Neuropeptide Y Gene Polymorphisms Confer Risk of Early-Onset Atherosclerosis

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Abstract

Neuropeptide Y (NPY) is a strong candidate gene for coronary artery disease (CAD). We have previously identified genetic linkage to familial CAD in the genomic region of NPY. We performed follow-up genetic, biostatistical, and functional analysis of NPY in early-onset CAD. In familial CAD (GENECARD, N = 420 families), we found increased microsatellite linkage to chromosome 7p14 (OSA LOD = 4.2, p = 0.004) in 97 earliest age-of-onset families. Tagged NPY SNPs demonstrated linkage to CAD of a 6-SNP block (LOD = 1.58–2.72), family-based association of this block with CAD (p = 0.02), and stronger linkage to CAD in the earliest age-of-onset families. Association of this 6-SNP block with CAD was validated in: (a) 556 non-familial early-onset CAD cases and 256 controls (OR 1.46–1.65, p = 0.01–0.05), showing stronger association in youngest cases (OR 1.84–2.20, p = 0.0004–0.09); and (b) GENECARD probands versus non-familial controls (OR 1.79–2.06, p = 0.003–0.02). A promoter SNP (rs16147) within this 6-SNP block was associated with higher plasma NPY levels (p = 0.04). To assess a causal role of NPY in atherosclerosis, we applied the NPY1-receptor-antagonist BBP-3226 adventitiously to endothelium-denuded carotid arteries of apolipoprotein E-deficient mice; treatment reduced atherosclerotic neointimal area by 50% (p = 0.03). Thus, NPY variants associate with atherosclerosis in two independent datasets (with strong age-of-onset effects) and show allele-specific expression with NPY levels, while NPY receptor antagonism reduces atherosclerosis in mice. We conclude that NPY contributes to atherosclerosis pathogenesis.

Introduction

The prevalence of early-onset cardiovascular disease in Americans (under 40 years of age) is approximately 10–15% [1] and the hereditary nature of coronary artery disease (CAD) is well-established [2]. The relative risk of developing CAD in a first degree relative is 3.8 to 12.1, with higher risk correlating with earlier age-of-onset [3]. Recent successes suggest that CAD genes can be identified through comprehensive genetic and functional studies [4–6]. However, the genetic architecture of CAD remains complex and poorly understood.

To identify genetic risk factors in early-onset CAD, we implemented a strategy combining the strengths of family-based studies with validation by case-control association, in conjunction with careful consideration of phenotype and functional data. In our own GENECARD linkage study of early-onset CAD, we have found five genomic regions of linkage with multipoint linkage odds (LOD) scores >1.0 [7]. The neuropeptide Y gene (NPY) is located adjacent to the peak microsatellite marker in the 7p14 peak. Because of its proximity to the peak marker, and because NPY has been implicated in disorders of vascular smooth muscle cell proliferation [8,9], we sought to examine NPY further as a candidate gene for early-onset CAD.

NPY is the most abundant peptide in the heart and brain, and is produced by sympathetic nerves, endothelial cells [10], and platelets [11]. NPY has diverse functions including roles in sympathetic nerve stimulation through co-release with norepinephrine; immune function [12]; regulation of food consumption [9]; and modulation of heart rate, vasocostriction, coronary blood flow and ventricular function [13]. These cardiovascular functions are primarily mediated through the NPY1 receptor [12,14,15]. In injured rat carotid arteries, non-atherosclerotic neointimal hyperplasia is aggravated by local delivery of NPY, and ameliorated by treatment with NPY1 receptor antagonist [8,9]. In
humans, NPY levels predict cardiovascular complications in end-stage renal disease [16], and NPY is implicated in congestive heart failure [17]. An NPY variant rare in most populations has been associated in Scandinavian populations with hyperlipidemia and carotid atherosclerosis [18,19], CAD in type 1 diabetics [20], and MI in hypertensive patients [21]; however, the effects of this variant on NPY expression remain obscure.

To date there have been no systematic studies of the role of the NPY gene, or of the functional consequences of genetic variation at this locus, in cardiovascular disease pathogenesis. In response to the results of the genome-wide linkage analyses, the phenotypic correlations, and the strong but limited prior published work, we proposed to test the hypothesis that NPY variants affect atherosclerosis through effects on NPY plasma levels. We pursued a comprehensive gene-wide approach to correlating NPY variants with CAD and plasma NPY levels in humans, and tested the effects of NPY1 receptor blockade on atherosclerosis in mice.

**Results**

Table 1 presents baseline characteristics of the three datasets: GENECARD (N = 946 affected, 37 unaffected individuals); CATHGEN (N = 556 cases, 256 controls); and GENECARD probands versus CATHGEN controls (N = 221 cases, 256 controls). Despite GENECARD families being selected on early age-of-onset, genetic heterogeneity manifest as differences in age-of-onset could still be present, as observed in the discovery of the BRCA1 breast cancer gene [22]. Hence, we performed ordered subset analysis (OSA) to understand the effect of age-of-onset on linkage to CAD. We found increased linkage on the chromosome 7p14 peak in a subset of 97 families with the youngest age-of-onset (overall LOD = 1.04; subset LOD = 4.22; p = 0.004 for increase, Figure 1). The mean age-of-onset in these families was 37.8 years, and they had significantly higher mean total- and LDL-cholesterol and were more often male, compared with affected members of the remaining 323 families. No other genomic regions showed a correlation between linkage and age-of-onset. The NPY gene resides within this linkage peak and is a strong biological candidate. As a result we aimed to evaluate NPY polymorphisms, NPY levels, and age-of-onset of CAD, along with evaluating the role of NPY in a mouse model system.

**Table 1. Baseline characteristics.**

| Variable       | GENECARD Affected (N = 946) | GENECARD Probands (N = 221) | CATHGEN Cases (N = 556) | CATHGEN Very-Young Cases (N = 74) | CATHGEN Controls (N = 256) |
|----------------|----------------------------|-----------------------------|-------------------------|-----------------------------------|-----------------------------|
| Age            | 51.5 (7.1)                 | 50.8 (6.6)                  | 51.1 (7.9)              | 43.4 (8.1)                       | 69.3 (6.5)                  |
| Age-of-onset   | 43.7 (5.8)                 | 43.8 (5.7)                  | 46.2 (6.4)              | 34.7 (3.3)                       | N/A                         |
| Sex (% male)   | 68.0%                      | 68.0%                       | 79.7%                   | 83.8%                            | 38.7%                       |
| Race           |                            |                             |                         |                                  |                             |
| % Caucasian    | 91.9%                      | 83.1%                       | 71.9%                   | 68.9%                            | 77.4%                       |
| % African-American | 2.3%                     | 7.4%                        | 20.5%                   | 21.9%                            | 16.7%                       |
| Dyslipidemia   | 82.4%                      | 83.7%                       | 72.5%                   | 81.2%                            | 41.8%                       |
| Lipids         |                            |                             |                         |                                  |                             |
| TC             | 205.6 (57.4)               | 216.3 (59.0)                | 194.2 (55.8)            | 201.1 (71.6)                     | 192.5 (50.3)                |
| TG             | 221.6 (166.5)              | 224.7 (149.5)               | 225.7 (263.2)           | 281.2 (391.8)                    | 163.4 (140.2)               |
| HDL            | 38.2 (10.7)                | 35.5 (9.6)                  | 39.8 (11.8)             | 38.5 (9.6)                       | 51.7 (17.7)                 |
| LDL            | 117.9 (45.9)               | 131.6 (53.0)                | 113.5 (43.0)            | 115.2 (46.3)                     | 106.3 (34.7)                |
| Hypertension   | 55.2%                      | 60.4%                       | 68.5%                   | 66.2%                            | 65.2%                       |
| Diabetes       | 20.9%                      | 25.6%                       | 31.7%                   | 27.0%                            | 16.4%                       |
| BMI (SD)       | 29.7 (5.7)                 | 30.6 (6.3)                  | 30.8 (6.5)              | 31.9 (6.5)                       | 28.8 (7.4)                  |
| Smoking        | 33.2%                      | 26.5%                       | 68.7%                   | 75.7%                            | 38.3%                       |
| History of MI  | 63.1%                      | 61.7%                       | 52.2%                   | 52.7%                            | 0%                          |
| Family history CAD | 100%                      | 100%                        | 54.1%                   | 70.3%                            | 22.3%                       |

*Continuous variables presented as mean (SD).

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Neuropeptide Y and Early-Onset Atherosclerosis

NPY Variants Are Associated with Early-Onset CAD

Twenty-four SNPs were genotyped and were in Hardy-Weinberg equilibrium. Rs5571 was monomorphic and not analyzed further. Consistent with microsatellite results, nine NPY SNPs had LOD >1.0 in GENECARD, with higher LODs for several SNPs in the subset of 97 very-young-age-of-onset OSA families. Six of these linked SNPs also showed family-based association with CAD by PDT in GENECARD (Table 2). These SNPs are in varying degrees of LD (Figure 2). Association with CAD was validated in the non-familial dataset CATHGEN for five SNPs within the 6-SNP block; the sixth SNP (rs16147) demonstrated borderline significance (Table 3). CATHGEN results also validated our GENECARD findings for age-of-onset effects, with stronger association in the youngest age-of-onset CAD cases (<38 years of age, threshold defined by the GENECARD OSA subset). Although the CATHGEN sample size decreased using this age-of-onset threshold, SNPs within the 6-SNP block remained significantly associated to CAD (Table 3), with odds ratios higher than those obtained with the full dataset, and with several SNPs showing greater significance than the overall cohort (e.g., rs16119, allelic OR 2.20, p = 0.0004). These very-young-age-of-onset cases had a higher prevalence of family history of CAD (p = 0.003) and a trend for higher prevalence of dyslipidemia (p = 0.07).

All six key NPY SNPs were also associated with early-onset CAD in the comparison of GENECARD probands with CATHGEN controls (Table 4). Although we had adjusted for race in our regression models, to further address the potential for population stratification we performed analyses stratified by race. In these analyses, the association between NPY SNPs and early-onset CAD remained consistent and often more significant in self-reported Caucasians (Table S1), suggesting that the results are not confounded by population stratification from race. However, there was no association between NPY SNPs and CAD in non-Caucasians, most likely due to the lower power to detect such a difference given the small sample size (N = 162 CATHGEN non-Caucasian CAD cases, 72 non-Caucasian controls). Haplotype analysis in the CATHGEN dataset showed association of several two-SNP haplotypes with CAD, recapitulating the LD structure of the individual SNPs, but not providing additional information. The haplotype most strongly associated was composed of rs5574 and rs16147 (p = 0.005).

Because rs16147 is a promoter SNP with established effects on expression of the gene [23–25], and all other key NPY SNPs are in LD with rs16147, we will focus the remainder of our results primarily on rs16147. However, as expected, findings were consistent across all six SNPs. As with the other five SNPs, there was consistency of the CAD-associated allele for rs16147 (G allele) and of its allele frequency across all datasets (CATHGEN cases 0.49; very-young CATHGEN cases 0.53; GENECARD probands 0.55; CATHGEN controls 0.43) (Table S2).

To test the possibility that associations between NPY SNPs with CAD are mediated through traditional CAD risk factors, we performed multivariable regression in our case-control datasets, using hypertension, dyslipidemia, diabetes, BMI, and smoking as covariates with NPY SNP genotype. In this analysis, the association of rs16147 with CAD remained significant in our comparison of GENECARD probands with CATHGEN controls (genotype OR 1.60 [95% CI 1.11–2.31], p = 0.01; allele OR 2.00 [1.11–3.59], p = 0.03), suggesting that NPY genotype contributes to CAD risk independently of traditional risk factors, at least in family-based cohorts. In the CATHGEN cohort, however, adjusting for risk factors attenuated association for rs16147 (genotype OR 1.15 [0.87–1.51], p = 0.43; allele OR 1.24 [0.82–1.80], p = 0.32), but the association of rs16120 with CAD intact (allele model, OR 1.54 [1.01–2.33], p = 0.04). Race-stratified analyses in Caucasians also showed attenuation of association between NPY SNPs and early-onset CAD, although three of the SNPs remained significantly associated (Table S1).

To understand which CAD risk factor(s) most mediate association of NPY genotype with CAD, we performed a forward stepwise logistic regression, first fitting a model with genotype and then adding each risk factor. The greatest attenuation of association was caused by dyslipidemia, with minimal additional attenuation by other variables. Table S3 lists risk factors significant in the multivariable model for the SNP remaining significant after adjustment (rs16120), demonstrating the independent strength of effect of genotype, as well as those for intermediate risk factors, in a full fitted model. Neither BMI nor cholesterol levels varied significantly by NPY genotype (data not shown). Thus, it appears that NPY SNPs may contribute to atherosclerosis risk either independently of traditional risk factors, especially in high-risk families, or via mechanisms that may relate to dyslipidemia in non-familial CAD.

NPY Risk Alleles Are Associated with a Younger Age-of-Onset

An alternate way to examine the relationship between quantitative covariates and genetic variants is comparing means of traits by genotype (measured genotype analysis). We performed this analysis in CATHGEN to elucidate further the influence of NPY variants on age-of-onset. We found that the risk allele for rs16147 is indeed associated with a younger age-of-onset (mean age-of-onset: 45.8 ± 6.5 years) in subjects with 1 or 2 copies of risk allele versus 47.3 ± 5.9 years in subjects with 0 copies, p = 0.03). These results support our GENECARD findings and suggest NPY may affect the course of atherogenesis such that atherosclerosis manifests earlier in life.
Allele-Specific Effects on NPY Levels

To examine allele-specific effects, we assessed the relationship of NPY variants with peripheral NPY levels in 220 subjects randomly selected from the CATHGEN case/control dataset. We found that rs16147 was associated with higher NPY levels for the minor (risk, G) allele compared with the major (A) allele (46.3 vs. 41.0 pmol/L, p = 0.04, Figure 3). Concordantly, NPY levels were higher in CAD cases compared with controls (46.8 vs. 41.1 pmol/L, p = 0.02).

Antagonism of NPY Reduces Atherosclerosis

To determine whether NPY-evoked signaling contributes to atherosclerosis, we attenuated the vascular effects of NPY [26] with an NPY1 receptor antagonist that does not cross the blood-brain barrier: BIBP 3226 (BIBP) [8,26,27], which has been shown to reduce non-atherosclerotic neointimal hyperplasia in rats [8,26]. In order to accelerate typical atherosclerosis in a focal manner, we employed carotid endothelial denudation in apoe$^{-/-}$ mice, as reported by other groups [28,29]. With this approach, we could apply BIBP just focally to the carotid artery in a peri-arterial Pluronic gel. Atherosclerosis developed typically in this carotid model, with abundant evidence of macrophage foam cells, SMC-like cells constituting fibrous caps of complex intimal lesions, and extracellular cholesteryl ester (Figure 4A–4C). Peri-carotid application of Pluronic gel by itself had no effect on extent of atherosclerosis (data not shown), and BIBP in the gel did not engender cell toxicity, as judged by apoptosis: cleaved caspase-3 levels were 70$^{\pm}$20% higher in control than in BIBP-treated carotids (p < 0.02, Figure 4D–4E). While control specimens demonstrated greater apoptosis than BIBP-treated specimens, they also demonstrated 1.7$^{\pm}$0.3-fold greater cell proliferation, as judged by immunofluorescence for proliferating cell nuclear antigen (PCNA, Figure 4F, 4G). However, while BIBP did not affect the prevalence of macrophages, SMCs or collagen in atherosclerotic plaques, it reduced total plaque cell number, collagen content and plaque area by 56% (p < 0.03, Figure 5). Thus, it appears that NPY, through the NPY1 receptor and perhaps other NPY receptors, contributes to atherogenesis.

Discussion

Our data provide the first evidence that NPY gene variants associate with CAD in humans, particularly those with early-onset CAD, and that NPY contributes to atherosclerosis in mice through its NPY1 receptor. Our results supporting the role of NPY in CAD pathogenesis emerge from multiple lines of evidence, including linkage, family-based and case-control association studies in multiple cohorts, as well as allele-specific differential NPY levels.

### Table 2. Linkage and family-based association of NPY SNPs with CAD (GENECARD).

| SNP       | Overall Two-Point LOD All Families (N = 420) | LOD OSA Very Young AOO Families (N = 97) | PDT | Geno-PDT |
|-----------|---------------------------------------------|------------------------------------------|-----|----------|
|           | Dom | Rec | Dom | Rec | Dom | Rec | P-value | P-value |
| RS7800861 | 0.25 | 0.09 | 0.43 | 0.35 | 0.49 | 0.50 |
| RS16148   | 0.38 | 0.25 | 0.45 | 0.31 | 0.10 | 0.25 |
| RS16147   | 1.88 | 1.39 | 2.10 | 1.16 | 0.05 | 0.03 |
| RS16143   | 0.20 | 0.05 | 0.47 | 0.18 | 0.69 | 0.50 |
| RS16478   | 0.16 | 0.17 | 0.27 | 0.21 | 0.41 | 0.29 |
| RS16142   | 0.24 | 0.08 | 0.19 | 0.11 | 0.41 | 0.29 |
| RS16141   | 1.62 | 1.15 | 1.92 | 1.19 | 0.12 | 0.17 |
| RS16140   | 0.01 | 0.01 | 0.27 | 0.19 | 0.62 | 0.67 |
| RS16139   | 0.03 | 0.01 | 0.03 | 0.02 | 1.00 | 1.00 |
| RS5572    | 0.89 | 0.46 | 0.24 | 0.31 | 1.00 | 1.00 |
| RS9785023 | 1.87 | 1.17 | 2.13 | 1.03 | 0.04 | 0.05 |
| RS16138   | 0.19 | 0.06 | 0.41 | 0.20 | 1.00 | 0.39 |
| RS1648271 | 0.09 | 0.26 | 0.16 | 0.68 | 0.78 | 0.78 |
| RS5574    | 2.13 | 1.26 | 2.73 | 1.37 | 0.02 | 0.05 |
| RS16132   | 0.40 | 0.39 | 1.07 | 0.92 | 0.17 | 0.17 |
| RS16131   | 1.42 | 0.86 | 1.09 | 0.68 | 0.08 | 0.08 |
| RS16475   | 0.10 | 0.29 | 0.70 | 0.76 | 0.16 | 0.16 |
| RS16126   | 0.02 | 0.03 | 0.01 | 0.00 | 1.00 | 1.00 |
| RS16474   | 2.72 | 2.26 | 1.72 | 1.13 | 0.04 | 0.02 |
| RS16473   | 1.91 | 1.62 | 1.56 | 0.94 | 0.08 | 0.18 |
| RS16120   | 1.63 | 1.33 | 1.32 | 0.73 | 0.03 | 0.04 |
| RS16119   | 1.58 | 1.14 | 1.62 | 0.85 | 0.04 | 0.08 |
| RS17374047| 0.07 | 0.28 | 0.18 | 0.16 | 0.74 | 0.57 |

PDT: Pedigree-Disequilibrium-Test. SNPs with PDT p-value $\leq 0.05$ in bold. SNPs listed in order of genomic location (3’→5’).

Dom: dominant; Rec: recessive.
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Because we observed increased linkage and association between \textit{NPY} SNPs and CAD in the very youngest age-of-onset cases, \textit{NPY} may make a particularly appealing therapeutic target for CAD prevention in families with early-onset disease. Together with our mouse data, our results support the following scenario by which \textit{NPY} variants promote atherosclerosis: presence of the rs16147 risk

Table 3. Association of \textit{NPY} SNPs with early-onset CAD in CATHGEN, adjusted for race and sex.

| SNP       | CATHGEN Cases |                      | CATHGEN Very-young AOO Cases |                      |
|-----------|---------------|----------------------|-----------------------------|----------------------|
|           | Genotype      | Allele               | Genotype                    | Allele               |
|           | OR (95% CI)   | p                    | OR (95% CI)                 | p                    |
| RS16147   | 1.25 (0.97–1.62) | 0.08 | 1.33 (0.91–1.94) | 0.15 | 1.55 (1.01–2.38) | 0.05 | 1.84 (0.91–3.72) | 0.09 |
| RS9785023 | 1.27 (0.99–1.63) | 0.06 | 1.46 (1.00–2.12) | 0.05 | 1.59 (1.04–2.43) | 0.03 | 1.98 (0.99–3.99) | 0.06 |
| RS5574    | 1.29 (1.01–1.66) | 0.04 | 1.48 (1.04–2.12) | 0.03 | 1.51 (0.99–2.30) | 0.06 | 2.01 (1.03–3.92) | 0.04 |
| RS16474   | 1.32 (1.03–1.70) | 0.03 | 1.57 (1.08–2.29) | 0.02 | 1.62 (1.06–2.47) | 0.03 | 1.93 (0.96–3.89) | 0.07 |
| RS16120   | 1.34 (1.05–1.73) | 0.02 | 1.65 (1.13–2.40) | 0.01 | 1.57 (1.03–2.40) | 0.04 | 2.07 (1.01–4.23) | 0.05 |
| RS16119   | 1.32 (1.02–1.70) | 0.03 | 1.57 (1.07–2.31) | 0.02 | 1.59 (1.07–2.35) | 0.02 | 2.20 (1.43–3.38) | 0.0004 |

AOO: age of onset; OR: Odds Ratio; 95% CI: 95% Confidence Interval; p: p-value. All case groups compared with CATHGEN controls.
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allele leads to increased plasma NPY levels, which, acting on NPY1 (and perhaps other) receptors, promotes arterial smooth muscle cell proliferation [30], thereby promoting atherogenesis.

To our knowledge, ours is the first study to take a gene-wide approach to NPY in cardiovascular disease. Furthermore, the NPY variant rs16147 has never been reported for any cardiovascular phenotypes. Because rs16147 is not in LD with upstream SNPs, it appears that the LD block tagged by rs16147 does not extend beyond the gene (www.hapmap.org). This inference is supported by our own data: the upstream rs7800861 is not in LD with rs16147 or other key SNPs.

The allele-specific effects of the NPY promoter SNP rs16147 (A/G) on NPY expression have been documented in three previous studies [23–25]. In accord with our work, two of these studies have observed that the rs16147 G allele (reverse strand C allele) increases NPY expression [23,24]. Furthermore, a putative SP1 transcription factor binding site within the rs16147 stretch of NPY sequence is lost with the rs16147 A allele [24]; consequently, we

| SNP       | GENECARD Probands | adjusted for race and sex. |
|-----------|-------------------|---------------------------|
| Genotype  | Allele            | OR (95% CI) | p-value | OR (95% CI) | p-value |
| RS16147   | 1.55 (1.15–2.09)  | 0.004       | 1.79 (1.12–2.88) | 0.016 |
| RS9785023 | 1.54 (1.15–2.06)  | 0.004       | 1.88 (1.18–3.00) | 0.008 |
| RS5574    | 1.57 (1.17–2.11)  | 0.003       | 1.89 (1.21–2.96) | 0.005 |
| RS16474   | 1.63 (1.21–2.19)  | 0.001       | 2.06 (1.28–3.31) | 0.003 |
| RS16120   | 1.58 (1.18–2.12)  | 0.002       | 2.02 (1.25–3.24) | 0.004 |
| RS16119   | 1.56 (1.15–2.11)  | 0.004       | 1.98 (1.21–3.24) | 0.007 |

OR: Odds Ratio; 95% CI: 95% Confidence Interval.

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Figure 3. Rs16147 is associated with higher plasma NPY levels. NPY levels were measured in fasting plasma (N = 220). Box-and-whisker plots are presented displaying mean (+), median, and 25–75th interquartile range (shaded boxes), according to number of risk alleles at rs16147. *P = 0.04 for comparison of 1 or 2 vs. 0 risk alleles.

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Figure 4. Mouse carotid atherogenesis: cellular effects of a NPY Y1 receptor antagonist. Immediately after wire-mediated endothelial denudation, carotid arteries of apoE−/− mice were encased in Pluronic gel lacking (“None,” “control”) or containing the NPY1 receptor-selective antagonist BIBP 3226 (“BIBP”). Carotid arteries were harvested 6 wk (A–C) or 2 wk (D–G) postoperatively, and processed as described in Methods. A, B, Typical atherosclerosis in a representative control-treated carotid artery. Frozen sections were stained for DNA and (immunofluorescently) with IgG specific for SMC α-actin and either the macrophage marker Mac3 (A), or an isotype negative control rat IgG (B). Green autofluorescence of the elastic laminae can be seen in both A and B. Alternatively, frozen sections were stained for cholesteryl ester with Sudan IV and hematoxylin counterstain (C). Scale bar = 100 µm. D, E, Carotid sections were stained with IgG for cleaved caspase-3 (apoptosis marker) and counterstained for DNA; specimens stained with non-immune rabbit IgG yielded no red immunofluorescence (not shown). Scale bar = 50 µm. F, G, Carotid sections were stained with IgG for proliferating cell nuclear antigen (PCNA), counterstained for DNA, and oriented with the lumen side upward. The closed arrow designates a single PCNA-positive nucleus in the tunica media, representative of ≥20 such nuclei in the field shown; specimens stained with non-immune rabbit IgG (not shown) yielded only elastic lamina autofluorescence, like that observed in panels F and G. IEL, internal elastic lamina, indicated by the open arrows. Scale bar = 50 µm. All images are representative of ≥4 independent specimens stained with each modality.

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cases, it follows that the G allele would result in increased NPY expression. Given previous studies showing a higher frequency of the rs16147 G allele in CAD patients compared to controls, this suggests a possible link between the rs16147 SNP and CAD risk. However, it is important to note that Zhou et al. [25] also found that haplotypes containing the rs16147 A allele result in higher, rather than lower, expression of NPY.

Furthermore, race-stratified analyses showed consistent, and often more significant, association between NPY SNPs and CAD in Caucasians, though our analyses were underpowered to assess such relationships in non-Caucasians. Importantly, there is no evidence of population stratification in previous GENECARD analyses [7] and, unlike the non-familial case-control regression analyses which are sensitive to population stratification, the family-based analyses [8] showed that the association between NPY SNPs and CAD is consistent across different populations. Concomitantly, we note that rs16147 is very common, and despite a modest relative risk conferred, this variant may have a higher population attributable risk in genetically diverse cohorts. In fact, 21% of our CATHGEN subjects are homozygous for the rs16147 risk allele. This prevalence is similar in magnitude to that observed with the recently identified chromosome 9p21 CAD susceptibility variant discovered through GWAS [5].

We cannot exclude the possibility that our CATHGEN data are affected by population stratification; however we adjusted for race and CATHGEN subjects were recruited from only one site. Furthermore, race-stratified analyses showed consistent, and often more significant, association between NPY SNPs and CAD in Caucasians, though our analyses were underpowered to assess such relationships in non-Caucasians. Importantly, there is no evidence of population stratification in previous GENECARD analyses [7] and, unlike the non-familial case-control regression analyses which are sensitive to population stratification, the family-based association analyses are robust to population stratification.

The allele frequencies of the NPY SNPs were remarkably similar in very-young-age-of-onset CATHGEN cases and GENECARD probands. Consequently, these independent cohorts appear to be genetically as well as clinically similar, in that they demonstrate a strong genetic component to their risk profiles. These very young individuals represent a prime target for prevention. In identifying the association between NPY variants and atherosclerosis, our results highlight the importance of pursuing a comprehensive gene-wide SNP survey. The replication of our GENECARD findings in our CATHGEN validation dataset is very consistent, not just for the NPY gene itself but also for the association of the individual associated alleles of each SNP, suggesting it is extremely unlikely that results are due to chance alone. It remains to be determined whether association between NPY and CAD is influenced by traditional CAD risk factors, particularly dyslipidemia, and whether a particular constellation of risk factors along with NPY SNPs is related to the very early-onset CAD associations observed in both GENECARD and CATHGEN. A more detailed analysis of these relationships is ongoing.

Figure 5. Reduction of atherosclerosis by antagonism of the NPY Y1 receptor. Mice treated identically to those in Figure 4 underwent carotid harvest 6 weeks after endothelial denudation, as described in Methods. A, Carotid sections from mice subjected to the indicated treatment were stained with a modified connective tissue stain (green = collagen, black = elastin, red = cytoplasm) [39]. Sections shown are representative of 3 obtained from each of 5 mice in each treatment group. Scale bar = 100 μm. B, Computerized morphometry was used to quantitate the indicated carotid artery dimension, as described in Methods; the means ± S.E. of 5 independent carotid arteries from each treatment group are shown. Compared with control: p<0.03. doi:10.1371/journal.pgen.1000318.g005

In summary, our data provide further support for a role for NPY in human atherosclerosis.
complete understanding of the relationship between NPY, CAD risk factors, and CAD will require additional studies.

In our study, the correlation between SNPs in the form of LD makes a Bonferroni correction for multiple comparisons too conservative. Using a method that corrects for LD [33] resulted in 11 minimally redundant SNPs (corrected p<0.0047). Thus, one SNP for our CATHGEN very-young-age-of-onset group (rs16119, p = 0.0004) and most SNPs for the GENECARD proband comparison group would remain significant. More importantly, we have used additional statistical and functional studies to demonstrate that association of NPY SNPs with CAD does not result from chance alone: the convergence of evidence from linkage and association, as well as replication of the same SNP/CAD association in independent cohorts. The possibility that NPY contributes to atherosclerosis is supported by the correlation of plasma NPY levels with NPY risk alleles, and the inhibition of murine atherosclerosis with an antagonist of the NPY1 receptor, which mediates most cardiovascular effects of NPY [8].

In summary, NPY is a strong candidate gene for early-onset CAD. NPY SNPs may help refine CAD risk estimates and target therapies for young members of families with CAD.

Methods

Study Sample and Phenotype Description

The GENECARD study enrolled 920 families with early-onset CAD (age-of-onset before 51 years for men, 56 years for women) to perform affected-sibling-pair linkage [7]. GENECARD families were recruited based on the presence of at least two siblings with early-onset CAD defined by: stress testing with ischemia; myocardial infarction (MI); coronary revascularization; or angiography with ≥50% stenosis in one major vessel. Unaffected family members had no clinical evidence of CAD and age ≥55 years for men (≥60 years for women). The 420 families from the initial linkage scan [7] are included in this report, including a limited number of unaffected family members (N = 37).

An independent non-familial validation cohort was selected from the CATHGEN biorepository, consisting of subjects recruited sequentially through the cardiac catheterization laboratories at Duke University Medical Center (Durham, NC). Fasting whole blood and plasma samples were obtained from the femoral artery during cardiac catheterization and frozen prior to use. CAD cases were defined as CAD-index≥32 (at least one vessel with ≥95% stenosis) with age-of-onset <56 years. CADI is a numerical summary of angiographic data directly related to outcome [36]. Age-of-onset was defined as age at first MI, coronary revascularization, or catheterization meeting CADI threshold. Controls were defined as age-at-catheterization >60 years; CAD-index≤23, no history of cardiovascular disease, and no clinically significant CAD. Dyslipidemia was defined as a previous diagnosis and/or treatment of hypercholesterolemia (yes/no), confirmed by review of medical records. We also constructed a third case-control group comprising GENECARD probands included in the original linkage scan from United States sites (N = 221) and CATHGEN controls. Institutional Review Boards approved all study protocols. Informed consent was obtained.

Genotyping and Plasma NPY Levels

Tagged NPY SNPs were identified using the SNPSselector program, which employs a linkage disequilibrium (LD) tagging algorithm that prioritizes functional SNPs [37], and were then genotyped using either Taqman or Illumina BeadArray (www.illumina.com). The 7900HT Taqman SNP genotyping system incorporates a standard PCR-based, dual fluor, allelic discrimination assay with a dual laser scanner. Assays were purchased through Applied Biosystems (www.appliedbiosystems.com). QC samples, composed of 12 reference controls, were included in each quadrant of the plate. Illumina BeadStation genotyping was performed using the 500G system. All SNPs examined were successfully genotyped for ≥95% of the individuals in the study. Error rate estimates for SNPs meeting QC benchmarks were <0.2%.

Plasma NPY levels were measured by an NPY-specific radioimmunoassay (Alpco Diagnostics, Salem NH) on 220 CATHGEN subjects, randomly selected (regardless of case/control status) from all subjects included in the genotyping studies. NPY levels were measured in fasting unextracted plasma collected at time of cardiac catheterization (prior to administration of supplemental anticoagulants if given), which was subsequently frozen and stored at −80°C, and analyzed in a single RIA run. Cross-reaction of the NPY RIA assay as reported by the company is: human NPY 100%; human PYY <2.0%; human pancreatic polypeptide (PP) <1.0%; NPY 1–21 <0.1%; NPY 20–36 <0.4%. The assay was characterized as a mean recovery of 82% (range 75–88%) for NPY-spiked plasma. Precision was characterized by an intra-assay CV of 2.6–3.9% in our samples, which were run in one batch. NPY levels were approximately normally distributed.

Mouse Atherosclerosis Studies

All animal experiments complied with institutional guidelines. Gender-matched apolipoprotein E-deficient (apoE−/−) C57BL/6 six mice, at the age of 10±2 weeks. To accelerate atherosclerosis, we performed endothelial denudation of the left common carotid artery with a 0.36-mm flexible angioplasty guidewire [Johnson and Johnson], inserted via the external carotid artery as described [28,38]. After ligating the external carotid artery, we encased the common carotid in 150 μl of 22.5% (w/v) Pluronic gel, containing either 1% (v/v) water that lacked (control) or contained the NPY1 receptor antagonist BIBP 3226 [27] [21 μmol/ L, final]; Sigma-RBI, Inc.). The skin was closed after the Pluronic gelled (virtually instantaneously). Postoperatively, mice were fed a Western diet (Harlan Teklad #88137) ad libitum for two-six weeks (until arterial harvest), and there were no differences in weight between mice treated with vehicle or BIBP 3226. BIBP 3226 does not affect blood pressure in rodents [26]. As we previously described [39], arteries were perfusion/ixed and embedded in parafﬁn, or embedded in OCT and frozen. Five-μm sections were stained with a modiﬁed connective tissue stain (parafﬁn sections), Sudan IV (frozen sections, for cholesterol ester), or immunostained (frozen sections) as previously described [38,39]; for macrophages (FITC-conjugated rat IgGk anti-mouse Mac3 or negative control FITC-conjugated rat IgGk (Pharmingen, Inc.)); smooth muscle cells (SMCs) (Cy3-conjugated 1A4 IgG targeting SMC α-actin (Sigma)); for apoptotic cells (monolonal rabbit IgG targeting cleaved (activated) caspase-3 (Cell Signaling, Inc.)); or for proliferating cell nuclear antigen (Santa Cruz Biotechnology, Inc.). All sections were digitally photographed with fixed light settings for all immunofluorescence specimens within a single staining batch, to facilitate quantitation [39]. Atherosclerosis, cellular (or protein antigen) prevalence, and PCNA-positive nuclear prevalence [38] were quantitated with Scion Image (www.scioncorp.com) or by manual counting by an observer blinded to specimen identity [39], from three cross sections obtained from the distal, middle, and proximal portions of each specimen, as described [39]. Data from these locations were averaged for each carotid specimen.

Statistical Analysis

Ordered subset analysis (OSA) examines the impact of covariates by defining homogeneous subsets of families for linkage
Neuropeptide Y and Early-Onset Atherosclerosis

[40], and has been successfully used for gene mapping in complex diseases [41–44]. Similar methods were used to identify the BRCA1 breast cancer susceptibility gene, where evidence for linkage was only present in earlier-onset families [22,45]. Subsets of families with earlier ages-of-onset may help evaluate genetic heterogeneity and define subgroups with a stronger genetic effect. A priori specification of the age-of-onset threshold is unnecessary, as OSA uses maximal linkage evidence to define subsets. OSA was conducted using nonparametric multipoint family-specific LODs from microsatellite genotypes from the original screen as input [7], with age-of-onset as a covariate. A permutation procedure provides empirical p-values for the significance of the increase in the maximum-subset-LOD from the overall LOD. To assess SNP linkage, two-point LODs were calculated using Merlin [46]. The Pedigree Disequilibrium Test (PDT) and Genotype-PDT were used for family-based association. PDT, an extension of the transmission-disequilibrium-test (TDT), allows incorporation of extended pedigrees and is valid even with population substructure [47]. Power calculations (QUANTO:http://hydra.usc.edu/) showed power ≥0.80 to detect effect sizes of ≥2.45 for the lowest allele frequency SNP (0.02, rs16139), and effect sizes ≥1.35 for the highest allele frequency SNP (0.49, rs16147).

In CATHGEN, association was assessed using logistic regression models adjusting for race and sex; and for race, sex, hypertension, diabetes, dyslipidemia, smoking and body-mass-index (BMI) (multivariable model). Measured genotype analysis using generalized linear models was performed to compare differences in means of quantitative traits (NPY levels, age-of-onset) by NPY genotype. Baseline differences were assessed using a chi-square or t-test. The Graphical Overview of Linkage Disequilibrium (GOLD) program [48] was used to assess LD. Haplotype analysis used HaploStats 1.1.0 (Mayo Clinic, Roche-Disequilibrium (GOLD) program [48] was used to assess LD. The maximum-subset-LOD from the overall LOD. To assess SNP linkage, two-point LODs were calculated using Merlin [46]. The Pedigree Disequilibrium Test (PDT) and Genotype-PDT were used for family-based association. PDT, an extension of the transmission-disequilibrium-test (TDT), allows incorporation of extended pedigrees and is valid even with population substructure [47]. Power calculations (QUANTO:http://hydra.usc.edu/) showed power ≥0.80 to detect effect sizes of ≥2.45 for the lowest allele frequency SNP (0.02, rs16139), and effect sizes ≥1.35 for the highest allele frequency SNP (0.49, rs16147).

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