterinary clinical and diagnostic significance of the current study should not be overlooked in light of its comparative and translational potential. Assays specific to the

HRAS p.Q61R allele, such as those described here, may offer veterinary diagnostic potential to distinguish CAA from other oral tumors with overlapping histological features but different biologic behavior, including OSCC.[6,19,20] Our confirmation of high frequency MAPK-activating alleles in CAA justifies the investigation of precision-based therapies for companion dogs that have the potential to mediate disease progression as an alternative to current invasive and costly surgical treatments.

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Table 1. Clinical information and *HRAS* mutation status of dogs included in this study.

Case No.	Dog breed	Sex	Age (years)	Diagnosis	Tumor/tissue location	HRAS status in tumor tissues	HRAS status in genomic DNA
1	Great Dane	Male	7	CAA	Rostral maxilla	Q61R	Wild-type
2	Samoyed	Male	12	CAA	Rostral maxilla	Q61R	Wild-type
3	Shiba Inu	Male	11	CAA	Rostral maxilla	Q61R	Not tested
4	Golden retriever	Male	10	CAA	Rostral maxilla	Q61R	Wild-type
5	Neapolitan mastiff	Male	10	CAA	Rostral mandible	Q61R	Wild-type
6	Mixed	Male	Unknown (adult)	CAA	Rostral mandible	Q61R	Not tested
7	Mixed	Male	6	CAA	Rostral mandible	Q61R	Not tested
8	Mixed	Male	12	CAA	Rostral mandible	Q61R	Not tested
9	Labrador retriever	Female	12	CAA	Rostral mandible	Q61R	Wild-type
10	Labrador retriever	Male	9	CAA	Rostral mandible	Q61R	Not tested
11	Airedale terrier	Female	10	CAA	Caudal mandible	Q61R	Wild-type
12	Labrador retriever	Male	9	CAA	Caudal mandible	Q61R	Not tested
13	Labrador retriever	Female	11	CAA	Caudal mandible	Q61R	Not tested
14	Labrador retriever	Female	8	CAA	Caudal mandible	Q61R	Wild-type
15	Staffordshire bull terrier	Female	9	CAA	Caudal mandible	Q61R	Not tested
16	Labrador retriever	Male	5	CAA	Caudal mandible	Wild-type	Not tested
17	German shorthair pointer	Female	12	OSCC	Caudal mandible	Wild-type	Not tested
18	Great Dane	Male	5	OSCC	Rostral mandible	Wild-type	Not tested
19	Boxer	Male	9	OSCC	Caudal maxilla	Wild-type	Not tested
20	Mixed	Male	12	OSCC	Rostral mandible	Wild-type	Not tested
21	Mixed	Male	4	OSCC	Rostral mandible	Wild-type	Not tested
22	Boxer	Male	12	OSCC	Rostral mandible	Wild-type	Not tested
23	Mixed	Female	10	OSCC	Caudal maxilla	Wild-type	Not tested
24	Mixed	Female	10	OSCC	Rostral maxilla	Wild-type	Not tested
25	Beagle	Male	3	Healthy gingiva	Rostral mandible	Wild-type	Not tested
26	Staffordshire bull terrier	Male	10	Healthy gingiva	Caudal mandible	Wild-type	Not tested
27	Pit-bull mix	Male	0.3	Healthy gingiva	Rostral maxilla	Wild-type	Not tested
28	Mixed	Female	Unknown (adult)	Healthy gingiva	Caudal maxilla	Wild-type	Not tested

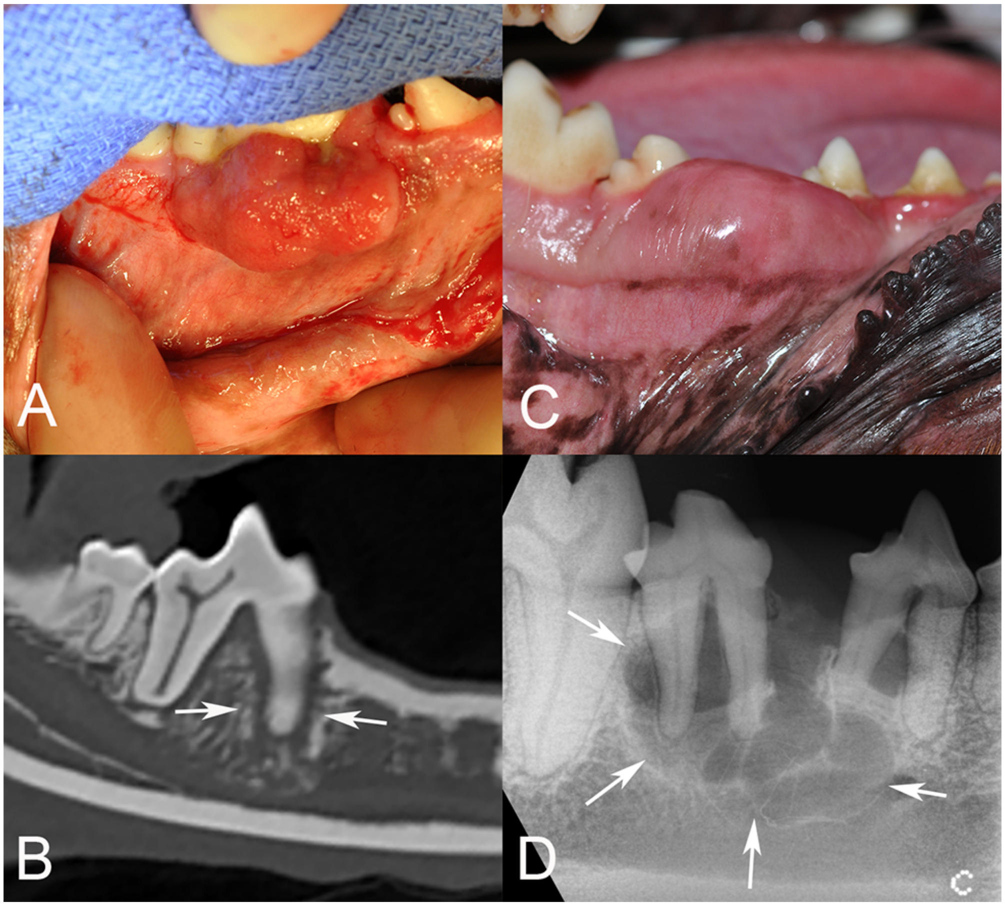
Figure legends

Figure 1. Clinical and radiographic features of CAA (A-B, dog No. 14; C-D, dog No. 16). (A) Soft tissue mass arising from periodontal tissues on the buccal aspect of the right mandibular first molar tooth. (B) A sagittal computed tomographic image of the same animal shows that the tumor infiltrates the entire alveolus of the mesial root of the same tooth (white arrows). (C) Solid mass under seemingly intact mucosa on the buccal aspect of the right mandibular third and fourth premolar teeth. (D) An intraoral radiograph of the same dog shows the tumor deeply invading the mandible resulting in a multilocular radiographic appearance (white arrows).

Figure 2. Histomorphologic features of CAA. (A) Gingival mucosal biopsy with an unencapsulated, locally infiltrative, densely cellular, multicystic exophytic neoplasm composed of broad, irregular, and anastomosing islands and trabeculae of odontogenic epithelial cells interspersed with fibrovascular stroma containing small foci of mineralized woven bone tissue (arrows). (B) Higher magnification showing polygonal neoplastic cells with distinct borders, moderate amount of eosinophilic cytoplasm and small round to oval nuclei with dispersed chromatin and 1-2 inconspicuous nucleoli. Note characteristic perpendicular palisading of neoplastic columnar epithelial cells with anti-basal nuclei (reverse polarization, thick arrow) at the periphery of an island and intercellular bridges of more central cells (thin arrow). Dog No. 11, H&E.

Figure 3. *HRAS* p.Q61R genotyping assays on CAA tumors and paired blood. (A) Sanger sequencing chromatograms of the antisense strand indicate the presence of a C nucleotide (top) causing the missense Q61R mutation (dog No. 5); this mutation is not evident in genomic DNA isolated from blood from the same dog (bottom). (B-D) Restriction fragment length polymorphism analyses rapidly characterize sample genotypes; case numbers are shown at the top of each lane. (B) HpaII cuts only the mutant allele (CC**G**G); (C) BstNI cuts the wild-type allele (CC**W**GG); (D) HpaII digest on genomic DNA from 7 paired blood samples confirmed that the *HRAS* p.Q61R mutation is somatic and not inherited (Size ladders: pBR322 MspI in panels 3B, 3C (center), 3D; 10bp ladder in panel 3C (left/right).

Figure 4. The percentage of oncogenic mutations in CAA (N = 16) and AM (N = 113). (A) *HRAS* p.Q61R mutations in CAA as reported in the present study (maxilla N = 3; mandible N = 12). One mandibular CAA case was wild-type (WT). (B) *BRAF* V600E, *HRAS*, *NRAS* or *KRAS*, *FGFR2*, *SMO* and wild-type in AM (maxilla N = 27, mandible N = 86) based on previous studies.[10-13]



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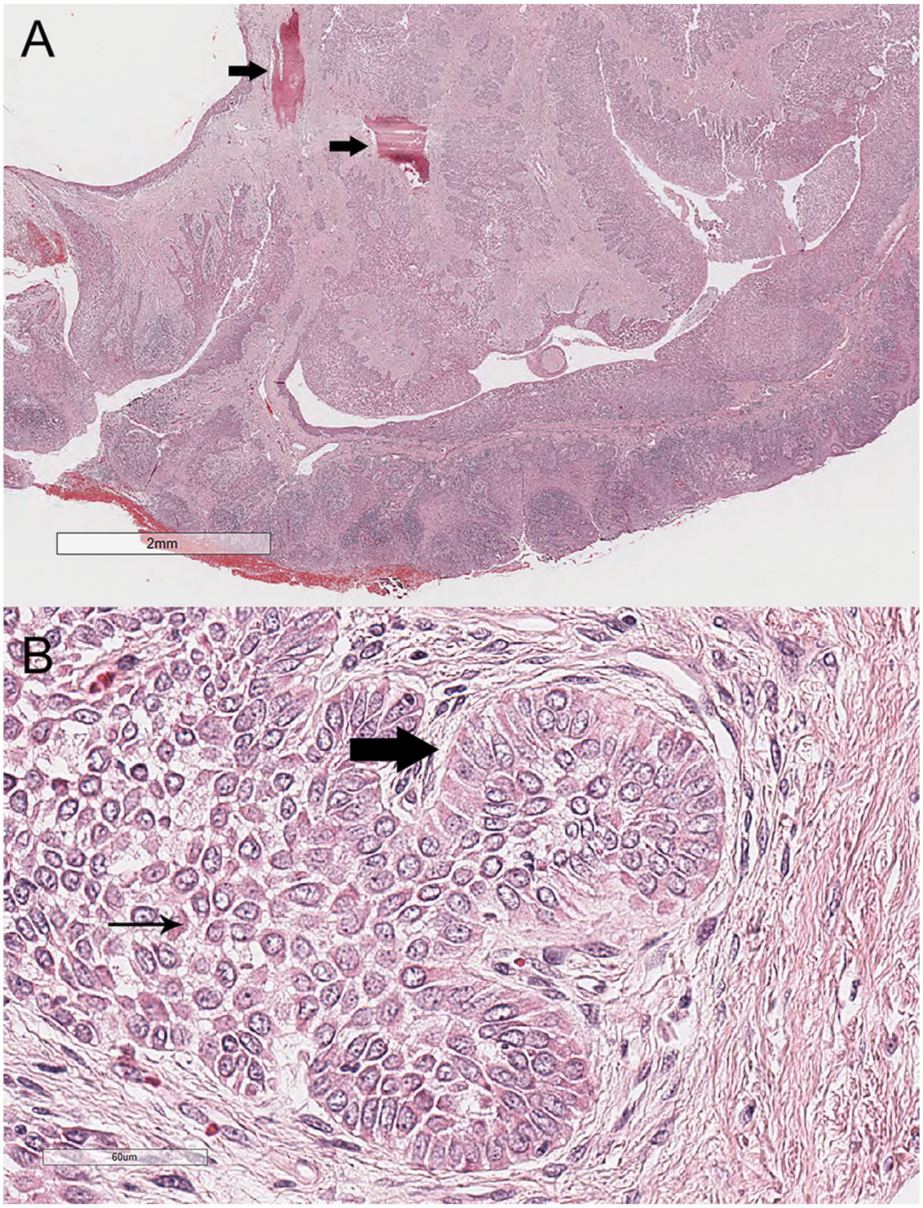

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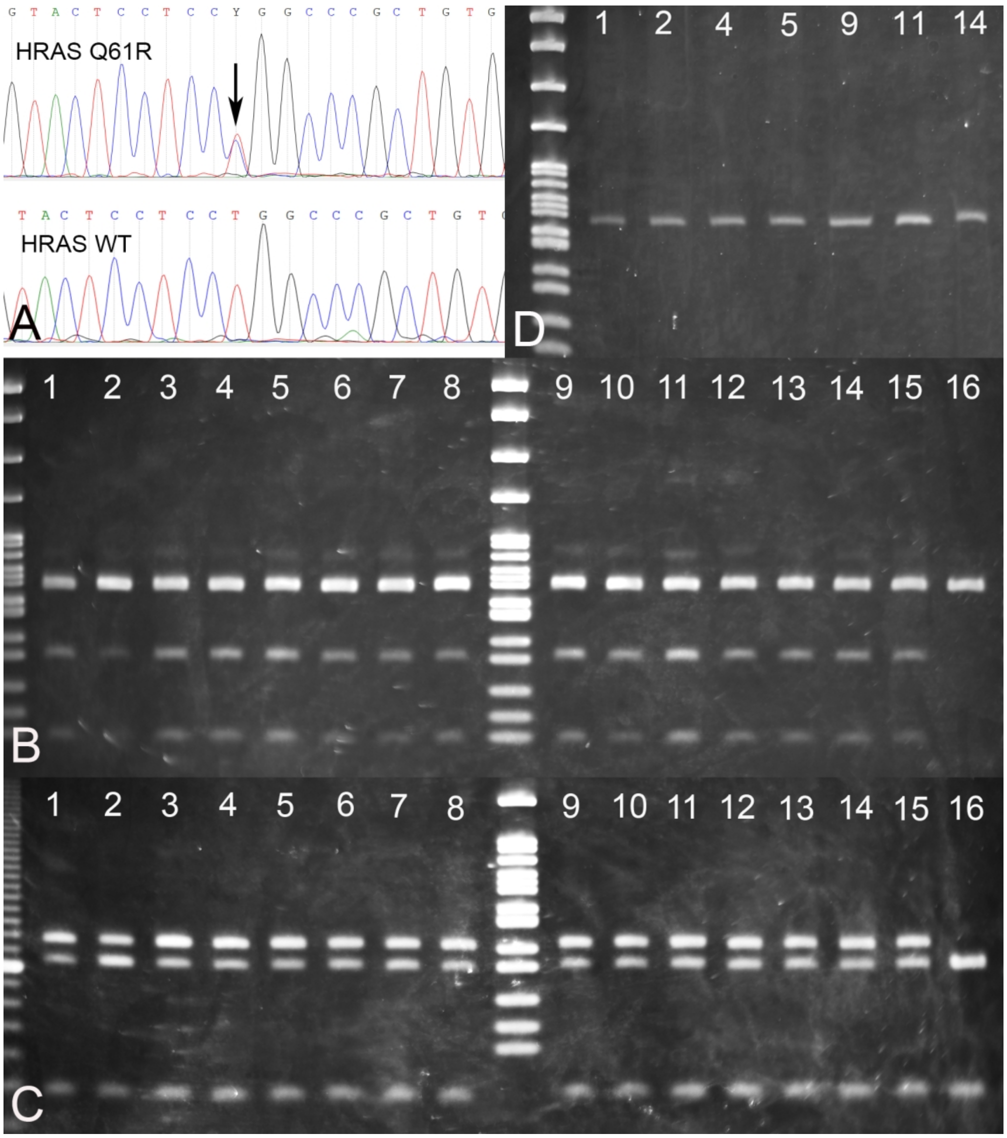

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