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DESIGN OF PRIMER FOR THYROTROPIN RELEASING HORMONE OF *MUS MUSCULUS* C57BL/6J FOR QRT-PCR

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Abstract: High fat simple carbohydrate fed C57BL/6J mice, a model for metabolic syndrome have shown significant disruption of feedback control between TSH of the pituitary and T3 hormone of the thyroid gland. In order to understand the exact mechanism involved, it is important to assess the level of the hypothalamic TRH. qRT-PCR is a method of choice when tissue sample is less. In the present study, we have designed C57BL/6J mice specific primers for TRH.

Keywords: TRH, Primer, qRT-PCR

I. INTRODUCTION

The metabolic syndrome, which is a set of lipid and non-lipid risk factors of metabolic origin linked with insulin resistance, is believed to be associated with an elevated risk for cardiovascular diseases [1-3]. Moreover, obesity, especially central obesity, is linked to many endocrine abnormalities including thyroid dysfunction [3]. There are reports of sub-clinical and overt hypothyroidism in metabolic syndrome patients. Sub-clinical and overt hypothyroidism is significantly associated with 16.4% metabolic syndrome patients [4]. Females have an increased risk of this association [5-7]. Thyrotropin-releasing hormone (TRH), produced in the paraventricular nucleus of the hypothalamus, stimulates the biosynthesis and secretion of Thyroid Stimulating Hormones (TSH) from the anterior pituitary [8, 9]. Further, TRH is present in many brain loci outside of the hypothalamus, supporting a potential role as a neuromodulator or neurotransmitter outside of traditional Hypothalamus- Pituitary- Thyroid (HPT) axis function [10, 11]. TRH has been also found outside the central nervous system (CNS) in the gastrointestinal tract, pancreas, reproductive tissues including placenta, ovary, testis, seminal vesicles, and prostate, retina, and blood elements [12]. The widespread distribution of TRH within and outside the CNS supports a diverse range of roles for this molecule, roles likely to involve many functions outside of the traditional HPT axis.

Our lab has developed an animal model for Metabolic syndrome (MetS) by feeding High Fat simple carbohydrate (HFSC) diet to C57BL/6J mice for a period of five months [13]. This model has displayed a significant disruption of feedback control between TSH and Triiodothyronine (T3) (unpublished data). In order to understand the exact mechanism involved, it is important to assess the level of the hypothalamic TRH. Qualitative real time Polymerase chain reaction (qRT-PCR) is a method of choice when the tissue sample is less.

Selection of oligonucleotide primers is often critical for the overall successes of polymerase chain reaction (PCR). The manual selection of optimal PCR primer set is a tedious process and not efficient. Bioinformatics database like NCBI was used to select target gene sequence and tools like BLAST and Primer 3 Plus was used for designing primer pairs.

II. MATERIALS & METHODS

A. Extraction of gene sequence for TRH from NCBI-

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NCBI- National Centre for Biological Information was used to extract the gene sequence for TRH of *Mus musculus* C57BL/6J. Since FASTA format is the simplest that can be used to align the sequence in various bioinformatics tools, the sequence was extracted in the same.

B. Use of BLAST tool to align sequence obtained above-

Basic Local Alignment Search Tool (BLAST) is the tool most frequently used for calculating sequence similarity. The BLAST algorithm is a heuristic program, which means that it relies on some smart shortcuts to perform the search faster. BLAST performs "local" alignments. The sequence database is then scanned for these “hot spots”. When a match is identified, it is used to initiate gap-free and gapped extensions of the “word”. BLAST uses statistical theory to produce a bit score and expect value (E-value) for each alignment pair. The bit score gives an indication of how good the alignment is; the higher the score, the better the alignment [14, 15].
Sequence extracted from NCBI was pasted into the textbox of the Nucleotide BLAST Web page. The button BLAST was clicked. The output was posted back in graphic and hit table format.

C. Primer design by Primer 3 Plus software-

Primer3 Plus is a computer program that suggests PCR primers for a variety of applications including radiation hybrid mapping [16], single nucleotide polymorphism discovery [17], sequencing reactions and qRT-PCR. In selecting oligos for primers in Primer3 Plus following parameters were considered. These include oligo melting temperature, length, GC content, 3’ stability, estimated secondary structure, the likelihood of annealing to or amplifying undesirable sequences (for example interspersed repeats), the likelihood of primer–dimer formation between two copies of the same primer, and the accuracy of the source sequence.
The online free software version of Primer 3 Plus was used as the web interface from www.bioinformatics.nl/cgi-bin/primer3plus/primer 3plus.cgi.

III. RESULTS AND DISCUSSION:

mRNA sequence in FASTA format for *Mus musculus* TRH with reference ID: NM_009426.3 was extracted from NCBI. The summary of the query suggests that this gene encodes a member of the thyrotropin-releasing hormone family which is a tripeptide hypothalamic regulatory hormone. TRH is involved in the regulation and release of TSH as well as prolactin. Disruption of this gene results in hypothyroidism, elevated TSH hormone levels, and hyperglycemia [18]. Studies from our lab on Metabolic Syndrome model revealed elevated TSH, prolactin as well as hyperglycemia (unpublished data) which is in agreement with the above.
We hypothesize that in Metabolic Syndrome TRH might be affected which could be confirmed only through qRT-PCR.

The above sequence (Fig.1) was used to carry out further alignment and primer designing.

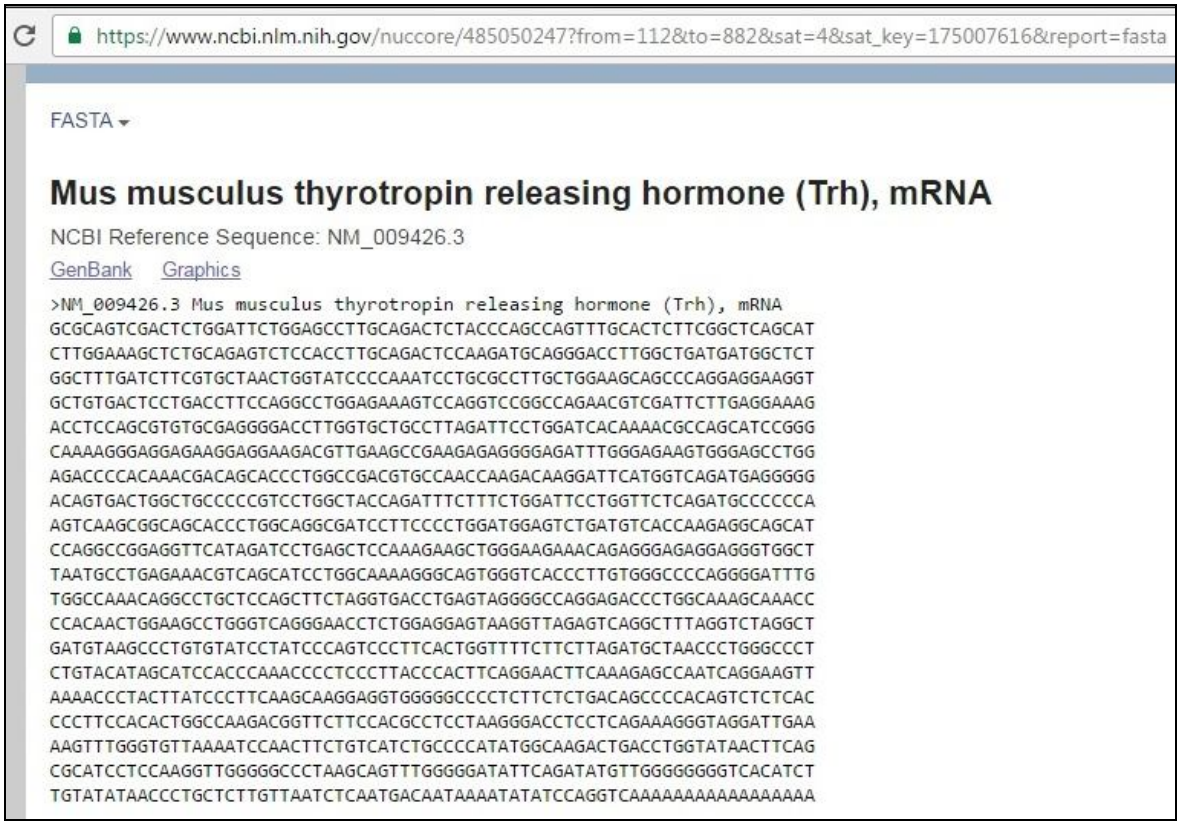

Figure 1. NCBI sequence for TRH specific for *Mus musculus*

BLAST alignment output gave nearly 33 similar sequences. But the query sequence was 100% similar to *Mus musculus* thyrotropin releasing hormone (Trh), mRNA (Fig.2) which is further confirmed by the E value that is zero (Fig.3).

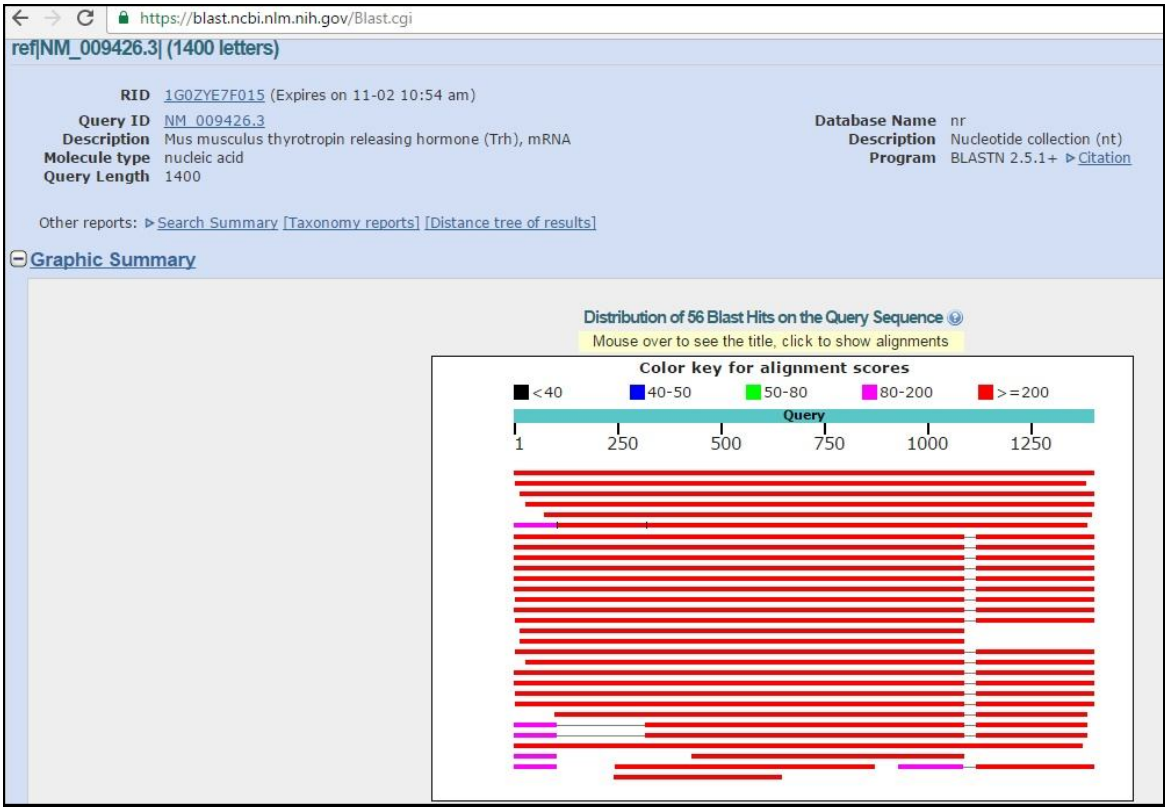

Figure 2. Graphic representation of the alignment.

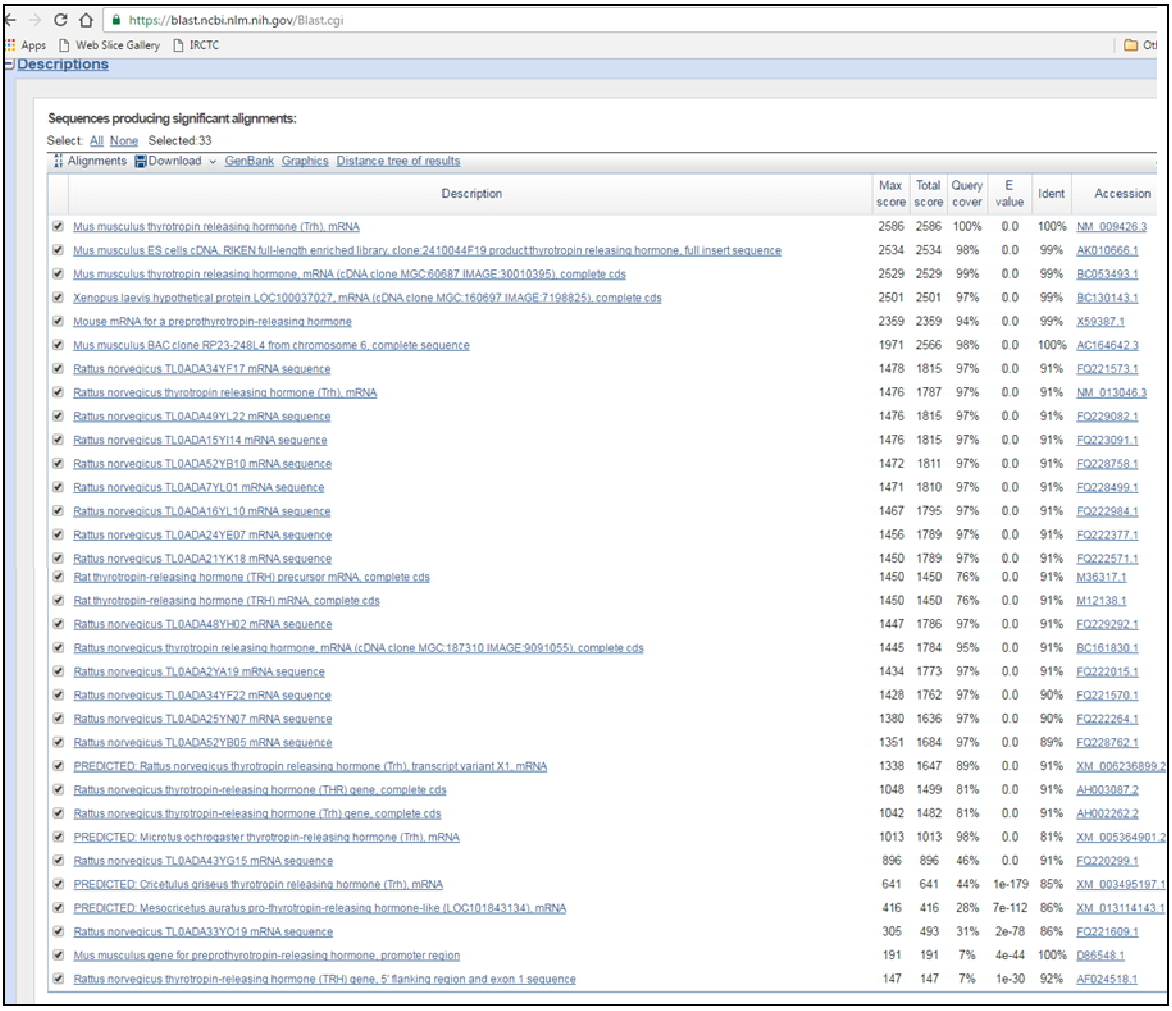

Figure 3. Hit table with score and E-value

Since the target sequence showed 100% sequence similarity with *Mus musculus* Trh mRNA, primer pairs for it were designed using Primer 3 Plus online software. The software considered 10728 of left primers, but GC content failed for 2, low tm for 1756, high tm for 6476, high-end complexity for 2, long poly-x sequence for 29 and high 3' stability for 260. It found 2203 primers were ok for primer designing. For right primer, it considered 10481 primers, GC content failed for 56, low tm for 2022, high tm for 6051, long poly-x sequence for 57and high 3' stability for 264. It found 2031 primers were ok for primer designing. In spite of the above-mentioned left and right primers, the software considered only 163 as primer pairs of which 148 were found unacceptable due to product size. Though only 15 were found ok, 5 were shortlisted for primer designing. The best primer among the given pairs of primers that satisfied all the necessary parameters was selected. The selected primer pair (Fig.4) had optimum GC% of 55 for forward and reverse primers. The optimum Tm was 60°C and the product length was 156bp.

Primer	Sequence	Length	Tm	GC%
Forward primer	GATTCTGGAGCCTTGCAGAC	20	60	55
Reverse primer	GGGGATACCAGTTAGCACGA	20	60	55

Figure 4. Selected primer pairs for TRH (*Mus musculus* C57BL/6J)

IV. CONCLUSION

The best primer pair specific for *Mus musculus* TRH was designed. This will be further validated by wet-lab experiments using mRNA isolated from hypothalamus of *Mus musculus* C57BL/6J which was fed with HFSC diet for a period of five months. The results of which will help to elucidate the HPT axis dysfunction in Metabolic Syndrome.

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