



Functional alteration of canine isocitrate dehydrogenase 2 (IDH2) via an R174K mutation

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ABSTRACT. Gliomas are common intracranial neoplasias in dogs. However, the underlying pathogenic mechanisms remain unclear. In humans, isocitrate dehydrogenase 2 (IDH2) is often mutated in gliomas. Although almost human IDH2 mutations have been identified at the Arg172 codon, few studies have reported structural, functional or mutational information for canine IDH2. In this study, we cloned the full-length canine IDH2 (cIDH2) cDNA and substituted wild type Arg174 (cIDH2 WT: corresponding to R172 of human IDH2) with Lys (cIDH2 R174K). The cIDH2 WT and R174K proteins were overexpressed in HeLa cells, and their presence was confirmed using an anti-human IDH2-WT mAb (clone: KrMab-3) and an anti-IDH2-R172K mAb (clone: KMab-1). The IDH2 activity between cIDH2 WT and cIDH2 R174K transfectants was compared by measuring the production of NADH and NADPH. NADPH production was lower for cIDH2 R174K than that for cIDH2 WT transfectants. Finally, we detected increased expression of hypoxia inducible factor-1 alpha (HIF-1 α) in cIDH2 R174K transfectants. This indicates that mutations at R174 can potentially induce carcinogenesis in canine somatic cells.

KEY WORDS: canine, glioma, IDH2, mutation

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Isocitrate dehydrogenases (IDHs) catalyse the oxidative decarboxylation of isocitrate to α -ketoglutarate (α -KG), producing NAD(P)H from NAD(P)⁺ [16]. Eukaryotes have both NAD⁺- and NADP⁺-dependent IDHs. IDH3 is NAD⁺-dependent and localised to the mitochondrial matrix and it plays a central role in aerobic energy production in the tricarboxylic acid (TCA) cycle. IDH1 and IDH2 are both NADP⁺-dependent and localised to the mitochondrial matrix [6, 9, 15, 18]. A recent mutational analysis of human cancers revealed predominant somatic mutations in human IDH2 (hIDH2) in gliomas [22]. Almost all the hIDH2 mutations were identified at Arg residue 172 (R172). A metabolite profiling analysis showed that hIDH2 mutants with mutations at R172 had reduced α -KG and increased 2-hydroxyglutarate (2-HG) production while converting NADPH to NADP⁺ [8]. 2-HG is known to accumulate in the inherited metabolic disorder 2-hydroxyglutaric aciduria [19]. The elevated levels of 2-HG in the brain increased reactive oxygen species, leading to a variety of downstream sequelae [21].

Although intracranial tumors in dogs, such as meningiomas and gliomas, are relatively common brain diseases [1, 2], the pathogenic mechanisms remain unclear. A mutation analysis of canine IDH1 (cIDH1) R132 and IDH2 (cIDH2) R238, using 25 cases of canine glioma, failed to detect any mutations involving these codons [17]. Most of the mutations in human IDH1 (hIDH1) and hIDH2 occur at R132 and R172, respectively, but there is no report investigating cIDH2 R174 (corresponding to R172 of hIDH2) mutations. Previously reported that residue 238 of canine IDH2, isolated from glioma cases were sequenced [17]. There was no variation compared to canine wildtype partial sequences. We hypothesised that there is a relationship between the development of gliomas and occurrence of mutations in cIDH2. In a human study, IDH mutations were identified by immunostaining with specific antibodies [5, 11–13, 20], but there are no data to support cross-reactivity of human antibodies with canine proteins.

In the present study, we cloned and sequenced the complete open reading frame (ORF) of the cIDH2 homologue, and induced a mutation at R174 in cIDH2. We also validated the cross-reactivity between human antibodies and cIDH2 mutants. Furthermore,

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Table 1. Primer sequences used in this study

Purpose	Base sequence	
	Forward	Reverse
Canine IDH2 amplified for sequence	5'-CCGGCGCCGTCTGCCTGTG-3'	5'-TAGCTCAGCCCCACCCGCCAC-3'
Canine IDH2 cloned into pMACS Kk HA-C	5'-CTCGAGACCACCATGGCTGGCTACCTGCGGGTCG-3'	5'-GAATTCCTGGCCAGAGCCTTGCCAGG-3'
R174K mutation primers	5'-ATTGGCAAACACGCCATGG-3'	5'-ATGGGCGTGTTTGCCAATGGTG-3'

IDH activity between wild type (WT) and mutant cIDH2 in HeLa cells was compared, and hypoxia inducible factor-1 alpha (HIF-1 α) expression in these cells was also assessed.

MATERIALS AND METHODS

cDNA cloning, sequencing, and mutagenesis of canine IDH2 (cIDH2)

RNA was obtained from total RNA of canine kidney cells (Zymogen, San Diego, CA, U.S.A.) and reverse-transcribed using SuperScript III (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.). Polymerase Chain Reaction (PCR) amplification was performed using PrimeSTAR (Takara, Tokyo, Japan) and dATP was added to the PCR products, using a 10 \times A-attachment kit (Toyobo, Osaka, Japan). PCR products were cloned into pGEM-T Easy (Promega, Madison, WI, U.S.A.) vector. The sequences were determined for at least five independent clones (ABI 3730; Applied Biosystems, Waltham, MA, U.S.A.). Nucleotide and amino acid (aa) sequences were analyzed and compared to the predicted canine, human and mouse sequences using Genetyx software (Genetyx Corp., Tokyo, Japan) and the ClustalW program. The functional domains of cIDH2 were queried using the protein family database (Pfam) program. To construct cIDH2 R174K, nucleotide substitutions were performed by PCR mutagenesis using a pGEM-T Easy vector carrying the wild type sequence as the template. Cloned cIDH2 WT and R174K were subcloned into haemagglutinin (HA)-tagged vector pMACS Kk HA-C (Miltenyi Biotec, Auburn, CA, U.S.A.). The PCR primer sets used in this study are described in Table 1.

Cell line and culture conditions

HeLa and MDCK cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, U.S.A.) and Hs68 human foreskin normal fibroblasts were purchased from RIKEN BRC (Tsukuba, Japan), and were maintained in Dulbecco's modified Eagle's medium (DMEM; Wako, Osaka, Japan) supplemented with 10% foetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin (Thermo Fisher Scientific Inc.) at 37°C in an atmosphere containing 5% CO₂.

Transfection and immunoblotting

Cells were plated and cultured to 80% confluency in 6-well plates, and transfected with the HA-tagged cIDH2 WT and cIDH2 R174K via FuGENE HD (Promega). Forty-eight hours after transfection, the cells were lysed using ice-cold RIPA buffer (Nacalai Tesque, Kyoto, Japan) and incubated for 15 min at 4°C. Insoluble fragments were removed by centrifugation at 16,000 \times g for 10 min at 4°C, and the supernatants were collected. Protein concentrations were determined using the Bicinchoninate Protein Assay kit (Nacalai Tesque). Approximately 10 μ g of the extracted protein was analyzed by western blotting (WB) with the following antibodies: anti-HA (561, 1:1,000, MBL, Nagoya, Japan), anti-IDH2 WT and mutant (clone KrMab-3; 1:1,000, MBL), anti-IDH2 R172K (clone KMab-1; 1:1,000, MBL) [10], anti-HIF-1 α (#3716, 1:1,000, Cell Signalling Technology, Beverly, MA) and β -actin (PM053, 1:2,000, MBL). Horseradish peroxidase-conjugated secondary antibodies (1:5,000) and EzWestLumi plus (ATTO, Tokyo, Japan) were used for the detection of antibody-bound proteins.

Immunocytochemistry

Cells were plated and cultured to 30–40% confluency in LabTek chambers (Nalgene, Rochester, NY, U.S.A.) and were transfected with the HA-tagged cIDH2 WT and cIDH2 R174K by FuGENE HD (Promega). Forty-eight hours after transfection, the cells were fixed with 4% paraformaldehyde in 100 mM phosphate buffer and blocked with 5% normal goat serum in phosphate-buffered saline (PBS). The cells were incubated with following monoclonal antibodies: anti-IDH2 WT and mutant (1:100), anti-IDH2 R172K (1:100), overnight at 4°C and then with Alexa Fluor 488-conjugated anti-mouse secondary antibody (1:500; Molecular Probes, Rockford, IL) for 1 hr at 25°C. To stain the nuclei, cells were incubated with Hoechst 33342 (Dojindo, Osaka Japan) for 15 min at 25°C. Normal rabbit IgG (Santa Cruz Biochemistry, Santa Cruz, CA, U.S.A.) was used as a negative control. The fluorescent staining was visualised under a fluorescence microscope (BZ-9000; KEYENCE, Tokyo Japan).

Measurements of isocitrate dehydrogenase activity

To measure the production of NADH and NADPH, cIDH2-transfected cells (5 \times 10⁴ cells) were processed using the Isocitrate Dehydrogenase Activity Colorimetric Assay Kit (BioVision, Milpitas, CA, U.S.A.) as per the manufacturer's instructions. The reaction mix was treated for 10 min and then the optical density at 450 nm was measured using an iMark™ microplate reader (BioRad, Hercules, CA, U.S.A.).

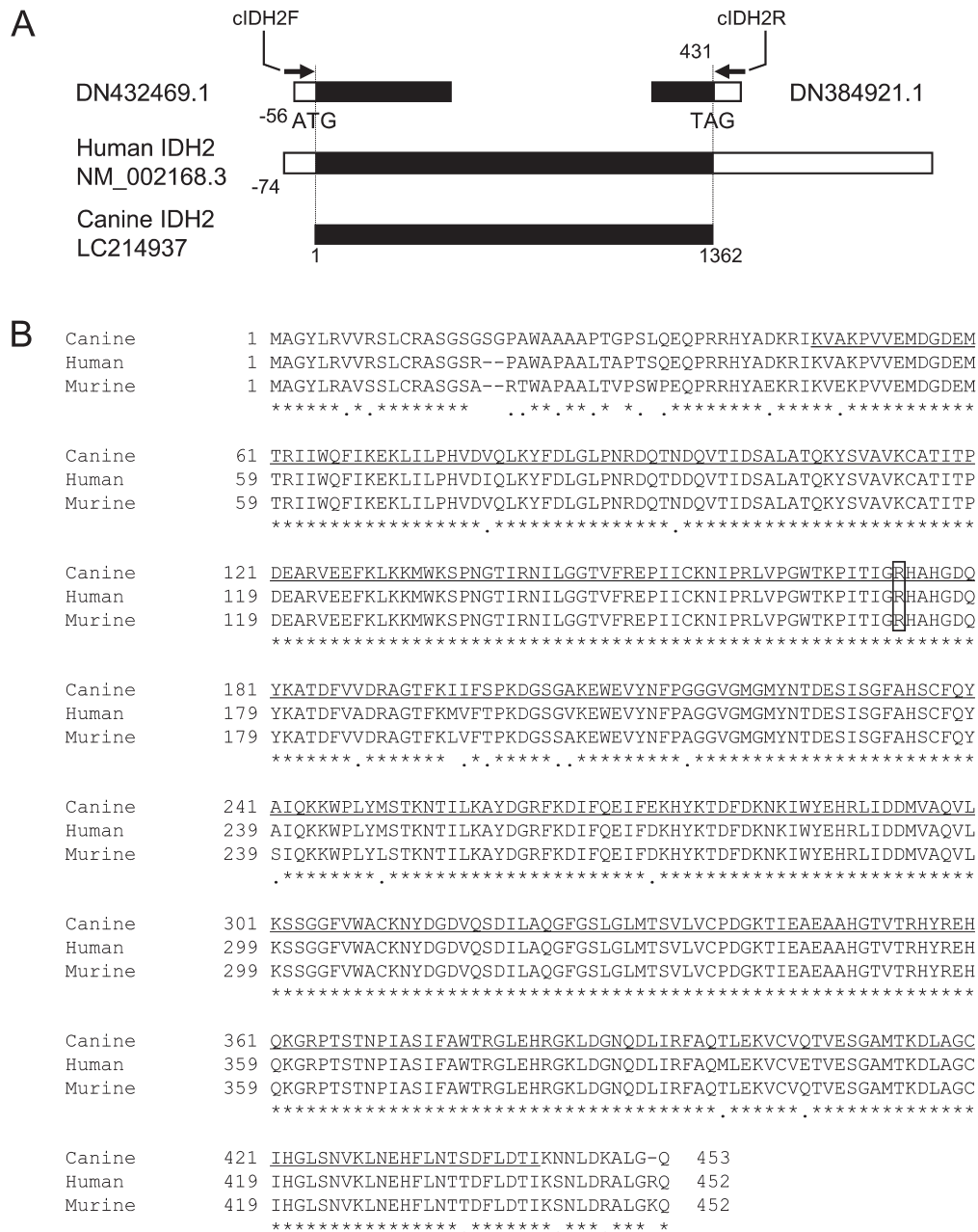


Fig. 1. Cloning strategy and sequence alignment of canine IDH2 (cIDH2). (A) Alignment of nucleic acid sequences identified in this study (LC214937) with the expressed sequence tag (EST) sequences (DN432469.1 and DN384921.1) and the human IDH2 (hIDH2) sequence (GenBank NM_002168.3). Identical sequences are indicated by black boxes. The start and stop codons are indicated with dotted lines. The primers used for cloning and sequencing (cIDH2F and R) are indicated by arrows. (B) Comparison of hIDH2 (NP_002159.2) and mIDH2 (NP_766599.2) amino acid (aa) sequences to the cIDH2 sequence (LC214937) that was identified in this study. The predicted aa sequence was aligned using ClustalW. The symbol '*' indicates single fully conserved aa sequences between all three species. The symbol '.' indicates conservation between two species. The potential isocitrate dehydrogenase region is underlined. The mutation hotspot for hIDH2 at R172 is boxed.

Statistical analysis

The data are shown as means \pm SD. Student's *t*-test was performed to assess the significance of differences between groups from four independent experiments. Differences were considered to be significant at $P < 0.01$.

RESULTS

Cloning and structural analysis of canine IDH2

The ORF of cIDH2 determined in this study (GenBank LC214937) was 1362-bp long and was predicted to code for 453 amino acids (Fig. 1A and 1B). The putative cIDH2 protein has one more residue than hIDH2 and murine IDH2 (mIDH2) (GenBank

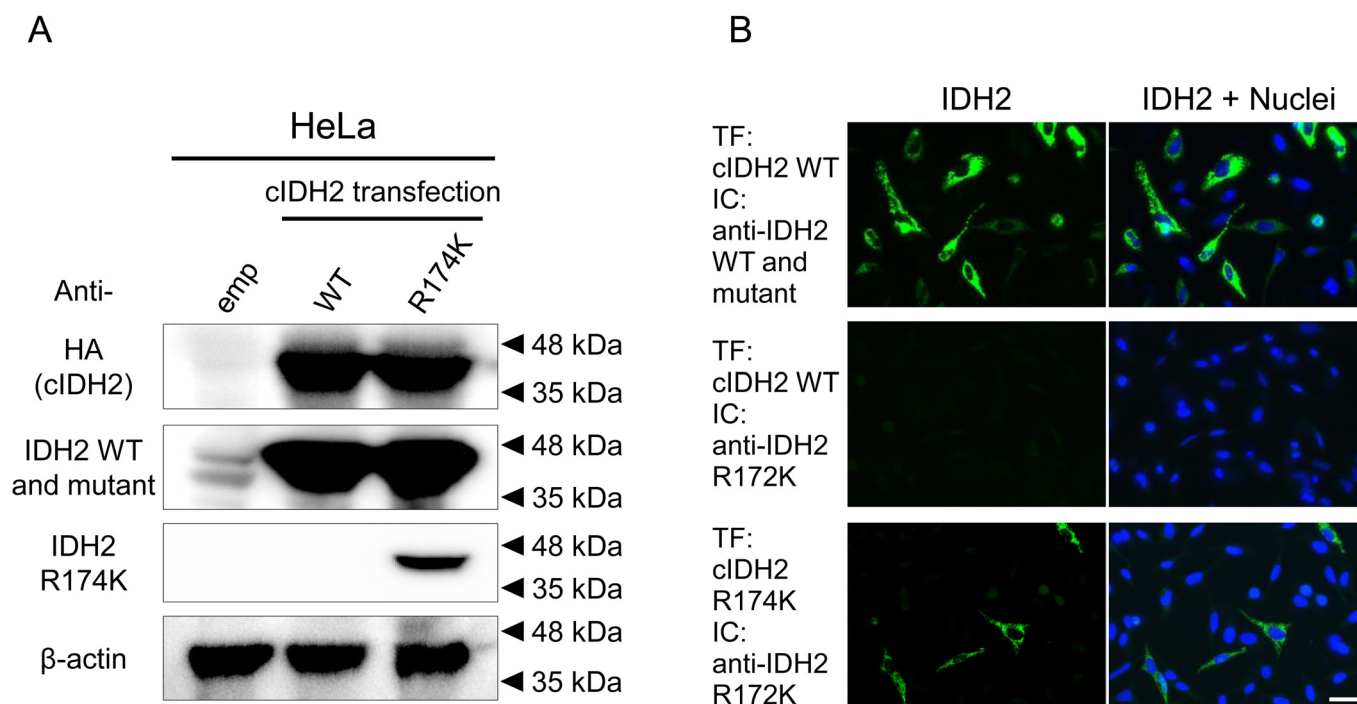


Fig. 2. Western blot and immunocytochemical analyses using anti-human IDH2 (anti-hIDH2) antibodies. (A) Lysates from HeLa cells expressing an empty haemagglutinin (HA)-tagged vector (emp), wild type canine IDH2 (cIDH2 WT), or cIDH2 R174K were electrophoresed using 5–20% PAGE gels. The separated proteins were transferred to a PVDF membrane (Bio-Rad Laboratories Inc.). After blocking with 10% skim milk and 6% glycine in TBS containing 0.1% Tween-20, western blotting was performed using anti-HA (to detect exogenous cIDH2), anti-hIDH2 WT (clone: KrMab-3), and anti-hIDH2 R172K (clone: KMab-1) antibodies. Furthermore, β -actin was used as a reference protein for normalisation of protein loading. (B) Immunocytochemical analysis using antibodies against hIDH2 WT or hIDH2 R174K in HeLa cells transfected with the respective constructs (TF). The secondary antibody used was anti-mouse IgG-Alexa 488 (green). Cells were also treated with Hoechst 33342 to stain nuclei (blue). Bar: 50 μ m.

NP_002159.2 and NP_766599.2, respectively). A putative isocitrate dehydrogenase sequence was conserved, which shows 94% homology with both hIDH2 and mIDH2. The cIDH2 protein includes an R174 residue identical to that present in hIDH2 and mIDH2. Therefore, we hypothesised that canine IDH2 has metabolic activity in the isocitrate- α -KG process, and that mutation involving R174 induces metabolic abnormality.

Western-blot and immunocytochemistry analysis of canine IDH2 transfectants using human IDH2 anti-R172K Mab

The antibodies against hIDH2 R172K (D328-3, 1:1,000, MBL) could specifically detect cIDH2 R174K in the transfectants by both WB and IC (Fig. 2A and 2B).

NADPH production was attenuated by R174K mutant of IDH2, but NADH was not affected

The mutated IDH2 R172K proteins lose normal catalytic activity for α -KG, resulting in reduced production of NADPH. Instead, the abnormal enzymatic activity produces 2-HG and consumes NADPH. Therefore, we investigated the formation of NADPH and NADH in HeLa, MDCK and Hs68 cells overexpressing cIDH2 WT and cIDH2 R174K via a colorimetric analysis. There were no differences in NADH production between HeLa cells expressing cIDH2 WT, cIDH2 R174K or the empty vector (negative control) (Fig. 3A). The production of NADPH was significantly different between HeLa, MDCK and Hs68 cells expressing cIDH2 WT versus those expressing cIDH2 R174K (Fig. 3B–D).

R174K mutation of canine IDH2 induces HIF-1 α expression

By western blot analysis, we investigated the induction of HIF-1 α expression as a result of the cIDH2 mutation. The expression of HIF-1 α was detected for the empty vector, cIDH2 WT and cIDH2 R174K transfectants, showing significantly increased levels of cIDH2 R174K in HeLa cells, and which were slightly elevated in MDCK and Hs68 cells (Fig. 4). Alterations to HIF-1 α have been reported to result from mutant human IDH2 protein expression [4], which caused oncogenic transformation.

DISCUSSION

In this study, we cloned, sequenced and analyzed the function of the full-length canine IDH2 ORF, for the first time. Primers for

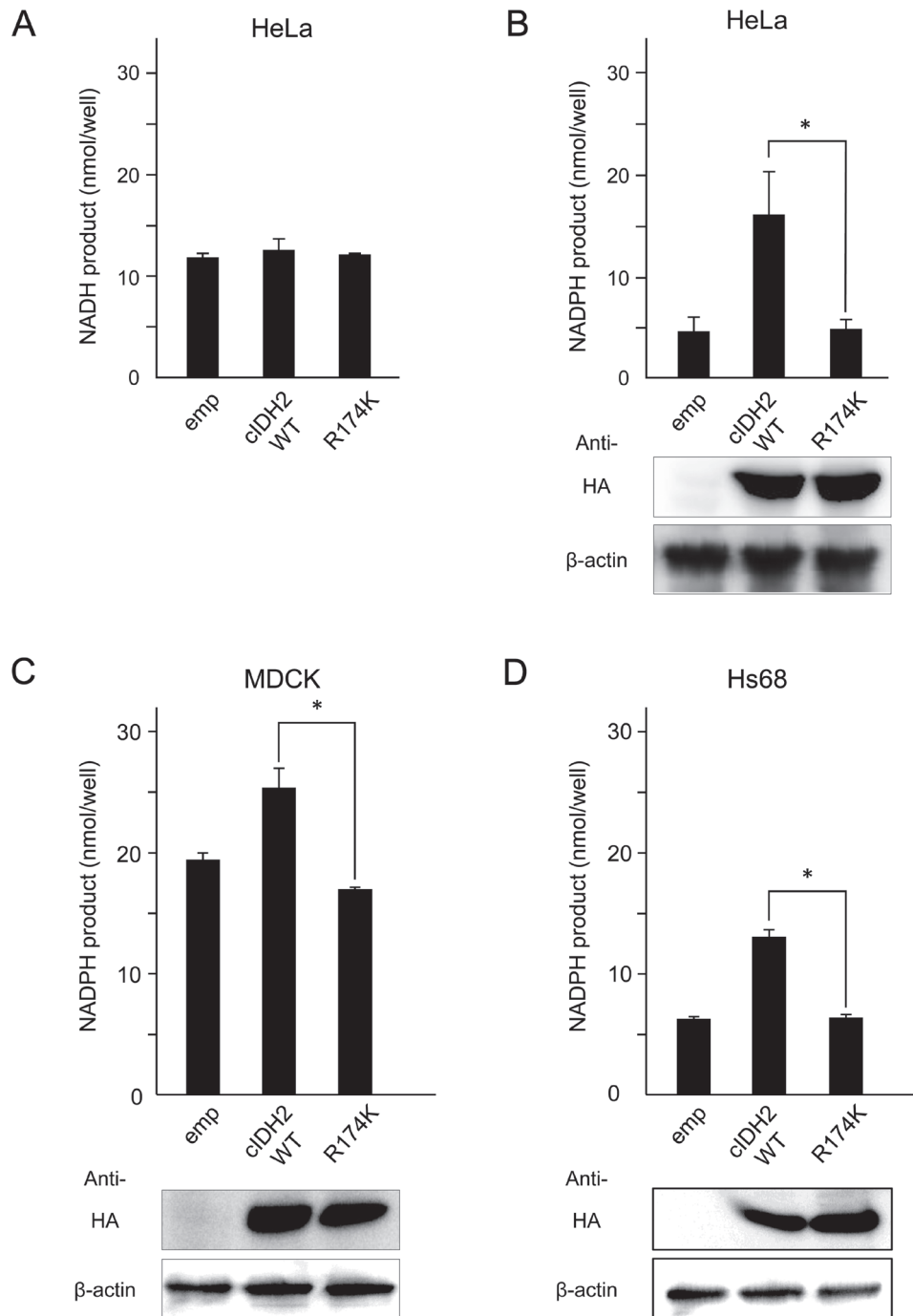


Fig. 3. Alteration of isocitrate dehydrogenase activities by mutations at R174 in canine IDH2 (cIDH2). (A) Measurements of isocitrate dehydrogenase activity by colorimetric assay. The production of NADH in HeLa cells transfected with cIDH2 WT or cIDH2 R174K is indicated in the upper graph. (B) (C) (D) The production of NADPH in HeLa (B), MDCK (C) and Hs68 (D) cells transfected with cIDH2 WT or cIDH2 R174K. The data are from four independent experiments. *, A significant difference ($P < 0.01$) was observed. Protein expression levels for each experiment were confirmed by western blotting using HA and are indicated in the lower panel.

cIDH2 amplification were designed using EST data. Compared to human and murine IDH2 proteins, canine IDH2 showed a highly conserved sequence and primary structure. Isocitrate dehydrogenase domains occupied a large proportion (approximately 80%) of canine IDH2 protein and are well conserved among species (e.g. human and mouse); hence, we predicted that canine IDH2 has isocitrate dehydrogenase activity.

Residue R172 in IDH2 is recognised as a mutation hotspot in human gliomas and other tumors such as AML [7]. In humans and dogs, there is conservation of R172 (corresponding to R174 of cIDH2), as well as the amino acid sequences around R172. Therefore, we anticipated that the antibodies used for detecting the specific mutation in R172 of humans could be used to detect

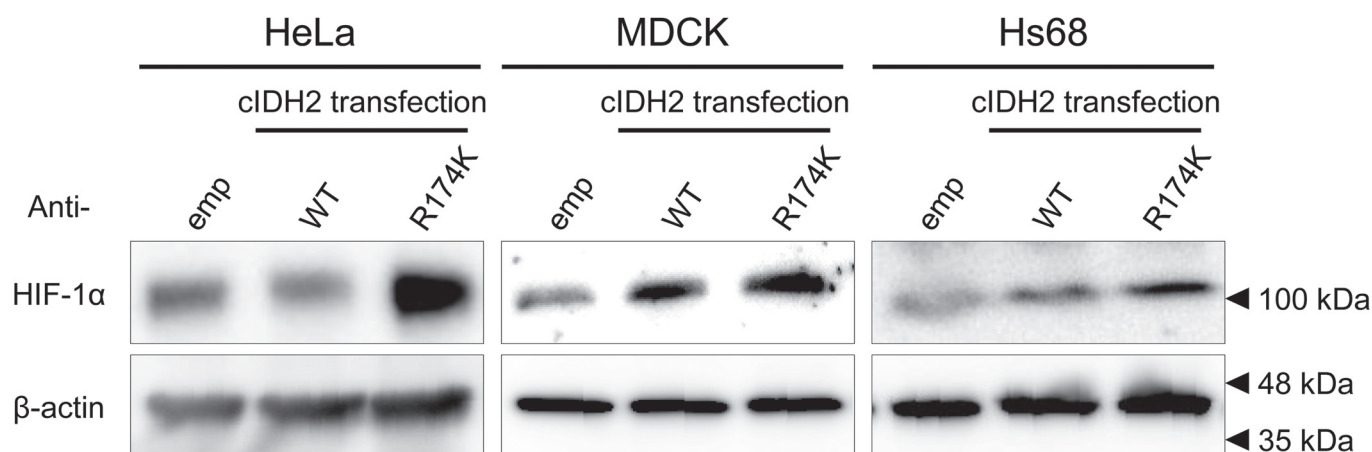


Fig. 4. Western blot analysis for detecting the hypoxia-responsible molecule induced by the overexpression of cIDH2 R174K. Cell lysates from HeLa, MDCK and Hs68 cells expressing an empty haemagglutinin (HA)-tagged vector (emp), cIDH2 WT, and cIDH2 R174K were electrophoresed using 5–20% PAGE gels. Western blotting was performed using anti-HIF-1 α antibodies, and β -actin was used as a reference protein for normalisation of protein loading.

the cIDH2 R174 mutations [10]. There were no reports for the detection of R174 mutants in canine cases, but we generated cIDH2 R172-mutated clones to investigate the cross-reactivity of an anti-human IDH2-R172K mAb (clone KMab-1) antibody. If clone KMab-1 showed cross-reactivity with cIDH2 R174 mutants, we could use KMab-1 for large-scale immunohistological screening of canine tumor tissues. In this study, antibodies against the human IDH2 R172K mutant could specifically detect cIDH2 mutations by both immunoblotting and immunocytochemical analysis. We expect that KrMab-3 and KMab-1 could also be used for the detection of cIDH2 WT and cIDH2 R174K in canine tissues. Therefore, we will perform histopathological analyses with canine gliomas and other tumor tissues in our future studies. In addition, another mutation, P162S, has been detected in hIDH2 [14]. Thus, there is a possibility that this mutation might also exist in cIDH2. In this study, we cloned the full-length ORF of cIDH2 to investigate the complete cIDH2 sequence.

In the human study, it was reported that the IDH2 WT enzyme catalyses the oxidative decarboxylation of isocitrate to α -KG, with the concomitant reduction of NADP⁺ to NADPH. However, the IDH2 R172 mutants increase the product of 2-HG instead of α -KG, while oxidising NADPH to NADP⁺ [8]. Decrease of α -KG with an increase of 2-HG causes the reduction of α -KG-dependent prolyl hydroxylases, such as those that regulate HIF-1 α levels. Alterations in HIF-1 α have been reported to result from mutant IDH2 protein expression [23], which caused oncogenic transformation. The cIDH2 R172K mutant showed a reduction in the NADPH production, but NADH production was not changed in tumor cells, immortalised cells or normal fibroblasts. We expect that NADH is produced by IDH3 in dogs, similar to that in humans; therefore, only the amount of NADPH was changed by cIDH2 R174K mutation. The decreased production of NADPH by the expression of cIDH2 R174K suggests that the R174K mutation in dogs induces 2-HG accumulation and causes tumorigenesis. To elucidate this prediction, in future experiments we will measure the production of 2-HG in cells expressing mutant cIDH2.

In the human study, IDH mutants (usually R132H of IDH1 and R172K of IDH2) lead to lower cellular α -KG levels, which may lead to inactivation of prolyl hydroxylases [3, 16]. In this study, HIF-1 α protein expression was increased in R174K transfectants of HeLa cells, and the NADPH production ability of the R174K mutant was lower than that of the WT. These results suggest that both relationships exist. Although, NADPH products were also reduced in the R174K transfectants of MDCK and Hs68 cells, HIF-1 α protein expression was slightly increased. In the previous study, HIF-1 α protein expression level was slightly increased in the glioblastoma cell line U-87MG when exogenous IDH1 R132H mutant [23], so slight increases in HIF-1 α may cause significant effects for tumorigenesis.

The mechanisms by which cIDH2 mutations cause canine gliomas and other tumors remain unclear, but findings in this study will make it easier to elucidate the relationship between cIDH2 mutations and tumor formation.

CONFLICT OF INTEREST. None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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