The Association of Thyroglobulin Single Nucleotide Polymorphism with Miniature Dachshunds-Specific Inflammatory Colorectal Polyps and its Involvement in Interleukin-6 Amplifier Induced Chronic Inflammation

（ミニチュアダックスフンドに特異的な炎症性結直腸ポリープに関連するサイログロブリンの一塩基多型とインターロイキン 6 増幅回路に誘導される慢性炎症への関与）

Teoh Yong Bin

Contents

ABBREVIATION TABLE

CD	Crohn's Disease
CCL2	C-C motif chemokine ligand 2
EDTA	Ethylenediaminetetraacetic acid
FBN1	Fibrillin-1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GWAS	Genome Wide Association Study
H4	Human neuroglioma
IBD	Inflammatory bowel disease
ICRP	Inflammatory Colorectal Polyp
IL-6	Interleukin 6
MD	Miniature Dachshund
mRNA	Messenger RNA
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RELA	Transcription factor p65
SDHA	Succinate Dehydrogenase Complex Flavoprotein Subunit A
siRNA	Silencing RNA
SNP	Single nucleotide polymorphism
STAT3	Signal transducer and activator of transcription 3
TG	Thyroglobulin
TNF-α	Tumor necrosis factor alpha
UC	Ulcerative Colitis
WES	Whole exome sequencing
WSAVA	World Small Animal Veterinary Association

NOTES

1.Publications related to the dissertation

1−1 An inflammatory bowel disease-associated SNP increases local thyroglobulin expression to develop inflammation in Miniature Dachshunds

1−1 is related to all three chapters of the dissertation.

GENERAL INTRODUCTION

Inflammatory bowel diseases (IBD) have begun to plaque all mammals so long as the history of man began, with the first case report in man filed in 1859 by Sir Samuel Wilks as ulcerative colitis (UC), one of the two most common IBD in humans[58] the other being Crohn's Disease (CD). It was not until a century later in 1992, where Jergens first coined the term IBD in dogs and cats which portrays clinical signs of refractory gastroenteritis, unresponsiveness to food trials and presence of cellular infiltrates in histological findings of gastrointestinal tract of IBD cases, similar yet distinct from humans[41]. Moving on to two decades after that, new findings have further classified IBD in dogs, specifically into a subset of diseases known as chronic enteropathies (CE)[86]. CE, with reference to its terminology on chronicity warrants the persistent manifestation of clinical signs such as emesis, diarrhea, weight loss or abdominal pain for at least 3 weeks, diagnosed after the exclusion of infectious diseases (parasitic or viral), extra-intestinal diseases (hepatic, pancreatic, renal, endocrine) or mechanical obstruction (intussusception, foreign body, intestinal tumor)[86]. The novel term CE has been introduced into the clinical settings of canine medicine due to the responsiveness of each patient with CE towards different treatment plans, which have resulted in classification of antibiotic responsive enteropathy (ARE), food responsive enteropathy (FRE), immunosuppressant-responsive enteropathy (IRE) and non-responsive enteropathy[25].

To put things into perspective, IBD falls into the sub-category of IRE, as dogs in this group usually respond poorly to dietary changes and antibiotic therapy, compelling clinicians into the mandatory prescription of immunosuppressive drugs such as steroids, antibodies, or antimetabolites to control gastrointestinal clinical signs[25]. While most immunosuppressants may control the signs of IBD, being a double-edged sword, their uses often couple with side-effects such as iatrogenic Cushing-disease in steroids or myelosuppression in antimetabolites, limiting their prolonged usages[79]. To complicate matters, some cases may develop resistance against the effects of immunosuppressants, consequently requiring concomitant prescription of different drug groups to control the disease[26]. Hence, the race to understand IBD more in both humans and dogs is still ongoing, where the hypothesis of pathogenesis in both species share a common

complexity in the interplay of host genetics, intestinal microenvironment from bacterial and dietary makeup, immunity, and triggers of gut inflammation from the environment[73].

Human domestication of the canine species over 15,000 years have successfully allowed the integration of dogs into human daily lives, even as we evolve[70]. This phenomenon is evident when we compare the ability of modern dogs in adapting towards human-like diet as compared to their ancestral grey wolves, where genome-wide sequencing studies have identified the presence of important genetic variants in domestic dogs contributing to all three stages of starch digestion[12]. While artificial selection in dogs were used to be governed by their functional phenotypes for specific involvement in human society such as gundogs, working dogs, herding dogs, hounds, and terriers; recent infatuation towards breed specifications of the dog has been geared by the needs of the pet industry in integrating ornamental dogs into human lives as family members, providing companionship with limited yet desirable extent of herding or guarding instincts[99]. Regardless of the selection objectives, the process has rendered the species into over 400 different modern breeds, with exaggerated disparity in phenotypic characteristics both anatomically and physiologically[11]. However, the deleterious effects of the uncompromising selection through inbreeding have not gone unnoticed, with several documented myriad of diseases prevalent in certain breeds due to the bottleneck effect of artificial selection[24]. Amongst those diseases, IBD in dogs has garnered an intense spotlight among veterinary clinicians and researchers alike, due to the frustrations and challenges in treating the disease, as well as the unique genetic composition of dog breeds which serve as an exemplary animal disease model in translating possible treatment options to human IBDs[82].

Breed specific IBDs in dogs have been reported since the early 2000s, such as histiocytic ulcerative colitis (HUC) predominantly reported in Boxer dogs[31]. Later, epidemiological studies of canine IBD in the United Kingdom in 2010s further fortified the hypothesis where some breeds are indeed more susceptible towards IBD compared to others, such as German Shepherd Dog, Weimaraner, Rottweiler and as expected, Boxer dogs as well[44]. These studies have induced a revelation in the interrogation of genetics in susceptible breeds, as sequencing technology improves with better affordability. Follow suit, genome-wide studies of IBDs in these breeds of interest gained more attention, even so when some of the disease-causing genes in dogs are

confirmed to be similar to humans, such as the identification of single nucleotide polymorphism (SNP) of toll-like receptor genes in German Shepherd dogs[43]. Unlike monogenetic diseases, such as very early onset IBDs in human infants, canine IBDs in general are clinically similar to the age onset of human IBDs at middle aged populations at about 5 to 7 years in dogs[20]. Such parallelism in clinical and genomic pattern among both species opened up countless opportunities in translational research in a One Health approach, allowing simultaneous unraveling of novel disease-associated mutations in genomes of either species, benefiting from the strengths of medicine in both fields seen in comparative oncology i.e. expedited clinical trials in dogs which can be translated to humans in osteosarcoma[77], as well as the novelty of innovative drugs developed in mice for human, which may be more accessible to canine patients during trial enrolments[46].

In Japan, the Miniature Dachshund (referred to as MD hereafter) dog breed garnered an immense popularity from the late 1990s to late 2000s[100] for its petite stature, suitable towards nuclear family household with smaller number of family members which are more common in Japan. An anecdotal hypothesis among the veterinary community in Japan has hypothesized that the boom in popularity in the 90s, coupled with unregulated inbreeding amongst the pedigree of MDs during that time has led to several inbreeding related diseases. This hypothesis became sounder in the recent years, as MD specific diseases which are seen in Japan, yet unreported in other regions of the world became more apparent in the clinical settings of Japan, especially those of immune mediated[91] or granulomatous in nature[45]. Amongst them, a novel inflammatory bowel disease known as the inflammatory colorectal polyp (ICRP) have stood out due to its clinical and pathological nature in disease presentation in MDs.

ICRP, as its name suggests is the emergence of a polyp at the colorectal region of the lower intestinal tract of an inflammatory origin. Previously reported gastrointestinal (GI) polyps in dogs summarized by Ohmi et al have shown to be mainly derivation of neoplasms, with adenomatous polyp and adenocarcinoma being over presented[65]. Unlike GI polyps of neoplastic origin, inflammatory polyps in dogs are rarely reported in the veterinary field[30]. The inflammatory nature of the disease can be explained in both clinical and histopathological findings, as some of the cases resolve with prudent usage of steroids (reported at 80%) and

immunosuppressive drugs[29]. However, the root of this breed-related disease has yet to be confirmed, as response to anti-inflammatories or immunosuppressants does not describe the relationship between breed and tendencies of inflammation. Hence, recent reports have exploited the involvement genes in terms of inflammatory panels such as IL-8[88], $CD4^+$ T cell cytokine related genes[66] and pattern recognition receptor genes such as *NOD2*[38] just to name a few. Although all reports concluded that ICRP is somehow related to pro-inflammatory genes, neither the molecular involvement of causative genes was elucidated, nor concrete evidence of breed-related genes were identified.

As ICRP has been reported to occur at the median age of 9.0 years old[65], its pathogenesis is thought to be very similar to those in human IBD, which occurs as a result of chronic inflammation[74]. The IL-6 amplifier is a peculiar mechanism which associates to development of chronic inflammation and distinct to non-immune cells including endothelial cells, fibroblasts, and keratinocytes through the hyperactivation of the NF-kB cascade[60,61]. This amplifier requires the synergistic activation of NF-kB and STAT3 in non-immune cells to be triggered, resulting in a continuous loop of inflammation through the recruitment of cytokines, chemokines, and their mediators[61,62]. Although IL-6 is one of very few stimulators of STAT3, there are multiple stimulators of NF-κB, including TNF-α, IL-17A, growth factors, noradrenaline, and TLR ligands, during inflammation development. Upon its advent, IL-6 amplifier has successfully described the drive behind chronic inflammation in several mouse disease models and human disorders such as autoimmune Sjögren's syndrome, keloids, uveoretinitis, chronic organ rejection and many more[3,4,6,9,28,33,35,47,51,55,59,60,64,68,69,76,84,87,89,90]. More importantly, a genome-wide screening in mouse along with human SNP-based association study has identified key regulatory genes of the IL-6 amplifier which are related to human inflammatory disease and disorders, providing a blueprint to future SNP-based research in chronic inflammation[60]. In dogs, there was only one past study which have identified SNPs which can be related to the pathogenesis of ICRP through the IL-6 amplifier, where the mutation of *PLG* was identified to be associated to the pathogenesis of ICRP through the MMP9-plasminogen axis [95]. However, since some MDs with wild-type allele of *PLG* also show the phenotype of ICRP, this signifies that ICRP may be a polygenic disease where multiple SNPs may induce its pathogenesis in MDs.

With that in mind, this research aimed to validate the genes which may be associated with ICRP in MDs, the involvement of the associated genes with inflammation, and the evidence of expression of such gene in Japanese MDs in three different parts. Firstly, the identified risk associated genes in a previous study were selected and the population of dogs screened were increased from 10 to at least 45 dogs in each gene. Next, as ICRP is believed and proven to be inflammatory driven, the association between risk genes with inflammatory pathways was sought through exploration of the IL-6 amplification pathway with non-immune cells using siRNA gene silencing and recombinant protein stimulation, where *TG* has been confirmed to be MD-specific and associated to IL-6 amplifier. Lastly, the local and systemic expression of TG was sought in cases with or without risk allele using qPCR and ELISA, as well its effect on the activation of NF-kB-induced chronic inflammation in MDs with ICRP.

CHAPTER 1

Validation of Risk Associated Gene in Miniature Dachshunds Diagnosed with Inflammatory Colorectal Polyps

1. INTRODUCTION

Past SNP studies have shown that next generation sequencing allow detection of novel breed-specific genes to explain breed-specific diseases, such as *SOD1* in degenerative myelopathy of Pembroke Welsh Corgi[10,22,83], chronic enteropathy in French Bulldog[63], as well as IBD in German Shepard Dogs[43]. In ICRP in MDs of Japan, previous studies have attempted to detect breed-specific genes involved in its pathogenesis with regards to inflammatory related genes, such as SNPs of *NOD2* or *TLR1*, *TLR2* and *TLR6*[38] or their local expression in lesion sites such as *IL-8*[88] or TLRs[98], usually pinpointing a specific set of genes which confined the studies in a narrow perspective. In our group, a recent WES study has revealed that when comparing 10 MDs against 10 dogs of other breeds, SNPs of *PLG, TCOF1, TG, COL9A2,* and *COL4A4* were specific to MDs, suggesting that ICRP may be a polygenic disease.

Hence, for the first chapter in this study, the potential risk alleles and genes in MDs which were identified in a previous study as mentioned above were compared to other dog breeds, then between MDs diagnosed with ICRP and age-matched MDs with relative lower risk towards ICRP though targeted genotyping.

2. MATERIALS AND METHODS

2.1 Animals

The whole exome sequencing data of a previous study was reviewed again to compare the SNPs of 2 MDs diagnosed with ICRP based on well-established histopathological findings[65] as compared to 2 beagles which are clinically healthy and owned by Hokkaido University. Blood samples or formalin-fixed paraffin embedded colonic samples from 146 MDs diagnosed with ICRP, 90 age-controlled MDs without ICRP, 36 juvenile MDs aged less than 1 year old, and 40 dogs of other various breeds (Table 1) were collected through venipuncture as part of routine blood test in the Hokkaido University Veterinary Teaching Hospital. DNA blood &Tissue Kit (QIAGEN, Hilden, Germany) were used to derive genomic DNA (gDNA) from the EDTA blood of the dogs stated above. Written informed consent has been retrieved from all dogs' owners prior to enrollment of this study.

2.2 Whole Exome Sequencing (WES) data analysis

Whole exome data which were not interrogated or analyzed in the previous study[95] was filtered with the publicly available canine genomic variant data such as iDog (https://ngdc.cncb.ac.cn/idog/) and European Variant Archives (https://www.ebi.ac.uk/eva/) for variants which were not detected in previous studies, or variants with unknown population ratio. Variants which were remained were further compared to the list of genes which are related to the IL-6 triggered positive feedback loop for NF-κB signaling known as the IL-6 amplifier to retrieve genes which have high suppression of IL-6 activities (>60% suppression) when knocked out in BC1 murine type 1 collagen^{+} cells[60]. The variants which fulfill the criteria above were then pursued for genotyping using Sanger sequencing.

2.3 Targeted Genotyping

Polymerase chain reaction (PCR) and Sanger sequencing were utilized to validate and genotype the variant filtered from WES data. Primer pairs used for variant validation were designed using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi; Table 2). Amplicons post PCR reaction were purified using a commercial clean up reagent, ExoSAP-IT Express (Thermo Fischer Scientific) and sequencing was performed using the Sanger method

utilizing BigDye Terminator v3.1(Thermo Fisher Scientific) and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). FinchTV was used to interrogate the reads generated and allele frequency was compared between groups using Fischer's exact test.

2.4 Statistical Analysis

Statistical analysis was performed using JMP Pro version 14.0 (SAS Institute Inc., Cary, NC, USA) and R studio (RStudio: Integrated Development for R. RStudio, PBC, Boston, MA). Fisher's exact test was used to analyze differences between two groups and proportion test was used to analyze differences of ratios between two groups. Value of $P < 0.05$ was considered as significant.

Table 1 Dog Signalment used for Targeted Genotyping

Case Number	Breed	Disease	Age	Sex
1	Miniature Dachshund	ICRP	9	SF
2	Miniature Dachshund	ICRP	9	CM
3	Beagle	Healthy	1	CM
4	Beagle	Healthy	1	SF
5	Miniature Dachshund	ICRP	8	CM
6	Miniature Dachshund	ICRP	12	SF
7	Miniature Dachshund	ICRP	11	CM
8	Miniature Dachshund	ICRP	10	SF
9	Miniature Dachshund	ICRP	14	SF
10	Miniature Dachshund	ICRP	7	IF
11	Miniature Dachshund	ICRP	6	IM
12	Miniature Dachshund	ICRP	12	CM
13	Miniature Dachshund	ICRP	12	
14	Miniature Dachshund	ICRP	10	IF
15	Miniature Dachshund	ICRP	7	SF
16	Miniature Dachshund	ICRP	11	IM
17	Miniature Dachshund	ICRP		CM
18	Miniature Dachshund	ICRP	12	SF
19	Miniature Dachshund	ICRP	12	IM
20	Miniature Dachshund	ICRP	7	SF
21	Miniature Dachshund	ICRP	9	CM
22	Miniature Dachshund	ICRP	9	CM
23	Miniature Dachshund	ICRP	10	IM
24	Miniature Dachshund	ICRP	8	IM
25	Miniature Dachshund	ICRP		SF
26	Miniature Dachshund	ICRP	15	IM
27	Miniature Dachshund	ICRP		IM
28	Miniature Dachshund	ICRP	8	IF
29	Miniature Dachshund	ICRP		SF
30	Miniature Dachshund	ICRP	9	CM
31	Miniature Dachshund	ICRP		IM
32	Miniature Dachshund	ICRP		CM
33	Miniature Dachshund	ICRP	9	
34	Miniature Dachshund	ICRP	15	SF
35	Miniature Dachshund	ICRP	12	SF

36	Miniature Dachshund	ICRP	7	IM
37	Miniature Dachshund	ICRP	12	IM
38	Miniature Dachshund	ICRP	13	SF
39	Miniature Dachshund	ICRP	9	SF
40	Miniature Dachshund	ICRP	8	IF
41	Miniature Dachshund	ICRP	0	
42	Miniature Dachshund	ICRP	11	
43	Miniature Dachshund	ICRP	9	SF
44	Miniature Dachshund	ICRP	10	IM
45	Miniature Dachshund	ICRP		IM
46	Miniature Dachshund	ICRP		SF
47	Miniature Dachshund	ICRP	14	IF
48	Miniature Dachshund	ICRP	14	SF
49	Miniature Dachshund	ICRP	8	CM
50	Miniature Dachshund	ICRP	13	SF
51	Miniature Dachshund	ICRP	10	SF
52	Miniature Dachshund	ICRP	13	CM
53	Miniature Dachshund	ICRP	16	IM
54	Miniature Dachshund	ICRP	12	IM
55	Miniature Dachshund	ICRP	10	SF
56	Miniature Dachshund	ICRP	6	CM
57	Miniature Dachshund	ICRP	13	CM
58	Miniature Dachshund	ICRP		IF
59	Miniature Dachshund	ICRP	12	CM
60	Miniature Dachshund	ICRP	13	SF
61	Miniature Dachshund	ICRP	7	SF
62	Miniature Dachshund	ICRP	8	IF
63	Miniature Dachshund	ICRP	11	IM
64	Miniature Dachshund	ICRP	10	SF
65	Miniature Dachshund	ICRP	11	SF
66	Miniature Dachshund	ICRP	13	IM
67	Miniature Dachshund	ICRP	8	IM
68	Miniature Dachshund	ICRP		IM
69	Miniature Dachshund	ICRP	10	CM
70	Miniature Dachshund	ICRP	12	SF
71	Miniature Dachshund	ICRP		
72	Miniature Dachshund	ICRP	7	CM
73	Miniature Dachshund	ICRP	6	IM

74	Miniature Dachshund	ICRP	14	CM
75	Miniature Dachshund	ICRP	12	IM
76	Miniature Dachshund	ICRP	7	IF
77	Miniature Dachshund	ICRP		SF
78	Miniature Dachshund	ICRP	10	SF
79	Miniature Dachshund	ICRP	9	CM
80	Miniature Dachshund	ICRP	6	IF
81	Miniature Dachshund	ICRP	16	IM
82	Miniature Dachshund	ICRP	9	IF
83	Miniature Dachshund	ICRP	9	IM
84	Miniature Dachshund	ICRP		SF
85	Miniature Dachshund	ICRP	5	CM
86	Miniature Dachshund	ICRP	13	SF
87	Miniature Dachshund	ICRP	3	IM
88	Miniature Dachshund	ICRP	9	IM
89	Miniature Dachshund	ICRP	10	SF
90	Miniature Dachshund	ICRP	14	CM
91	Miniature Dachshund	ICRP	11	SF
92	Miniature Dachshund	ICRP	8	SF
93	Miniature Dachshund	ICRP	12	CM
94	Miniature Dachshund	ICRP	7	IM
95	Miniature Dachshund	ICRP		SF
96	Miniature Dachshund	ICRP	14	CM
97	Miniature Dachshund	ICRP		
98	Miniature Dachshund	ICRP	12	CM
99	Miniature Dachshund	ICRP	11	CM
100	Miniature Dachshund	ICRP	11	SF
101	Miniature Dachshund	ICRP	10	IM
102	Miniature Dachshund	ICRP		IM
103	Miniature Dachshund	ICRP	12	IM
104	Miniature Dachshund	ICRP	5	SF
105	Miniature Dachshund	ICRP	11	CM
106	Miniature Dachshund	ICRP	9	CM
107	Miniature Dachshund	ICRP	6	IM
108	Miniature Dachshund	ICRP	8	SF
109	Miniature Dachshund	ICRP	10	CM
110	Miniature Dachshund	ICRP	11	CM
111	Miniature Dachshund	ICRP		SF

112	Miniature Dachshund	ICRP	6	IF
113	Miniature Dachshund	ICRP	5	IF
114	Miniature Dachshund	ICRP	6	SF
115	Miniature Dachshund	ICRP	8	SF
116	Miniature Dachshund	ICRP	7	CM
117	Miniature Dachshund	ICRP	8	IF
118	Miniature Dachshund	ICRP	9	CM
119	Miniature Dachshund	ICRP	7	CM
120	Miniature Dachshund	ICRP	11	CM
121	Miniature Dachshund	ICRP	5	CM
122	Miniature Dachshund	ICRP	10	SF
123	Miniature Dachshund	ICRP	10	IF
124	Miniature Dachshund	ICRP	5	CM
125	Miniature Dachshund	ICRP	12	IF
126	Miniature Dachshund	ICRP		IM
127	Miniature Dachshund	ICRP	6	CM
128	Miniature Dachshund	ICRP	7	CM
129	Miniature Dachshund	ICRP	7	IM
130	Miniature Dachshund	ICRP		CM
131	Miniature Dachshund	ICRP	8	SF
132	Miniature Dachshund	ICRP	9	IF
133	Miniature Dachshund	ICRP	13	SF
134	Miniature Dachshund	ICRP	7	SF
135	Miniature Dachshund	ICRP	5	IM
136	Miniature Dachshund	ICRP	5	SF
137	Miniature Dachshund	ICRP	9	IF
138	Miniature Dachshund	ICRP	8	CM
139	Miniature Dachshund	ICRP	6	
140	Miniature Dachshund	ICRP	10	IF
141	Miniature Dachshund	ICRP	9	CM
142	Miniature Dachshund	ICRP	7	CM
143	Miniature Dachshund	ICRP	9	CM
144	Miniature Dachshund	ICRP	11	IM
145	Miniature Dachshund	ICRP	6	IM
146	Miniature Dachshund	ICRP	10	IM
147	Miniature Dachshund	ICRP	8	SF
148	Miniature Dachshund	ICRP	10	CM
149	Miniature Dachshund	ICRP		CM

150	Miniature Dachshund	ICRP	13	SF
151	Miniature Dachshund	Healthy	8	SF
152	Miniature Dachshund	Gastrointestinal Stromal Tumor	12	SF
153	Miniature Dachshund	Chronic Lymphocytic Leukemia	9	CM
154	Miniature Dachshund	Granulomatous Meningioencephalitis	13	IM
155	Miniature Dachshund	Cholelithiasis	12	SF
156	Miniature Dachshund	Pituitary Dependant Hyperadrenocorticism	13	CM
157	Miniature Dachshund	Oral Squamous Cell Carcinoma	14	IF
158	Miniature Dachshund	Hypothyroidism	12	SF
159	Miniature Dachshund	Non Regenerative Immune Mediated Anemia	12	SF
160	Miniature Dachshund	Hypothyroidism	13	SF
161	Miniature Dachshund	Immune Mediated Poly Arthritis	10	SF
162	Miniature Dachshund	Fever of Unknown Origin	12	SF
163	Miniature Dachshund	Chronic Hepatitis	11	IM
164	Miniature Dachshund	Chronic Rhinitis	12	IM
165	Miniature Dachshund	Immune Mediated Poly Arthritis	12	IF
166	Miniature Dachshund	Suspected Prostate Adenoma	10	SF
167	Miniature Dachshund	Healthy	10	SF
168	Miniature Dachshund	Healthy	10	SF
169	Miniature Dachshund	pyometra	15	CM
170	Miniature Dachshund	Healthy	13	SF
171	Miniature Dachshund	Healthy	4	SF
172	Miniature Dachshund	Chronic Kidney Disease	17	SF
173	Miniature Dachshund	Progressive Retinal Atrophy	14	CM
174	Miniature Dachshund	Healthy	5	CM
175	Miniature Dachshund	Healthy	8	SF
176	Miniature Dachshund	Healthy	10	CM
177	Miniature Dachshund	Healthy	10	IM
178	Miniature Dachshund	Healthy	9	SF
179	Miniature Dachshund	Healthy	9	IF
180	Miniature Dachshund	Healthy	12	SF
181	Miniature Dachshund	Canine Atopic Dermatitis	12	SF
182	Miniature Dachshund	Intervertebral Disc Disease	6	CM
183	Miniature Dachshund	Healthy	11	CM
184	Miniature Dachshund	Healthy	11	
185	Miniature Dachshund	Intervertebral Disc Disease	13	IF
186	Miniature Dachshund	Intervertebral Disc Disease	7	IM
187	Miniature Dachshund	Healthy	5	IM

188	Miniature Dachshund	Healthy		CM
189	Miniature Dachshund	Splenic Mass	7	SF
190	Miniature Dachshund	Healthy	6	CM
191	Miniature Dachshund	Healthy		CM
192	Miniature Dachshund	Healthy	8	SF
193	Miniature Dachshund	Healthy	13	SF
194	Miniature Dachshund	Healthy	7	IM
195	Miniature Dachshund	Elevated Liver Enzyme	14	IF
196	Miniature Dachshund	Healthy	9	SF
197	Miniature Dachshund	Hematochezia	11	SF
198	Miniature Dachshund	Rectal Adenocarcinoma	14	SF
199	Miniature Dachshund	Lymphocytic Plasmocytic Enteritis	14	CM
200	Miniature Dachshund	Lymphocytic Plasmocytic Enteritis	7	SF
201	Miniature Dachshund	Urinary Bladder Polyp	14	CM
202	Miniature Dachshund	Suspected Systemic Lupus Erythematosus	15	SF
203	Miniature Dachshund	Pancreatitis	11	IM
204	Miniature Dachshund	Malignant neoplasia	13	IF
205	Miniature Dachshund	Splenic mass, melena	10	SF
206	Miniature Dachshund	Pancreatic cyst	10	SF
207	Miniature Dachshund	Malignant Hepatic Mass	17	IM
208	Miniature Dachshund	Acute Pancreatitis	14	CM
209	Miniature Dachshund	Hepatic cycst	17	SF
210	Miniature Dachshund	Suspected Non Regenerative Immune Mediated Anemia	15	SF
211	Miniature Dachshund	Metastatic Adrenal tumor, pancreatitis	17	IF
212	Miniature Dachshund	Pancreatitis, Dmelitus	11	SF
213	Miniature Dachshund	Gall Bladder Mucocoele	14	CM
214	Miniature Dachshund	Benign Liver nodule	15	SF
215	Miniature Dachshund	Pancreatits	13	SF
216	Miniature Dachshund	Susp Pituitary Tumor	13	SF
217	Miniature Dachshund	Lymphocytic Plasmocytic Enteritis	14	CM
218	Miniature Dachshund	Suspc Histiocytic Sarcoma	12	SF
219	Miniature Dachshund	Chronic Kidney Disease	12	SF
220	Miniature Dachshund	Suspected Gastric Mass	13	CM
221	Miniature Dachshund	Squamous Cell Carcinoma	17	IM
222	Miniature Dachshund	Idiopathic Seizure	16	CM
223	Miniature Dachshund	Immune Mediated Hemolytic Anemia	16	IF
224	Miniature Dachshund	Pancreatitis	15	IM
225	Miniature Dachshund	Liver Tumor	12	IF

226	Miniature Dachshund	Intervertebral Disc Disease	11	IM
227	Miniature Dachshund	Hydrocephalus	11	IM
228	Miniature Dachshund	Hemangiosarcoma	15	SF
229	Miniature Dachshund	Immune Mediated Thrombocytopenia	11	IM
230	Miniature Dachshund	Benign Prostatic Hyperplasia	9	IF
231	Miniature Dachshund	Splenic mass	16	SF
232	Miniature Dachshund	Malignant Melanoma	11	SF
233	Miniature Dachshund	Pancreatitis	14	CM
234	Miniature Dachshund	Idiopathic CRP elevation	12	SF
235	Miniature Dachshund	Intervertebral Disc Disease	12	CM
236	Miniature Dachshund	Pancreatitis	16	SF
237	Miniature Dachshund	Malignant Liver Neoplasia	16	CM
238	Miniature Dachshund	Nodular Hyperplasia	16	SF
239	Miniature Dachshund	Idiopathic Epilepsy	13	CM
240	Miniature Dachshund	Inappetance	16	SF
241	Miniature Dachshund	Juvenile	< 1	IM
242	Miniature Dachshund	Juvenile	< 1	IM
243	Miniature Dachshund	Juvenile	< 1	IM
244	Miniature Dachshund	Juvenile	< 1	IM
245	Miniature Dachshund	Juvenile	< 1	IM
246	Miniature Dachshund	Juvenile	< 1	IF
247	Miniature Dachshund	Juvenile	< 1	IF
248	Miniature Dachshund	Juvenile	< 1	IM
249	Miniature Dachshund	Juvenile	< 1	IM
250	Miniature Dachshund	Juvenile	< 1	IM
251	Miniature Dachshund	Juvenile	< 1	IM
252	Miniature Dachshund	Juvenile	< 1	IF
253	Miniature Dachshund	Juvenile	< 1	IF
254	Miniature Dachshund	Juvenile	< 1	IF
255	Miniature Dachshund	Juvenile	< 1	IM
256	Miniature Dachshund	Juvenile	< 1	IM
257	Miniature Dachshund	Juvenile	< 1	IM
258	Miniature Dachshund	Juvenile	< 1	IM
259	Miniature Dachshund	Juvenile	< 1	IM
260	Miniature Dachshund	Juvenile	< 1	IM
261	Miniature Dachshund	Juvenile	< 1	IM
262	Miniature Dachshund	Juvenile	< 1	IM
263	Miniature Dachshund	Juvenile		IM

264	Miniature Dachshund	Juvenile	< 1	IF
265	Miniature Dachshund	Juvenile	< 1	IF
266	Miniature Dachshund	Juvenile	< 1	IM
267	Miniature Dachshund	Juvenile	< 1	IF
268	Miniature Dachshund	Juvenile	< 1	IM
269	Miniature Dachshund	Juvenile	< 1	IF
270	Miniature Dachshund	Juvenile	< 1	IM
271	Miniature Dachshund	Juvenile	< 1	IM
272	Miniature Dachshund	Juvenile	< 1	IF
273	Miniature Dachshund	Juvenile	< 1	IM
274	Miniature Dachshund	Juvenile	< 1	IM
275	Miniature Dachshund	Juvenile	< 1	IM
276	Miniature Dachshund	Juvenile	< 1	IF
277	Border Collie	Leukemia	11	IF
278	Toy Poodle	Chronic Kidney Disease	11	SF
279	Labrador Retriever	Chronic Myeloid Leukemia	9	IF
280	Miniature Schnauzer	Chronic Kidney Disease	14	SF
281	Shibainu Dog	Chronic Kidney Disease	15	SF
282	Beagle	Healthy	3	IF
283	Beagle	Healthy	2	IF
284	Shih-Tzu	Idiopathic Vestibular Disease		SF
285	Shih-Tzu	Idiopathic Vestibular Disease	12	SF
286	Pembroke Welsh Corgi	Idiopathic Emesis	14	CM
287	Pembroke Welsh Corgi	Addison Disease	13	CM
288	Jack Russel Terrier	Tracheobronchomalacia	14	SF
289	Chihuahua	Intervertebral Disc Disease	11	SF
290	Cavalier King Charles Spaniel	Mitral Regurgitation	10	IM
291	Chihuahua	MD	11	SF
292	Shih-Tzu	Malassezia Dermatitis	10	CM
293	Whippet	Intervertebral Disc Disease	12	SF
294	Pembroke Welsh Corgi	Suspected Interstitial Pneumonia	15	CM
295	Papillon	Perineal Hernia	14	IM
296	Labrador Retriever	Thoracic Fibrosarcoma	5	IF
297	Labrador Retriever	Healthy	10	SF
298	Toy Poodle	Colitis	10	SF
299	Mix	Colonic Adenoma	8	IM
300	Yorkshire Terrier	Lymphocytic Plasmocytic Enteritis	12	CM
301	Shih-Tzu	Oral Melanoma	10	SF

302	French Bulldog	Suspected Vertebral Diseases	7	SF
303	Toy Poodle	Suspected Pancreatic Tumor	11	SF
304	Italian Greyhouund	Suspected Colitis	11	SF
305	Jack Russel Terrier	Vestibular disease	14	CM
306	Chihuahua	Plasmacytoma	15	SF
307	Mix	Exocrine Pancreatic Insufficiency	1	SF
308	Rottweiler	Addison Disease	10	SF
309	Miniature Schnauzer	Cutaneous Lymphoma	14	SF
310	American Cocker Spaniel	Immune Mediated Hemolytic Anemia	13	SF
311	Beagle	Healthy	4	IF
312	Mix	Healthy	3	IM
313	Beagle	Healthy	2	IF
314	Beagle	Healthy	2	IF
315	Beagle	Healthy	2	IF
316	Beagle	Healthy	2	IF

**CM: castrated male, IM: intact male, SF: spayed female, IF: intact female

Table 2 Chromosomal Position and Primers used for PCR

Gene	Chromosome	Position	Genbank accession number	Variant (mRNA)	Variant (protein)	Forward Primer (5' - 3')	Reverse Primer (5' - 3')
TG	13	29406010	NM_001048104	c.4567C>T	p.R1523W	GATGGGCGGTGAAGGGTTAA	GCACACAGCCCACAAGAAAG
FBN1	30	14742477	NM_001287085	c.1205C>T	p.P402L	AAGGGGCCAATTGAGAAACT	GTGCTCTGTCCCCTTGGTAA

3. RESULTS

3.1 Whole Exome Sequencing analysis

From the previous WES data, nonsynonymous, insertion or deletion variants in 2 MDs with ICRP and 2 healthy beagles detected totaled at 726, 865, 590 and 771. Common variants between both MDs which were not detected in the 2 beagles totaled at 118. Further analysis of the variant was performed by screening with the publicly available canine genomic variant data and the list of genes which are related to the IL-6 amplifier, resulting in two potential genes (Table 2), *TG* c.4567C>T and *FBN1* c.1205>T. When referred to the publicly available variant data on European Variant Archives, the frequency of *TG* c.4567C>T was found as heterozygotes in 3 of 24 dogs, without any frequencies of homozygous at a minor allele frequency of 0.062[49)] while *FBN1* c.1205>T was detected to be novel without any data available.

3.2 Case-control analysis of the variant frequency in of *TG* c.4567C>T and *FBN1* c.1205C>T

When all MDs regardless of age or disease status were compared to dogs of other various breeds, the frequency of *TG* c.4567C>T (p.1523R>W) allele in MDs (n = 272) was at 68.01% (45 homozygous and 140 heterozygous), which was significantly higher than dogs of other breeds (n = 40), which was 22.5% (1 homozygous and 8 heterozygous) ($P < 0.0001$, Figure 1A). When we compared the allele frequency of *TG* between age-controlled MD-ICRP (median age = 9.5) and MD-Controls (median age = 12), the frequency was significantly higher in MD-ICRP, at a 83.56% (31 homozygous, 91 heterozygous, n = 146) while MD-controls had only 47.78% (9 homozygous, 34 heterozygous, n = 90) ($P < 0.0001$, Figure 1B). Proportion test comparing the T/T risk allele frequencies between MD-ICRP and MD-Control showed that the ratios were significantly different ($P = 0.0282$). When aligned with amino acids to selected mammals other than the dog, it was found that only the amino acid in this specific position is not conserved in dogs, but well conserved in other mammals (Figure 2C). For *FBN1* c.1205C>T (p.P402L), even though MDs showed a significantly higher allele frequency (n = 90) at 52.22% (43 homozygous and 39 heterozygous) as compared to non-MDs (n = 40) at 7.69% (2 heterozygous) ($P = 0.0002$, Figure 3A), no significant difference was seen when comparing MD-ICRP (allele frequency at 55.56%, 7 homozygous and 18 heterozygous) and MD-Controls (allele frequency at 48.89%, 1

homozygous and 21 heterozygous) (Figure 3B), and there also no significant difference in proportion test of the ratio between T/T risk allele of two groups ($P = 0.0793$).

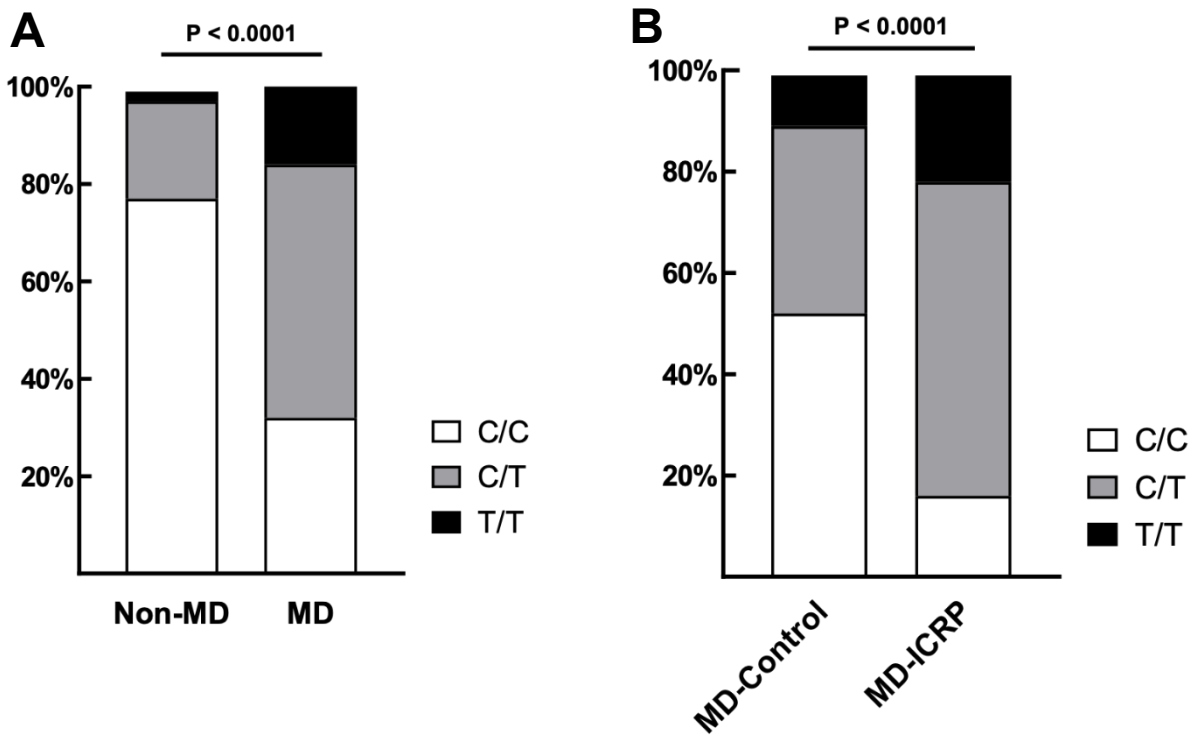

Figure 1. *TG* SNP is associated with the pathology of ICRP in MDs
(A) The frequency of variant *TG* c.4567C>T (p.R1523W) was significantly higher in MDs (n = 272) when compared to other breeds (n = 40), with $P < 0.0001$
(B) Among MDs, age-matched case-control showed the same variant was significantly higher in MD-ICRP (n = 146) when compared to MD-Control (n = 90), with $P < 0.0001$

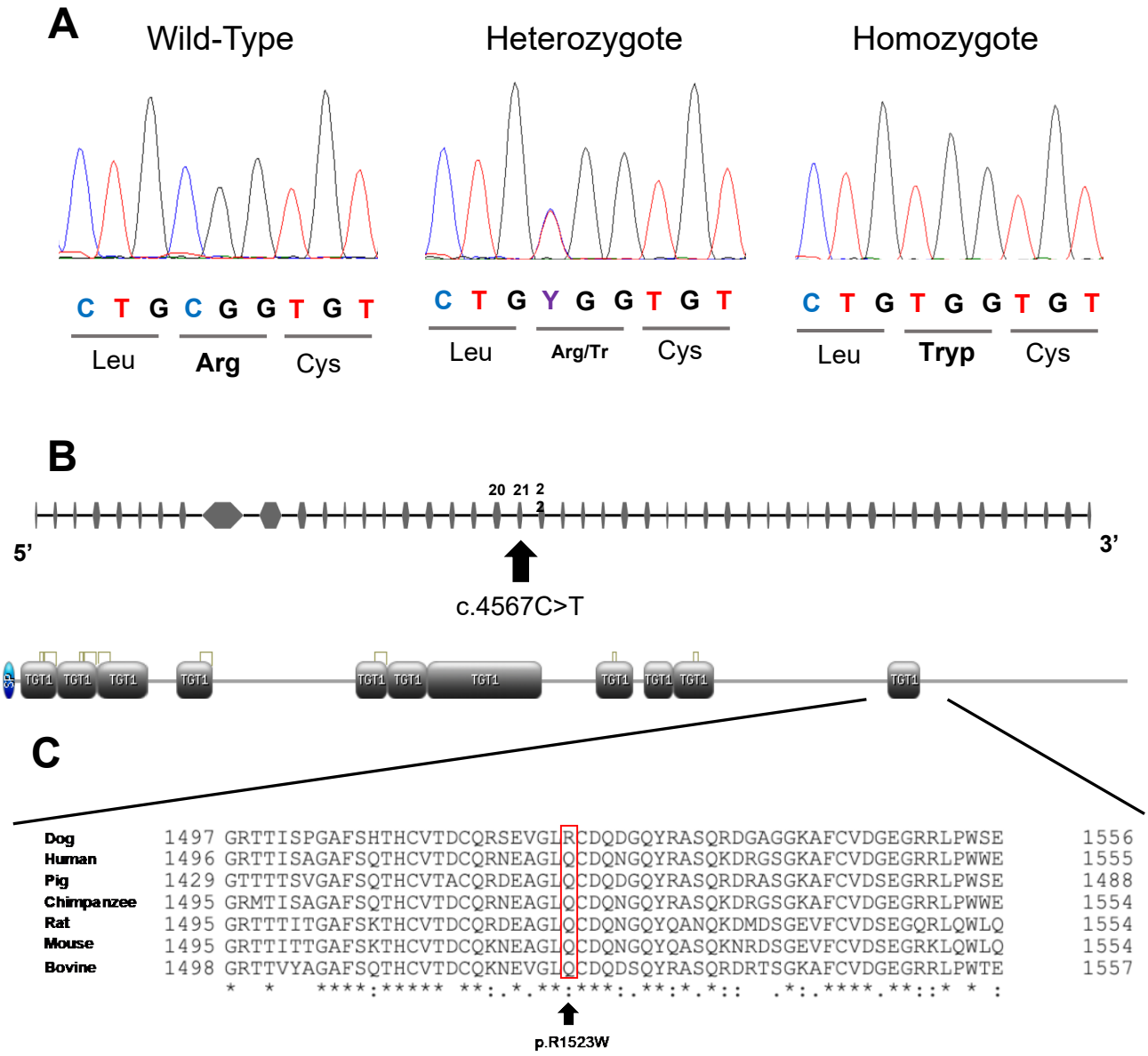

Figure 2. Polymorphism of *TG* gene
(A) The sequence chromatogram of *TG* c.4567C>T (p.R1523W) in wild-type, heterozygote and homozygote variant samples.
(B) A schematic diagram of the canine *TG* gene and thyroglobulin protein. SP: Signal protein. TGT1: Thyroglobulin Repeat Type 1.
(C) Alignment of amino acid sequence in the final thyroglobulin type-1 repeat domain of canine thyroglobulin against selected mammals using the UniProt repository (https://www.uniprot.org/). Asterisk (*): completely conservation. Colon (:): strong similar properties. Period (.): weak similar properties. R1523 in dogs has strong similar properties to other mammals but highly conserved among other mammals.

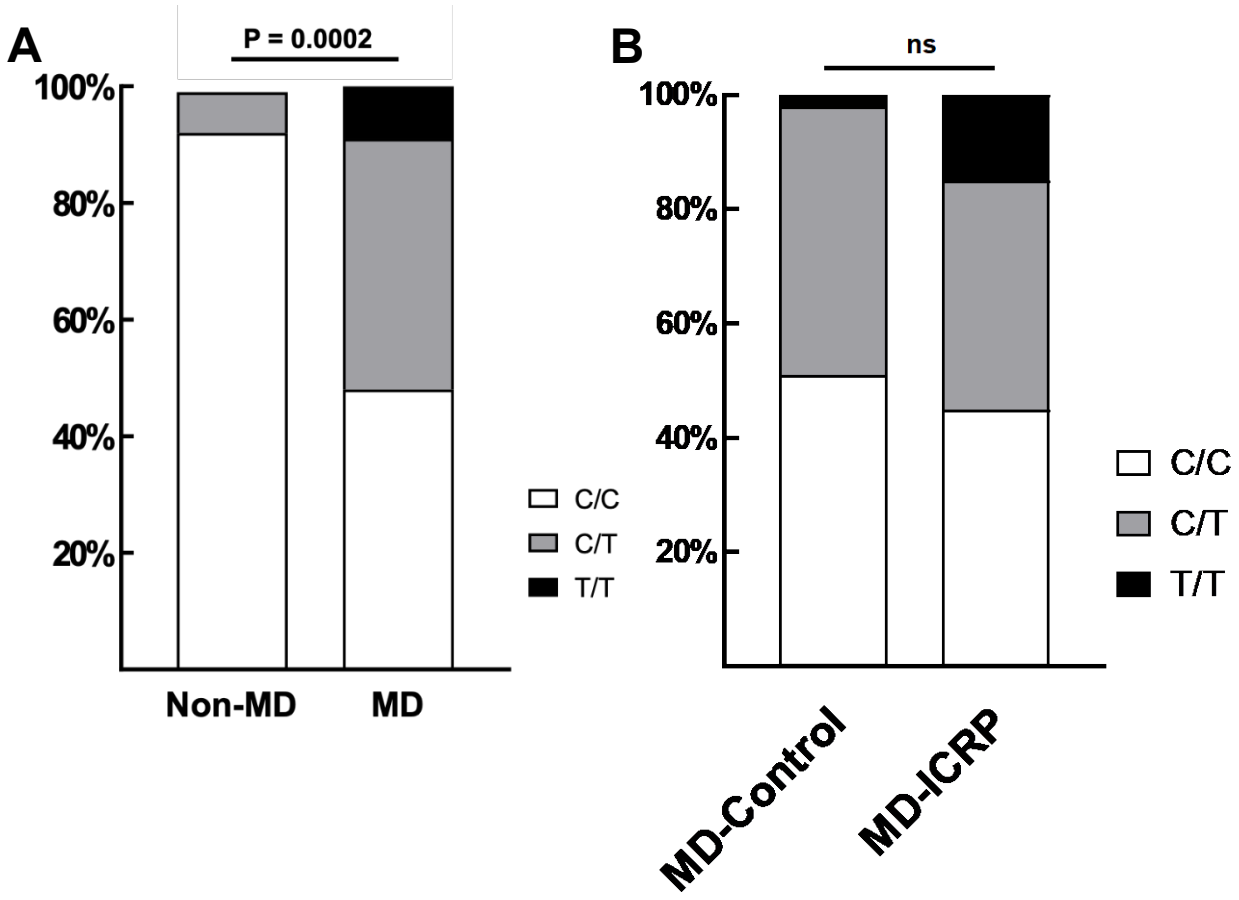

Figure 3. *FBN1* SNP is breed specific to MD but not associated with pathology of ICRP
(A) The frequency of variant *FBN1* c.1205C>T (p.402P>L) was significantly higher in MDs (n = 90) when compared to other breeds (n = 40).
(B) However, among MDs, age-matched case-control analysis showed no significant difference between MD-ICRP (n= 45) and MD-control (n = 45).

4. DISCUSSION

By reviewing the WES data from a previous study, novel SNPs which have yet to be explored were identified. Although IBD in humans may differ between geographical regions, most studies point towards a peak in incidences in 15-29-year-old group[42]. Unlike very early onset IBDs which is presented as Mendelian monogenic disorders in children less than 6 years old, adult onset IBDs are known to be polygenic complex disorders affected by multiple genetic variances influencing patient's immunity, intestinal immunopathological process and their responses towards environmental stimuli[54,71]. ICRP is an IBD which affects middle-aged MDs (median age of 9.0)[65] , where our cohort bears resemblance with a median age of 10, with a range from 6 to 16 years old, which suggest the disease required time for the effects of gene-environment interaction to induce pathology development. Furthermore, no familial effects of ICRP have been reported in previous studies to suggest it as a Mendelian disease, hence ICRP is also generally assumed to be a polygenic disease in nature.

This study has validated two novel SNPs which are significantly higher in MDs as compared to other breeds, but they are not exclusive to MDs. Although ICRP has been reported to be breed specific with an odds-ratio of 24.6 as compared to other breeds, sporadic occurrences in other breeds have also been reported[65]. Hence, the presence of such SNPs in the population of other dog breeds is not surprising. Intriguingly, despite being detected in the population of Cane Corso Italiano dog breed including 232 dogs collected over 25 countries which did not include Japan, homozygous risk allele of *TG* c.4567C>T was not detected in that population[49]. In contrast, our study has detected homozygous risk alleles

among the dog population in Japan (Figure 1A), which is significantly higher in MDs and in MD-ICRP groups, for which this breed is significantly predisposed to ICRP. Proportion testing further confirmed that the ratio of risk allele T/T is indeed higher in MD-ICRP, proving that this SNP is important in the pathogenesis of ICRP. Similarly, no studies have reported the presence of risk allele *FBN1* c.1205C>T outside of Japan, which we have picked up as a novel SNP in our study, which is significantly higher in MDs, although not significantly different between ICRP and control group. Considering that ICRP is a disorder confined within the archipelago of Japan, these SNPs which are only detected within the population of dogs in Japan may be strongly associated to the pathology of ICRP, which warrants further investigation in their functionality in disease pathogenesis.

The greatest limitation in this study is the number of samples recruited for the WES study. WES was only performed in 2 MDs and 2 beagles, which may have limited the detection power for susceptible genes. Most human studies in GWAS to detect susceptible genes in IBD included immensely high number of individuals per cohort, up to 86640 individuals per study[52]. Next generation sequencing is expected to be more affordable soon, future studies may recruit more samples to improve the detection power of subsequent analysis.

5. SUMMARY

In Chapter 1, we have confirmed that two previously discovered SNPs in a whole exome sequencing analysis are indeed breed-specific towards MD, and *TG* SNP has a higher frequency of risk allele T/T in the MD-ICRP group as compared to MD-Control, signifying its importance in the pathogenesis of ICRP. Hence, these SNPs are valid candidates for further exploration of their functions and involvement in the IL-6 amplifier cascade which may be an important pathogenesis mechanism of ICRP in the subsequent chapters.

CHAPTER 2

Mechanistic Analysis of Risk Associated Genes and Their Involvement with IL-6 Mediated Inflammatory Response *in vitro*

1. INTRODUCTION

Despite knowing that activation of inflammatory cytokines and infiltration of inflammatory immune cells are involved in the lesion sites of ICRP[37,38,48,66,88,97,98], no studies have managed to bridge the link between the genetic predisposition of ICRP in MDs and the inflammatory pathophysiology of ICRP. This may be contributed by the specificity of past studies in focusing on the expression and mutation of selected inflammatory cytokines such as *IL-8*[88], *NOD2*[38], *TLR*s[97] and *LRG*[67], without a genome wide association study data which provides information on risk alleles to the disease.

Past studies on mouse silencing models and human GWAS have proven that specific genes can be effective regulators of the IL-6 amplifier cascade, where they act as factors in stimulating NF-kB or STAT3 in non-immune cells leading to hyperactivation of NF-kB, resulting in chronic inflammation and disorders[4,5,7,8,33,34,47,50,55,56,59–62,64,69,72,76,84,87,89,90]. However, no GWAS studies in canine inflammatory or auto-immune disorders have been done to assess the effects of regulatory genes in activating the IL-6 amplifier in dogs, apart from the previous study in our group which have shown MMP-9, an NF-kB target is able to breakdown a mutated PLG protein more readily in MDs and humans with IBDs[96].

The data from chapter 1 showed that *TG* and *FBN1* are both MD specific, and particularly *TG* has shown to be associated with the pathology of ICRP. This chapter aims to investigate the missing link between the genetic predisposition of MD in developing ICRP with the inflammatory nature behind the risk associated genes found in the previous chapter. By using the technology of silencing RNA, the involvement of *TG* and *FBN1* in the inflammatory response in non-immune cells, in specific human neuroglioma cell-line (H4), with regards to the IL-6 amplifier cascade were investigated. The effects of *TG* and *FBN1* on the activation of IL-6 amplifier were also explored through treatment of recombinant protein of each gene on non-immune cells.

2. MATERIALS AND METHODS

2.1 Stimulation conditions

H4 human cancer cell line was purchased from ATCC (Sumitomo Dainippon Pharma, Osaka, Japan). All cell lines were cultured in DMEM (Thermo Fisher Scientific, Waltham, MA) enriched with 10% fetal bovine serum (Thermo Fisher Scientific) and treated with 1% penicillin and streptomycin at 37°C under 5% CO2. For cytokine stimulation, cells were seeded in 96-well plates (1 x 10^4 cells/well) and stimulated with human IL-6 (100ng/mL; Toray Industries, Tokyo, Japan) plus human soluble IL-6 receptor (100ng/mL; R&D Systems, Minneapolis, MN) and TNF- α (50ng/mL; PeproTech, Tokyo, Japan) for 3 hours after 2 hours of serum starvation in Opti-MEM (Thermo Fisher Scientific, Waltham, MA). For TG stimulation, cytokine stimulation was modified to include cells stimulated with 1/5 of cytokine with addition of serial increment of recombinant human TG (1μg/mL, 5μg/mL, 10μg/mL) reconstituted as per manufacturer recommendation (Cloud-Clone Corp., USA). After stimulation, the cells were lysed, and total RNA was retrieved for real-time PCR. For FBN1 stimulation, cytokine stimulation was modified to include cells stimulated with 1/5 of cytokine with addition of serial increment of recombinant human FBN1 (1μg/mL, 5μg/mL, 10μg/mL) reconstituted as per manufacturer recommendation (Bon Opus Biosciences., USA). After stimulation, the cells were lysed, and total RNA was retrieved for real-time PCR.

2.2 Human small interfering RNAs

siRNAs were transfected into H4 cells using Lipofectamine RNAiMax (Thermo Fischer Scientific). The sequences for the sense oligonucleotides of the knockdown constructs were human si-TG (1: CCUUAUGAGUUCUCACGGAtt and 2: GCUGCUACAUGGUAUUACUtt; Ambion Silencer Select siRNA, Thermo Fisher Scientific), human si-FBN1 human (1: GGACAGUGCAAUGAUCGUAtt and 2: GGGCUUUCAUGUUACACGAtt; Ambion Silencer Select siRNA, Thermo Fisher Scientific), si-p65 (Ambion Silencer Select RELA siRNA, Thermo Fisher Scientific), and human si-non-target (Ambion Negative Control #1 siRNA, Thermo Fisher Scientific).

2.3 Quantitative Real-Time PCR

The Bio-Rad CFX96 real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA) and THUNDERBIRD SYBR qPCR Mix (TOYOBO Co. Ltd., Osaka, Japan) were used to quantify the levels of target mRNA and internal control mRNA (glyceraldehyde-3-phosphate dehydrogenase, *GAPDH*). Total RNA was prepared from cells using SuperPrep Cell Lysis Kit for qPCR (TOYOBO). Primer pairs used for qPCR are described in Table 1. The conditions for real-time PCRs were 40 cycles at 94 °C for 15 s followed by 40 cycles at 60 °C for 60 s.

2.4 Statistical Analysis

Statistical analysis was performed using JMP Pro version 14.0 (SAS Institute Inc., Cary, NC, USA). Two-tailed Student T-test was used to analyze differences between two groups. Value of $P < 0.05$ was considered as significant.

Table 1 Primers used for qPCR

Gene	Forward Primer (5' - 3')	Reverse Primer (5' - 3')
IL6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC
GAPDH	GAGTCAACGGATTTGGTCGT	CGCTCCTGGAAGATGGTG
TG	CCAGTGGCTTCTCTTCCTGACT	CCTTGGAGGAAGCGGATGGTTT
FBN1	GGATACACAGGTGATGGCTTCAC	GTCGCATTCACAGCGGTATCCT

3. RESULTS

3.1 *TG* is a strong target for IL-6 amplifier activation in *in vitro* models

Cytokine stimulation of H4 cells has induced a sequential increase in *TG* mRNA expression (Figure 1B) as the strength of stimulation increases, similar to the *IL*6 mRNA expression (Figure 1A) as seen in the IL-6 amplifier mechanism, signifying TG as a strong target in the IL-6 amplifier activation cascade. However, *FBN1* mRNA expression (Figure 1C) did not show equal significance in the cytokine stimulation model.

3.2 *TG* is critical for IL-6 amplifier activation in *in vitro* models

Small interfering (siRNA) knockdown of *TG* has significantly inhibited the NF- kB induced *IL6* expression in H4 cells (human neuroglioma cells) (Figure 2A, B), showing that *TG* is crucial in NF- kB activation in non-immune cells upon stimulation with cytokines. Similarly, when recombinant human TG was sequentially co-stimulated with 5 x diluted cytokines, NF- kB induced *IL6* expression in H4 cells have shown remarkable increment (Figure 2C) as the concentration of recombinant TG was increased.

3.3 FBN1 showed minimal effect in IL-6 amplifier activation in *in vitro* models

Small interfering (siRNA) knockdown of *FBN1* has only inhibited the NF- kB induced *IL6* expression in 2 out of 3 siRNA from the library transfected into H4 cells (Figure 2A, B), showing that *FBN1* is association in the NF- kB activation in non-immune cells upon stimulation with cytokines, but may not be absolute. However, when recombinant human FBN1 was sequentially co-stimulated with 5 x diluted cytokines, NF- kB induced *IL6* expression in H4 cells have shown no significant increment (Figure 2C) as the concentration of recombinant FBN1 was increased, differing substantially from the results seen in TG. Hence, further interrogation of the effects of this gene was not pursued.

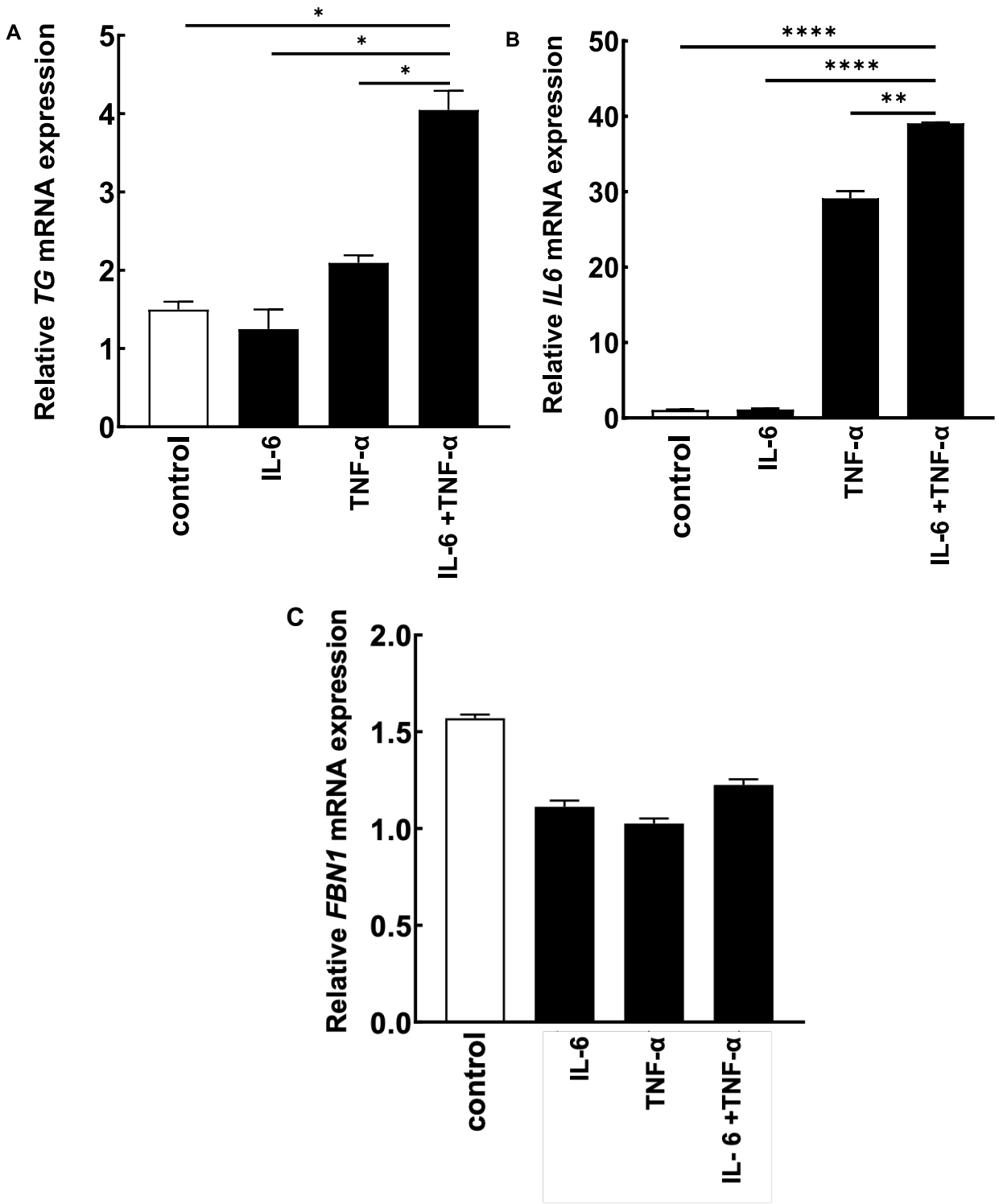

Figure 1. *TG* is a strong target for IL-6 amplifier activation *in vitro*
(A, B, C) H4 cells were stimulated with cytokines. Relative *IL6*, *TG* and *FBN1* mRNA expression levels were assessed. Mean ± SEMs are shown. * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$.

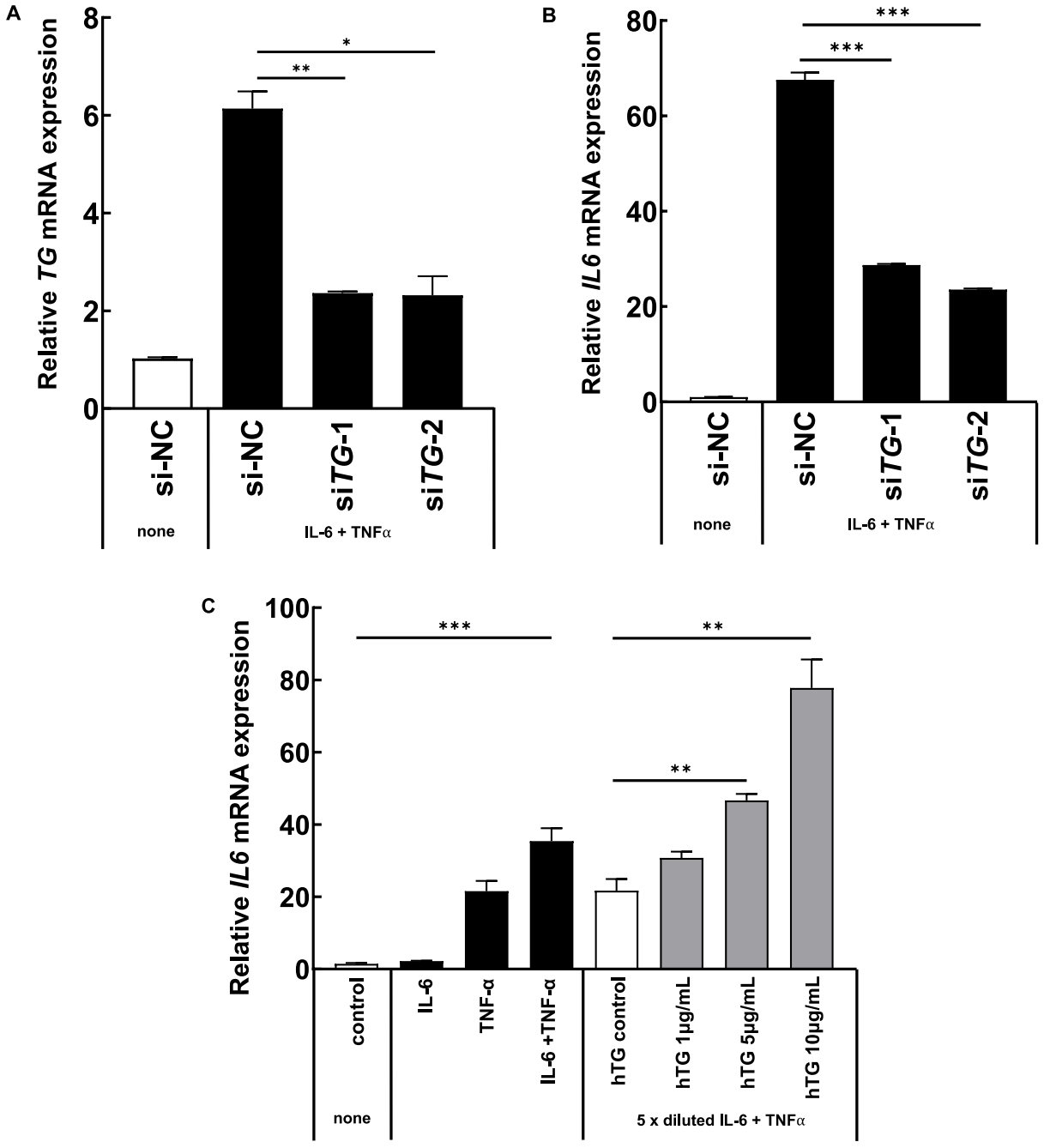

Figure 2. *TG* is critical for IL-6 amplifier activation *in vitro*
(A, B) H4 cells were treated with three different siRNAs for all *TG* variants or control. *IL-6* levels and *TG* knockdown efficiency were assessed. Mean ± SEMs are shown. * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$. (C) H4 cells were stimulated with cytokines and recombinant human TG. *IL-6* levels were assessed. Mean ± SEMs are shown. * $p < 0.05$, ** $p < 0.01$, ***$p < 0.005$.

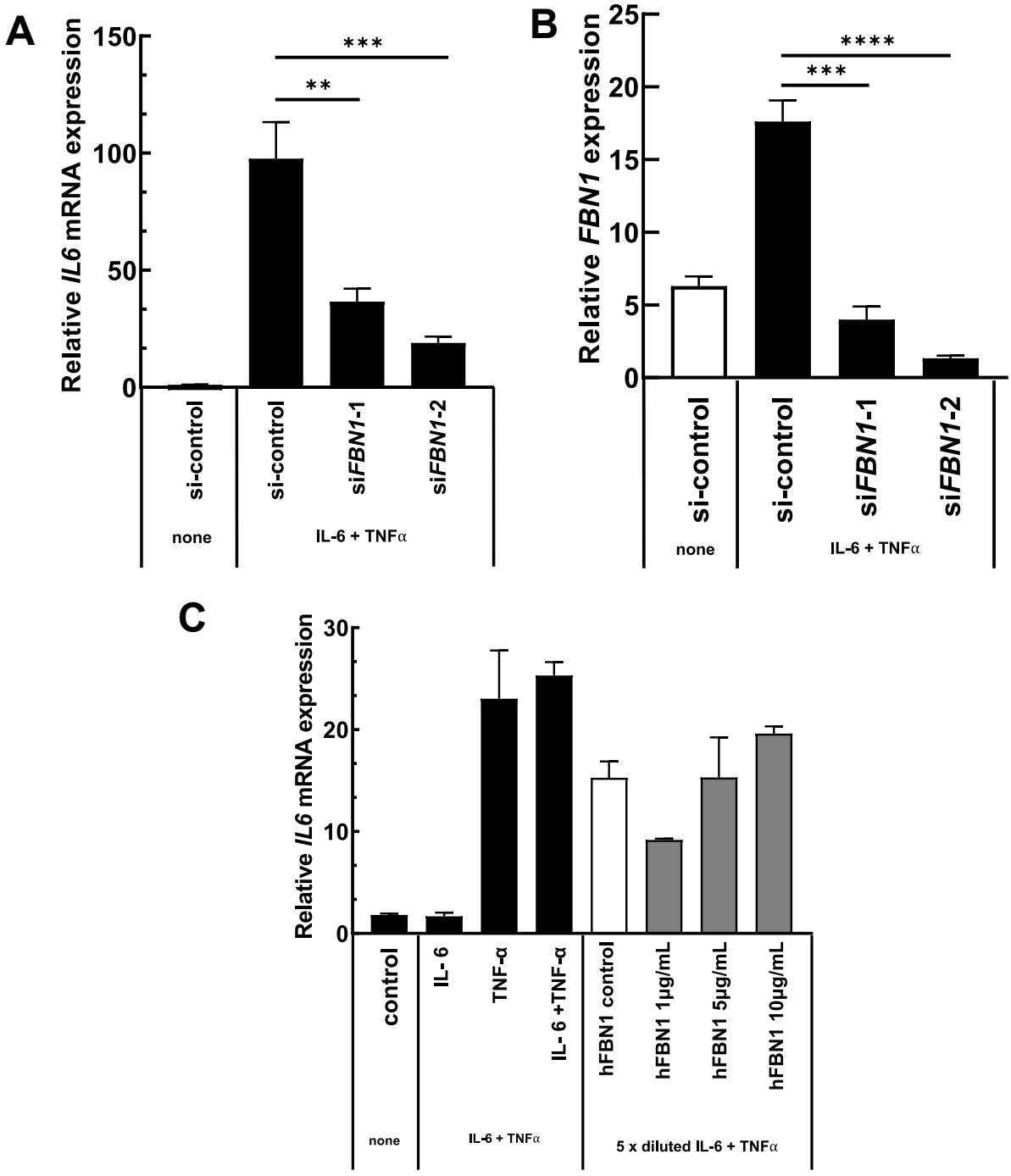

Figure 3. *FBN1* is only partially related to the IL-6 amplifier activation *in vitro*
(A, B) H4 cells were treated with three different siRNAs for all *FBN1* variants or control. *IL-6* levels and *FBN1* knockdown efficiency were assessed. Mean ± SEMs are shown. * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$. (C) H4 cells were stimulated with cytokines and recombinant human FBN1. *IL-6* levels were assessed. Mean ± SEMs are shown. H4 cells were stimulated with cytokines and relative *FBN1* mRNA expression levels were assessed. Mean ± SEMs are shown.

4.0 DISCUSSION

From the previous chapter, *TG* has been speculated to be involved in the pathogenesis of ICRP, but it was not certain if this gene was involved in the inflammatory pathway. As *TG* has not been reported to have much involvement in the inflammatory pathway, the investigation for involvement of *TG* in inflammation was the prime priority in this study. In sooth, functional analysis of *TG* through cytokine stimulation, silencing RNA and recombinant protein stimulation has all shown that *TG* is indeed involved in the IL-6 amplifier mechanism. To the author's knowledge, this study is the first to note the connection between *TG* and IL-6 amplifier, which may connect the missing link between high concurrent prevalence of IBD in human thyroidal patients as well. The activation cascade involved in this mechanism is still unknown, but the effects of thyroid hormones on colitis have been reported in both hypothyroid and hyperthyroid experimental animal models. Ísman et al reported in 2003 that methimazole-induced hypothyroidism in rats have improve oxidative damage induced in experimental colitis of trinitrobenzene sulfonic acid, where they concluded that the hypothyroid state led to reduced reactive oxygen intermediates which resulted in lowered colonic oxidative damage[40]. On the other hand, Bagaturiya et al in 2019 have shown that in mice experimental L-thyroxine induced hyperthyroidism model, hypertrophy of the mucous membrane of the colon was evident with infiltration of plasma cells and lymphocytes[13]. While they suggested that thyroid status may module inflammation through immune cell interaction, direct association was not indicated in their study. Interestingly, both studies did not measure the levels of *TG* either locally or systematically, which indicates such study may be needed in the future to determine if *TG* may play important role in mouse or rat colitis models, since such association in dogs have been shown in this study.

FBN1 while showed breed specificity, had no significant association between MD-ICRP and MD-Control in the previous chapter. While siRNA technology showed that the siRNA silencer sequences managed to suppress *IL-6* mRNA expression, stimulation of H4 cells with cytokines and sequential increment of *FBN1* did not show favorable changes in *IL-6* mRNA expression. With the above two results, it has been concluded that *FBN1* may not be as important in the pathogenesis of ICRP, hence further investigation was not continued.

5. SUMMARY

In Chapter 2, we have confirmed that TG is important in the activation of the IL-6 amplifier cascade using *in vitro* studies, utilizing the power of si-RNA through gene silencing and recombinant TG through protein stimulation in H4 cells. These results strongly suggest that the *TG* SNP validated in chapter 1 has a high probability to function as a initiator in chronic inflammation through the activation of IL-6 amplifier in the pathogenesis of ICRP in MDs. On the other hand, despite promising results in gene silencing studies, FBN1 was shown to have minimal relationship with the activation of IL-6 amplifier as sequential stimulation of recombinant FBN1 showed no changes in *IL6* mRNA expression, nor IL-6 stimulation affects the changes in *FBN1* mRNA expression *in vitro*. These results have confirmed that *TG* SNP is a strong candidate gene in the elucidation of pathogenesis of ICRP in MDs, and the function of this gene should be investigated in case samples to confirm the presence of IL-6 amplifier activation in the subsequent chapter.

CHAPTER 3

Evaluation Of Local and Systemic Expression of The Risk Associated Gene TG as well as Its Effect On The Activation Of IL-6 Amplifier In MDs With ICRPs

1. INTRODUCTION

Thyroglobulin (*TG*) is commonly known as the precursor protein to thyroid hormones T3 and T4, which regulates multiple metabolic pathways in the mammalian body[23]. Nevertheless, recent advances have shown that *TG* may function both inside and outside of the thyroid, which is no surprise since *TG* expression was detected in multiple non-thyroidal cells such as human and mouse kidney cells[94]. Other human tissues such as testis, suprarenal gland, appendix, lung, thymus, and hypophysis too were proven to express *TG* mRNA by Bojunga et al in 2000[18]. Interestingly, *TG* mRNA expression was also detected in the blood[19] and blood cells including lymphocytes and leukocytes[2,17].

In humans, thyroidal involvement in inflammatory bowel disease have been detected since 1995, where sub-clinical increment in thyroid volume and serum free thyroxine levels were shown to be present in IBD patients[16]. However, this study did not explain the pathophysiology behind this result, where they only concluded that IBD is a systemic disease which involves several organs and thyroid is one of the affected organs. On the other hand, Autoimmune thyroidal diseases (AITDs) in humans are categorized into two main diseases, Hashimoto's Disease (HD) and Graves' Disease (GD), where their concurrency with IBDs have been reported multiple times[16,21,39,80,85]. Intriguingly, serum TG elevation has been detected in AITDs as well[1,57,81,93]. In dogs, serum TG is not routinely measured as compared to autoantibodies against thyroglobulin (TgAA), as the latter is used to diagnosed canine lymphocytic thyroiditis (CLT), a canine homolog to human HD[15]. CLT is characterized by the destruction of thyroid glands due to infiltration of B and T lymphocytes, commonly accompanied by elevation in circulatory TgAA[32,53]. Unlike human, concurrent AITDs and IBDs reports in dogs are scarce, but it may or may not be due to under-reports.

In the previous chapters, it has come to light hat *TG* is both associated to pathology of ICRP and IL-6 amplifier, which are both related to local chronic inflammation. In this chapter, the function of *TG* c.4567C>T SNP and the expression of *TG* systemically and locally in dogs with ICRP was analyzed.

2. MATERIALS AND METHODS

2.1 Study Population

For systemic TG expression study, serum sample samples from 22 MD diagnosed with ICRP, 22 control MDs and 36 juvenile MDs were enrolled in this study. Blood samples were retrieved from 44 MDs which were referred to the Hokkaido University Veterinary Teaching Hospital between November 2016 and December 2020 for detailed investigation of various diseases, summarized in Table 1. 22 MDs were diagnosed with ICRP (MD-ICRP) like the study population as described above, while 22 control MDs (MD-Control) were diagnosed with diseases apart from MD with similar age with MD-ICRP. The median age of MD-ICRP was 9.5 (ranged 6-12) years old while in MD-Control was 13 (ranged 8 to 14.25), with no significant statistical difference. There were 12 males (6 intact, 6 castrated) and 10 females (3 intact, 7 spayed) in the MD-ICRP group while there were 9 males (4 intact, 5 castrated) and 13 females (2 intact, 11 spayed) in the MD-Control group. For the juvenile samples, blood samples were retrieved from a small and medium-sized enterprise pet store, with all aging less than 1 year old, and a population of 25 intact males and 11 intact females. Blood was collected by a veterinarian, or a veterinary student supervised by a licensed veterinarian through jugular venipuncture as a part of routine blood workup for screening or diagnosis purposes. Signed consent form was obtained from the owner of all owned dogs for sample collection and usage in this study.

For local *TG* expression study, colorectal mucosa samples were retrieved from 7 MDs which were referred to the Hokkaido University Veterinary Teaching Hospital between September 2013 and December 2020 for detailed investigation of hematochezia, tenesmus, mucoid feces and presence of polypoid mass upon rectal palpation. All the dogs were diagnosed with ICRP through endoscopic and histopathological findings which were established before in previous reports[65,75,92)]. Among the population, 4 MDs have had prednisolone treatment initiated (0.15mg-1mg/kg/day) prior to presentation. The median age of the dogs was 11 (ranged 6-12) years old and the median weight was 5.46 (ranged 4.4-7.7) kg. There were 4 males (3 castrated, 1 intact) and 3 spayed females in this population. Routine blood test which included complete blood count, serum biochemistry, fecal test and abdominal ultrasonogram showed no remarkable changes in these cases, apart from the apparent increase in serum C-reactive protein

(CRP >5mg/dl) in 2 dogs. Written informed consent was retrieved from all the owners of dogs enrolled in this study.

2.2 Tissue Sample Collection

Endoscopic examinations were performed under general anesthesia for all dogs to retrieve the colon tissues. Each dog was administrated with midazolam (0.1 mg/kg) and butorphanol tartrate (0.2mg/kg) intravenously as pre-medication, subsequently with propofol (4 to 6 mg/kg) with the same route. Anesthesia was then maintained through inhalation of isoflurane with oxygen, where additional butorphanol were administrated when necessary. Necessary monitoring parameters such as pulse oximetry, electrocardiography, capnograph, arterial blood pressure and rectal temperature were monitor throughout anesthesia to ensure smooth endoscopic procedures. Endoscopic procedures were completed within 2 hours in all dogs, and all dogs recovered uneventfully. In all MDs, polyp lesions which were deemed inflammatory on gross appearance were collected endoscopically with forceps or through polypectomy. Normal colorectal mucosae were collected endoscopically from the colorectal region adjacent which were deemed normal on gross appearance. At least 6 tissue samples were collected from both polyp lesion site and normal colorectal mucosal region in MDs with ICRP. All biopsy specimens were assessed by a board-certified veterinary pathologist (Y.K.) according to the histopathologic standards established by the World Small Animal Veterinary Association Gastrointestinal Standardization Group[27]. Each type of tissue sample was stored in -80°C for protein analysis; and RNA*Later* RNA Stabilization Solution (Ambion Inc., Austin, TX, USA) for 24 hours in 4°C to allow penetration and stabilization, subsequently in -80°C for prolonged storage indefinitely.

2.3 Enzyme-Linked Immunosorbent Assay (ELISA) detection of serum *TG* levels

Serum samples separated after centrifugation were stored in -80°C until TG analysis. Serum TG concentrations were measure as per protocol provided by the manufacturer using the Canine Thyroglobulin ELISA Kit (MyBioSource, San Diego, CA, USA, Catalogue No: MBS2608140). Intra-assay coefficient of variability between sample replicates were less than 20%.

2.4 Quantitative Real-Time PCR

For qPCR using canine colonic mucosa samples, seven MD-ICRP (3 with T/T allele and 4 with C/C allele) which visited HUVTH between April 2017 to April 2021 were included, total RNA was extracted using a RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and genomic DNA was removed using a RNase-free DNase Set (Qiagen) following the manufacturer's instructions. cDNA synthesis was then performed using oligo (dT) and M-MLV reverse transcriptase (Promega, Madison, Wisconsin, USA) from 1 μg total RNA as per the manufacturer's recommendation. Real-time Taqman qPCR was performed using a commercially available set of a pre-designed probe and primers (Applied Biosystems) for *TG* (product no: Cf02701382_m1) with an endogenous control for colonic samples using *SDHA* (product no: Cf02664981_m1) confirmed by a previous study (46). Real-time qPCR was performed using TaqMan PCR probe master mix (KAPA Biosystems) and the following cycle conditions: 40 cycles at 94 °C for 3 seconds and 40 cycles at 60 °C for 30 seconds. The relative *TG* mRNA expression levels were normalized to the level of *SDHA* mRNA expression. THUNDERBIRD SYBR qPCR Mix (TOYOBO Co. Ltd., Osaka, Japan) was used to quantify the levels of the target mRNA (IL6, CCL2) and internal control mRNA (Succinate dehydrogenase complex, subunit A; SDHA). The condition for real-time qPCR was 40 cycles at 94 °C for 15 s followed by 40 cycles at 60 °C for 60 s. Relative *IL6* and *CCL2* mRNA expressions were normalized to the level of SDHA mRNA expression. The primers and their sequences for the qPCR are described in Table 3 and reported previously.

2.4 Statistical Analysis

Statistical analysis was performed using JMP Pro version 14.0 (SAS Institute Inc., Cary, NC, USA). Two-tailed Student T-test was used to analyze differences between two groups. Value of $P < 0.05$ was considered as significant.

Table 1 Signalmen of dog recruited for serum *TG* ELISA assessment.

Case Number	Breed	Disease	Age	Sex	TG Genotype
1	Miniature Dachshund	ICRP	11	CM	T/T
2	Miniature Dachshund	ICRP	6	CM	T/T
3	Miniature Dachshund	ICRP	9	CM	T/T
4	Miniature Dachshund	ICRP	12	IM	T/T
5	Miniature Dachshund	ICRP	9	SF	T/T
6	Miniature Dachshund	ICRP	10	IF	T/T
7	Miniature Dachshund	ICRP	13	FS	T/T
8	Miniature Dachshund	ICRP	8	CM	T/T
9	Miniature Dachshund	ICRP	8	IM	C/T
10	Miniature Dachshund	ICRP	8	SF	C/T
11	Miniature Dachshund	ICRP	11	CM	C/T
12	Miniature Dachshund	ICRP	9	SF	C/T
13	Miniature Dachshund	ICRP	7	SF	C/T
14	Miniature Dachshund	ICRP	7	IM	C/T
15	Miniature Dachshund	ICRP	8	IM	C/T
16	Miniature Dachshund	ICRP	14	SF	C/T
17	Miniature Dachshund	ICRP	13	IM	C/T
18	Miniature Dachshund	ICRP	12	SF	C/T
19	Miniature Dachshund	ICRP	10	IF	C/C
20	Miniature Dachshund	ICRP	10	CM	C/C
21	Miniature Dachshund	ICRP	14	IF	C/C
22	Miniature Dachshund	ICRP	8	IM	C/C
23	Miniature Dachshund	Pancreatic cyst	10	SF	T/T
24	Miniature Dachshund	Malignant Hepatic Mass	17	IM	T/T
25	Miniature Dachshund	Acute Pancreatitis	14	IF	T/T
26	Miniature Dachshund	Metastatic Adrenal Tumor	17	IM	T/T
27	Miniature Dachshund	Gall Bladder Mucoceole	14	SF	T/T
28	Miniature Dachshund	Hemangiosarcoma	15	IM	T/T
29	Miniature Dachshund	Splenic mass, Melena	19	CM	C/T
30	Miniature Dachshund	Anemia, Hepatic cycst	17	SF	C/T
31	Miniature Dachshund	Benign Liver nodule	15	SF	C/T
32	Miniature Dachshund	Susp Pituitary Tumor	13	SF	C/T
33	Miniature Dachshund	Chronic Kidney Disease	12	SF	C/T
34	Miniature Dachshund	Intervertebral Disk Disease	11	CM	C/T
35	Miniature Dachshund	Intervertebral Disk Disease	12	SF	C/T
36	Miniature Dachshund	Urinary Bladder Polyp	14	SF	C/C
37	Miniature Dachshund	Squamouse Cell Carcinoma	17	CM	C/C

38	Miniature Dachshund	Idiopathic Seizure	16	SF	C/C
39	Miniature Dachshund	Liver Tumor	12	IM	C/C
40	Miniature Dachshund	Hydrocephalus	11	IF	C/C
41	Miniature Dachshund	Bening Prostatic Hyperplasia	9	IM	C/C
42	Miniature Dachshund	Nodular Hyperplasia	16	SF	C/C
43	Miniature Dachshund	Idiopathic Epilepsy	13	CM	C/C
44	Miniature Dachshund	Inappetance	16	SF	C/C

**CM: castrated male, IM: intact male, SF: spayed female, IF: intact female

3. RESULTS

3.1 Systemic TG expression were not significantly different between groups

No significant difference was found in the serum TG levels between MD-ICRP and MD-Control groups as shown in Figure 1A. Then, the serum TG levels between genotypes across both groups (Figure 1B) were also compared, yet there were no remarkable differences either. As systemic drugs such as anti-inflammatory may also induce changes in systemic TG, the comparison of serum TG of cases with or without anti-inflammatory treatment showed that two groups were not different significantly. (Figure 1C). Hypothesizing that both ICRP and non-ICRP diseases may inflict certain degree of inflammation systematically and affect serum TG levels, we speculate that young MDs without underlying diseases may provide a better clarity in the effect of the SNP on systemic TG levels. However, the serum TG remained insignificant when compared across genotypes within the population of juvenile MDs (Figure 1D).

3.2 *TG* is highly expressed locally in colonic mucosa of ICRP dogs with risk allele

We hypothesized the *TG* SNP perhaps will function to increase TG expression locally instead, since ICRP is a localized IBD. To test this hypothesis, we then investigated the expression levels of *TG* in the non-inflammatory colonic mucosa of MD-ICRP. We found that TG expression in samples with the risk T/T SNP was significantly higher than those with the non-risk C/C SNP (Figure 2A), suggesting that the TG SNP is an expression quantitative trait locus (eQTL) in the colon of MDs.

3.3 IL-6 amplifier activation is enhanced in the MD-ICRP with risk allele

We next investigated whether TG is induced by the IL-6 amplifier in colon. Because the IL-6 amplifier reflects the activation of NF-kB in nonimmune cells, we analyzed the expression of two NF-κB targets, *IL6* and *CCL2*, in the same colon samples. We found that *IL6* and *CCL2* were also enhanced in colon samples with the risk T/T SNP (Figure 2B, C). These results indicate that the risk T/T SNP of *TG* is an eQTL and enhances *TG* expression. They also indicate that TG is involved in activation of the IL-6 amplifier in the colon of MD-ICRP. Therefore, the risk T/T SNP of TG may be critical in the pathogenesis of ICRP through activation of the IL-6

amplifier. Interestingly, despite statistically insignificant, polypoid lesions showed similar pattern of expression in *IL6* and *CCL2* (Figure 3A, B) in MD-ICRP with risk T/T SNP.

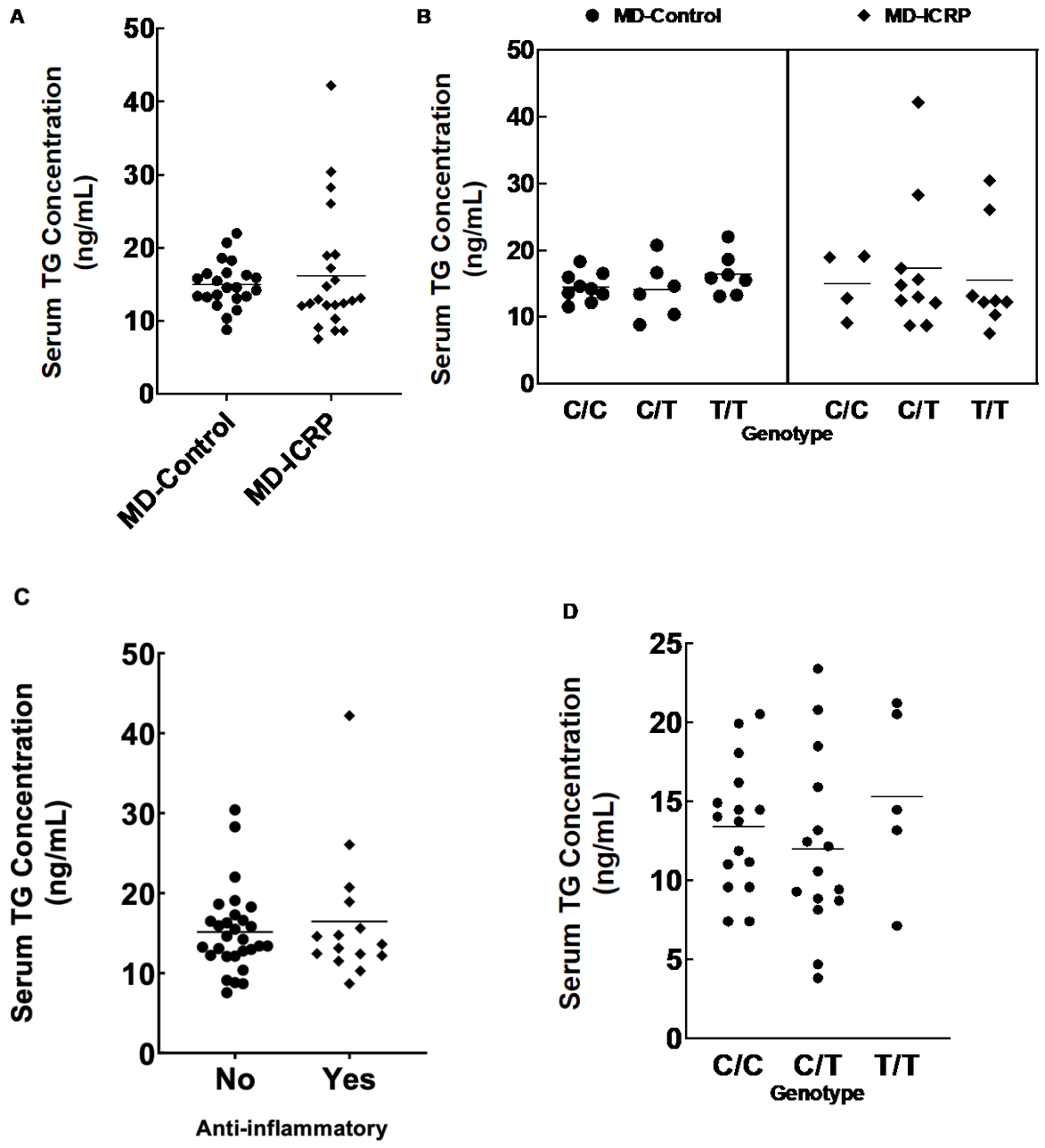

Figure 1. The TG SNP does not affect systemic TG levels

(A) Serum TG concentration between age-controlled MD-Control and MD-ICRP groups.

(B) Serum TG concentration between genotypes in MD-Control and MD-ICRP groups.

(C) Serum TG concentration between MDs treated with or without anti-inflammatory drugs.

(D) Serum TG concentration between genotypes in MDs less than 1 year old.

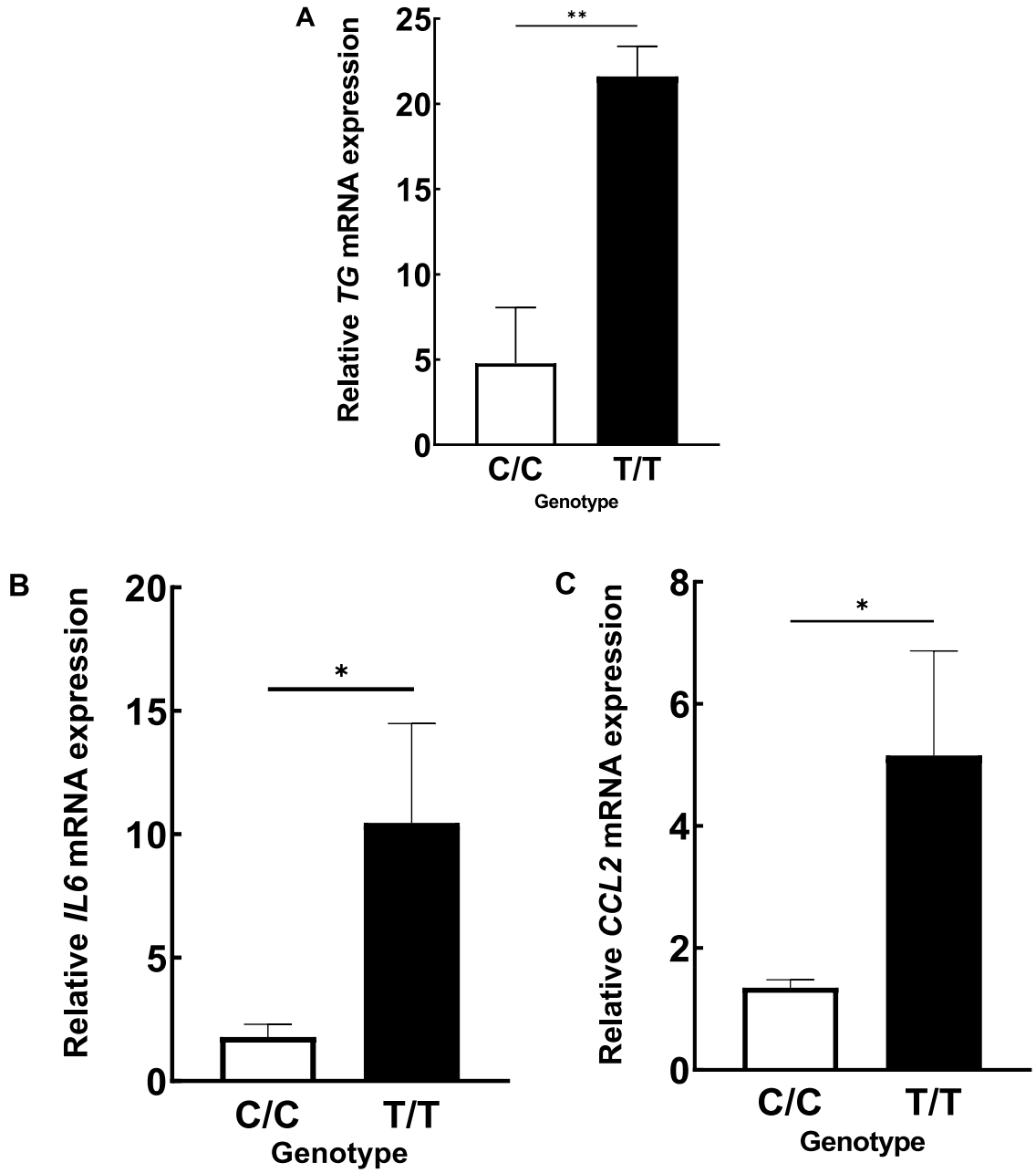

Figure 2. ICRP-MDs with risk alleles have a higher expression of TG and NF-κB-related chemokines in non-inflammatory colonic mucosa

(A) Relative TG, (B) IL6, and (C) CCL2 mRNA expressions in canine non-inflammatory colonic mucosa adjacent to the ICRP lesion site and diagnosed as normal histopathologically in MDs with wild-type (C/C) and risk (T/T) alleles. Means ± standard error of means is shown. * $p < 0.05$, ** $p < 0.005$.

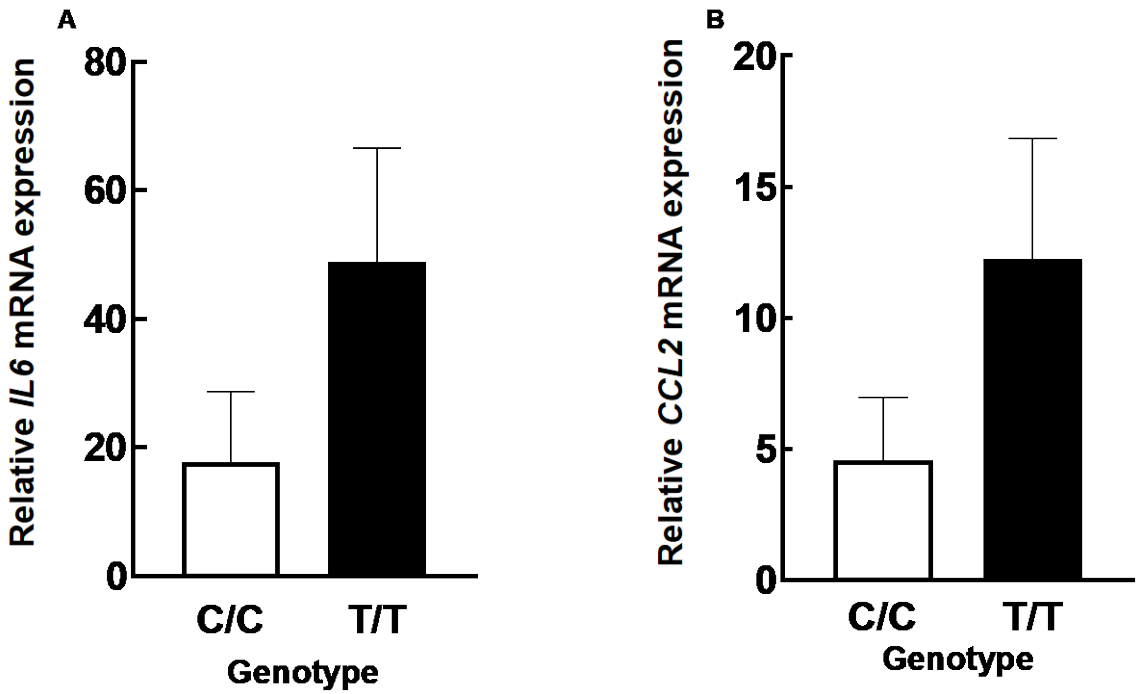

Figure 3: **ICRP-MDs with risk alleles may also have a higher expression of TG and NF-κB-related chemokines in non-inflammatory colonic mucosa**

(A) Relative *IL6* and (B) *CCL2* mRNA expressions in canine ICRP lesion in MDs with wild-type (C/C) and risk (T/T) alleles. Means ± standard error of means is shown.

4. DISCUSSION

Elevated serum *TG* level is reported to be related with autoimmune thyroid disease (AITD), where a study has shown increased *TG* levels can reduce the inhibitory function of regulatory T-cells (Treg) with a compensatory increment in Treg proportion in the thyroid due to suppressive effects of *TG* on the expression of *FOXP3*, *IL-10* and *TGF-β* in Tregs [36]. As ELISA results showed no significant difference in systemic *TG* levels between risk and wild type allele groups, effects of the *TG* c.4567C>T SNP is suggested to be confined locally in the colon of MDs. Interestingly, a past study have shown that *FOXP3* expression is lower in non-inflammatory colonic mucosa of MD-ICRPs as compared to normal control beagles [48], which suggests *TG* may have effects on suppressing expression of *FOXP3* in MD-ICRPs, leading to limited Treg function and enhanced inflammation.

Localized of *TG* expression was found to be elevated in MD-ICRP with the risk allele T/T, signifying that this SNP indeed functions as an eQTL to induce a higher local expression of TG in the colon, but not in the circulation. This localization of *TG* expression in the colon mucosa with T/T risk allele also had a significant increase in NF-kB targets such as *IL6* and *CCL2*, showing TG possesses a biological role in the activation of NF-kB, which is a critical component of the IL-6 amplifier. This result has answered the question on why ICRP only occurs in the colorectal region, but not the other regions of the gastrointestinal tract. However, our study did not compare the expression of *TG* in other regions of gastrointestinal tract of the ICRP cases, although more study of the expression of TG and other NF-kB targets in other organs, including other regions of the gastrointestinal tract, with or without the risk allele T/T of TG SNP is needed. To our surprise, even the inflammatory polypoid lesions showed similar expression patterns of *IL6* and *CCL2* expression despite insignificant statistically. This was because we assumed inflammatory lesions would have similar magnitude of cytokine and chemokine expression, disrupting the true phenotypical expression of TG-induced NF-kB activation, which was seen in the insignificance in *TG* expression of polypoid lesions (data not shown).

This study has few limitations which are inevitable. Sample numbers in local expression studies of the qPCR were low for each genotype group due to difficulties in retrieving samples,

as the samples were inherited from predecessors of the study and were retrieved from previously stored sample archives. Secondly, even though local *TG* expression was detected in the colonic mucosa, the reason behind the local expression was not able to be explained, as systemic levels of TG were not significantly different between risk allele group and wild type group. Perhaps circulating thyroglobulin antibody (TgAb) may have interfered with the TG measurement in the cases, as presence of TgAb has been reported to render TG levels into false elevation or lowering in the serum of human patients[14,78)], which may result in the absence of difference between allele groups.

5. SUMMARY

In the final chapter, we have confirmed the function of the *TG* SNP using clinical samples from MDs with or without MDs both systematically and locally. TG concentration measured with commercial ELISA kits have proven that there was no significant difference between MD-ICRPs and MD-Controls, across the genotypes of these groups and across the genotypes of MDs less than 1 year old. This prove that the SNP does not affect the production of TG protein systematically. However, when we measured the mRNA expression of *TG* in the normal colonic mucosa of MD-ICRP, those with risk allele T/T showed a higher expression locally, along with the IL-6 amplifier related genes such as *IL6* and *CCL2*, proving that the *TG* SNP functions to increase mRNA expression at a local scale. This also showed that IL-6 amplifier is indeed involved in the pathogenesis of ICRP, as ICRP is a localized chronic inflammatory disease.

GENERAL CONCLUSION

ICRP is a unique disease which occurs and has been reported only in the dog population in Japan. Despite sporadic reports of occurrence in non-MD breeds have been reported, the over-presentation of ICRP in MDs is undeniably high, which have strongly suggested a genetic factor leading to this significant breed-specificity. This study has successfully shown that there are genes which are important in the pathology of ICRP which are both specific in MDs and induces inflammation.

Beginning in chapter 1, the identified risk gene data from a previous study was reviewed once more to pick up more susceptible genes which are associated with the breed-specificity of ICRP in MDs. Compared to similar previous studies, we expanded the number of samples in non-MD, MD-ICRP and in MD-Control group to detect potential genes, and surprisingly two more MD specific SNP were managed to be picked up, *TG* and *FBN1*, with *TG* showing a significant association with ICRP pathology.

After the discovery of susceptible candidate genes which may contribute to the pathogenesis of ICRP, in chapter 2 the involvement of these genes with the IL-6 amplifier activation cascade was next assessed, as they have yet to be assessed in dogs but well established in mice disease models and human disorders related to chronic inflammation and auto-immunity. ICRP is known to be of inflammatory in nature due to the histopathological findings with infiltration of neutrophils, lymphocytes and macrophages and goblet cell hyperplasia, along with the prompt response to anti-inflammatory or immunosuppressive treatment in the cases. Using silencing RNA, knockdown of *TG* has proven to inhibit the expression of *IL-6* mRNA in non-immune cell line (H4 cell line), one of the key cytokines in the IL-6 amplifier cascade. Furthermore, treatment of non-immune cells with sequential increment of recombinant human TG has also induced a remarkable induction of *IL6* mRNA expression. These results in combination have concluded that TG, despite the traditional thought of a protein involved only in

thyroid hormonogenesis, too is a key regulator gene in IL-6 amplifier which affects chronic inflammation in non-immune cells.

Finally in chapter 3, functional analysis of the *TG* c.4567C>T SNP was performed systemically in the serum using ELISA kit and locally using qPCR quantification of *TG* in non-inflamed colonic mucosa of MDs affected with ICRP. As polypoid lesions are usually erratic due to the excessive inflammatory signaling from infiltration of immune cells, colonic mucosa samples which were diagnosed as normal from naked eye in endoscopy and histopathology were assessed in the local expression study. Although circulatory serum TG levels did not differ neither between case-control groups nor risk allele groups in both adult and juvenile population, by quantifying using Taqman probe quantitative PCR, *TG* mRNA expression in the non-inflamed colonic mucosa showed significant difference between T/T and C/C allele groups. Furthermore, we have also proven that the IL-6 amplifier is also activated in the MD-ICRP patients with T/T risk allele, as seen in increased expression of *IL6* and *CCL2*, targets of NF-κB. This has further strengthened the fact that *TG* expression is critical in inducing the activation of IL-6 amplifier which is essential in driving chronic inflammation in pathogenesis of ICRP.

To conclude, this study has proven to establish the genetic link between breed-specificity of MD with ICRP to the inflammatory pathogenesis of ICRP through the functional evaluation of the *TG* c.4567C>T SNP with respect to IL-6 amplifier. What is more exciting is that this study has managed to show that thyroglobulin, a non-traditional inflammatory molecule, is indeed related to chronic inflammation, which may also be exploited to explain the high concurrent thyroidal disease and IBD in human patients. This has also proven that studies in animal disorders have immense potential in translating the findings to studies in human disorders, with respect to the ideology of zoobiquity.

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JAPANESE SUMMARY (和文要旨)

The Association of Thyroglobulin Single Nucleotide Polymorphism with Miniature Dachshunds-Specific Inflammatory Colorectal Polyps and its Involvement in Interleukin-6 Amplifier Induced Chronic Inflammation

（ミニチュアダックスフンドに特異的な炎症性結直腸ポリープに関連するサイログロブリンの一塩基多型とインターロイキン 6 増幅回路に誘導される慢性炎症への関与）

犬の炎症性結直腸ポリープ（Inflammatory colorectal polyp, ICRP）は、本邦のミニチュアダックスフンド（Miniature Dachshund, MD）に好発する特徴的な炎症性腸疾患である。症例は主に血便、しぶりおよび粘液便などの臨床徴候を呈し、下部消化管内視鏡検査では下行結腸から直腸の粘膜において、出血を伴う孤立性または多発性の炎症性ポリープが観察される。病理組織検査では好中球、マクロファージ、リンパ球浸潤を伴う杯細胞の過形成を認め、この組織像および免疫抑制療法に良好な反応を示すことから、ICRP の病態には遺伝的な免疫異常が関与していることが示唆されている。近年、病態解明を目的とした ICRP の病変部における炎症性サイトカインの発現を調べた研究が多数報告されているものの、MD に特異的かつ炎症を誘導する遺伝子は明らかになっていない。そこで本研究は、MD に特異的かつ炎症を引き起こす遺伝子を明らかにし、その炎症機序を解明することを目的とした。

第 1 章では、先行研究で特定されたリスク遺伝子データを再検討した。MD に特異的な ICRP の疾患に関連するより多くの感受性遺伝子を検出するため、非 MD、MD-ICRP および MD-Control 群それぞれにサンプルを追加した。以前の研究と比較して、TG (exon22:c.C4567T:p.R1523W)と FBN1 (exon10:c.C1205T:p.P402L)の SNP が追加で検出され、TG に ICRP の発症機序との有意な関連性が示された。

第２章では、マウスの疾患モデルや慢性炎症、ならびに医学領域の自己免疫疾患で確立されている炎症経路であるインターロイキン 6 増幅回路(IL-6 アンプ)と、第１章で同定された ICRP の病因候補遺伝子の関与を評価した。サイレンシング RNA を用いて TG をノックダウンしたところ、非免疫細胞系である H4 細胞において IL-6 mRNA の発現が抑制されることが証明された。さらに、非免疫細胞に対して recombinant TG を処理したところ、濃度依存的な IL-6 mRNA の発現が顕著に誘導された。また、TG は IL-6 アンプを介する NF-κB の標的分子であったため、TG のポジティブフィードバックループが炎症を増悪する可能性が示唆された。これらの結果から、TG は、従来、甲状腺ホルモン生成にのみ関与するタンパク質と考えられていたものの、免疫細胞以外の慢性炎症に影響を与える IL-6 アンプの主要な調節遺伝子であることが示された。

最後に、第３章では ICRP に罹患した MD において全身的あるいは局所的な TG の発現を調べるため、末梢血の TG 濃度および非炎症性大腸粘膜における TG の発現を ELISA キットと qPCR で解析した。サンプルは ICRP 罹患犬とコントロール犬の血清および結腸組織とした。ポリープ状病変には、免疫細胞の浸潤による過剰な炎症が引き起こされていると予想されたため、内視鏡検査と病理組織学的検査により正常と診断された大腸粘膜サンプルを qPCR の発現解析の対象とした。末梢血の TG 濃度には、ICRP 罹患群およびコントロール群で有意な差は認められなかった。さらに成犬および子犬のアレル群間での比較においても明らかな差は認められなかった。一方で結腸組織においては、Taqman プローブ定量 PCR を用いた非炎症性大腸粘膜中の TG mRNA 発現量には、T/T および C/C アレル群間で有意な差が見られた。さらに、IL-6 アンプも ICRP 罹患群の T/T リスクアレルを有する場合に活性化されることが証明され、NF-κB の標的である IL-6 および CCL2 の発現が増加することが示された。以上のことから、TG 発現が IL-6 アンプの活性化を誘導する上で重要であり、ICRP の発症機序に関与していることが示唆された。

本研究では、TG c.4567C>T SNP の機能評価を通じて、MD の犬種特異性と ICRP の遺伝的関連性が ICRP の炎症性病態の確立につながることが証明された。さらに興味深

いことに、非典型的な炎症分子である TG が慢性炎症に関連していることが示された。これは医学領域で甲状腺疾患を有する患者に IBD が高頻度で発生することの根拠となる可能性がある。また、本研究を通じて、動物の疾患の研究は、汎動物学の考え方に沿って、人の疾患の研究に発展する可能性があることも示唆された。