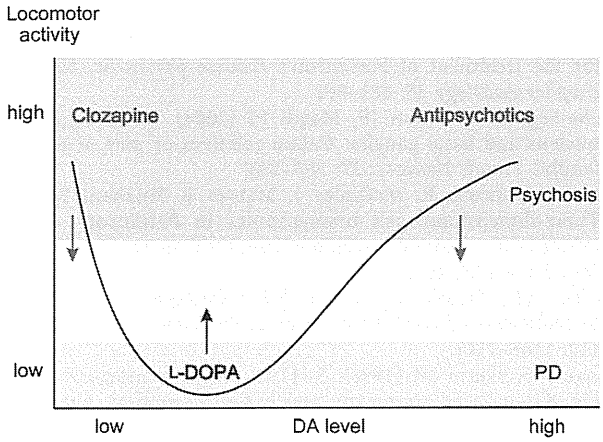


**Figure 5** Illustrations showing possible relationships between locomotor activity and DA levels. Hyperlocomotion that occurs at high DA levels is ameliorated by antipsychotic drugs. Hypolocomotion that occurs at low DA levels is ameliorated by L-DOPA. Hyperlocomotion that occurs at extremely low DA levels is ameliorated by clozapine. Hyperlocomotion would be related to psychosis, and hypolocomotion would be related to PD.

in DD mice. DA depletion that is induced by α-methyl-*p*-tyrosine and reserpine was shown to decrease basal ACh release in the striatum (Bertorelli *et al*, 1992). In addition, ChAT activity has been reported to be normal or reduced in the striatum in PD patients (Hornykiewicz and Kish, 1987). Striatal cholinergic interneuron activity and ACh release were also reported to be modulated by DA neurons (Pisani *et al*, 2007). These previous results support the present finding that the ACh neurotransmission system is suppressed when the DA system becomes dysfunctional.

Dysfunctions in the interactions between DA and ACh may have important implications for various neurological and neuropsychiatric disorders (Lester *et al*, 2010). A reduction of dopaminergic input to the striatum causes relative cholinergic overactivity that in turn increases inhibitory output from the basal ganglia to thalamus, resulting in impaired motor function in PD (Graybiel, 2005). Motor function in PD patients is improved by anticholinergic drugs. In DD mice, hyperactivity was ameliorated by stimulation of the cholinergic system. Therefore, ACh may consistently reduce motor activity in PD patients and DD mice. Furthermore, the basal ganglia are interconnected with the pedunculopontine nucleus (PPN; Mena-Segovia *et al*, 2004). The PPN is thought to be involved in the initiation and modulation of gait and other stereotyped movements (Pahapill and Lozano, 2000). The loss of cholinergic neurons in the PPN has been reported in PD patients (Hirsch *et al*, 1987; Rinne *et al*, 2008). Cholinergic neurons in the PPN might also be altered because of low DA in DD mice as well as in PD patients.

DD mice lack DA in all brain regions, and determining the specific brain region responsible for hyperactivity may be difficult only by pharmacological approach. Injection of dopaminergic and cholinergic agents into specific brain regions, molecular and optogenetic approach, or viral rescue experiments may lead to a better understanding of the mechanisms that underlie this hyperactivity.

In conclusion, we investigated locomotion in mice with extremely low levels of DA and observed hyperactivity that is inconsistent in prior theories but consistent with kinesia paradoxia and typical antipsychotic drug-resistant symptoms in schizophrenia. We also pharmacologically found that reduced ACh levels may be involved in this hyperactivity.

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## Author Contributions

YH, KK, HYM, and KI conceived and designed the experiments and wrote the paper. YH performed the microdialysis experiments and the behavioral experiments. SS, HY, and DY performed the kinematic analysis of hindlimb movements. SK performed the gene expression experiments. MF and MH performed immunoblot analysis and immunohistochemistry. KK provided the DD mice.

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RESEARCH ARTICLE

# Prediction Formulas for Individual Opioid Analgesic Requirements Based on Genetic Polymorphism Analyses

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## Abstract

### Background

The analgesic efficacy of opioids is well known to vary widely among individuals, and various factors related to individual differences in opioid sensitivity have been identified. However, a prediction model to calculate appropriate opioid analgesic requirements has not yet been established. The present study sought to construct prediction formulas for individual opioid analgesic requirements based on genetic polymorphisms and clinical data from patients who underwent cosmetic orthognathic surgery and validate the utility of the prediction formulas in patients who underwent major open abdominal surgery.

### Methods

To construct the prediction formulas, we performed multiple linear regression analyses using data from subjects who underwent cosmetic orthognathic surgery. The dependent variable was 24-h postoperative or perioperative fentanyl use, and the independent variables were age, gender, height, weight, pain perception latencies (PPL), and genotype data of five single-nucleotide polymorphisms (SNPs). To examine the utility of the prediction formulas, we performed simple linear regression analyses using subjects who underwent major open abdominal surgery. Actual 24-h postoperative or perioperative analgesic use and the predicted values that were calculated using the multiple regression equations were incorporated as dependent and independent variables, respectively.

### Results

Multiple linear regression analyses showed that the four SNPs, PPL, and weight were retained as independent predictors of 24-h postoperative fentanyl use ($R^2 = 0.145$, $P = 5.66 \times 10^{-10}$) and the two SNPs and weight were retained as independent predictors of perioperative fentanyl use ($R^2 = 0.185$, $P = 1.99 \times 10^{-15}$). Simple linear regression analyses showed

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that the predicted values were retained as an independent predictor of actual 24-h postoperative analgesic use ($R^2 = 0.033$, $P = 0.030$) and perioperative analgesic use ($R^2 = 0.100$, $P = 1.09 \times 10^{-4}$), respectively.

## Conclusions

We constructed prediction formulas, and the possible utility of these prediction formulas was found in another type of surgery.

## Introduction

Opioid analgesics are widely used for the treatment of moderate to severe pain during the perioperative period. However, the analgesic efficacy of opioids is well known to vary widely among individuals [1]. For example, the minimal effective analgesic concentration of fentanyl that is required for satisfactory analgesia varies from 0.2 to 2.0 ng/ml among patients [2]. Thus, effective pain treatment is often hampered by significant differences in opioid sensitivity (S1 Fig.). Inadequate pain relief because of insufficient doses of opioids and adverse side-effects caused by unnecessarily high doses of opioids (e.g., nausea, vomiting, and constipation) are often observed in clinical settings [3]. The proper administration of opioids that meets the needs of individual patients is crucial.

Individual differences can be attributed to both environmental and genetic factors, although the relative influence of each of these factors can be variable [4]. For example, this variation in postoperative pain and analgesic consumption is reportedly affected by environmental factors including hepatic or renal function, type of surgery, and anesthesia method, demographic factors including gender, age, and ethnic origin, and preexisting psychophysical factors including anxiety and preoperative pain [4–6]. Additionally, a recent study that included identical and fraternal twins revealed that an estimated 60% of the variance in cold-pressor pain and 26% of the variance in heat pain were genetically mediated. Thus, some individual differences are likely to be attributable to genetic factors [7]. To date, various candidate genetic polymorphisms related to individual opioid sensitivity have been revealed and in particular several genetic polymorphisms within or close to regions of the *OPRM1* gene that encodes opioid receptor, mu 1, *CACNA1E* gene that encodes calcium channel, voltage-dependent, R type, alpha 1E subunit, *ADRB2* gene that encodes adrenoceptor beta 2, surface, *GIRK2* (*KCNJ6*) gene that encodes potassium inwardly-rectifying channel, subfamily J, member 6, and *CREB1* gene that encodes cyclic adenosine 3',5'-monophosphate responsive element binding protein 1, have been reported to be strongly associated with opioid requirements in patients who underwent cosmetic orthognathic surgery with postoperative pain [8–10].

Although various factors related to individual differences in opioid sensitivity have been identified, a prediction model that calculates appropriate opioid analgesic requirements has not yet been developed. Therefore, the present study sought to construct prediction formulas for individual opioid analgesic requirements based on five genetic polymorphisms described above and clinical data using patients who underwent cosmetic orthognathic surgery. We investigated patients who underwent mandibular sagittal split ramus osteotomy, which is highly standardized at our institute regarding surgical procedures, duration of surgery, and the skill of the surgeons. Because these patients are usually young and healthy and expected to have similar pain after surgery, they may be ideal for evaluating the analgesic effects of opioids [1, 8]. We

also validated the utility of the prediction formulas in another type of surgery, major open abdominal surgery.

## Materials and Methods

### Ethics statement

The study protocol was approved by the Institutional Review Boards at Tokyo Dental College (Tokyo, Japan), the Institute of Medical Science, The University of Tokyo (Tokyo, Japan), Toho University Sakura Medical Center (Sakura, Japan), and the Tokyo Institute of Psychiatry (currently Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). All of the subjects and the parents when the subjects were minors provided informed, written consent for the genetics studies.

### Subjects

Enrolled in the initial study to construct the prediction formulas for individual opioid analgesic requirements were 354 healthy patients (American Society of Anesthesiologists Physical Status I, age 15–52 years, 126 males and 228 females) who were scheduled to undergo cosmetic orthognathic surgery (mandibular sagittal split ramus osteotomy) for mandibular prognathism under general anesthesia at Tokyo Dental College Suidoubashi Hospital. Patients with chronic pain, those taking pain medication, and those who experienced Raynaud's phenomenon were excluded. Peripheral blood samples were collected from these subjects for the gene analysis. The detailed demographic and clinical data of the subjects are provided in Table 1 and S1 Table.

The subjects who were included in the second analysis to examine the utility of the prediction formulas that were constructed based on multiple regression analysis were different 145 patients (American Society of Anesthesiologists Physical Status I or II, age 28–80 years, 83 males and 62 females) who underwent major open abdominal surgery, mostly gastrectomy for gastric cancer and colectomy for colorectal cancer under combined general and epidural anesthesia at the Institute of Medical Science (The University of Tokyo) or Toho University Sakura Medical Center. Peripheral blood or oral mucosa samples were collected from these subjects for the gene analysis. The detailed demographic and clinical data of the subjects are provided in Table 2 and S2 Table.

**Table 1. Demographic and clinical data of the subjects who underwent cosmetic orthognathic surgery.**

| | |
|---|---|
| Age (years) | 25.9 ± 7.6 (15–52) |
| Male/Female | 126/228 |
| Height (cm) | 164.6 ± 8.8 (143–190) |
| Body weight (kg) | 57.7 ± 10.9 (38–128) |
| PPL (s) | 14 [9, 23] (2–150) |
| *OPRM1* (rs9384179) AA/AG, GG | 283/71 |
| *CACNA1E* (rs3845446) AA/AG, GG | 167/187 |
| *ADRB2* (rs11959113) AA, AG/GG | 153/201 |
| *GIRK2* (rs2835859) TT/TC, CC | 305/49 |
| *CREB1* (rs2952768) CC/TC, TT | 35/319 |

The data are expressed as numbers, mean ± SD (range), or median [interquartile range].
PPL, pain perception latency.

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**Table 2. Demographic and clinical data of the subjects who underwent major open abdominal surgery.**

| | |
|---|---|
| Age (years) | 63.8 ± 9.9 (28–80) |
| Male/Female | 83/62 |
| Height (cm) | 158.3 ± 8.5 (133–175) |
| Body weight (kg) | 56.3 ± 10.5 (30–80) |
| *OPRM1* (rs9384179) AA/AG, GG | 117/28 |
| *CACNA1E* (rs3845446) AA/AG, GG | 68/77 |
| *ADRB2* (rs11959113) AA, AG/GG | 63/82 |
| *GIRK2* (rs2835859) TT/TC, CC | 132/13 |
| *CREB1* (rs2952768) CC/TC, TT | 17/128 |

The data are expressed as numbers and mean ± SD (range).

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## General anesthesia and postoperative pain management

For the subjects who underwent cosmetic orthognathic surgery, the surgical protocol and subsequent postoperative pain management were fundamentally the same as in previous studies [8, 9]. The cold pressor-induced pain test was performed before the induction of anesthesia as previously described [11, 12]. Briefly, crushed ice cubes and cold water were blended 15 min before testing in a 1 L isolated tank, and the mixture was stirred immediately before each test to ensure a uniform distribution of temperature (0°C) within the tank. The dominant hand was immersed up to the wrist. The subjects were instructed to keep their hand calm in the ice-cold water and withdraw it as soon as they perceived any pain. The same investigator conducted the test for all of the patients. The baseline latency to pain perception, defined as the time of immersion of the hand in the ice water (pain perception latency [PPL]), was recorded. A cut-off point of 150 s was set to avoid tissue damage. After the induction of anesthesia, peripheral blood samples were collected from these subjects for the gene analysis. After emergence from anesthesia and tracheal extubation, 1.25 mg droperidol was administered intravenously to prevent nausea/vomiting, and intravenous patient-controlled analgesia (PCA) with fentanyl (1 mg fentanyl and 5 mg droperidol diluted in normal saline in a total volume of 50 ml) commenced using a CADD-Legacy PCA pump (Smiths Medical Japan, Tokyo, Japan). The PCA settings included a bolus dose of 20 μg fentanyl on demand and a lockout time of 10 min. Continuous background infusion was not used. Droperidol was coadministered with fentanyl to prevent nausea/vomiting because our preliminary study found a high incidence (up to 30%) of nausea/vomiting with PCA fentanyl in young females. Patient-controlled analgesia was continued for 24 h postoperatively. Intraoperative fentanyl use and postoperative PCA fentanyl use during the first 24-h postoperative period were recorded. The doses of fentanyl administered intraoperatively and postoperatively were normalized to body weight.

For the subjects who underwent major open abdominal surgery, the surgical protocol and subsequent postoperative pain management were fundamentally the same as in previous studies [13, 14]. Postoperative pain was managed primarily with continuous epidural analgesia with fentanyl or morphine. Fentanyl or morphine was diluted with 0.25% bupivacaine in a total volume of 100 ml and infused at a constant rate of 2 ml/h through a catheter placed in the lower thoracic or upper lumbar epidural space. Whenever the patient complained of significant postoperative pain despite continuous epidural analgesic, appropriate doses of opioids, including morphine, buprenorphine, pentazocine, and pethidine, and/or nonsteroidal antiinflammatory drugs (NSAIDs), including diclofenac and flurbiprofen, were administered as rescue analgesics at the discretion of the surgeons based on the patient's request. The doses of rescue

analgesics (opioids and/or NSAIDs) administered during the first 24-h postoperative period and dose of analgesics administered during the intraoperative period were recorded. To allow intersubject comparisons of the analgesic doses required during the intraoperative period and first 24 h postoperative period, the doses of opioids and NSAIDs administered as rescue analgesics during these periods were converted to the equivalent dose of systemic fentanyl according to previous reports [13–24]. The total dose of analgesics administered was calculated as the sum of the systemic fentanyl-equivalent doses of all of the opioids and NSAIDs administered to patients as analgesics during the intraoperative period and first 24-h postoperative period.

Peripheral blood or oral mucosa samples were collected from these subjects after surgery for the gene analysis.

## Genotyping

Genomic DNA was extracted from the oral mucosa or whole-blood samples using the Wizard Genomic DNA Purification Kit (Promega, Tokyo, Japan) or the QIAamp DNA Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions [25].

For genotyping the five selected single-nucleotide polymorphisms (SNPs; rs9384179 in the human *OPRM1* gene, rs3845446 in the human *CACNA1E* gene, rs11959113 in the human *ADRB2* gene, rs2835859 in the human *GIRK2* [*KCNJ6*] gene, and rs2952768 around the human *CREB1* gene), direct sequencing, the TaqMan allelic discrimination assay, and Infinium assay II were used. Genotype data from whole-genome genotyping, which was performed using Infinium assay II and an iScan system (Illumina, San Diego, CA, USA), were used for the rs3845446, rs11959113, rs2835859, and rs2952768 SNPs. Direct sequencing was conducted to genotype the rs9384179 SNP as described in a previous report [8]. The TaqMan allelic discrimination assay was conducted to genotype the rs3845446, rs2835859, and rs2952768 SNPs as described in previous reports [9, 10] and the rs11959113 SNP. To perform the TaqMan assay with a LightCycler 480 (Roche Diagnostics, Basel, Switzerland), we used TaqMan SNP Genotyping Assays (Life Technologies, Carlsbad, CA, USA) that contained sequence-specific forward and reverse primers to amplify the polymorphic sequence and two probes labeled with VIC and FAM dye to detect both alleles of the rs3845446, rs11959113, rs2835859, and rs2952768 SNPs (Assay ID: C___7539287_30, C__27108051_10, C__16076710_10, and C__11510543_20, respectively). Real-time polymerase chain reaction was performed in a final volume of 10 μl that contained 2× LightCycler 480 Probes Master (Roche Diagnostics), 40× TaqMan SNP Genotyping Assays, 5–50 ng genomic DNA as the template, and $H_2O$ (Roche Diagnostics). The thermal conditions were the following: 95°C for 10 min, followed by 45 cycles of 95°C for 10 s and 60°C for 60 s, with final cooling at 50°C for 30 s. Afterward, endpoint fluorescence was measured for each sample well, and each genotype was determined based on the presence or absence of each type of fluorescence. The details of Infinium assay II for whole-genome genotyping were provided in a previous report [9].

## Statistical analysis

Three hundred fifty-four subjects who underwent painful cosmetic surgery were used for the initial analysis to construct prediction formulas for individual opioid sensitivity. As an index of opioid sensitivity, postoperative PCA fentanyl use during the first 24-h postoperative period was used because analgesic requirements likely reflect the efficacy of fentanyl in each individual patient. Prior to the analyses, the quantitative values of PPL (in seconds) and postoperative fentanyl requirements (μg/kg) were natural-log-transformed for approximation to the normal distribution according to the following formulas: *Value for analyses = Ln (1 + endpoint PPL value [s])* and = *Ln (1 + postoperative fentanyl requirement [μg/kg])*, respectively. To predict 24-h

postoperative fentanyl requirements from the clinical or genomic parameters that may affect the analgesic efficacy of fentanyl (i.e., age, gender, height, weight, PPL, and genotype of the five SNPs), multiple linear regression analysis using the stepwise method of bidirectional selection was performed. The cut off *P* value for inclusion was set at 0.05. Moreover, to confirm the utility of including the five SNPs in the predictive models of 24-h postoperative and perioperative fentanyl use, we examined whether adding the five SNPs to other patient characteristics in the models improves its predictive ability using forced entry method. The dependent variable was 24-h postoperative fentanyl use, and the independent variables were age, gender, height, weight, pain perception latencies, and genotype data of five single-nucleotide polymorphisms (SNPs). For the analysis, the gender and genotype data for each SNP were used as dummy variables based on the previous analyses (S3 and S4 Tables) by replacing females and the genotypes associated with greater fentanyl use with 1, and males and the genotypes associated with lesser fentanyl use with 0. Therefore, we replaced homozygous carriers of the A allele of the rs9384179 SNP with 1 and non-carriers with 0, homozygous carriers of the A allele of the rs3845446 SNP with 1 and non-carriers with 0, carriers of the A allele of the rs11959113 SNP with 1 and homozygous carriers of the G allele with 0, homozygous carriers of the T allele of the rs2835859 SNP with 1 and non-carriers with 0, and homozygous carriers of the C allele of the rs2952768 SNP with 1 and non-carriers with 0, respectively. Similar analysis using subjects who underwent cosmetic orthognathic surgery was also conducted for analgesic requirements during the perioperative period instead of during the postoperative period because the analgesic effect of the intermediate-acting analgesics, administered pre- and intraoperatively, could outlast the duration of surgery and thus affect postoperative analgesic use, especially in patients who received large doses of analgesics intraoperatively. Perioperative fentanyl use was calculated as the sum of the intraoperative and postoperative doses of fentanyl or fentanyl-equivalent doses of analgesics.

Second analyses were conducted using the different 145 subjects who underwent major abdominal surgery. The total dose of rescue analgesics administered during the first 24-h postoperative period was used as an index of opioid sensitivity. Prior to the analyses, the quantitative values of postoperative analgesic requirements (μg/kg) were natural-log-transformed for approximation to the normal distribution according to the following formula: *Value for analyses = Ln (1 + postoperative analgesic requirement [μg/kg])*. To explore the regression of the actual value of 24-h postoperative analgesic requirements after major abdominal surgery to the predicted value that was calculated using the multiple regression equation constructed in the initial analysis, simple linear regression analysis was performed. Actual 24-h postoperative analgesic use (μg/kg; log-transformed) and the predicted value that was calculated were incorporated as dependent and independent variables, respectively. Similar analysis using subjects who underwent major abdominal surgery was also conducted for analgesic requirements during the perioperative period instead of during the postoperative period. Perioperative fentanyl use was calculated as the sum of the intraoperative and postoperative doses of fentanyl or fentanyl-equivalent doses of analgesics.

For all of the statistical analyses described above, IBM SPSS v.20.0 for Windows software (IBM Japan, Tokyo, Japan) was used.

## Results

The $R^2$ value was improved from 0.062 to 0.169 and from 0.127 to 0.208 by adding the five SNPs to other patient characteristics in the predictive models of 24-h postoperative and perioperative fentanyl use, respectively. Initial multiple linear regression analysis of the postoperative period using the stepwise method showed that the genotype of the four SNPs i.e. rs2952768

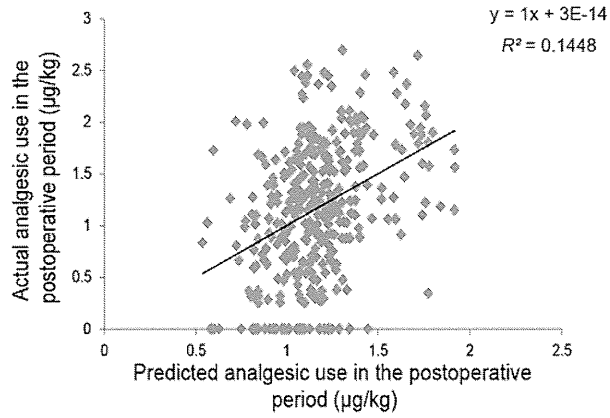


**Fig 1. Regression analysis of 24-h postoperative fentanyl use after cosmetic orthognathic surgery.** The figure shows a scatterplot for the actual value of 24-h postoperative fentanyl use (μg/kg; log-transformed) in patients who underwent cosmetic orthognathic surgery and the predicted value that was calculated. Each point represents an individual patient. The solid line in the scatterplot represents the regression line, and the mathematical formula represents the regression equation.

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($P = 1.3 \times 10^{-6}$), rs2835859 ($P = 0.003$), rs9384179 ($P = 0.042$), and rs11959113 ($P = 0.018$), PPL ($P = 0.016$), and weight ($P = 0.044$) were retained as independent predictors of 24-h postoperative fentanyl use for cosmetic orthognathic surgery ($R^2 = 0.145$, $P = 5.66 \times 10^{-10}$; Fig. 1). The detailed multiple regression equation is shown in Fig. 2. Similar multiple linear regression analysis of the perioperative period considering the analgesic effect of intermediate-acting

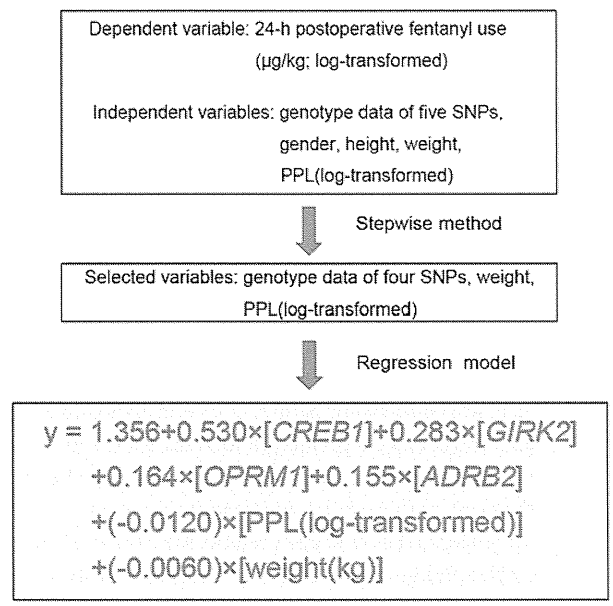


**Fig 2. Construction of prediction formula.** Multiple linear regression analysis during the postoperative period using the stepwise method was performed to construct a prediction formula for individual opioid sensitivity. "y" represents the predicted value of 24-h postoperative fentanyl requirements (μg/kg; log-transformed) that was calculated using the multiple regression equation.

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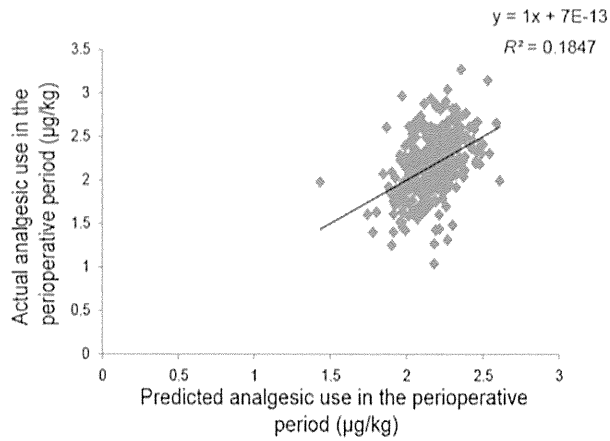


**Fig 3. Regression analysis of perioperative fentanyl use after cosmetic orthognathic surgery.** The figure shows a scatterplot for the actual value of perioperative fentanyl use (μg/kg; log-transformed) in patients who underwent cosmetic orthognathic surgery and the predicted value that was calculated. Each point represents an individual patient. The solid line in the scatterplot represents the regression line, and the mathematical formula represents the regression equation.

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analgesics administered pre- and intraoperatively showed that the genotype of the two SNPs, rs2952768 ($P = 9.1 \times 10^{-5}$) and rs3845446 ($P = 0.001$), and weight ($P = 2.8 \times 10^{-12}$) were retained as independent predictors of perioperative fentanyl use for cosmetic orthognathic surgery ($R^2 = 0.185$, $P = 1.99 \times 10^{-15}$; Fig. 3). The multiple-regression equation was the following: *predicted value of perioperative fentanyl requirements (μg/kg; log-transformed) = 2.749 + 0.221 × [CREB1] + 0.109 × [CACNA1E] + (-0.011) × [weight (kg)].*

Second simple linear regression analysis of the postoperative period using subjects who underwent major abdominal surgery showed that the predicted value that was calculated was retained as an independent predictor of actual 24-h postoperative analgesic use for major abdominal surgery ($R^2 = 0.033$, $P = 0.030$; Fig. 4). Similar simple linear regression analysis of the perioperative period showed that the predicted value that was calculated was retained as an independent predictor of actual perioperative analgesic use for major abdominal surgery ($R^2 = 0.100$, $P = 1.09 \times 10^{-4}$; Fig. 5).

## Discussion

A prediction formula for individual opioid analgesic requirements during the first 24-h postoperative period (Fig. 2) was constructed using multiple linear regression analysis in healthy subjects who underwent painful cosmetic orthognathic surgery. By conducting similar analysis, we also constructed a prediction formula for the perioperative period, although the variables that were retained as independent predictors after the analysis of the perioperative period were not the same as those of the postoperative period (Fig. 3). The $R^2$ index in the prediction formula for perioperative analgesic requirements was higher than the $R^2$ index for the first 24-h postoperative analgesic requirements. Furthermore, by conducting simple linear regression analyses in subjects who underwent major open abdominal surgery, we demonstrated the possibility that the prediction formulas may be useful in another type of surgery (Figs. 4 and 5), although the predictability of the formula remains to be verified in future studies. Using the prediction formulas and the patients' genetic polymorphisms and clinical data, better analgesia could be provided to individual patients. Based on the predicted values, less fentanyl could be

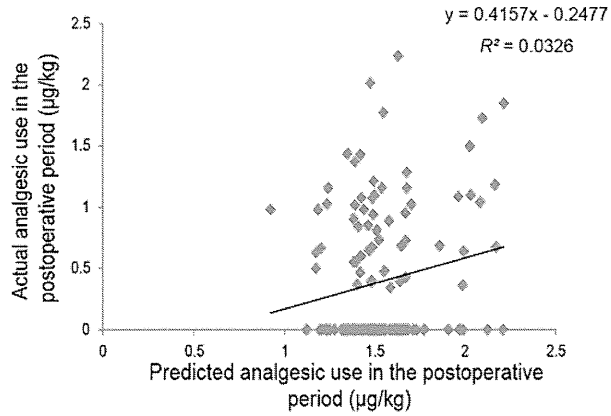


**Fig 4. Regression analysis of 24-h postoperative analgesic use after major abdominal surgery.** The figure shows a scatterplot for the actual value of 24-h postoperative analgesic use (μg/kg; log-transformed) in patients who underwent major abdominal surgery and the predicted value that was calculated. Each point represents an individual patient. The solid line in the scatterplot represents the regression line, and the mathematical formula represents the regression equation.

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administered to patients with higher opioid sensitivity, and more fentanyl could be administered to patients with lower opioid sensitivity. For example, setting the appropriate dose of analgesics for individual patients by setting an upper level has great potential for safe, personalized pain control while minimizing potential side effects.

Empirical approaches to the effective treatment of pain are currently limited because the initial drug selection and subsequent dosage titrations, drug additions, and switches in therapy are driven by only a few patient-specific clinical features [26]. Thus, pharmacogenetic studies are being developed to advance personalized medicine. Pharmacogenetics is defined as the use of pharmacogenomic or pharmacogenetic tests in conjunction with drug therapy and has the potential to change the way in which healthcare is provided by stratifying patients into likely

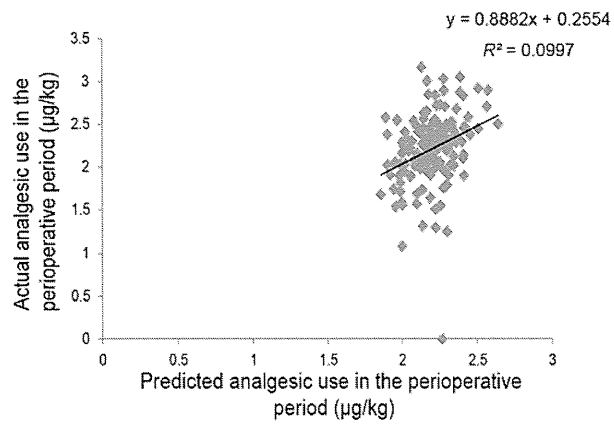


**Fig 5. Regression analysis of perioperative analgesic use after major abdominal surgery.** The figure shows a scatterplot for the actual value of perioperative analgesic use (μg/kg; log-transformed) in patients who underwent major abdominal surgery and the predicted value that was calculated. Each point represents an individual patient. The solid line in the scatterplot represents the regression line, and the mathematical formula represents the regression equation.

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responders, likely non-responders, and likely to experience adverse drug reactions [27]. The use of pharmacogenomic principles represents an opportunity to enhance public health and patient care. Therefore, prediction formulas can be constructed for individual analgesic requirements based on genetic polymorphisms.

Recently, methods of predicting drug requirements using genetic tests may be adopted for drugs for which dose adjustments are difficult because of large individual differences in sensitivity. For example, a dosing algorithm has been constructed for warfarin, consisting of the patient's age, body surface area, state of amiodarone co-administration, and genotype of three SNPs ($R^2 = 0.434$) [28]. A prospective study reported that an algorithm guided by pharmacogenetic and clinical factors improved the accuracy and efficiency of warfarin dose initiation, although a reduction of out-of-range prothrombin time international normalized ratios (INRs) was not achieved [29]. Thus, prediction formulas have also been constructed and verified for medications other than opioid analgesics. Although the precision of our prediction formula for individual opioid analgesic requirements appears to be relatively low in terms of the $R^2$ index compared with the warfarin dosing algorithm, such algorithms are expected to be improved in future studies and contribute to personalized medicine.

Gene and opioid-effect relationships are difficult to determine from data that are sampled from patients with cancer pain because the mechanism, severity, and nature of cancer pain can differ substantially between patients [30]. Thus, patients with acute postoperative pain after standardized surgical procedures in the present study may be optimal subjects for investigating such relationships [1]. The precision of our prediction formulas for individual opioid analgesic requirements was relatively low in terms of the $R^2$ index for major open abdominal surgery compared with cosmetic orthognathic surgery, and several factors may have contributed to this imprecision. The first consideration is differences in the surgical procedures and the degree of invasiveness. The present study included patients with various diseases and pathologies that required major abdominal surgery. Another consideration is the analgesics employed and their routes of administration. Various analgesics and routes of administration are used in major abdominal surgery, and analgesic action, absorption, metabolism, and excretion can vary considerably among analgesics and routes of administration. For example, buprenorphine is long-acting and antagonizes other opioids, which may prevent the effects of continuous epidural opioids and other rescue opioids. A third consideration is the pathways of pain. Major abdominal surgery involves visceral pain, and some pain pathways may not be exactly the same as those associated with cosmetic orthognathic surgery. Therefore, to apply our formulas to other types of surgery, further investigation is necessary. Future studies should verify the utility of the prediction formulas in surgeries that have fewer variations with regard to patients and surgical procedures.

Additionally, studies that investigate the genes that encode opioid metabolizing enzymes and transporters are also worth performing. Because most opioid drugs are metabolized by cytochrome P450 enzymes (CYPs), including CYP2D6, glucuronidated by UDP-glucuronosyltransferases (UGTs), and transported between the blood and brain by ATP-binding cassette, sub-family B (MDR/TAD), member 1 (ABCB1), the genes that encode these metabolic enzymes and transporters are worth examining with regard to differences in analgesic requirements [2, 31, 32]. The onset of negative effects (e.g., nausea, vomiting, and constipation) may also be a useful and interesting outcome because such effects can cause some patients to stop requesting analgesics despite not actually achieving full analgesia. However, we did not construct prediction formulas for negative effects because the number of patients with adverse side-effects was small in the present study. Additionally, other variables, such as cigarette smoking status and pre-existing opioid tolerance, can influence opioid analgesic requirements. We found that some SNPs were associated with self-reported pain level (data not shown), and pain level in addition to analgesic requirements may be predicted by gene analyses.

Moreover, gene-gene interactions and gene-environment interactions can also affect opioid sensitivity, although the present study assumed that each factor was independent. Thus, other prediction formulas should be constructed by taking these factors into account. Such considerations should aid in the development of more effective personalized pain treatment for patients who suffer from cancer pain or postoperative pain after various surgeries by predicting opioid sensitivity based on simple genetic tests.

In conclusion, by conducting multiple linear regression analyses of healthy subjects who underwent painful cosmetic orthognathic surgery, we constructed prediction formulas for individual opioid analgesic requirements during the first 24-h postoperative period and perioperative period. Furthermore, by conducting simple linear regression analyses in subjects who underwent major open abdominal surgery, we found that these prediction formulas may be useful for other types of surgery. Although further validation is needed, our data provide valuable information for the individualization of appropriate fentanyl doses to achieve adequate pain control and open new avenues for personalized pain treatment.

## Supporting Information

**S1 Fig. Illustration of personalized medicine in patients with high and low opioid sensitivity.** The minimal effective analgesic concentration (MEAC) is 5- to 10-fold different among individuals, and this is a purported cause of wide variations in the clinical response to opioids among individuals. The difference between the MEAC and maximum concentration with pain (MCP) is low among individuals. Purple, pink, blue, and brown zones indicate ranges of the blood opioid concentration associated with severe pain (no analgesia), pain (insufficient analgesia), satisfactory analgesia, and side-effects, respectively. Conventional pain control (dashed line) can result in an overdose (associated with side-effects) in patients with high opioid sensitivity or under-dose (associated with persistent pain) in patients with low opioid sensitivity. Personalized pain control (solid line) provides satisfactory pain relief in both patients.
(TIF)

**S1 Table. Detailed data of the subjects who underwent cosmetic orthognathic surgery.**
(XLSX)

**S2 Table. Detailed data of the subjects who underwent major open abdominal surgery.**
(XLSX)

**S3 Table. Actual fentanyl use after cosmetic orthognathic surgery stratified by genotype of the five SNPs.**
(DOCX)

**S4 Table. Actual analgesic use equivalent to systemic fentanyl after major open abdominal surgery stratified by genotype of the five SNPs.**
(DOCX)

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## Author Contributions

Conceived and designed the experiments: KY DN KI. Performed the experiments: KY DN. Analyzed the data: KY DN T. Ichinomiya. Contributed reagents/materials/analysis tools: T. Ichinohe MH KF. Wrote the paper: KY DN KI.

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原著

# ノルエピネフリントランスポーター遺伝子多型とアルコール依存症との関連研究

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(受付：平成26年6月19日；受理：平成26年9月9日)

# Association between norepinephrine transporter gene polymorphism and alcohol dependence in Japanese

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## Summary

Several studies have suggested that the norepinephrine transporter (NET) may play an important role in the pathogenesis of alcohol dependence. Therefore, in this study, we investigated whether the NET gene polymorphism is a susceptibility factor for alcohol dependence in 64 alcoholics and 73 healthy controls. In addition, we examined whether the combination of the NET and serotonin transporter genotypes are associated with alcohol dependence. The

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NET (1287G/A, -182T/C, and -3081A/T) and serotonin transporter (5-HTT3'UTR) genotypes were determined by the polymerase chain reaction (PCR) - restriction fragment length polymorphism (RFLP) method. No significant differences in genotype and allele frequencies of the NET and serotonin transporter gene polymorphisms were found between alcoholics and controls. The haplotype frequencies of the NET gene polymorphisms were not also significantly different between them. Furthermore, the combination of the NET and serotonin transporter genotypes had not significant effects on alcohol dependence. The present study suggests that the polymorphisms of 1287G/A, -182T/C and -3081A/T in NET gene are not risk factors in alcohol dependence.

Key words: norepinephrine transporter, serotonin transporter, gene polymorphism, combination, alcohol dependence
ノルエピネフリントランスポーター，セロトニントランスポーター，遺伝子多型，組み合わせ，アルコール依存症

## はじめに

モノアミン神経伝達物質であるノルエピネフリン（NE）は，自律神経系の制御を含む生命維持活動や睡眠，記憶・学習，情緒に関与していると言われる[1]．モノアミン神経終末の細胞膜には，放出された伝達物質を$Na^+/Cl^-$依存的に再取り込みして神経伝達を終了させるモノアミントランスポータータンパクが存在し，NEを特異的に取り込むものとしてはノルエピネフリントランスポーター（NET）が知られている[2]．NETは膜12回貫通型で細胞内にN末端とC末端を持ち，膜貫通第3領域と第4領域の間に数箇所の糖鎖結合部位を有する大きな細胞外ループを持つ構造となっている[1]．また，向精神薬や三環系抗うつ薬の標的分子であることから，中枢神経疾患との関連が予想されている[1]．

これまでの研究で，NETと依存性物質の一つであるアルコールとの関連を示す知見がいくつか示されており，エタノール抑制作用の調節にNETが重要な役割を果たしている可能性があることや[3]，動物の脳を画像化した研究において，脳内のNETレベルの低下は高いアルコール嗜好性に関連したという報告がある[4]．さらに最近の研究では，ヒトNET遺伝子（*hSLC6A2*）上に存在する一塩基多型（single nucleotide polymorphism: SNP）とアルコール依存症との間に関連を認めている[5]ことから，NET遺伝子多型がアルコール依存症脆弱性の個人差に寄与する可能性があると考えられる．ヒトNETをコードしているNET遺伝子は染色体16q12.2に存在し[6]，エクソン14個から構成されている[7]．マウスの8番染色体に位置するげっ歯類のNET遺伝子（gene symbol: *Slc6a5*）はエタノール感受性をつかさどる量的形質座位であり，この部分がヒト染色体の16qに対応することから[8]，NET遺伝子とアルコール感受性との間に何らかの関連があるのではないかと考えられる．NET遺伝子上に存在する主なSNPとしては，第9エクソンに位置する1287G/A（rs5569）[9]やプロモーター領域に位置する-182T/C（rs2242446）[10]，-3081A/T（rs28386840）[11]などが知られており，あらゆる精神疾患との関連研究が行われている．なかでも1287G/Aと-182T/Cに関しては，他集団においてアルコール依存症との関連研究が行われているが，有意な関連は認められていない[12,13]．その一方で，-3081A/Tにおいてはこれまでにアルコール依存症との関連研究は我々の知る限りでは行われていない．-3081A/T多型

は機能性を有する多型であり，T alleleはA alleleと比較してNET遺伝子のプロモーター機能の低下を誘導することが報告されている[11]．

また，過去の研究においてHallらは，NETまたはセロトニントランスポーター（5-HTT）のどちらか一方をノックアウトさせたマウスではコカイン報酬効果に変化を認めなかったが，それら両方をノックアウトさせたマウスではコカイン報酬効果の増強を認めたと報告している[14]．一方，5-HTTはアルコール摂取における抑制効果にも関与しているとされており，5-HTT (-/-) マウスでは5-HTT（+/+）マウスに比べ，アルコール摂取量が有意に低下したという報告[15]があることから，NETが5-HTTとの相互作用によりアルコール依存形成に相加効果をもたらす可能性があるのではないかと考えた．ヒト5-HTT遺伝子（hSLC6A4）は，31kbの長さからなり，染色体17q11.1-12に位置する[16,17]．5-HTT遺伝子の3'非翻訳領域（3'UTR）には第14エクソンの終止コドンより689塩基下流におけるグアニン（G）からチミン（T）へのSNP（rs3813034）が存在し[18]，この多型は異常なポリアデニル化がmRNAの安定と細胞質への移動の促進を抑制する可能性があるといわれている[19]．これまでに，5-HTT3'UTR多型とアルコール依存症の関連性を検討した症例対象研究は我々の知る限りでは行われていない．

本研究では，アルコール依存形成の遺伝子要因解明の一助として，NET遺伝子多型（1287G/A，-182T/C，-3081A/T）がアルコール依存症の形成に影響を与えているかどうか検討を行い，さらにNET遺伝子多型と5-HTT遺伝子多型（5-HTT3'UTR）の相互作用（組み合わせ）の影響についても同様に検討を行った．

## 対象と方法

書面においてインフォームド・コンセントの得られたアルコール依存の経歴のない健常者73人（男性22人; 女性51人; 平均年齢35.52 ± 9.13），DSM-IVの診断基準においてアルコール依存症と診断されたアルコール依存症患者64人（男性50人; 女性7人; 不明7人; 平均年齢57.34 ± 10.18）を対象とした．公徳会佐藤病院にてこれらの対象者から採血を行い，東京都医学総合研究所によって指名された管理者において匿名化を行った．すべての対象者は山形県在住である．本研究は麻布大学ヒトゲノム・遺伝子解析研究に関する倫理審査委員会，東京都医学総合研究所および公徳会佐藤病院の承認を得ている．

NET遺伝子多型1287G/A，-182T/Cおよび-3081A/Tの解析は，Huangら，Suzukiらの方法に従い，Polymerase Chain Reaction（PCR）- Restriction Fragment Length Polymorphism（RFLP）法により，それぞれ制限酵素 *Sau96* I（New England BioLabs），*Sty* I（New England BioLabs），*BsrS* I（Promega）を用いて行った[12,20]．また，5-HTT遺伝子多型5-HTT3'UTRの解析は，PCR法ではBattersbyら[18]，RFLP法ではAokiら[21]の方法に従い，制限酵素 *Tru1* I（MBI Fermentas）を用いて行った．なお，-3081A/Tおよび5-HTT3'UTRについては，解析できたアルコール依存症患者63人で検討を行った．

統計学的解析は，アルコール依存症患者群と健常者群において，NET遺伝子多型の遺伝子型および対立遺伝子の頻度に差があるかどうか検討するために$\chi^2$検定およびYatesの補正を行った．また，NETと5-HTTの両遺伝子多型の相互作用（組み合わせ）とアルコール依存症との関連性については，マイナーアレルの有無により群分けし，$\chi^2$検定およびYatesの補正を行った．$\chi^2$検定およびYatesの補正にはy-statを用いた[22]．さらに，NET遺伝子多型に関しては連鎖不平衡およびハプロタイプ解析を行い，gPLINK v. 2.050とHaploview v. 4.2を用いた[23,24]．有

意水準は$p < 0.05$とした．

## 結　果

解析の結果，NET遺伝子多型1287G/A，-182T/C，-3081A/Tおよび5-HTT遺伝子多型5-HTT3'UTRの健常者群のそれぞれの遺伝子型頻度分布において，Hardy-Weinberg平衡からの有意な逸脱は認められなかった（1287G/A: $\chi^2(1) = 2.605$, $p = 0.107$; -182T/C: $\chi^2(1) = 0.223$, $p = 0.637$; -3081A/T: $\chi^2(1) = 1.036$, $p = 0.309$; 5-HTT3'UTR: $\chi^2(1) = 0.041$, $p = 0.839$）．

両群におけるNETおよび5-HTT遺伝子多型の遺伝子型と対立遺伝子頻度をTable 1に示した．解析したすべての遺伝子多型において両群間の遺伝子型頻度に有意な関連は認められなかった（1287G/A: $\chi^2(2) = 0.232$, $p = 0.890$; -182T/C: $\chi^2(2) = 0.011$, $p = 0.995$; -3081A/T: $\chi^2(2) = 0.658$, $p = 0.720$; 5-HTT3'UTR: $\chi^2(2) = 1.366$, $p = 0.505$）．同様に，対立遺伝子頻度に関しても有意な関連は認められなかった（1287G/A: $\chi^2(1) = 0.056$, $p = 0.814$; -182T/C: $\chi^2(1) = 0.069$, $p = 0.793$; -3081A/T: $\chi^2(1) = 0.318$, $p = 0.573$; 5-HTT3'UTR: $\chi^2(1) = 1.707$, $p = 0.191$）．また，男女別の解析も行ったが有意な関連は認められなかった（data not shown）．

次に，両群におけるNET遺伝子多型1287G/A，-182T/C，-3081A/Tのハプロタイプ頻度をTable 2に示した．同一染色体上の対立遺伝子の組み合わせからなるハプロタイプは7種類存在し，すべてのハプロタイプにおいて両群間での出現頻度に有意な関連は認められなかった（$\chi^2(6) = 3.869$, global $p = 0.6943$）．また，Fig. 1にNET遺伝子の上記3多型間の連鎖不平衡の指標値を示した．1287G/Aと-182T/C（$D' = 0.079$, $r^2 = 0.004$）および1287G/Aと-3081A/T（$D'$

Table 1　Genotype and allele frequencies of the *SLC6A2* and *SLC6A4* polymorphisms in alcoholics and controls.

| Polymorphism | Subject | n | Genotype (%) | | | Allele (%) | |
|---|---|---|---|---|---|---|---|
| | | | G/G | G/A | A/A | G | A |
| 1287G/A | Alcoholic | 64 | 38 (59.4) | 22 (34.4) | 4 ( 6.2) | 98 (76.6) | 30 (23.4) |
| (rs5569) | Control | 73 | 44 (60.3) | 22 (30.1) | 7 ( 9.6) | 110 (75.3) | 36 (24.7) |
| | | | $X^2$ (2) = 0.232, $p$ = 0.890 | | | $X^2$ (1) = 0.056, $p$ = 0.814 | |
| | | | T/T | T/C | C/C | T | C |
| -182T/C | Alcoholic | 64 | 27 (42.2) | 30 (46.9) | 7 (10.9) | 84 (65.6) | 44 (34.4) |
| (rs2242446) | Control | 73 | 32 (43.8) | 34 (46.6) | 7 ( 9.6) | 98 (67.1) | 48 (32.9) |
| | | | $X^2$ (2) = 0.011, $p$ = 0.995 | | | $X^2$ (1) = 0.069, $p$ = 0.793 | |
| | | | A/A | A/T | T/T | A | T |
| -3081A/T | Alcoholic | 63 | 16 (25.4) | 31 (49.2) | 16 (25.4) | 63 (50 ) | 63 (50 ) |
| (rs28386840) | Control | 73 | 18 (24.7) | 32 (43.8) | 23 (31.5) | 68 (46.6) | 78 (53.4) |
| | | | $X^2$ (2) = 0.658, $p$ = 0.720 | | | $X^2$ (1) = 0.318, $p$ = 0.573 | |
| | | | G/G | G/T | T/T | G | T |
| 5-HTT3'UTR | Alcoholic | 63 | 44 (69.8) | 19 (30.2) | 0 ( 0 ) | 107 (84.9) | 19 (15.1) |
| (rs3813034) | Control | 73 | 45 (61.6) | 25 (34.3) | 3 ( 4.1) | 115 (78.8) | 31 (21.2) |
| | | | $X^2$ (2) = 1.366, $p$ = 0.505 | | | $X^2$ (1) = 1.707, $p$ = 0.191 | |

*SLC6A2*: Norepinephrine transporter gene, *SLC6A4*: Serotonin transporter gene.

Table 2 Haplotype frequencies of the *SLC6A2* polymorphisms in alcoholics and controls.

| Haplotype | 1287G/A (rs5569) | -182T/C (rs2242446) | -3081A/T (rs28386840) | Frequency Alcoholic | Frequency Control | $X^2$ | Haplotype $p$ value[a] |
|---|---|---|---|---|---|---|---|
| 1 | A | T | A | 0.103 | 0.0636 | 1.398 | 0.2371 |
| 2 | G | C | A | 0.02232 | 0.03287 | 0.2767 | 0.5988 |
| 3 | G | T | A | 0.3747 | 0.3693 | 0.008389 | 0.927 |
| 4 | A | C | T | 0.0746 | 0.1059 | 0.7979 | 0.3717 |
| 5 | A | T | T | 0.05254 | 0.07707 | 0.6629 | 0.4155 |
| 6 | G | C | T | 0.2364 | 0.19 | 0.8738 | 0.3499 |
| 7 | G | T | T | 0.1364 | 0.1613 | 0.3275 | 0.5671 |

*SLC6A2*: Norepinephrine transporter gene.

[a] Haplotype distributions were not significantly different between alcoholics and controls (global $p$ = 0.6943).

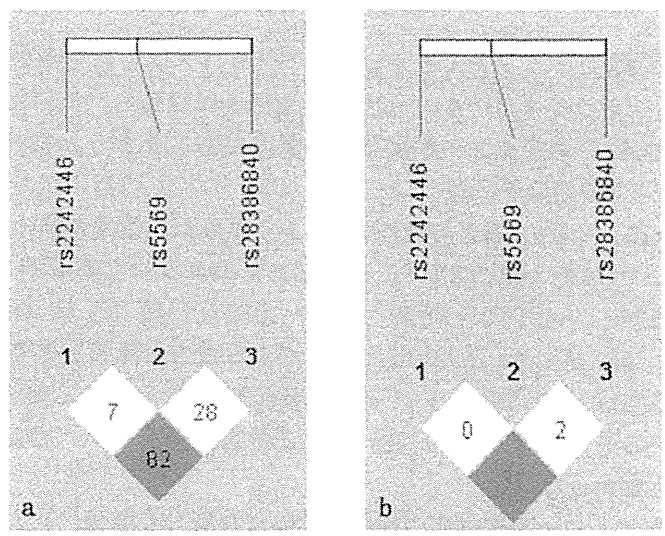


Fig. 1 Linkage disequilibrium (LD) map of the *SLC6A2* locus in this study. a: $D'$ value, b: $r^2$ value, rs5569: 1287G/A polymorphism, rs2242446: -182T/C polymorphism, rs28386840: -3081A/T polymorphism, *SLC6A2*: Norepinephrine transporter gene.

= 0.282, $r^2$ = 0.023）の組み合わせでは相関は低かったが，-182T/Cと-3081A/Tの組み合わせにおいては比較的強い相関が認められた（$D'$ = 0.821, $r^2$ = 0.31）．

さらに，両群におけるNETおよび5-HTT遺伝子多型の相互作用（組み合わせ）による遺伝子型頻度をTable 3に示した．両群間で1287G/A，-182T/Cおよび-3081A/T多型の各遺伝子型と5-HTT3'UTR多型の遺伝子型との組み合わせに有意な関連性は認められなかった（1287G/A and 5-HTT3'UTR: $\chi^2$ (3) = 0.598, $p$ = 0.897; -182T/C and 5-HTT3'UTR: $\chi^2$ (3) = 0.432, $p$ = 0.934; -3081A/T and 5-HTT3'UTR: $\chi^2$ (3) = 0.678, $p$ = 0.878)．なお，他の組み合わせパター