Detection of genetic association and a functional polymorphism of dynamin 1 gene (*DNM1*) with nicotine dependence in European and African Americans

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Abstract

Although it has been documented that dynamin 1 gene (*DNM1*) is significantly modulated by nicotine in animal models, its association with nicotine dependence (ND) in human population remained to be unexplored. To determine whether *DNM1* is associated with ND, in this study, we genotyped seven single-nucleotide polymorphisms (SNPs) within this gene in 602 nuclear families of either African-American (AA) or European-American (EA) origin. Individual SNP-based association analysis revealed a significant association of SNP rs3003609 with Smoking Quantity (SQ; $P = 0.0031$) and Heaviness of Smoking Index (HSI; $P = 0.0042$) in the EA sample. Furthermore, our haplotype-based association analyses indicated that haplotypes T-G-T, formed by rs2502731-rs2229917-rs3003609 (at a frequency of 54%), G-T-A, formed by rs2229917-rs3003609-rs16930313 (at a frequency of 52%), and T-A-G, formed by rs3003609-rs16930313-rs7022174 (at a frequency of 52%) are significantly associated with SQ ($Z = -2.44 \sim -2.92$; $P = 0.015 \sim 0.0055$) and HSI ($Z = -2.52 \sim -2.67$; $P = 0.012 \sim 0.0076$) in the EA sample. In the AA sample, another haplotype, G-T-A, formed by rs7875406-rs2502731-rs2229917, at a frequency of 12% was significantly associated with SQ ($Z = -2.58$; $P = 0.0098$). Finally, by using *in vitro* gene expression assays, we demonstrated that the T allele of rs3003609 in the exon 9 of *DNM1* significantly decreases the expression of dynamin 1, by 27.1% at the mRNA and 22.0% at the protein level, suggesting that rs3003609 represents a functional polymorphism affecting *DNM1* expression and may partly contributed to the observed association of the gene with ND in our samples. Taken together, our findings indicate that dynamin 1 is likely involved in the etiology of ND and represents a plausible candidate for further investigation in independent samples.

Keywords

nicotine; smoking; genetic association; dynamin 1; functional SNP

Introduction

Smoking is the leading cause of preventable death in the United States, causing approximately 438,000 premature deaths and $157 billion in health-related costs annually (Mokdad *et al*, 2004; USDHHS, 2000). Numerous earlier studies have demonstrated that nicotine is the primary addictive substance in tobacco (Stolerman and Jarvis, 1995; USDHHS, 2000; WHO, 2002). Twin and family studies reveal that nicotine dependence (ND) is a complex trait determined by genetic and environmental factors, as well as their interactions (Carmelli *et al*, 1992; Swan *et al*, 2003; Swan and Lessov, 2004). A meta-analysis of 17 reported twin studies indicates that the weighted mean heritability for ND is 0.59 in male and 0.46 in female smokers, with an average of 0.56 for all smokers (Li *et al*, 2003).

A number of plausible candidate genes has been implicated and investigated for potential association with ND in human studies (Li, 2006). A variety of genes and proteins has also been revealed in microarray and proteomic studies that are modulated in response to nicotine stimulation (Hwang and Li, 2006; Wang *et al*, 2008). Previously, using two-dimensional electrophoresis combined with mass spectrometry, we identified a number of proteins whose expressions were significantly modulated in the brain regions of nicotine-treated rats compared with controls. Of these proteins, dynamin 1 was modulated significantly by nicotine in four of five brain regions we examined, suggesting that this protein plays a significant role in neuronal activities responding to nicotine stimulation (Hwang and Li,, 2006).

Dynamin 1 belongs to the GTPase superfamily that is involved in various cell processes and has important functions in endocytosis (Schmid *et al*, 1998), apoptosis, and signaling (Frank *et al*, 2001; Hislop *et al*, 2001; Kashiwakura *et al*, 2004). As one of the best-characterized family members, dynamin 1 is expressed exclusively in the central nervous system and is believed to function in synaptic vesicle recycling in nerve terminals (Powell and Robinson, 1995; Sontag *et al*, 1994), a process important for neural activities and plasticity. Study of a temperature-sensitive mutant of dynamin in *Drosophila* shows a clear role of dynamin in synaptic-vesicle retrieval in nerve terminals (Koenig and Ikeda, 1989). Similarly, in rat, dynamin 1-mediated processes appear to be necessary for normal neuronal morphogenesis and dynamin 1 is indispensable for vesicle endocytosis at fast central nervous system synapses (Torre *et al*, 1994; Yamashita *et al*, 2005). Moreover, several recent studies revealed that reduced dynamin 1 expression may impair neuronal transport and vesicle trafficking by interactions with other endocytic accessory proteins in hippocampal neurons (Jiang *et al*, 2006; Kelly and Ferreira, 2006; Kitzmueller *et al*, 2004). Whereas a study of dynamin 1-knockout mice found that dynamin 1-independent mechanisms can support limited synaptic vesicle endocytosis, the protein is essential during high levels of neuronal activity (Ferguson *et al*, 2007).

Given that dynamin 1 is modulated significantly nicotine in rat brain regions (Hwang and Li,, 2006) and plays an important role in endocytosis and neuronal activities, we investigated genetic association of its encoding human gene, *DNM1*, with ND and its potential involvement in the etiology of ND.

Materials and Methods

Participants and ND measures

Participants were of either African-American (AA) or European-American (EA) ancestry and were recruited primarily from the states of Tennessee, Mississippi, and Arkansas during 1999–2004. Proband smokers were required to be at least 21 years old, to have smoked for at least five years, and to have consumed an average of 20 cigarettes per day for the last 12 months. Siblings and parents of a proband were recruited whenever possible, regardless of their smoking status. Extensive data were collected on each participant, including demographics (e.g., sex, age, race, biological relationships, weight, height, years of education, and marital status), medical history, smoking history and current smoking behavior, ND, and selected personality traits, assessed by various questionnaires/interviews available at the NIDA Genetics Consortium Website (http://zork.wustl.edu/nida). All participants provided informed consent. The study protocol and forms/procedures have been approved by all participating Institutional Review Boards.

For each smoker, ND was ascertained by three measures commonly used in the tobacco research field: Smoking Quantity (SQ; defined as the number of cigarettes smoked per day), the Heaviness of Smoking Index (HSI; 0–6 scale), and the Fagerström Test for ND score (FTND; 0–10 scale) (Heatherton *et al*, 1991). The SQ provides a simple, quantified index of consumption (using a 0–3 point compressed format), whereas HSI includes one item addressing quantity (SQ) and another assessing urgency. The FTND score includes the HSI plus other indicators of behavioral propensity to smoke in various situations. Given the presence of overlap in the content of the three ND measures, there exist fairly robust correlations among them ($r = 0.88–0.94$) in both the AA and EA samples.

Of the 2,037 participants, the average age was 39.4 ± 14.4 (SD) years for the AA and 40.5 ± 15.5 years for the EA participants. The average nuclear family size was 3.14 ± 0.75 for AAs and 3.17 ± 0.69 for EAs. The average number of cigarettes smoked per day, his, and FTND scores of smokers were 19.4 ± 13.3, 3.7 ± 1.4, and 6.26 ± 2.15 for AA smokers (N = 1053) and 19.5 ± 13.4, 3.9 ± 1.4 and 6.33 ± 2.22 for EA smokers (N = 515).

DNA extraction, SNP selection, and genotyping

Genomic DNA was isolated from blood sample of each participant using the QIAamp DNA Blood Maxi kit (Qiagen, Valencia, CA). The SNPs used for genotyping were selected from the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=snp). Two SNPs (rs2229917 and rs3003609) were selected specifically because of their locations at exons 4 and 9, respectively. To obtain a uniform coverage of the gene, the other five were selected on the basis of their high heterozygosity with a minor allele frequency (MAF) > 0.15 (Table 1). All SNPs were genotyped using the *Taq*Man assay in a 384-well microplate

format (Applied Biosystems, Foster City, CA). Briefly, 15 ng of DNA was amplified in a total volume of 7 μl, which contains a set of *Taq*Man primers and probes and 2.5 μl of *Taq*Man universal PCR master mix. The amplification procedure was 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 95°C for 25 sec and 60°C for 1 min. Allelic discrimination analysis was performed on the ABI Prism 7900HT Sequence Detection System. To ensure the quality of the genotyping, eight positive and negative controls were added to each microplate. Genotyping data were accepted only when all control samples produced consistent results; otherwise, all samples of the microplate under investigation were re-genotyped.

Association analyses

We used the PedCheck program (O'Connell and Weeks, 1998) to detect genotyping inconsistencies for Mendelian inheritance. One hundred fifty inconsistencies, with 95 in the AA sample and 25 in the EA sample, were detected from approximately 14,300 assays (i.e., 0.8% genotyping error) for seven SNPs across all DNA samples and were excluded from subsequent statistical analysis. Pair-wise linkage disequilibrium (LD) between all possible SNP pairs was estimated using the program Haploview (Barrett *et al*, 2005). Associations between individual SNP and the three ND measures were determined by the PBAT program using generalized estimating equations (Lange *et al*, 2003). Associations between each ND measure and haplotypes from all possible haplotypes in consecutive three-SNP combinations were calculated using the FBAT program with the option of computing the *P*-value of the *Z*-statistic using Monte Carlo sampling under the null distribution of no linkage and no association (Horvath *et al*, 2004). Three genetic models (additive, dominant, and recessive) were tested for both individual and multi-locus SNPs (i.e., haplotypes). For all PBAT and FBAT association tests, sex and age were used as covariates in the EA and AA samples and sex, age, and ethnicity as covariates in the combined sample. The three ND measures, SQ, HSI, and FTND, were analyzed individually. All significant associations were corrected for multiple testing according to the SNP spectral decomposition (SNPSpD) approach (Nyholt, 2004) for individual SNP analysis and using Bonferroni correction by dividing the significance level by the number of major haplotypes (frequency >5.0%) for haplotype-based association analysis.

In consideration of high correlations between the three ND measures and among the results under different genetic models, we chose not to correct for testing of the three highly-correlated ND measures or genetic models to avoid being over-conservative. This is because there is no generally accepted methodology capable of handling the problem of highly correlated multiple testing. However, if one wishes to correct for multiple testing for the three highly-related genetic models and/or phenotypes, a justified P-value can be obtained by dividing the corrected P-value after correction for major multiple testing given in the note #1 of Table 2 by 6, i.e., 2 accounting for two independent tests for the three genetic models multiply by 3 ND measures.

Plasmid construction and mutagenesis

The full-length cDNA clone of human *DNM1* was purchased from Open Biosystems (Huntsville, AL). The coding region of *DNM1* was amplified with a pair of primers, 5′-

GCCG<u>GAATTC</u>GGATGGGCAACCGCGGCATGGAAGATC-3′ (forward) and 5′-
CATGG<u>GCGGCCGC</u>TCAGGGGTCACTGATAGTGATTCTG-3′ (reverse), and was
subcloned into the pCMV-HA vector (Clontech, Mountain View, CA), using the *Eco*RI and
*Not*I two cloning sites. The yielded construct was sequence confirmed to contain
rs2229917G and rs3003609C and was thus designated pE4G-E9C. Allele combinations of
SNPs rs2229917 and rs3003609 were then generated with the QuikChange II XL Site-
Directed Mutagenesis kit (Stratagene, La Jolla, CA), and designated pE4A-E9C, pE4G-E9T,
and pE4A-E9T, separately (Figure 2A). All four constructs were verified by sequencing.

Cell culture and transfection

Human neuroblastoma SH-SY5Y cells were purchased from the American Type Culture
Collection (Manassas, VA), and grown in the DMEM supplemented with 10% fetal bovine
serum (Invitrogen, Carlsbad, CA) at 37°C with 5% CO_2. Cells were transfected with *DNM1*
constructs using Lipofectamine 2000 (Invitrogen) in accordance to the manufacturer's
protocol. Cells were grown for additional 24 hours after transfection and were harvested for
RNA isolation and protein extraction, respectively.

Expression analysis of haplotype-specific *DNM1*

Total RNA in the transfected cells was isolated with the Trizol reagent (Invitrogene) and
treated with RNA-free DNase I (Ambion, Austin, TX) to remove any potential remaining
plasmid DNA prior to reverse transcription. For quantitative real-time reverse transcriptase-
polymerase chain reaction (RT-PCR), RNA was first reverse transcribed with SuperScript II
RNase H⁻ and random hexamer (Invitrogen). Then a *Taq*Man probe specifically designed to
detect the HA-fused dynamin-1 gene: 5′-GATGTTCCAGATTACGCTCTTATGG-3′
(forward), 5′-CAGCGGGATGAGATCTTCCA-3′ (reverse), and 5′-FAM-
CGAATTCGGATGGGCA-MGB-3′ (probe), was applied for the standard *Taq*Man PCR
procedures in the ABI Prism 7900HT Sequence Detection System. Ribosome RNA (18S)
was used as a control for data normalization. Proteins in the transfected cells were extracted
by the RIPA buffer, and sonicated and quantified using the Bio-Rad Protein Assay (Bio-
Rad, Hercules, CA). The same amount of total protein was applied in the SDS-PAGE
separation. The protein expression of haplotype-specific *DNM1* was detected by using a
primary antibody against HA tag, a horseradish peroxidase-conjugated secondary antibody
(Covance, Princeton, NJ), and the SuperSignal West Pico chemiluminescent substrate
(Pierce, Rockford, IL) in Western blotting analysis.

mRNA stability analysis of haplotype-specific *DNM1*

To evaluate the effect of SNP rs3003609 alleles (C/T) on the mRNA stability, pE4G-E9C
and pE4G-E9T were transfected separately into SH-SY5Y cells. One day after transfection,
cells were treated with actinomycin D at a final concentration of 5 μg/ml for 0, 1, 3, 5, 7, or
9 hours, respectively. Total RNA was then isolated for quantitative real-time RT-PCR assay.

Results

Association analysis of individual SNPs with ND

Association of individual SNPs with the three ND measures (SQ, HSI, FTND) was determined with PBAT-GEE (Lange *et al*, 2003). We analyzed the EA and AA samples separately, because 1) the potential genetic differences were reported in nicotine metabolism and smoking behavior among racial groups (Benowitz *et al*, 1999) and 2) we noticed significant differences in the allele frequencies of several SNPs between the EA and AA samples (Table 2).

In the EA sample, we found significant associations under the dominant model of rs2229917 with the HSI measure ($P = 0.043$), of rs3003609 with all three ND measures ($P = 0.0031\sim0.011$), and of rs16930313 with the SQ measure ($P = 0.039$). However, only the associations of rs3003609 with SQ and HSI remained significant after correction for multiple testing (adjusted *P*-value at the 0.05 significance level = 0.007) based on the SNPSpD approach (Nyholt, 2004). We found no significant association of individual SNPs in *DNM1* with any ND measure in the AA sample (Table 2). Nonetheless, we also tested these individual SNPs in the EA and AA combined sample. We only detected rs7022174 that was marginally associated with the FTND measure ($P = 0.05$), which was no longer significant after correction for multiple testing.

Haplotype block structure

Figure 1 shows the pair-wise D′ values for the seven selected SNPs within *DNM1* that were determined in the EA and AA populations using the HaploView algorithm (Barrett *et al*, 2005). The haplotype block in the EA sample, a length of 5 kb extending from rs7875406 to rs2502731, was predicted according to the block definition proposed by Gabriel and colleagues (Gabriel *et al*, 2002). No haplotype block was found in the AA sample.

Haplotype-based association analysis

Since we found no large haplotype block within *DNM1*, we performed haplotype-based association analysis with the FBAT program for all possible haplotypes in consecutive three-SNP combinations and the three measures in the EA and AA samples. As shown in Table 3, in the EA sample, four major haplotypes showed significant associations with at least two of three ND measures, of which three major haplotypes remained significant for the SQ and HSI after Bonferroni correction. The first haplotype, T-G-T, formed by SNPs rs2502731, rs2229917 and rs3003609, at a frequency of 54% showed a significant inverse association with SQ ($Z = -2.76$, $P = 0.0058$; Global $P = 0.015$) and HSI ($Z = -2.66$, $P = 0.0079$; Global $P = 0.027$) under the additive model. The second haplotype, G-T-A, formed by rs2229917, rs3003609 and rs16930313, at a frequency of 52% showed a significant inverse association with SQ ($Z = -2.92$, $P = 0.0035$; Global $P = 0.033$) and HSI ($Z = -2.67$, $P = 0.0076$; Global $P = 0.056$) under the dominant model. The last one, T-A-G, formed by rs3003609, rs16930313 and rs7022174, at a frequency of 52% also exhibited a significant inverse association with SQ and HSI under both the additive and dominant models. By examining the haplotype associations in the EA sample (Table 3), we found that all significant haplotypes contain the T allele of SNP rs3003609, which always responds to the

protective haplotypes. This indicates that rs3003609 may represent a functional polymorphism contributing to the observed association of the gene with ND in the EA sample.

Although we detected no significant association of individual SNPs with ND in the AA sample, interestingly, we did find several haplotypes that were significantly associated with the SQ and HSI under the recessive model. However, only the haplotype G-T-A, formed by SNPs rs7875406, rs2502731, and rs2229917, at a frequency of 12%, remained a significant association with SQ ($Z = -2.58$; $P = 0.0098$; Global $P = 0.11$) after correction for multiple testing (Table 3).

Biological effect of SNPs rs2229917 and rs3003609 on the expression of dynamin 1

To define the functional significance of exonic SNPs rs2229917 (A/G) and rs3003609 (C/T) for the expression of dynamin 1, we constructed four plasmids for the possible allele combinations (i.e., pE4G-E9C, pE4A-E9C, pE4G-E9T, and pE4A-E9T) by site-directed mutagenesis from a complete cDNA clone and then transfected them into human neuroblastoma SH-SY5Y cells. Following the transient transfection, we measured the mRNA and protein expression levels for each construct using the quantitative real-time RT-PCR and Western blotting analysis, respectively (Figure 2).

After normalization to the corresponding 18S ribosome RNA of each sample, we found that constructs pE4G-E9C and pE4A-E9C showed significantly higher mRNA expression than pE4G-E9T and pE4A-E9T ($\sim$27.0%; $P < 0.05$). On the other hand, no difference was observed between pE4G-E9C and pE4A-E9C or between pE4G-E9T and pE4A-E9T (Figure 2B). At the protein level normalized to the corresponding tubulin expression of each sample, almost the same expression trends were found, in that pE4G-E9T and pE4A-E9T showed significantly less expression than was seen from the other two constructs ($-22.2\sim-29.2\%$; $P < 0.05$; Figure 2D). These results indicate that the two alleles of rs2229917 at exon 4 have little or no impact on the expression of *DNM1* and that all differences observed among the four constructs were attributable primarily to rs3003609 at exon 9, of which the C allele caused higher expression than does the T allele, indicating that this SNP is a functional polymorphism contributing at least partly to the observed association of the gene with ND in our samples.

Regulatory effect of rs3003609 on mRNA stability of *DNM1*

As demonstrated above, the constructs with the T allele of rs3003609 caused significantly less expression of dynamin 1 than did those with the C allele. Given that the same trends occurred at both the mRNA and protein levels, a possible explanation is the stability difference of the allele-specific mRNAs. To test this speculation, we transfected SH-SY5Y cells with constructs pE4G-E9C and pE4G-E9T, treated cells with actinomycin D for a different period of time, and measured the mRNA expression level with real-time RT-PCR. We found that the estimated half-life of *DNM1* mRNA was about 3.2 hours for pE4G-E9C and 3.6 hours for pE4G-E9T (Figure 3). No significant difference was detected in the mRNA half-life between the two constructs, although the mRNA level of pE4G-E9T was significantly lower at each time point than that of pE4G-E9C, implying that the expression

difference of *DNM1* with different rs3003609 alleles may be caused at the transcription level.

Discussion

Previously, we reported in a proteomics study that dynamin 1 is modulated significantly by nicotine in several rat brain regions (Hwang and Li, 2006). By using different molecular approaches in independent animal experiments, we subsequently confirmed this finding (Xu and Li, unpublished data). Given these consistent results, we performed a genetic association study in two independent ethnic samples to explore whether *DNM1*, encoding dynamin 1, is associated with ND. Our individual SNP analysis revealed that SNPs rs2229917, rs3003609, and rs16930313 are significantly associated with at least two ND measures in the EA sample (Table 2). Further, our haplotype analysis revealed three major haplotypes, T-G-T, G-T-A and T-A-G, formed by SNPs rs2502731, rs2229917, rs3003609, and rs16930313, that have significant inverse associations with at least two ND measures in the EA sample (Table 3). Together, this indicates that *DNM1* is significantly associated with ND in the EA population. In contrast, we found no significant association of individual SNPs with ND in the AA sample, but identified a haplotype, G-T-A, formed by SNPs rs7875406, rs2502731, and rs2229917, that has a significant inverse association with SQ in the AA sample (Tables 2 and 3). It suggests that the association in the AA sample is potentially attributable to linkage disequilibrium with a more centromeric genetic variant in the near region.

Although we examined the association with three ND measures in the EA and AA combined sample as well, we only found marginal significant associations in the individual SNP and haplotype analyses. These associations were no longer significant after correction for multiple testing (data not shown). This, from another point of view, reflects the significant difference in the associations of *DNM1* with ND in the two ethnic groups, which is most likely attributable to the ethnic-specific characteristics of some SNPs within *DNM1* (Tables 1 and 2). Also, we like to point out that our association results for both individual SNPs and haplotypes did not correct for testing of the three highly correlated ND measures and genetic models, or multiple genes that we found to be associated with ND previously in the samples. This is because Bonferroni correction is considered to be over-conservative and there is no generally accepted method for handling such highly correlated multiple testing. Regarding genetic models, we chose to use the three models because in some cases we found that dominant and/or recessive model produce better results than additive model likely due to the presence of heterosis that has not received much attention in almost all human genetic association analyses. Regardless of these rationale, we like to consider our association results of ND with dynamin 1, especially for the AA sample, as exploratory and suggest that more replication in independent samples is greatly needed in future studies.

Polymorphisms rs2229917 and rs3003609 are two exonic synonymous variants, residing at exons 4 and 9, respectively. Because both SNPs demonstrated consistently associations with ND in our individual SNP and haplotype analyses in the EA sample (Tables 2 and 3), we speculate that they may represent two functional polymorphisms to affect *DNM1* expression. By expressing plasmid constructs containing different allele combinations of rs2229917 and rs3003609 in human neuroblastoma SH-SY5Y cells, we found that the plasmid constructs

bearing rs3003609T allele produced significantly lower expression levels of mRNA and protein than those with rs3003609C allele, whereas no significant difference was detected between the constructs with the G or A allele of rs2229917 (Figure 2). We thus conclude that SNP rs3003609, but not rs2229917, is a potential causative polymorphism involved in the regulation of *DNM1* expression. Of note, we revealed all negative *Z*-value for the haplotypes containing rs3003609T allele (Table 3), indicating that the T allele of rs3003609 may play a protective role against the development of ND.

Non-synonymous SNPs change amino acids in protein sequence and can directly influence protein function. The effects of no-synonymous polymorphisms are thus relatively easy to study and have been widely characterized for associations with human diseases (Yampolsky *et al*, 2005). In contrast, synonymous SNPs are more common in human genome but do not produce altered coding sequences. Their characterization is relatively difficult. Recently, studies have revealed that synonymous SNPs can also modulate gene expression by altering mRNA secondary structure (Nackley *et al*, 2006) or influence protein function by altering protein conformation in the presence of a rare codon marked by the polymorphism (Kimchi-Sarfaty *et al*, 2007). Our data in this report thus provide the new evidence of synonymous SNPs as potential functional variants in human genetics. However, although we demonstrated the differential expression of rs3003609 allele-specific *DNM1*, subsequently we detected no significant difference in their mRNA stability or half-life. We reasoned that endogenous expressed dynamin 1, exclusively confined in neurons (Praefcke and McMahon, 2004), might interfere with our results, despite that we employed an exogenous gene-specific *Taq*Man probe for mRNA quantification and an antibody against HA tag for protein detection. Nonetheless, further study is required to determine how polymorphism rs3003609 modulates the expression of *DNM1*.

In summary, we provided the first evidence that *DNM1* is associated with ND, more significant in the EA sample than in the AA sample. Further, we demonstrated that SNP rs3003609 at exon 9 represents a functional variant, of which the C allele causes greater *DNM1* expression at both the mRNA and protein levels than does the T allele. On the basis of these findings, along with our previous findings of nicotine-modulated dynamin 1 expression in the brain regions (Hwang *et al*, 2006) and the well-known function of dynamin 1 in neuronal activities and plasticity (Ferguson *et al*, 2007; Praefcke *et al*, 2004), we conclude that dynamin 1 is an important player in the etiology of ND. Further genetic association study of this gene in other independent samples is warranted.

Acknowledgments

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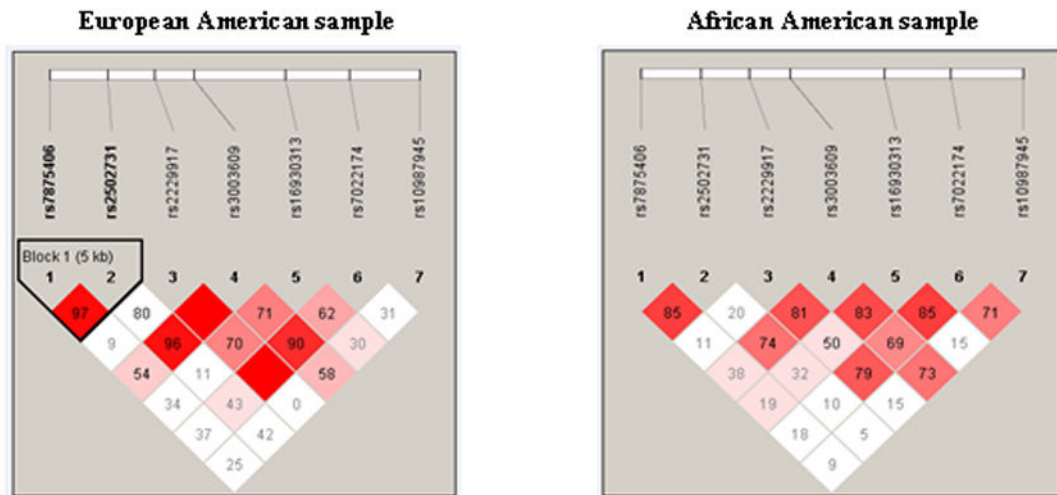
Figure 1.
Haploview-generated LD maps of the seven SNPs within *DNM1* in the EA and AA samples. Regions of high LD (D′ = 1 and LD >2) are shown in dark gray. Markers with LD (0.21 < D′ < 1 and LOD >2) are shown in dark through light gray, with the color intensity decreasing with decreasing D′ values. Regions of low LD and low LOD scores (LOD <2) are shown in white. The number within each box indicates the D′ statistic between the two SNPs. Haplotype blocks in the two samples were produced by the HaploView program using the block definitions proposed by Grabiel et al.
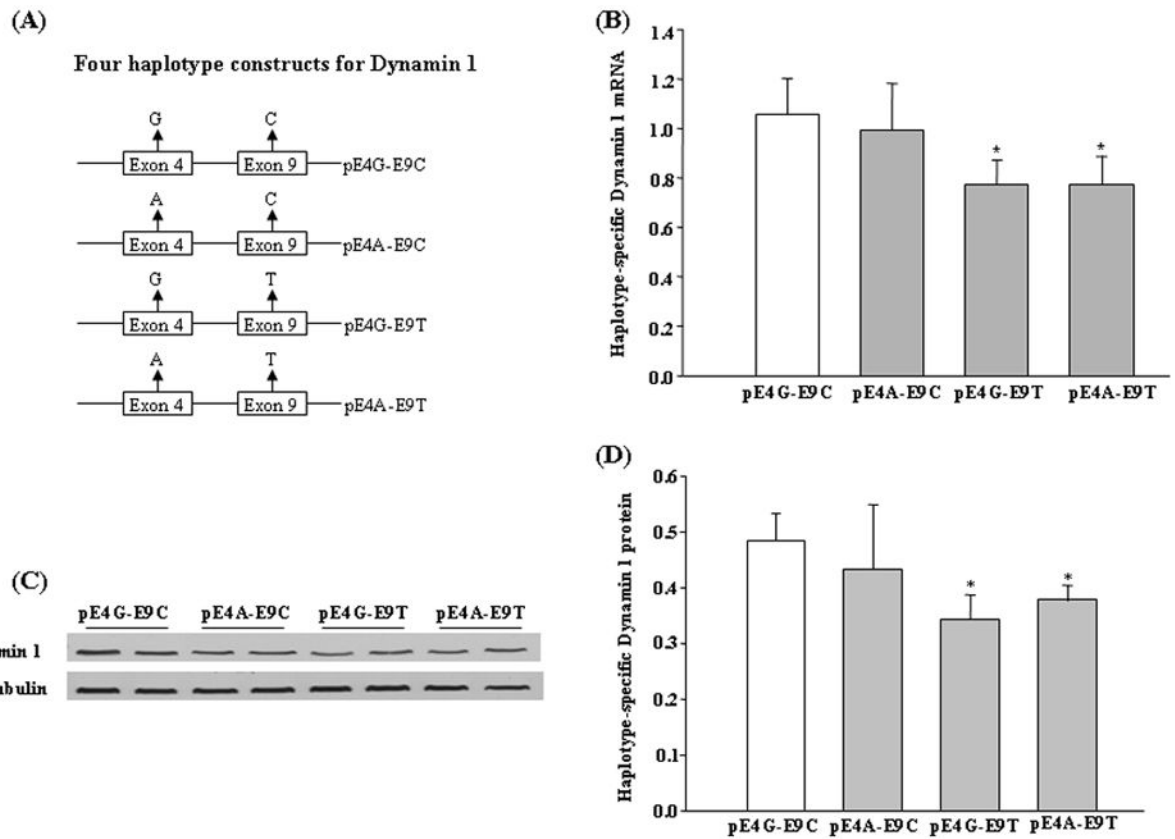
Figure 2.
Expression analyses of allele combinations of SNPs rs2229917 at exon 4 and rs3003609 at exon 9. (A) Illustration of four plasmids constructed for dynamin 1 expression analysis. (B) Statistical analysis of real-time RT-PCR data for the four plasmids. 18S RNA was used to normalize expression of each *DNM1* construct. Compared with pE4G-E9C, pE4G-E9T and pE4A-E9T showed lower expression of dynamin 1, by 27.1% and 26.8%, respectively. No significant difference was detected between constructs pE4G-E9C and pE4A-E9C. (C) Representative Western blotting images for dynamin 1 and tubulin. Tubulin was used to normalize expression of each *DNM1* construct. (D) Statistical analysis of the protein expression levels of four *DNM1* constructs. Similar to the mRNA expression data in (C), pE4G-E9T and pE4A-E9T showed decreased dynamin 1 protein expression, by 29.2% and 22.2%, respectively, compared with pE4G-E9C. Data in Figures B and D are given as means ± S.E.M. (* *P* <0.05; n = 3/group).
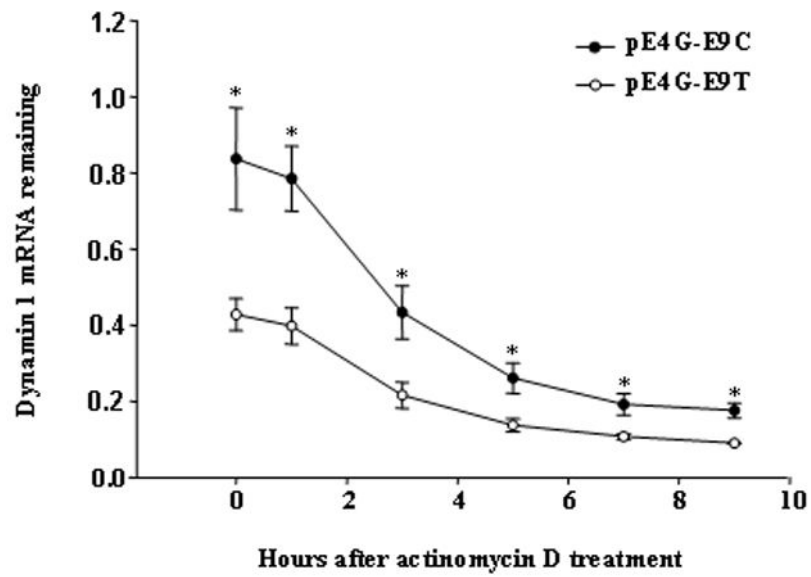
Figure 3.
Comparison of mRNA stability of *DNM1* from pE4G-E9C and pE4G-E9T constructs.
Measured half-life of mRNA was about 3.2 hours for the former and 3.6 hours for the latter.
No significant difference in the half-life of *DNM1* mRNA was found in the two constructs.
Data are given as means ± S.E.M. (* P <0.05; n = 3/group).

Table 1

Information of seven SNPs within *DNM1* selected for this study

SNP number	dbSNP ID	SNP location	Chromosome position	Allele	Frequency (EA)*	Frequency (AA)*	Sequences of *Taq*Man primers and MGB probes (5′→3′)
1	rs7875406	Intron 2	130010839	A/G	0.96/0.04	0.69/0.31	F: GGAGGCTTGCGGCTGAT R: AGCCTCCTTCAAAATACACGACAA P₁: AAATCCACAAAGAGCACGT P₂: ATCCACAAAGGGCACGT
2	rs2502731	Intron 2	130016378	T/C	0.72/0.28	0.51/0.49	F: TGCCCAGCAAGCTATCGTTATTTAT R: ACATCAGTAAAATGGGTTGCTGTGA P₁: TTCAGCCATCAGTCTGTATA P₂: TCAGCCATCAGTTTGTATA
3	rs2229917	Exon 4	130020758	G/A	0.91/0.09	N/A	F: GGAGGTGCGCCTTGAGATC R: GCGAGATGCCCTTGTTGGT P₁: CCTGTCGGTTTCGGCCT P₂: CTGTCGGTCTCGGCCT
4	rs3003609	Exon 9	130024576	T/C	0.62/0.38	0.01/0.99	F: ACCACTCTCCCACCAGGAT R: AGCCCTCAATGCGCTTCT P₁: CAAAGTCTACGGCGAACT P₂: AAAGTCTACGGCAAACT
5	rs16930313	Intron 11	130033218	A/G	0.88/0.12	0.54/0.46	F: AGGCATGCGCCACCAT R: CCTCCTTTTAGACCAAAAGCTTTCCT P₁: TCTGTGTGATTTTTATGAGCA P₂: CTGTGTGATTTTTGTGAGCA
6	rs7022174	Intron 12	130039293	G/C	0.79/0.21	0.13/0.87	F: TTGTCCCATCTGGAAAGTCATTCTC R: GACCCTTGCAAACGATTAAAATGTGA P₁: ATCACTACTCAATTCC P₂: TCACTACTGAATTCC
7	rs10987945	Intron 16	130045847	G/T	0.96/0.04	0.34/0.66	F: GCCAGGGAGACCATGTGA R: CCCCTCCCCCACAGTGT P₁: CGGTCTCCGGCCCT P₂: CGGTCTCAGGCCCT

Table 2

Allele frequency and *P*-value of individual SNPs for association with three ND measures in the EA and AA samples

dbSNP ID	Allele	EA sample				AA sample			
		Frequency	SQ	HSI	FTND	Frequency	SQ	HSI	FTND
rs7875406	A/G	0.63/0.37	0.10^d	0.10^d	0.19^d	0.53/0.47	0.42^d	0.44^r	0.56^r
rs2502731	T/C	0.63/0.37	0.08^a	0.11^a	0.21^a	0.44/0.56	0.43^d	0.46^d	0.42^a
rs2229917	G/A	0.94/0.06	0.05^r	0.04^d	0.05^r	0.78/0.22	0.69^a	0.77^d	0.60^r
rs3003609	T/C	0.55/0.45	$\mathbf{0.0031^d}$	$\mathbf{0.0042^d}$	0.011^d	0.11/0.89	0.07^d	0.13^d	0.12^d
rs16930313	A/G	0.86/0.14	0.04^d	0.05^d	0.14^d	0.64/0.36	0.22^r	0.31^r	0.50^r
rs7022174	G/C	0.79/0.21	0.39^r	0.21^r	0.32^r	0.29/0.71	0.10^d	0.12^d	0.08^d
rs10987945	G/T	0.95/0.05	0.25^a	0.34^a	0.32^a	0.50/0.50	0.43^d	0.73^a	0.62^d

Notes:

[1] Significant *P*-value after correction for multiple testing is given in bold and the adjusted *P*-value at the 0.05 significance level is 0.007 for both the EA and AA samples. Given that the three genetic models and three ND measures are so highly related to each other, we feel that it is too conservative to apply a Bonferroni correction to these comparisons. However, if one wishes to correct for them, the adjusted P value at the 0.05 significance level becomes 0.0012 for both the samples. This approach can be applied to Table 3 as well.

[2] Superscripts indicate genetic models used for analysis: a = additive; d = dominant; and r = recessive.

[3] Age and sex were used as covariates in the analyses for both the EA and AA samples.

Table 3

P- and Z-values of haplotypes that are significantly associated with three ND measures in the EA and AA samples

Haplotype 1	2	3	4	5	6	Freq.	SQ P-value	Z-value	Global P-value	No. of families	HIS P-value	Z-value	Global P-value	No. of families	FTND P-value	Z-value	Global P-value	No. of families
EA sample																		
T	G		T			0.54	**0.0058**[a]	−2.76[a]	0.015[a]	79[a]	**0.0079**[a]	−2.65[a]	0.027[a]	80[a]	0.31[a]	1.02[a]	0.15[a]	71[a]
							0.013[d]	−2.49[d]	0.12[d]	45[d]	0.033[d]	−2.13[d]	0.19[d]	48[d]				
		G	T	A		0.52	**0.015**[a]	−2.44[a]	0.11[a]	82[a]	0.018[a]	−2.36[a]	0.13[a]	83[a]	0.031[d]	−2.15[d]	0.092[d]	51[d]
							0.0035[d]	−2.92[d]	0.033[d]	51[d]	**0.0076**[d]	−2.67[d]	0.056[d]	51[d]				
			C	A	G	0.24	0.033[a]	2.41[a]	0.14[a]	64[a]	0.031[a]	2.13[a]	0.10[a]	65[a]	0.046[a]	1.97[a]	0.25[a]	65[a]
			T	A	G	0.52	**0.0071**[a]	−2.69[a]	0.14[a]	87[a]	**0.0078**[a]	−2.66[a]	0.10[a]	88[a]	0.047[a]	−2.00[a]	0.25[a]	88[a]
							0.0041[d]	−2.87[d]	0.07[d]	60[d]	**0.012**[d]	−2.52[d]	0.078[d]	61[d]	0.049[d]	−1.98[d]	0.19[d]	61[d]
AA sample																		
G	T	A				0.12	**0.0098**[r]	−2.58[r]	0.11[r]	16[r]	0.037[r]	−2.09[r]	0.31[r]	16[r]	0.05[r]	−1.96[r]	0.34[r]	16[r]
T	A	C				0.15	0.014[r]	−2.46[r]	0.062[r]	18[r]	0.052[r]	−1.94[r]	0.28[r]	18[r]	0.090[r]	−1.69[r]	0.36[r]	18[r]

Notes:

[1] Only the major haplotypes with P-value < 0.05 in at least one ND measure are shown

[2] At the 0.05 significance level, significant P-value after Bonferroni correction for three major haplotypes in the EA sample is 0.0167; for five major haplotypes in the AA sample is 0.01.

[3] Superscripts indicate the genetic models used in the analysis: a = additive; d = dominant; and r = recessive.

[4] Age and sex were used as covariates in both the EA and AA samples.