A *PYY* Q62P variant linked to human obesity

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ABSTRACT

Peptide YY (PYY) has been implicated in the control of food intake through functional studies in rodents and humans. To investigate whether genetic alterations within this gene result in abnormal weight in humans, we sequenced its coding exons and splice sites in a large cohort of extremely obese [n=379; average body mass index (BMI) 49.0 kg/m^2] and lean (n=378; average BMI 19.5 kg/m^2) individuals. In total, three rare non-synonymous variants were identified, only one of which, *PYY* Q62P, exhibited familial segregation with body mass. Through serendipity, previous cell culture based studies revealed this precise variant to have altered receptor binding selectivity *in vitro*. We further show using mouse peptide injection experiments that while the wild-type PYY peptide reduces food intake, the mutant PYY 62P had an insignificant effect in reducing food intake *in vivo*. Taken together, these results are the first to support that rare sequence variants within *PYY* can influence human susceptibility to obesity.

INTRODUCTION

The most convincing evidence for a genetic component for obesity comes from twin and adoption studies supporting that the genetic transmission of obesity is at least as important as the non-genetic factors (1). Using genetic approaches, a number of candidate genes for obesity have been identified and the importance of several of these genes was ascertained through genetically engineered mice (2). Overall, genes that may contribute to obesity susceptibility can be considered in three broad areas. These include genes that i) regulate food intake (3), ii) participate in adipogenesis (4), and iii) influence energy expenditure including mitochondrial proton leak and adaptive thermogenesis (5).

In the category of food intake regulation, functional studies both in humans and rodents indicate a potentially important role for peptide YY (PYY) in decreasing food intake (6-8). Following food intake and in proportion to meal size, PYY is secreted into the blood stream from L-cells in the gastrointestinal tract in two forms, PYY_{1-36} and PYY_{3-36} (9), and binds to neuropeptide receptors within the brain to reduce food intake. PYY_{3-36} was previously shown to bind *in vitro* with highest affinity to the neuropeptide Y receptor Y2 (NPY2R) (10), an observation that was further established *in vivo* using *Npy2r*-deficient mice, in which peripheral administration of PYY_{3-36} failed to reduce food intake (7). In rodents, intravenous administration of PYY_{3-36} led to a reduction in food intake (6, 7, 11) and in both obese and lean human subjects, PYY_{3-36} infusion markedly decreased food intake (8). Combined, these studies support an important physiological role for PYY in the regulation of feeding behavior.

In humans, little is known of the effects of genetic variation in *PYY* on susceptibility to obesity. In one limited study, DNA sequence analysis of *PYY* and its receptor, *NPY2R*, in 83 extremely obese Pima Indians suggested that certain common variants may be associated with severe obesity in males (12). However, in a separate study performed in 101 individuals with severe early onset obesity, no association was found between common or rare nucleotide variants in *PYY* and obesity (13). To directly address the effects of variations in the coding sequences of *PYY* on human body weight we sequenced a large cohort of subjects at the two extremes of body mass index.

RESULTS

We sequenced the human *PYY* (NCBI accession no. NM_004160) coding exons and their splice sites in 379 obese and 378 lean individuals with a mean BMI of 49.0 and 19.5 kg/m^2 respectively. Sequence analysis revealed no significant frequency differences between two of the three common variant (>1% allele frequency) genotypes in the obese versus lean population (Table 1). For R72T we observed a marginally significant (p-value=0.02;chi-square test) genotype frequency difference between our two cohorts, though this variant has been previously reported to lack association with BMI when tested in 952 Caucasians (13), which are of similar ethnicity as our population. In addition, three rare (<1% allele frequency) non-synonymous variants unique to either the obese or lean population were discovered (Table 1). Two variants, P9H and Q62P, were identified in two isolated obese individuals while a nonsense variant, Y49*, was found in a single lean individual.

Based on the severe nature of the Y49* nonsense substitution, we assessed this variant for segregation in the proband's available extended pedigree. However, we found no relationship between this variant and BMI (Supplemental Figure 1). Nevertheless, we further examined the subjects bearing the PYY stop mutation to determine if such a heterozygous nonsense change effects circulating PYY_{3-36} plasma levels. Fasting and postprandial plasma PYY concentrations were measured by immunoassay (Supplementary methods) for carriers (n=5) and non-carriers (n=5) closely matched for age, sex and BMI. These studies demonstrated significantly lower mean PYY_{3-36}

concentrations for carriers versus controls in the fasting state and a strong trend at the 90 minute postprandial time-point (Table 2). Detailed analysis of cognitive dietary restraint, disinhibition and hunger (Three Factor Eating Questionnaire) as well as subjective phenomena related to appetite pre and post meal (Visual Analogue Scores) did not reveal significant differences between carriers and non-carriers of this variant (Supplementary Tables 1-4 and Supplementary Methods) (14). These data suggest that subtle differences in plasma PYY_{3-36} concentrations do not have a major effect on appetite or eating behavior and that haploinsufficiency of PYY is not associated with a BMI phenotype.

Familial segregation analysis of the obese variants showed no significant correlation between variant P9H and BMI in the small kindred that was available for genotyping (Supplementary Figure 1B). On the other hand, *PYY* Q62P was found in four individuals in an extended family (Figure 1A), and these had an average BMI adjusted for age and sex at the 87^{th} percentile (including a 4 year old child with congenital heart disease, which may restrict an obesity phenotype) compared to an average BMI at the 52^{nd} percentile for 5 non-carriers ($p=0.0158$, 2-tailed t-test with Welch correction). In terms of an obesity phenotype, the Q62P proband was hypertensive but normolipidemic as well as euglycemic and, in comparison to other age, sex and BMI matched subjects, lost weight quickly on a 900 kcal liquid formula diet, implying that his obese state was secondary to hyperphagia rather than low energy expenditure. These data were suggestive of a possible role for *PYY* Q62P in influencing obesity susceptibility and encouraged further functional studies on its protein product.

Serendipitously, the exact Q62P amino acid change had been previously studied *in vitro* (15). PYY amino acid position Q62, which represents Q34 in the secreted form of PYY, is extremely evolutionarily conserved both in PYY and NPY paralogs, while in PPY this residue is substituted by a proline (Figure 1B). This observation of a paralogous protein amino acid change led several groups to conduct experiments on the effect of substituting PYY_{1-36} Q34 to a proline (10). Using nuclear magnetic resonance (NMR) and circular dichroism spectroscopy (CD spectra) studies as well as numerous cell culture assays, a profound functional consequence of this change, resulting in an altered binding affinity and specificity to several neuropeptide Y receptors, was documented (10). Combined, our limited family segregation data and these previously established *in vitro* properties of PYY Q62P led us to test the effects of this variant on food intake *in vivo*.

Based on previous publications implicating PYY_{3-36} as the major PYY peptide to reduces food intake in rodents (6), we generated a mutant PYY_{3-36} P34 peptide and sought to determine its effects on food intake in mice. Following a previously described protocol (11), we injected wild-type (Q34) and mutant (P34) PYY peptide into mice after a 24-hour fast. We found that 6 hours post-injection the wild-type peptide significantly decreased food intake in comparison to saline control as previously described (11). In contrast, the mutant peptide had no significant effect on food consumption compared to saline control ($p=0.26$, one-way ANOVA) (Figure 2). In addition, we co-injected both the wild-type and mutant peptide and observed a consequence similar to mutant P34 alone (Figure 2). This abolishment of wild-type peptide feeding inhibition in the co-injection experiments suggests P34 has a gain of function mechanism of action thereby

overcoming the wild-type peptide effect. Together, these results support a functional role for the PYY Q62P variant on the regulation of food intake.

DISCUSSION

PYY has been functionally implicated in the control of food intake primarily through rodent-based studies. Here we explored the potential genetic contribution of this gene to human body weight through DNA resequencing in two extreme populations followed by familial and functional investigation. Based on our relatively large sample size, and previous reports (12, 13), it appears that rare non-synonymous changes in this gene in the obese population are infrequent and their contribution to widespread weight differences in humans is likely to be minor. In addition, while our analysis was not designed as an exhaustive genetic association study between common variants in the *PYY* genomic region and BMI, we found 3 common variants, one of which showed marginal frequency differences between the obese and lean groups (Table 1). Whether other common noncoding variants in the extended genomic region may more strongly impact on human BMI remains to be further explored. Nevertheless, the finding of *PYY* Q62P variant and the demonstration of its functional importance suggest that a small subset of variants in *PYY* might be detrimental and potentially important in the etiology of weight differences.

Mechanistically, we can speculate on how the 62P variant might lead to a change in PYY function. Previous i*n vitro* studies carried out using a mutant PYY_{1-36} P34 peptide demonstrated that this residue is of importance in terms of the binding specificity to other neuropeptide Y receptors, specifically NPY1R (16, 17), NPY4R (18), and NPY5R (19). Since NPY2R ligand binding is predicted to result in appetite suppression, and NPY1R and NPY5R [also possibly NPY4R (20)] in appetite induction [reviewed in ref. (21)], it is

possible that the P62 variant shifts PYY function *in vivo*. This could be either through P62 activation of NPY1R and NPY5R (and/or NPY4R) and/or its competitive blockage of their natural peptide ligands, thus leading to a gain of function effect. We should point out that since our functional analysis was done in mice and not in humans, and previous receptor binding assays were carried out *in vitro*, our functional data can only be suggestive regarding the causative nature of this variant in human obesity.

Finally, the finding of a nonsense variant, Y49*, in a lean individual and several of his lean to normal weight family members indicates that PYY haplo-insufficiency in humans does not promote obesity. In addition, the observation of decreased levels of fasting and postprandial plasma PYY_{3-36} levels in Y49* carriers versus controls support the notion that subtle changes in PYY are unlikely to profoundly influence human weight. Taken together, these data suggests that rare genetic changes in *PYY* may influence isolated cases of human obesity most likely through a gain of function mutation mechanism. Further mutation screening in *PYY* in large obese cohorts will provide a better estimate of the prevalence of such nucleotide changes and their effects on human body weight.

MATERIALS AND METHODS

Subjects

Obese Caucasian subjects were recruited from the patient population of the University of Ottawa Weight Management Clinic and the Heart Institute Lipid Clinic. Criteria for inclusion included a BMI > 36 kg/m^2 and a history of obesity for at least 10 years of adult life. Exclusion criteria included treatment with oral glucocorticoids, anti-psychotics, lithium or medical conditions including major depression, bipolar affective disorder or psychosis. Lean subjects of the same ethnic background were recruited from the Ottawa community. BMI for obese and lean subjects was categorized according to the population percentiles for age and sex using the Canadian Heart Health Survey data for subjects over the age of 18 years (data on file; Health Canada) and NHANES data for children (22). Inclusion criteria for the lean subjects include a BMI $\leq$ the 10^{th} percentile for age and sex, with no prior history of having had a BMI > 25^{th} percentile for age and sex for more than a two year consecutive period. Exclusion criteria include medical conditions affecting weight gain including hyperthryroidism, anorexia nervosa, bulimia, major depression or malabsorption syndromes. This study was approved by the institutional review boards of the University of Ottawa Heart Institute and the Ottawa Hospital and informed written consent was obtained from all participants. Genomic DNA was extracted from white blood cells by standard methods (23) or from saliva samples collected in Oragene Kits (DNA Genotek).

Sequencing and data analysis

Primers were designed to give a maximum product size of 500bp and a minimum of 40bp flanking the splice sites using the Exon Locator & eXtractor for Resequencing program (EXLR) (http://mutation.swmed.edu/ex-lax/). An M13F tag (gttttcccagtcacgacgttgta) and M13R tag (aggaaacagctatgaccat) was added to forward and reverse primers respectively. 10ng of DNA from each sample was amplified in a 10ul PCR reaction using AmpliTaq Gold® (Applied Biosystems) and cleaned using Tetra-Ethylene Glycol (TET)(http://www.jgi.doe.gov/sequencing/protocols/ BETcleanup.doc). Sequencing reactions were performed using the M13 primers along with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) (http://www.jgi.doe.gov/sequencing/ protocols/BigDye3.1auto1.0.doc) and cleaned again with TET before separation on a 3730xl DNA Analyzer (ABI). Base calling, quality assessment and assembly were carried out using the Phred, Phrap, Polyphred, Consed software suite (www.phrap.org). All sequence variants identified were verified by manual inspection of the chromatograms and by a second independent sequencing reaction.

Mouse peptide experiments

Two-month old 129/Sv male mice were individually housed in cages for two weeks prior to experimentation. Mice were housed in a temperature-controlled room under a 12-hour light and dark cycle, given free access to water and fed *ad libitum* on a standard chow. After the 2-week period, the mice were fasted for 24 hours and then injected intraperitoneally with 10µg per 100gr of body weight PYY_{3-36}, PYY_{3-36} P34 (both from Bachem), or saline at the onset of the dark cycle (18:00). When both PYY_{3-36} and PYY_{3-}

$_{36}$ P34 were injected, equal amounts were adjusted so combined they would give 10µg per 100gr of body weight. Food intake was measured at 6, 24, 48 hours following injection by measuring the pre-weighed portions of food dispensed from wire cage tops. Cages were carefully monitored for evidence of food spillage or grinding, which were negligible. Experiments were performed in duplicate with an average of 8 mice per study.

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CONFLICT OF INTEREST STATEMENT

None declared.

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FIGURE LEGENDS

Figure 1: PYY Q62P. A) Familial segregation of *PYY* Q62P (the arrow indicates the individual sequenced in the cohort). Black shaded individuals in the pedigree are carriers of the 62P variant, while the lone gray shaded individual is inferred to be an obligate carrier. B) ClustalW (24) and boxshade protein alignment analysis of the secreted PYY paralogs, human NPY and PPY. Amino acids PYY Q62/Q34 and PPY P63/P36 are marked with black arrows.

Figure 2: A bar chart showing the mean food intake in mice 6 hours post peptide injection. Error bars depict the standard error. 'N' below the chart indicates the number of mice, followed by the mean food intake after 6 hours and the standard error in each experiment. **, $P < 0.01$; *, $P < 0.05$ versus wild-type PYY_{3-36}, one-way ANOVA.

Table 1. *PYY* variants identified through resequencing.

Variant	Location*	Gen.	Obese	Lean	Sequence
P9H	567	CC AC AA	367 1 0	364 0 0	aggccgtggc(c/a)cgccttgacc
Y49*	688	CC CG GG	374 0 0	372 1 0	accgctacta(c/g)gcctccctgc
A50A	691	CC CT TT	373 1 0	372 1 0	gctactacgc(c/t)tccctgcgcc
Q62P	726	AA AC CC	374 1 0	373 0 0	gtcacccggc(a/c)gcggtatggg
R72T	757	GG GC CC	174 153 47	166 173 34	ggcccggaca(g/c)gcttctttcc
IVS3+68	NA	CC CT TT	293 51 6	299 67 5	ggcaacatca(c/t)ttaacgacgt
3'UTR	877	CC CT TT	354 0 0	370 1 0	accacgccca(c/t)gtcatttgca
3'UTR	984	CC CA AA	328 26 0	335 33 3	tcggtgcccc(c/a)gccccctggg

*Genotype and variant numbering are based on NCBI accession no. NM_004160.

Table2. Fasting and postprandial PYY3-36 concentrations in PYY Y49* carriers versus matched controls.

	Baseline (after 12 h fast) pg/ml	**Postprandial (45 min) pg/ml**	**Postprandial (90 min) pg/ml**
PYY Y49* carriers n=5	56.5 ± 2.7	91.9 ± 5.7	89.9 ± 7.5
Matched Controls n=5	72.6 ± 5.1	105.0 ± 7.4	116.5 ± 8.1
P value	0.005	0.065	0.054

Data are provided as mean ± SEM; Student's one-tail t-test for paired samples.

Figure 1

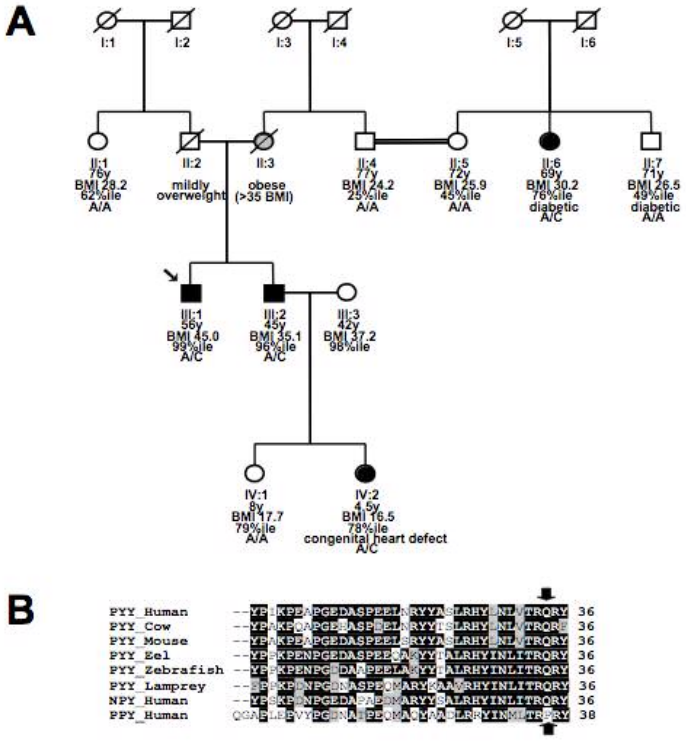
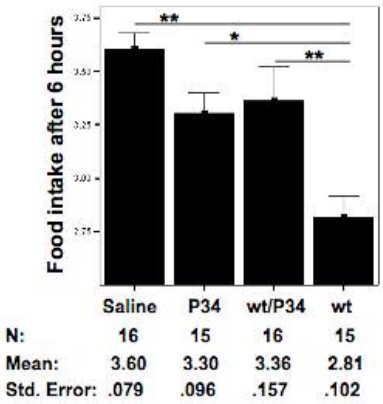

A
I:1
I:2
I:3
I:4
I:5
I:6
II:1
76y
BMI 28.2
62%ile
A/A
II:2
mildly
overweight
II:3
obese
(>35 BMI)
II:4
77y
BMI 24.2
25%ile
A/A
II:5
72y
BMI 25.9
45%ile
A/A
II:6
69y
BMI 30.2
76%ile
diabetic
A/C
II:7
71y
BMI 26.5
49%ile
diabetic
A/A
III:1
56y
BMI 45.0
99%ile
A/C
III:2
45y
BMI 35.1
96%ile
A/C
III:3
42y
BMI 37.2
98%ile
IV:1
8y
BMI 17.7
79%ile
A/A
IV:2
4.5y
BMI 16.5
78%ile
congenital heart defect
A/C
B
PYY_Human
PYY_Cow
PYY_Mouse
PYY_Eel
PYY_Zebrafish
PYY_Lamprey
NPY_Human
PPY_Human

Figure 2


Food intake after 6 hours
**
*
**
Saline
P34
wt/P34
wt
N: 16 15 16 15
Mean: 3.60 3.30 3.36 2.81
Std. Error: .079 .096 .157 .102

ABBREVIATIONS

PYY= Peptide YY

BMI= body mass index

NPY2R= Neuropeptide Y receptor Y2

kcal= Kilocalorie

NMR= Nuclear magnetic resonance

CD spectra= Circular dichroism spectroscopy

ANOVA= Analysis of variance

NPY1R= Neuropeptide Y receptor Y1

NPY4R= Neuropeptide Y receptor Y4

DNA= Deoxyribonucleic acid

PCR= Polymerase chain reaction

pg/ml= picograms per milliliter

SEM= Standard error of the mean