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Linkage disequilibrium analyses of natriuretic peptide precursor B locus reveal risk haplotype conferring high plasma BNP levels.

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A short title: NPPB SNP and plasma BNP

4 Tables and 3 Figures

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## ABSTRACT

**Background:** Brain natriuretic peptide (BNP) has been widely used for the diagnosis and prognostic evaluation of chronic heart failure (CHF). In the present study, we performed association study of single nucleotide polymorphisms (SNPs) surrounding the natriuretic peptide precursor B (NPPB) gene with plasma BNP levels in 2970 adult Japanese. **Methods and Results:** Association analysis between SNPs of the NPPB gene and plasma BNP revealed significant associations of the 8 SNPs surrounding the entire NPPB gene with plasma BNP levels. For instance, as to SNP rs198389 (T-381C), plasma BNP levels among the three genotypic categories, i.e., 2189 homozygous T-allele carriers (BNP  $26.4 \pm 0.6$  pg/ml), 697 heterozygous carriers ( $35.0 \pm 1.1$  pg/ml) and 52 homozygous C-allele carriers ( $46.0 \pm 4.1$  pg/ml) indicated a co-dominant effect of the minor C-allele on elevating plasma BNP levels ( $P < 0.0001$ ). Linkage disequilibrium (LD) analysis among the 8 SNPs revealed that the region consisted of two, 5' major and 3' minor, LD blocks. Haplotype-based association analysis demonstrated that plasma BNP levels were associated closely with the haplotypes-1 and -2 of the major LD block. **Conclusion:** These results suggest that genetic variation at the primary locus NPPB gene, represented by definition of risk haplotypes, may be an important determinant of plasma BNP levels.

**Key words:** BNP; NPPB; SNP

## INTRODUCTION

Despite the significant reduction in mortality achieved in clinical trials, chronic heart failure (CHF) patients still have a poor prognosis (1). The role of cardiac biomarkers in the evaluation and risk stratification of patients presenting to the hospital with possible heart failure continues to increase in importance (2). Biomarkers can serve to confirm the diagnosis in symptomatic patients, help in the assessment of patients who present with nonspecific symptoms, and evaluate prognosis of patients. Brain natriuretic peptide (BNP) is a 32-amino acid protein secreted from cardiac ventricles in response to mechanical overload (3). BNP plays an important role in regulating blood pressure and body fluid volume. In addition, the diagnostic and prognostic value of plasma BNP levels in CHF is supported in many studies (4-6). Plasma BNP level monitoring has been proposed for the treatment optimization in patients with CHF (7). However, the actual mechanisms that control this cardiac hormone have not yet been clarified.

Single nucleotide polymorphism (SNP) represents the most frequent type of human population DNA variation (8, 9). The world wide Human Genome Project and HapMap project have revealed the existence of numerous numbers of SNPs and ethnic differences of frequencies of SNPs among races in human population (10, 11). One of the main goals of

SNP research is to understand the genetics of the human phenotype variation (12).

In this study, we found significant association of 8 polymorphisms surrounding the natriuretic peptide precursor B (NPPB) gene including T-381C polymorphism in the promoter region with plasma BNP levels in a large general population of Japanese adults. We also evaluated linkage disequilibrium (LD) and haplotype structure within this gene.

## METHODS

### *Subjects*

This study is a part of the ongoing molecular epidemiological study utilizing the regional characteristics of the 21<sup>st</sup> Century Centers of Excellence (COE) program in Japan. The survey population in the present study is the general population over 40 years old in Takahata Town, Yamagata, Japan as described previously (13). Briefly, from 2004 to 2005, 2970 subjects (1343 males and 1626 females, mean age  $63.0 \pm 10.2$  years) participated in the program and agreed to join the study (Table 1). The study protocol was approved by the Institutional Ethical Committee. All subjects gave written informed consent.

### *SNP typing*

Analysis of genetic polymorphisms SNP genotyping was performed by Invader assay (Third Wave Technologies, Madison, WI, USA) (14, 15) and TaqMan Allelic discrimination assay (16). Reagents were purchased from Applied Biosystems (Foster City, CA, USA). TaqMan probes were designed and synthesized by Applied Biosystems, and distinguish the SNPs at the end of a polymerase chain reaction. One allelic probe was labeled with the fluorescent FAM dye and the other with the fluorescent VIC dye. Polymerase chain reaction was performed by TaqMan Universal Master Mix with polymerase chain reaction primers at

concentrations of 225 nM and TaqMan MGB probes at concentrations of 50 nM. Reactions were performed in 382-well formats in a total reaction volume of 3  $\mu$ L using 3.0 ng of genomic DNA. The plates were then placed in a GeneAmp PCR system 9700 (Applied Biosystem) and heated at 95 °C for 10 min, followed by 40 cycles at 92 °C for 15 sec and at 60 °C for 1 min, with a final soak at 25 °C. The plates were read by the Prism 7900HT instrument (Applied Biosystems) where the fluorescence intensity in each well of the plate was read. Fluorescence data file from each plate were analyzed by the SDS 2.0 allele calling software (Applied Biosystems). Several data (signal intensity) were eliminated to preserve the reliability of the assay system (missing data were less than 1.1%).

#### ***Assay of plasma BNP levels***

Blood samples were obtained for measurements of plasma concentrations of BNP. These samples were transferred to chilled tubes containing 4.5 mg of ethylenediaminetetraacetic acid disodium salt (Na-EDTA) and aprotinin (500 U/ml), and centrifuged at 1,000 *g* for 15 min at 4 °C. The clarified plasma samples were frozen, stored at −70 °C and thawed just before assays (17). BNP concentrations were measured using a commercially available specific radioimmunoassay for human BNP (Shiono RIA BNP assay kit, Shionogi Co. Ltd, Tokyo, Japan). The analytical range of assay was 4-2000 pg/ml.

*Statistical analysis*

The Hardy-Weinberg's equilibrium of alleles at the individual loci was evaluated using a chi-square test ( $P > 0.05$ ) as implemented in R 2.5 package "genetics" (<http://www.r-project.org/>). The association between the genotypes and plasma BNP levels was tested by regression analysis and analysis of variance (ANOVA) using SPSS version 15.0.1J (SPSS, Inc., Chicago IL, USA). Linkage disequilibrium (LD) for all possible two-way combinations of the SNPs was tested with  $D'$  and  $r^2$  by using Haploview Ver. 3.32 (18). Haplotypes were inferred, and haplotype frequencies were estimated using the modified expectation-maximization (EM) method of haplotype inference included in haplo.stats program (19). Haplotype-based association with clinical traits was performed using the haplo.stats program, output global score statistics and haplotype-specific scores derived from generalized linear models. In covariance, we estimated the relation genotype or haplotype and plasma BNP levels adjusted for age. The statistical significance was assigned at P value of less than 0.05.



## RESULTS

### *Association of polymorphisms at NPPB with plasma BNP levels*

To examine the relationship between the NPPB locus and plasma BNP levels, we performed association analysis with 8 SNPs that display minor allele frequencies  $> 0.1$  (Figure 1). Nature of each SNP is summarized in Table 2. No deviation of genotypic frequencies from Hardy-Weinberg's equilibrium was observed ( $P < 0.05$ ). We observed extremely significant association ( $P < 0.0001$ ) of all the 8 SNPs at the NPPB gene with plasma BNP levels when the individuals were categorized into three groups for each of the SNPs (Table 3). For instance, as to the promoter SNP rs198389 (T-381C), plasma BNP levels among the three genotypic categories, i.e., 2189 homozygous T-allele carriers (BNP  $26.4 \pm 0.6$  pg/ml), 697 heterozygous carriers ( $35.0 \pm 1.1$  pg/ml) and 52 homozygous C-allele carriers ( $46.0 \pm 4.1$  pg/ml) indicated a co-dominant effect of the minor C-allele on elevating plasma BNP levels ( $P < 0.0001$ ) as shown in Figure 2.

### *LD and haplotype structure at the NPPB locus*

We investigated LD for all possible two-way comparisons among the 8 selected SNPs in NPPB. Analysis of  $D'$  revealed two highly structured LD blocks ( $|D'| > 0.8$ ), a major LD block (SNPs rs6676300, rs198389, rs198388, rs6668352, rs198375, and rs632793) and a

minor LD block (SNPs rs5063 and rs198358) as shown in Figure 3. Haplotypes were constructed for the major LD block on the basis of genotypes for all sequence variations spanning the region, and haplotype frequencies in each of the three genotypic populations were estimated using the EM algorithm with phase-unknown samples. Two common haplotypes (frequency more than 5%) were observed within the region (Table 4). A haplotype-1 consisting of TTGCAT from SNP1 to SNP6 showed significantly negative haplotype-specific score (-6.2576,  $P < 0.0001$ ), suggesting that this haplotype was inversely correlated with plasma BNP levels. In contrast, haplotypes-2 carrying the identical genotypic combination CCATGC from SNP1 to SNP6 appears to be the haplotypes responsible for risk of elevating effect on plasma BNP levels positive (haplotype specific score 7.0991,  $P < 0.0001$ ).

## DISCUSSION

In the present study, both single SNP analysis and haplotype-based analysis revealed that the population having the NPPB SNPs is associated with plasma BNP levels. Mean BNP levels were  $26.4 \pm 0.6$ ,  $35.0 \pm 1.1$ , and  $46.0 \pm 4.1$  pg/ml in population having NPPB rs198389 T/T, T/C, and C/C ( $P < 0.0001$ ), respectively. LD profiles among SNPs revealed that the region analyzed consisted of 2 LD blocks. Haplotype-based association analysis demonstrated that plasma BNP levels were associated closely with the haplotypes 'TTGCAT' and 'CCATGC' in the major LD block.

It has been reported that plasma BNP levels are increased with aging (20) and inversely correlated with body mass index (BMI) in individuals with (21) and without (22) CHF. Plasma BNP concentrations are higher in women than in men (23). In the present study, we showed that the NPPB gene is a key source of inter-individual variation in plasma BNP levels. The fact that the strongest relationship was seen with a 5' flanking variant suggests that this effect may be due to differences in transcriptional regulation. The regulatory regions of the NPPB gene have been previously reported (24). Although the T-381C variant (rs198389) does not fall into verified regulatory sequences, it is in close proximity to known transcriptional factor binding sites. Recently, it has been reported that -381C allele was associated with higher BNP promoter activity in reporter gene assays (25).

Meirhaeghe et al. have assessed T-381C polymorphism (rs198389) and risk of type 2 diabetes. They found that individuals bearing the -381CC genotype had lower fasting glucose levels than -381TC or -381TT individuals in European study population (25). Moreover, the -381CC genotype was less frequent in individuals with type 2 diabetes (13.6%) or with impaired fasting glucose (12.9%) compared to normoglycemic individuals (17.8%). On the other hand, it has been reported that plasma BNP levels are higher in individuals with essential hypertension than in normotensive individuals (26). Therefore, Kosuge et al. assessed the relationship between polymorphisms in the NPPB gene and essential hypertension (27). They found that the 16-repeat allele of the variable number of tandem repeat polymorphism in the 5'-flanking region of NPPB is associated with essential hypertension among Japanese female.

Our data have potential implications for diagnostic and prognostic values of plasma BNP levels. The fact that variants in NPPB gene are associated with alterations in plasma BNP levels suggests that these variants may change the predictive abilities of BNP for diagnosing heart failure, determining prognosis, and responses to optimal medical therapy. Genetic analyses may be able to explain the wide variability of plasma BNP levels in the study population and better define the clinical utility of this important biomarker. Further study is necessary to define the impact of genetic variation on the clinical interpretation of plasma BNP levels.

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## FIGURE LEGENDS

**Figure 1.** The 8 polymorphisms in the NPPB gene with surrounding polymorphisms examined in the present study.

**Figure 2.** Comparisons of plasma BNP levels among genotypically determined groups according to one NPPB variation, rs198389.

**Figure 3.** Major haplotypes of the major LD block of the NPPB locus consisting of 6 SNPs.

**Table 1. Baseline clinical characteristics of the study population.**

|                          | n=2970       | Male (n=1343) | Female (n=1626) |
|--------------------------|--------------|---------------|-----------------|
| Age (year)               | 63.0 ± 10.2  | 63.4 ± 10.4   | 62.7 ± 10.1     |
| height (cm)              | 156.6 ± 9.0  | 163.4 ± 6.9   | 151 ± 6.2       |
| weight (kg)              | 57.8 ± 10.2  | 62.8 ± 9.8    | 53.7 ± 8.6      |
| BMI (kg/m <sup>2</sup> ) | 23.5 ± 3.2   | 23.5 ± 2.9    | 23.5 ± 3.4      |
| SBP (mm Hg)              | 134.2 ± 15.8 | 136.0 ± 15.5  | 132.7 ± 16.0    |
| DBP (mm Hg)              | 79.3 ± 10.0  | 81.8 ± 9.8    | 77.3 ± 9.8      |
| BNP (pg/ml)              | 28.8 ± 31.0  | 28.1 ± 34.2   | 29.3 ± 27.9     |

\*The sex of one subject was unknown and was excluded from analysis.

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; BNP, brain natriuretic polypeptide.

Values are presented as mean ± SD.

**Table 2. The 8 polymorphisms in the NPPB gene with surrounding polymorphisms examined in the study.**

| SNPID | Gene<br>Symbol | NCBI SNP<br>Reference | SNP Type        | Chr | Public Location<br>Position (B36.2) | Allele 1 | Allele 2 | Allele frequency |             | Genotype |     |    |                |
|-------|----------------|-----------------------|-----------------|-----|-------------------------------------|----------|----------|------------------|-------------|----------|-----|----|----------------|
|       |                |                       |                 |     |                                     |          |          | Allele<br>1      | Allele<br>2 | 11       | 12  | 22 | Heterozygosity |
| SNP1  | -              | rs6676300             | Intergenic      | 1   | 11798170                            | T        | C        | 0.86             | 0.14        | 2158     | 722 | 66 | 0.248          |
| SNP2  | NPPB           | rs198389              | Promoter        | 1   | 11792217                            | T        | C        | 0.86             | 0.14        | 2189     | 697 | 52 | 0.236          |
| SNP3  | NPPB           | rs198388              | 3' near gene    | 1   | 11790644                            | G        | A        | 0.86             | 0.14        | 2191     | 712 | 57 | 0.240          |
| SNP4  | -              | rs6668352             | Intergenic      | 1   | 11789021                            | C        | T        | 0.87             | 0.13        | 2267     | 641 | 51 | 0.220          |
| SNP5  | -              | rs198375              | Intergenic      | 1   | 11786566                            | A        | G        | 0.84             | 0.16        | 2081     | 808 | 76 | 0.271          |
| SNP6  | -              | rs632793              | 3'-untranslated | 1   | 11784631                            | T        | C        | 0.86             | 0.14        | 2212     | 701 | 52 | 0.235          |
| SNP7  | NPPA           | rs5063                | Coding          | 1   | 11780518                            | C        | T        | 0.91             | 0.09        | 2451     | 495 | 18 | 0.163          |
| SNP8  | -              | rs198358              | Intergenic      | 1   | 11777433                            | T        | C        | 0.89             | 0.11        | 2356     | 582 | 29 | 0.192          |

NCBI, National Center for Biotechnology Information; NPPA, natriuretic peptide precursor A; NPPB, natriuretic peptide precursor B; Chr, chromosome; Allele 1, major allele; Allele 2, minor allele.

**Table 3. The association analysis of each SNP examined in the study with the plasma BNP level using parametric measure.**

| SNP ID | NCBI reference ID | ANOVA          | Post-hock (LSD) <i>p</i> value |           |           | mean $\pm$ SE  |                |                | genotype |     |     |
|--------|-------------------|----------------|--------------------------------|-----------|-----------|----------------|----------------|----------------|----------|-----|-----|
|        |                   | <i>p</i> value | 11 vs. 12                      | 11 vs. 22 | 12 vs. 22 | 11             | 12             | 22             | 11       | 12  | 22  |
| SNP1   | rs6676300         | < 0.0001       | < 0.0001                       | < 0.0001  | 0.019     | 26.4 $\pm$ 0.6 | 34.5 $\pm$ 1.1 | 43.4 $\pm$ 3.6 | T/T      | T/C | C/C |
| SNP2   | rs198389          | < 0.0001       | < 0.0001                       | < 0.0001  | 0.010     | 26.4 $\pm$ 0.6 | 35.0 $\pm$ 1.1 | 46.0 $\pm$ 4.1 | T/T      | T/C | C/C |
| SNP3   | rs198388          | < 0.0001       | < 0.0001                       | < 0.0001  | 0.004     | 26.5 $\pm$ 0.6 | 34.0 $\pm$ 1.1 | 45.6 $\pm$ 3.9 | G/G      | G/A | A/A |
| SNP4   | rs6668352         | < 0.0001       | < 0.0001                       | < 0.0001  | 0.001     | 26.9 $\pm$ 0.6 | 33.9 $\pm$ 1.2 | 48.0 $\pm$ 4.1 | C/C      | C/T | T/T |
| SNP5   | rs198375          | < 0.0001       | < 0.0001                       | < 0.0001  | 0.031     | 26.7 $\pm$ 0.6 | 33.0 $\pm$ 1.0 | 40.6 $\pm$ 3.3 | A/A      | A/G | G/G |
| SNP6   | rs632793          | < 0.0001       | < 0.0001                       | < 0.0001  | 0.003     | 26.8 $\pm$ 0.6 | 33.8 $\pm$ 1.1 | 46.0 $\pm$ 4.0 | T/T      | T/C | C/C |
| SNP7   | rs5063            | < 0.0001       | < 0.0001                       | NS        | NS        | 27.4 $\pm$ 0.6 | 35.0 $\pm$ 1.3 | 37.6 $\pm$ 6.8 | C/C      | C/T | T/T |
| SNP8   | rs198358          | < 0.0001       | < 0.0001                       | NS        | NS        | 27.4 $\pm$ 0.6 | 33.7 $\pm$ 1.2 | 36.5 $\pm$ 5.4 | T/T      | T/C | C/C |

Abbreviations as in Table 2.

**Table 4. Association of major haplotypes (frequency more than 5%) comprised from SNP1 to SNP6 with the plasma BNP levels.**

| Haplotypes |           |          |          |           |          |          | Frequencies | Haplotype-Specific<br>Score Statistic | p-value  |
|------------|-----------|----------|----------|-----------|----------|----------|-------------|---------------------------------------|----------|
|            | SNP1      | SNP2     | SNP3     | SNP4      | SNP5     | SNP6     |             |                                       |          |
|            | rs6676300 | rs198389 | rs198388 | rs6668352 | rs198375 | rs632793 |             |                                       |          |
| Hap-1      | T         | T        | G        | C         | A        | T        | 0.8154      | -6.2576                               | < 0.0001 |
| Hap-2      | C         | C        | A        | T         | G        | C        | 0.1155      | 7.0991                                | < 0.0001 |

Global Score Statistics      Global Statistics 52.03      df = 2      Global p-value < 0.0001

# Chromosome 1

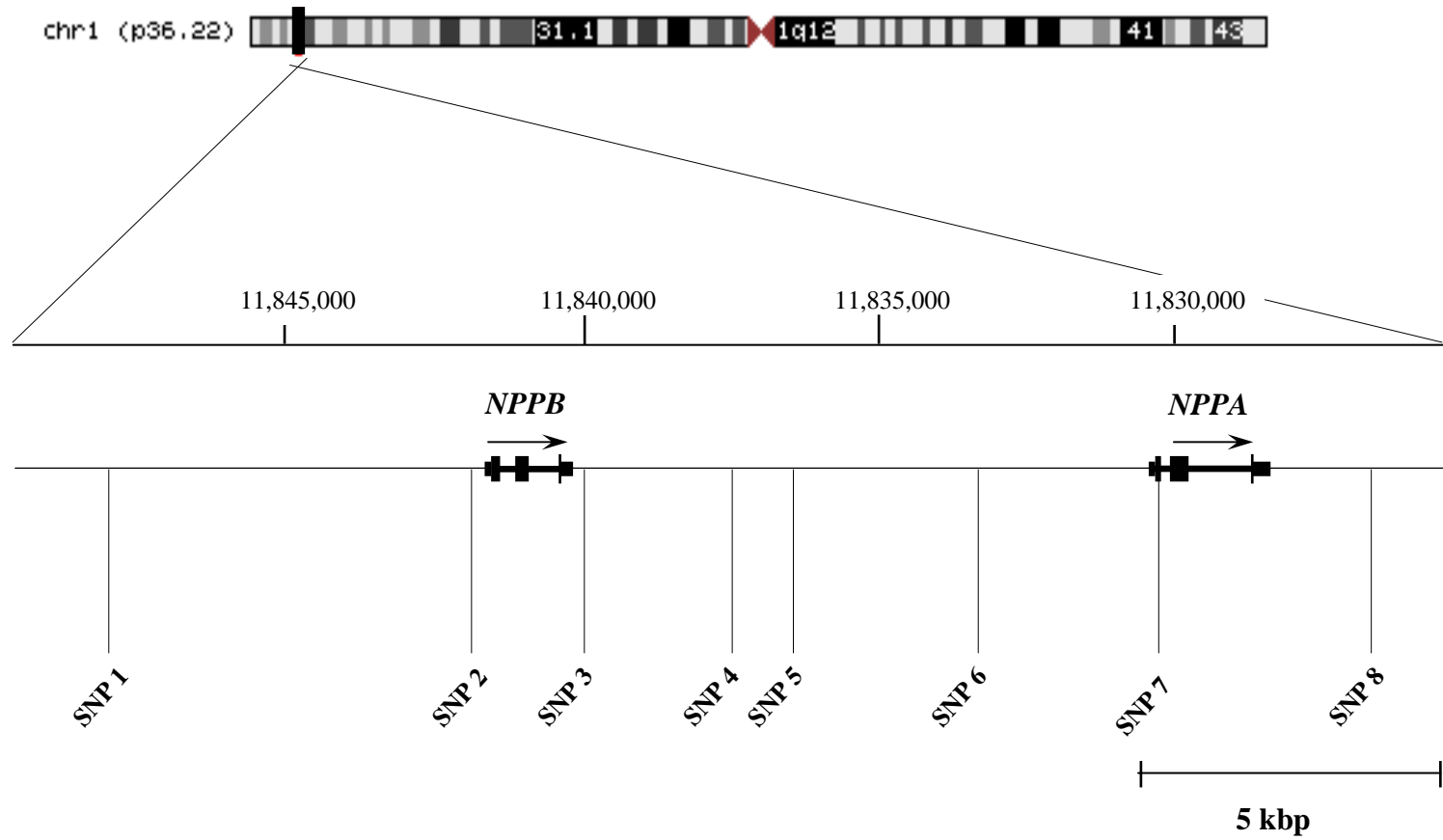


Figure 1



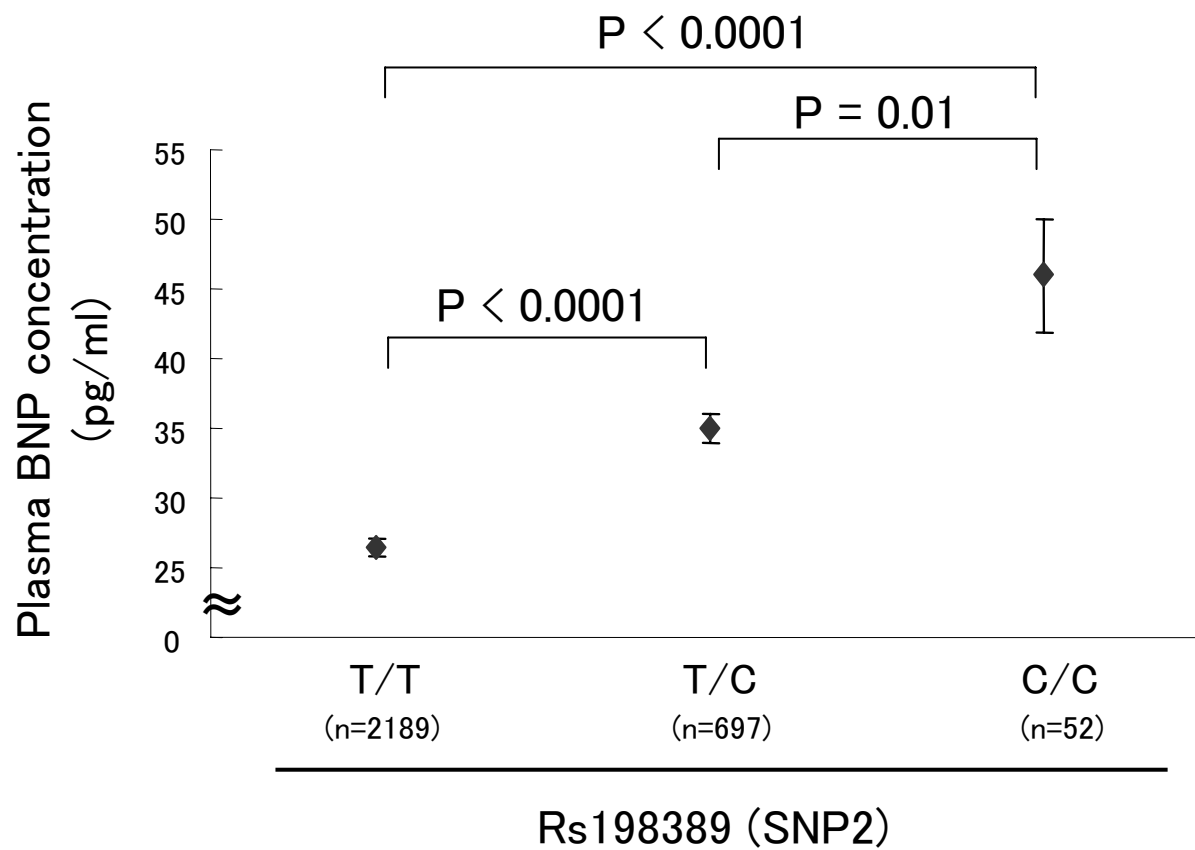


Figure 2

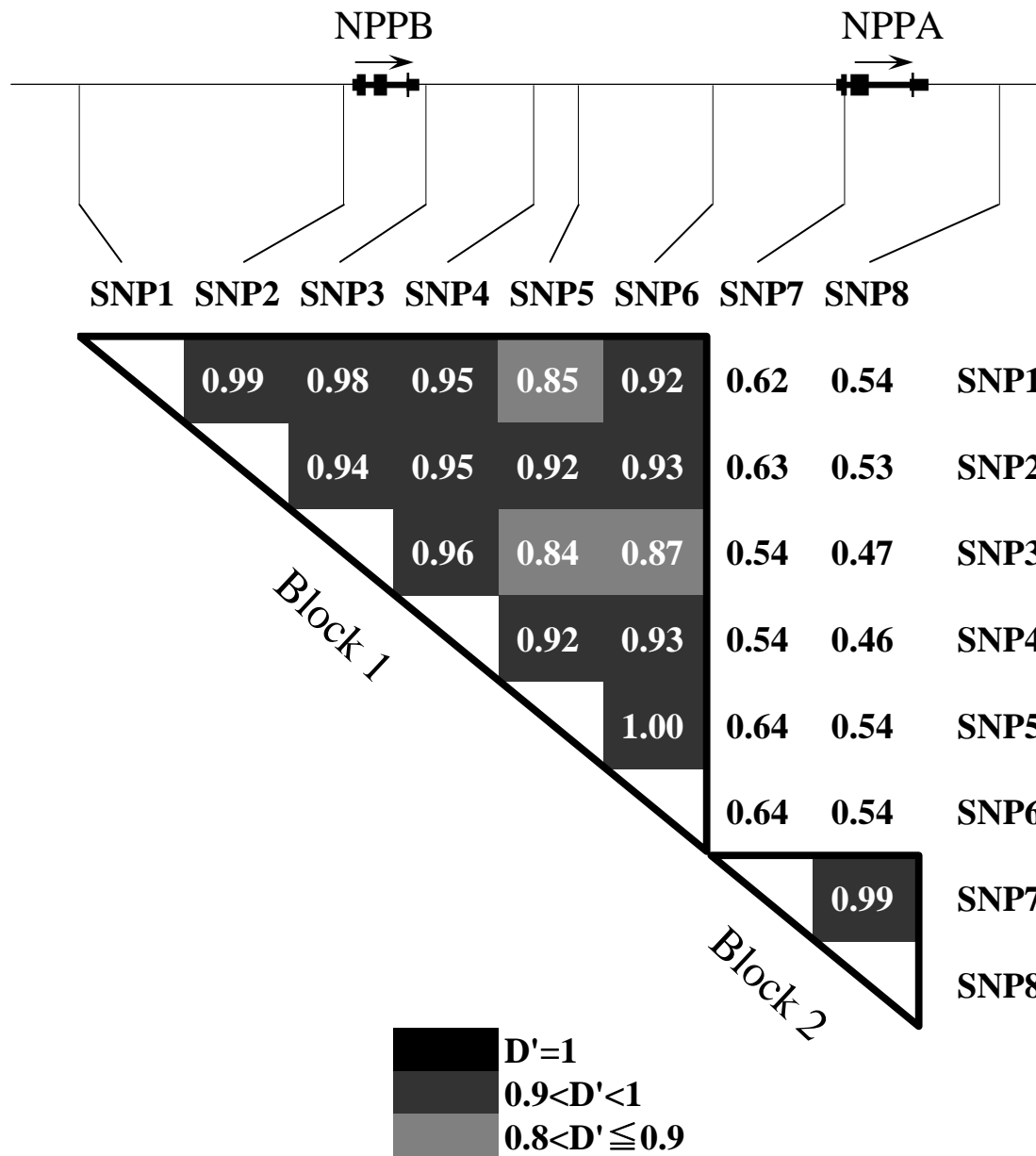


Figure 3