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Detection and characterization of interleukin-6 gene variants in *Canis familiaris*: Association studies with periodontal disease

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ABSTRACT

Periodontal disease (PD) is the most common inflammatory disease of the oral cavity of domestic carnivores. In Human Medicine molecular genetics research showed that several genes play a role in the predisposition and progression of this complex disease, primarily through the regulation of inflammatory mediators, but the exactly mechanisms are poorly understood. This study aims to contribute to the characterization of the genetic basis of PD in the dog, a classically accepted model in Periodontology. We searched for genetic variations in the interleukin-6 (*IL6*) gene, in order to verify its association with PD in a case–control study including 25 dogs in the PD case group and 45 dogs in the control group. We indentified and characterized three new genetic variations in *IL6* gene. No statistically significant differences were detected between the control and PD cases groups. Our results do not support an evidence for a major role contribution of these variants in the susceptibility to PD in the analyzed population. Nevertheless, the sequence variant I/5_g.105 G>A leads to an amino acid change (arginine to glutamine) and was predicted to be possibly damaging to the IL6 protein. A larger cohort and functional studies would be of extreme importance in a near future to understand the possible role of IL6 variants in this disease.

1. Introduction

Periodontal disease (PD) is an inflammatory disease induced by bacterial plaque in periodontium. This pathology includes two different inflammatory stages: an initial form called gingivitis and an advanced form called periodontitis. The second form progresses with bone resorption, cementum necrosis and gingival recession or hyperplasia, and ultimately with a permanent loss of tooth support, increasing teeth mobility and teeth loss (Niemiec, 2008). The importance of PD in Veterinary and in Human Medicine is clear, due to its enormous prevalence and life threatening implications on systemic health. Some studies show that, around two years of age, about 70% of cats and 80% of dogs already have PD (Niemiec, 2008). In dogs, the association of the PD with renal, liver and cardiovascular diseases (Pavlica et al., 2008; Glickman et al., 2009; Glickman et al., 2011) has been proposed. In humans, it is estimated that 30% of the adult population is affected by chronic periodontitis (Nares, 2003) and PD has been related to premature births, low birth weight and neonatal morbidity (Rakoto-Alson et al., 2010; Vogt et al., 2010), diabetes (Choi et al., 2011), osteoarticular (Shum et al., 2010), respiratory (Sharma and Shamsuddin, 2011) and cardiovascular diseases (Kim et al., 2010). These facts have contributed to the increased interest in the investigation of factors related to PD development.

PD presents a multifactorial aetiology: microbial, behavioural, environmental, systemic and genetic factors contribute to the susceptibility and clinical expression of the disease (e.g. Meng et al., 2007; Laine et al., 2010; Stabholz et al., 2010). The mechanisms involved in the development of PD are still unclear and it is necessary to develop new studies that can inter-relate these susceptibility factors. Early

Abbreviations: PD, Periodontal disease; IL6, interleukin-6; IL1, interleukin-1; TNFα, tumor necrosis factor-alpha; MMPs, matrix metalloproteinases; VDR, vitamin D receptor; TLRs, toll-like receptors; IGF1, insulin like growth factor-1; TGFβ, transforming growth factor-beta; BMPs, bone morphogenetic proteins; mRNA, messenger RNA; SNPs, single nucleotide polymorphisms; PCR, polymerase chain reaction; UTR, unstranslated region; OR, odds ratio; CI, confidence interval; LRT, likelihood-ratio test.

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identification of these risk indicators, such as the genetic factors, can be important to detect the predisposed individuals, to establish more effective preventive strategies and personalized therapies (e.g. Takashiba and Naruishi, 2006; Yoshie et al., 2007). In recent years, the molecular and genetic research showed that genes play a key role in the predisposition and progression of PD, primarily through the regulation of inflammatory mediators (Meng et al., 2007). However, these studies were performed, mainly, in humans and many of them showed inconsistent results (Taylor et al., 2004; Laine et al., 2010). The development of an animal model that can offer ideal characteristics to study the genetic component associated with PD pathogenesis, is an important contribution for the knowledge of this disease. The dog is a classic model used in periodontal research and its contribution for the knowledge in periodontics is considerable. This model has been used to study the prevalence, severity and aetiological factors of PD (e.g. Gad, 1968; Sorensen et al., 1980); the clinical, histological and histopathological features associated with the disease (Lindhe et al., 1975; Hamp and Lindberg, 1977; Berglundh et al., 1991); analyze the efficiency of new therapeutic protocols (Jansen and Pilot, 1981; Haney et al., 1995); evaluate the regenerative potential of periodontal tissues (e.g. Iwata et al., 2009; Kwon et al., 2010) and utilized in comparative studies on the inflammatory response associated with the pathogenesis of the disease in dog an human (Skaleric et al., 1997; Papadimitriou et al., 2006). Despite the long history of use of this animal model in the periodontology research, it remains a very actual model, especially in studies involving the development of new methodologies or therapeutic protocols (e.g. Polimeni et al., 2009; Min et al., 2011). The results of these studies allow a reflection on the usefulness of dogs in other research lines, such as the genetic factors associated to PD susceptibility.

The role of genetics in the canine PD development is unknown. However, the disease in these animals has several similarities to the human and it can be speculated that genetics is one factor that justifies the huge difference in susceptibility/resistance to disease among individuals (Pavlica, 2006). In addition, the dog appears as a promising animal model in the intense medical research currently developed about the genetic basis of complex diseases (Parker et al., 2010; Shearin and Ostrander, 2010). To validate the applicability of the canine model in genetic studies of PD it is urgent to develop research in this area to find candidate genes involved in the pathogenesis of this disease in these animals.

In PD, the genetic variations may affect important regulators of the disease, influencing the tissue structure (innate immunity), antibody responses (adaptive immunity) and inflammatory mediators (non-specific inflammation) (Kinane et al., 2005), changing the response against periodontopathogenic bacteria and, ultimately, leading to periodontal destruction (Laine et al., 2010). The studies developed in human and animals have demonstrated the association of several candidate genes with host-response patterns, inflammatory and immune reactions (e.g. IL1, IL6, TNFα, MMPs, VDR, TLRs) (Meng et al., 2007; Yoshie et al., 2007; Laine et al., 2010) and tissue regeneration (e.g. IGF1, TGFβ and BMPs) (Ivanovski et al., 2007). The genes and pathways associated with regulation of cytokines have received the most attention in the genetic studies of complex diseases, including PD (Smith and Humphries, 2009). There is a complex interaction of pro- and anti-inflammatory cytokines which plays a central role in the development and progression of the disease by regulating of inflammatory processes and adaptive immune responses (Taylor et al., 2004). The cytokine gene polymorphisms associated to alterations in the regulation and immune biochemical pathways of cytokines are related with different susceptibility/resistance profiles in human PD. In our study we used a dog model to analyze a potential candidate gene, the inteleukin-6 (*IL6*).

IL6 is a pleiotropic cytokine synthesized in diverse cells, like macrophages and neutrophils, and has different functions, namely the B cell differentiation, the T cell proliferation and acute phase protein production (Irwin and Myrillas, 1998; Hirano, 2010; Scheller et al., 2011). This interleukin is crucial in the inflammatory response against infectious agents, particularly, gram-negative bacteria (Dalrymple et al., 1996), and has an important role in the local regulation of bone turnover (Ishimi et al., 1990; Okada and Murakami, 1998; Taylor et al., 2004). In periodontal lesions with inflammatory origin it was demonstrated that IL6 is expressed in multiple cells types (T cells, macrophages, endothelial cells and fibroblasts) by analyzing the mRNA and protein levels (Matsuki et al., 1992; Takahashi et al., 1994). It is speculated that the increase of B cells/plasma cells observed in periodontal lesions may result from an increased expression of IL6 in the locations affected (Fujihashi et al., 1993) and therefore to influence the bone resorption (Ishimi et al., 1990; Irwin and Myrillas, 1998; Okada and Murakami, 1998). Several single nucleotide polymorphisms (SNPs) in the promoter region of the *IL6* gene have been associated with human PD susceptibility (e.g. Nibali et al., 2008a, 2008b; Nibali et al., 2009). These variations are associated with plasma IL6 level increase and transcriptional activity of *IL6* gene (Fishman et al., 1998; Hulkkonen et al., 2001), and appear to influence the predisposition to the colonization of periodontopathic bacteria (*A. actinomycetemcomitans*, *P. gingivalis*and *T. forsythensis*) (Nibali et al., 2007; Nibali et al., 2008b; Nibali et al., 2011). Consequently, the response to periodontal treatment may be influenced in the predisposed individuals. All these authors reported the need to develop further studies to clarify the role of IL6 in the inflammatory process of PD.

The present work aims to contribute to the characterization of the genetic basis of PD in the dog. We performed a molecular analysis of *IL6* gene in order to identify genetic variations and verify its association with PD in a case–control study. We pretend to amplify the knowledge about the canine PD and contribute to the implementation of a potential animal model in the study of the genetic basis of human PD.

2. Material and methods

2.1. Clinical evaluation and inclusion criteria

The clinical periodontal examination of population (70 dogs), performed between January and May of 2009, was based on the classification followed by the American Veterinary Dental College (AVDC) (http://www.avdc.org/nomenclature.html#periostages). Animals were submitted to general clinical examination, previously, in order to clarify its health condition. After an informed consent of the owners and meeting all the standards of animal welfare, the dogs were sedated by an intramuscular administration of butorphanol (Torbugesic 1%; Fort Dodge, The Netherlands) and acepromazine (Vetranquil; CEVA Sante Animal, France). Anesthesia was achieved by an intravenous administration of diazepam (Diazepam MG; Labesfal, Portugal), ketamine (Imalgene 1000; Merial, France) and propofol (Lipuro 2%; Braun, Germany) and was maintained using isoflurane (IsoFlo; Abbott Animal Health, USA), administered in oxygen through an endotracheal tube. Then, all animals were subjected to a systematic odonto-stomatological evaluation to ascertain the presence or absence of PD and to determine its degree.

We selected the population considering the following parameters: dogs with similar feeding habits (mix of home-prepared rations and commercial pet foods) and mesocephalic skull type. None of them had undergone dental preventive measures (e.g. tooth brushing, dental diets) or dental treatment before. The dogs analyzed are unrelated/unfamiliar individuals. The population was separated into two groups. The cases group included 25 dogs with a PD degree range of gingivitis to advanced periodontitis. The control group included 45 dogs with healthy periodontium. All animals were mixed-breed with body weight ranging from 9 to 18 kg and ages varying between 2 and 8 years. A more detailed characterization of the population is presented in Table 1.

2.2. Sample collection and DNA extraction

After examination we collected 3 ml of blood samples from all the animals. The DNA extraction was performed from figurative elements of the blood with QuickGene DNA whole blood kit S (DB-S) (Fujifilm