Development of a Novel PCR Protocol to Study Epigenetic Regulation of IL-4, IL-5 and IL-13 in Cats

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Introduction

- Epigenetics is the study of heritable changes that alter gene expression without altering DNA sequence. It is a relatively new field of study, which focuses on the relationship between genetics and environmental influences on disease.
- Several studies have demonstrated a relationship between methylation status in the promoter region of several genes and the development of asthma in humans and mouse models.
- Areas rich in a cytosine nucleotide followed by a guanine nucleotide (CpG dinucleotides) can be found in gene promoter regions and are referred to as CpG islands.
- Asthma is a chronic respiratory disease of the lower airways that is mediated by a T helper type 2 (Th2) immune response to inhaled aeroallergens. Cats spontaneously develop allergic asthma and share similar pathological features as humans, including eosinophilic airway inflammation, airway hyperresponsiveness and airway remodeling.
- Currently no research has been done to explore if epigenetic regulation present in humans and mice translates to the cat.
- In humans and mice, several genes for key cytokines that play a role in the Th2 response have shown differences in CpG island methylation of the promoter region. (Figure 1)
- In both humans and in mouse models of asthma, epigenetic changes at the promoter region of the gene for Interleukin (IL)-13 have been detected in asthmatics and correlate with the severity of symptoms.
- Further, we will explore the epigenetic regulation of cytokines IL-4 and IL-5 based on their role in the Th2 immune mediated response.

Hypothesis

- Based on knowledge of the importance of epigenetic regulation of the promoter region of the genes coding for Th2 cytokines in human asthmatics and murine asthma models, we will investigate methylation patterns in the analogous promoter region of the feline IL-4, IL-5 and IL-13 genes.

Methods

- DNA sequence for feline IL-4, IL-5 and IL-13 promoter region was attained through the NCBI Nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore) from a whole genome shotgun sequence of Cinnamon, an Abyssinian cat.
- Maverix Genome Browser(https://wedge.maverixbio.com) was utilized to detect any single nucleotide polymorphisms (SNP), which have been identified from 14 cats that have been sequenced in that database. Those SNPs, which were identified, were excluded from primer design to decrease the likelihood of primer failure.
- CpG islands were located using Methprimer, a program that locates CpG islands in DNA sequence.
- Methprimer was used to design primers for two types of methylation PCR, Methylation-Specific PCR (MSP) and Combined Bisulfite-Restriction Analysis (COBRA).
- Bisulfite Conversion was performed by treating DNA with bisulfite. (Fig. 2)
- Bisulfite converted DNA is used in COBRA and MSP. (Fig. 3)
- A temperature gradient PCR was conducted to determine the optimal annealing temperature for each primer in order to ensure reproducibility of the PCR and optimize the PCR product. (Fig. 4)
- Splenic DNA was used in all initial PCR tests. DNA was extracted using an alcohol extraction method and was used for the development of the primers and PCR protocol for IL-4, IL-5 and IL-13.

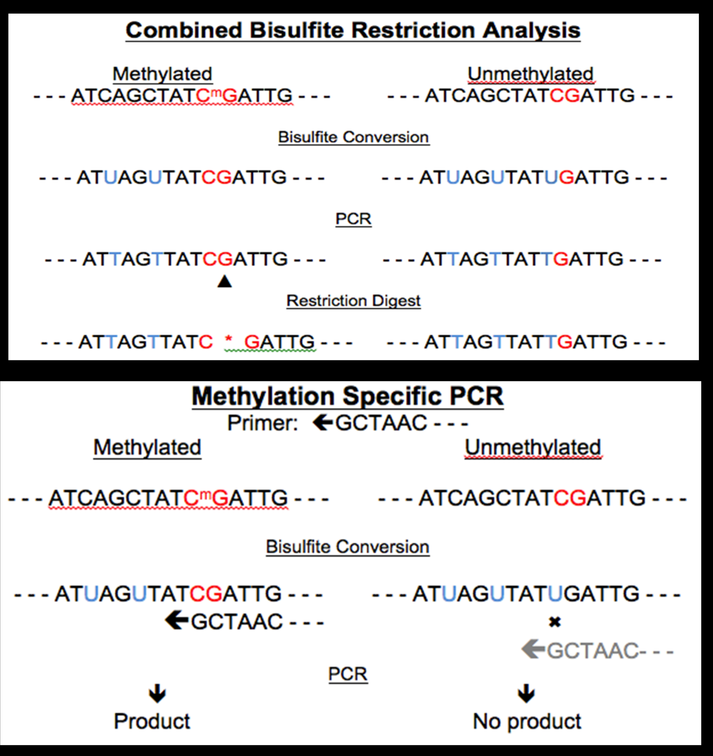

Figure 3. Both COBRA and MSP are run with bisulfite converted DNA. COBRA uses restriction enzymes to distinguish differences in methylation within the sequence of interest by only cleaving at sites of methylation. MSP primers used for PCR are designed to include CpG dinucleotides close to their 3' ends. Primers are designed to amplify either methylated CpG or unmethylated CpG after bisulfite conversion to TpG, but not both. If product is formed and a band is seen when using the methylated-specific primers that would indicate that the sequence was methylated; conversely, a band would not be expected with use of the unmethylated-specific primers and vice versa.

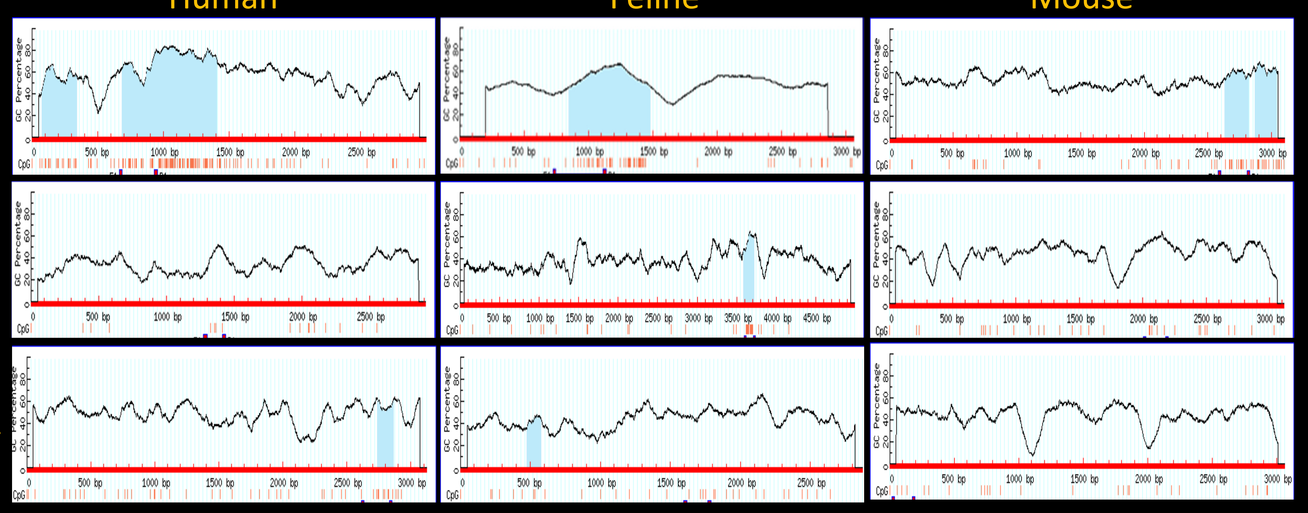

Figure 1. Several promoter regions for Th2 cytokine genes including IL-4, IL-5 and IL-13 were compared across human, mouse and cat to look for the presence of CpG islands using MethPrimer software. CpG islands were identified as areas that have a greater than expected ratio of cytosine and guanine as well as at least 50% of the sequence consisting of guanine-cytosine pairs. There were differences in the presence of CpG islands in all species suggesting that the cat will have specific and different patterns of methylation than the human and mouse.

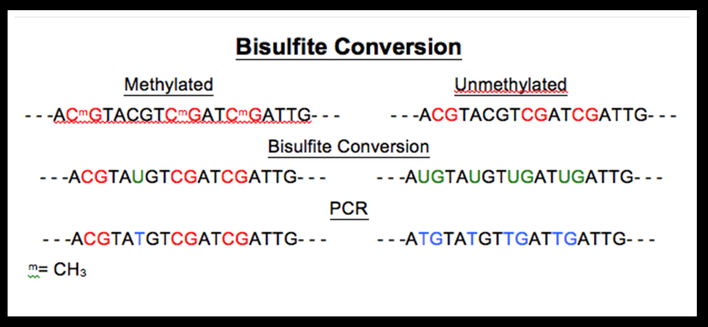

Figure 2. DNA is treated with bisulfite which will convert unmethylated cytosine to uracil. In subsequent PCR amplification, a thymine will be incorporated in place of the uracil in the amplified sequence. Methylated cytosine will remain unconverted.

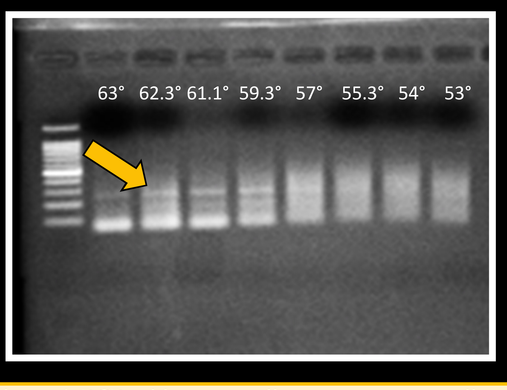

Figure 4. PCR with an annealing temperature gradient of 53-63 degrees Celsius was performed to determine the optimal annealing temperature for the primers used. For this primer pair the optimal annealing temperature was determined to be 61 degree Celsius. A gold arrow points to the expected product.

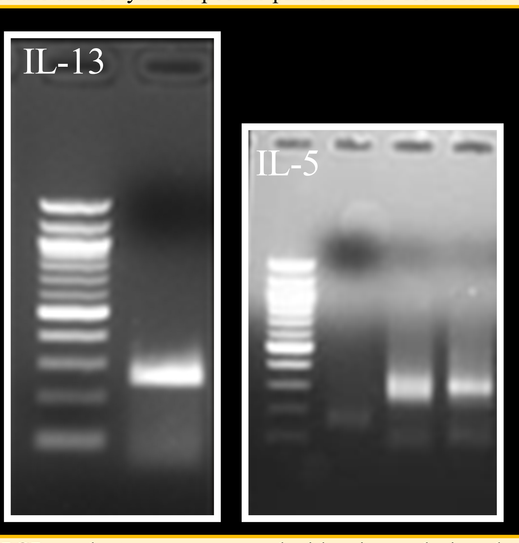

Figure 5. PCR products were generated with primers designed to amplify an area within a CpG island in the promoter region of feline IL-13 and IL-5. Two different primer sets were used to generate the products shown for IL-5.

Results

- Comparison of human, mouse and cat DNA showed differences in CpG island location and presence within the gene promoter regions.
- Based on the finding of CpG islands in IL-13 as well as IL-5, we designed primers to amplify sequences that spans the CpG island found in each promoter region. (Fig. 5)
- Further work is needed to develop primers to analyze IL-13 using MSP and to amplify the IL-4 promoter region.

Future Directions

- Epigenetics is a rapidly growing field of research and will have important clinical applications.
- Currently, the diagnosis of asthma in feline patients has presented considerable challenges. The identification of specific methylation profiles in asthmatic cats could lead to the development of a non-invasive diagnostic tool.
- Determining the role of epigenetic regulation in asthma will increase understanding of the pathogenesis and expand future research avenues for novel therapeutics.

Acknowledgements

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