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Research paper

Gastrokine mRNA expression in gastric tissue from dogs with helicobacter colonisation but without inflammatory change during treatment

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ABSTRACT

Gastrokines (GKNs) are bioactive substances secreted by gastric cells. Evidence supports functional roles for GKNs in gastric homeostasis, immune responses and tumour suppression. Down-regulation has been reported in *Helicobacter pylori* associated gastritis and other inflammatory gastrointestinal conditions in mice and people. The aim of this study was to evaluate GKN gene expression in dogs positive for other *Helicobacter* spp. both before and after treatment.

Expression of *Gkn*-1 and *Gkn*-2 mRNA was studied in endoscopic biopsy samples collected from seven healthy dogs over three time-points pre- (T0) and at 1 and 18 weeks post-treatment for *Helicobacter* spp. colonisation (T1 & T2). The relative expression software tool (REST) was used to provide efficiency corrected expression ratios for comparisons between groups and these results were compared to a standard 2ΔΔCT methodology.

Compared with T1 *Gkn1* and *Gkn2* mRNA expression was greater at T0 by a mean factor of 2.53 (SE = 1.83–3.54) for *Gkn1* (P = 0.000) and 2.85 (SE = 2.23–3.75) for *Gkn2* (P = 0.000). This difference was attenuated when comparisons were made between T0 and T2. Histopathological evidence of gastritis was not present in any *Helicobacter* spp. positive sample.

When compared to post-eradication samples *Gkn* gene expression is increased in the presence of *Helicobacter* spp. in dogs without evidence for concurrent inflammation. Further evaluation is required to determine the relevance of this finding, however given a suspected role in gastric homeostasis, up-regulation of GKN1 and GKN2 could limit development of gastritis in *Helicobacter* spp. positive dogs.

1. Introduction

Gastrokines (GKNs) are bioactive molecules that are highly conserved across species; are secreted by specific gastric cells and that contain the BRICHOS domain (Menheniott et al., 2013; Yoon et al., 2013; Yoon et al., 2014). Other proteins within the BRICHOS superfamily have been linked to dementia and cancer, where BRICHOS domain function may confer roles related to intracellular trafficking, propeptide processing, chaperone function and secretion (Menheniott et al., 2013; Yoon et al., 2014). The precise functional role of the BRICHOS domain for GKNs is not established, however recently Yoon et al. (2013) demonstrated that for GKN1 it was associated with reduced cellular viability, proliferation and colony formation of atypical glandular cells (AGS) (Yoon et al., 2013; Yoon et al., 2014). Therefore whilst definitive functional roles for gastrokines have not yet been confirmed, current evidence supports their role in gastric homeostasis, immune responses and tumour suppression (Menheniott et al., 2013; Rippa et al., 2011; Toback et al., 2003; Xing et al., 2012). GKNs appear to maintain close associations with trefoil factors (TFFs) and together these may play important roles in cellular differentiation, be cytoprotective and have resultant anti-inflammatory effects by modulating NF-κβ signaling pathways; therefore aiding and promoting epithelial restoration (Mao et al., 2012b; Menheniott et al., 2013; Rippa et al., 2011; Toback et al., 2003; Yoon et al., 2013). Alteration of GKN gene expression and subsequent deficiency of GKN1 & GKN2 has been identified in a variety of clinical situations including gastric inflammation, aspirin and non-steroidal anti-inflammatory (NSAID) mediated mucosal injury and the development of gastric neoplasia in people (Mao et al., 2012a; Martin et al., 2008; Menheniott et al., 2013; Nardone et al., 2008; Oien et al., 2004). For the latter, loss of GKN1 and GKN2 may be prognostic (Menheniott et al., 2013; Nardone et al., 2008; Oien et al., 2004).

It is likely that a complex interplay of aberrantly expressed genes contributes to the development of *Helicobacter pylori* related disease,

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however various studies have identified altered GKN expression in *H. pylori* associated gastritis, with consistent down-regulation observed during infection. This includes studies utilizing a proteomic approach and these findings in particular have led to increased interest in elucidating the role of GKNs (Menheniott et al., 2013; Nardone et al., 2008; Nardone et al., 2007; Peterson et al., 2010; Resnick et al., 2006). It is unclear why altered expression occurs, but suppression of GKNs could reflect a method by which *H. pylori* is able to avoid clearance and perpetuate disease (Peterson et al., 2010). Alternatively down-regulation could aid potentiation of appropriate immune responses in a host-mediated attempt to clear organisms (Menheniott et al., 2013). Universal loss of gene expression is reported within gastric tumours and in gastric cancer cell lines (Menheniott et al., 2013; Nardone et al., 2008; Oien et al., 2004; Peterson et al., 2010). It remains unclear whether gastrokine deficiency is a pre-requisite for oncogenic transformation, however the identified association between *H. pylori* organisms and suppression of *Gkn1* and *Gkn2* could indicate a method by which this process is potentiated (Menheniott et al., 2013; Nardone et al., 2008; Oien et al., 2004; Peterson et al., 2010).

A number of non-*Helicobacter pylori* Helicobacter (NHPH) are known to colonise the stomach of dogs (Eaton et al., 1996; Hwang et al., 2002; Jalava et al., 1998; Neiger and Simpson, 2000; Neiger et al., 1999). However the relevance of NHPH to the development of gastric inflammation and neoplasia in this species remains uncertain (Ali Shabestari et al., 2010; Baele et al., 2009; Buczolits et al., 2003; Eaton et al., 1996; Jalava et al., 1998; Neiger and Simpson, 2000; Neiger et al., 1999; Polanco et al., 2011). Given the findings in people with *H. pylori*, and the proposed role of GKN1 and GKN2 in gastric homeostasis, differences in the expression of these genes could also be identified in dogs and could help further define their role. Alteration in gene expression might precede development of gastric inflammation. If alterations occur in the absence of gross inflammatory change, this could also further support a direct role for helicobacters in the alteration of gene expression. Alternatively identification of differences in dogs compared with people could explain why inflammatory disease and neoplastic transformation is an uncommon sequelae to colonisation with NHPH in the majority of infected animals.

The aim of the current study was to evaluate relative gene expression of *Gkn1* and *Gkn2* from *Helicobacter* spp. positive dogs without evidence for concurrent inflammation and to compare this with data obtained from the same dogs following successful clearance of organisms.

2. Materials and methods

2.1. Animals and prior treatment

Samples obtained from seven clinically healthy young adult mix-breed research colony dogs (4 M, 3F) included in a previously medical treatment study were evaluated. Dogs had been housed within the University of Melbourne's dog colony; and prior to inclusion had been identified as healthy based on a combination of physical examination, routine haematology and serum biochemistry and abdominal ultrasound. Vaccinations and parasite prophylaxis were current for all dogs. The University of Melbourne's Animal Ethics Committee approved all dog use according to National Health and Medical Research Council guidelines (Institutional Animal Care and Use Committee Approval numbers 1112209). Dogs included in the study had received one of two treatment protocols during the study period. Treatment Protocol A (n = 3 dogs) included amoxicillin (20 mg/kg q12 h PO) and omeprazole (1 mg/kg q12 h PO). Treatment Protocol B (n = 4 dogs) included amoxicillin (20 mg/kg q12 h PO), clarithromycin (7.5 mg/kg q8 h PO) and bismuth subsalicylate (3.8 mg/kg q8 h PO).

Endoscopic biopsy samples collected from the gastric body at each of three time-points were analysed. These time-points represented Arrival (Baseline = T0), Week 3 (T1) and Week 18 (T2) of the study; where T1 was immediately post-clearance and T2 represented the last time-point at which dogs were reassessed in the study. For the purpose of analysis samples were also designated as being either *Helicobacter* spp. positive or negative, with infection status determined on the basis of a combination of standard histopathology, confocal endomicroscopy (CEM), polymerase chain reaction (PCR) against the 16 s rRNA gene and fluorescence *in situ* hybridisation (FISH) as described elsewhere (Sharman et al., 2016; Sharman et al., 2014).

2.2. Endoscopy procedure

Standard white light endoscopy (WLE) and CEM gastroduodenoscopy were performed as previously described (Sharman et al., 2012; Sharman et al., 2013, 2014). At each of the included time-points dogs were fasted for 12 h prior to anaesthesia and following completion of endoscopic evaluations multiple gastric mucosal endoscopic pinch biopsy specimens were collected (6–10 per dog). Endoscopic biopsy samples included in the current analysis were obtained from the greater and lesser curvature of the gastric body. Samples were preserved in 10% buffered formalin for histological processing and in RNA-*Ice* (Invitrogen Life Technologies) for later quantitative real-time polymerase chain reaction (qPCR). For histologic examination serial sections (4–5 μm) of paraffin embedded samples were obtained and stained with haematoxylin and eosin (H & E) and were examined by a board-certified veterinary pathologist.

Samples for qPCR were stored for up to 12 months at −80 °C in RNA-*Ice* until used in the current study.

2.3. RNA isolation and cDNA synthesis

For each dog, at each time-point a single endoscopic biopsy was homogenized in lysis buffer with 2% β-mercaptoethanol (Buffer RLT, Qiagen, Chadstone, VIC, Australia) using a homogeniser (Polytron PT3100, Kinematica, Switzerland). RNA extractions were then performed using the MicroRNEasy extraction kit (Qiagen, Chadstone, VIC, Australia) according to the manufacturer's instructions. To eliminate genomic DNA (gDNA) contamination a DNase step was included for each set of reactions. The yield of RNA in each extraction sample was measured using the NanoDrop Spectrophotometer ND-1000 (NanoDrop; Thermoscientific, Scoresby, VIC, Australia) at optical density (OD) A260/280 nm.

For mRNA quantification cDNA was generated by using 1 μg of RNA with a QuantiTect Kit (Qiagen, Chadstone, VIC, Australia) and according to manufacturer's instructions. To further eliminate gDNA contamination RNA was first incubated with gDNA WipeOut Buffer at 42 °C for two minutes. Following generation cDNA was stored at −80 °C until use.

2.4. Primer design

Primers for each canine GKN gene were designed using the NCBI Primer-Design tool. For this, predicted sequences within the NCBI gene database were used based upon analysis of the whole canine genome to identify analogous gene regions compared with various other species (Menheniott et al., 2013). All primers were designed to amplify a 60–250 bp region of each gene of interest, spanning an exon–exon junction. Primers were tested by NCBI Primer-BLAST analysis against the Refseq mRNA database.

For the reference gene, β-Actin, primers previously validated within canine splenic tissue were used (Table 1) (Lage et al., 2007).

2.5. Real-time PCR optimisation

For initial assay optimisation cDNA was generated as described from gastric biopsy samples obtained from one dog enrolled in the described study but with an incomplete dataset over the three time-