7. Ideally, for further analyses, postoperative pain should be managed with a standardized protocol employing a single opioid analgesic (e.g., i.v. PCA fentanyl). However, if multiple opioids were used postoperatively, intersubject comparisons of postoperative opioid requirements are possible by converting the dose of one opioid to an equivalent dose of another, based on published data showing equipotent doses of various opioid analgesics. For example, epidural fentanyl 100 μg, systemic morphine 10 mg, epidural morphine 2 mg, systemic pentazocine 30–60 mg, and systemic buprenorphine 333 μg can be converted to an equivalent systemic fentanyl dose of 100 μg (7). The total opioid requirements in the first 24 h postoperative period are determined as the sum of equivalent systemic fentanyl doses of all opioids used during this period.
8. If postoperative pain scores are not documented in hospital records, the researchers can ask the research subjects, by mail, to rate the pain intensity they had at rest during the particular period (e.g., during the first 24 h postoperative period) using a 5-point verbal pain rating scale (0 = no pain, 1 = mild pain, 2 = moderate pain, 3 = severe pain, 4 = the most severe pain imaginable).

3.2. Preparation of Genomic DNA

Before genotyping specific polymorphisms, genomic DNA of the subjects should be collected and purified. Although genomic DNA can be extracted from various cells or tissues in humans, we describe here the methods of extracting it from cells of oral mucosa. Only a small amount of DNA is usually extracted from the oral mucosa of each subject; therefore, the oral mucosa might not be durable for repeated use intended to genotype many candidate polymorphisms. However, recent whole genome amplification technology has enabled us to investigate genotypes of many genetic polymorphisms in the candidate loci without repeatedly collecting DNA samples from subjects.

3.2.1. Collection of Oral Mucosa

1. Rinse mouth with clean water.
2. Press the first swab onto the upper right buccal mucosa and roll it on the mucosa 25 times, slightly changing its position (see Fig. 2).
3. Put the swab into a centrifuge tube. Hold the tube and swab upright and let the swab fall down directly onto the bottom of the tube, avoiding contact between the swab and the inner wall of the tube (see Fig. 2).
4. Similarly, press the second, third, and forth swabs onto the lower left, lower right, and upper left buccal mucosa, respectively, and roll each of them 25 times on the mucosa of each site. Put the swab sticks, one at a time, into the same test tube as the first swab (see Fig. 2). Rinse mouth with clean water each time.

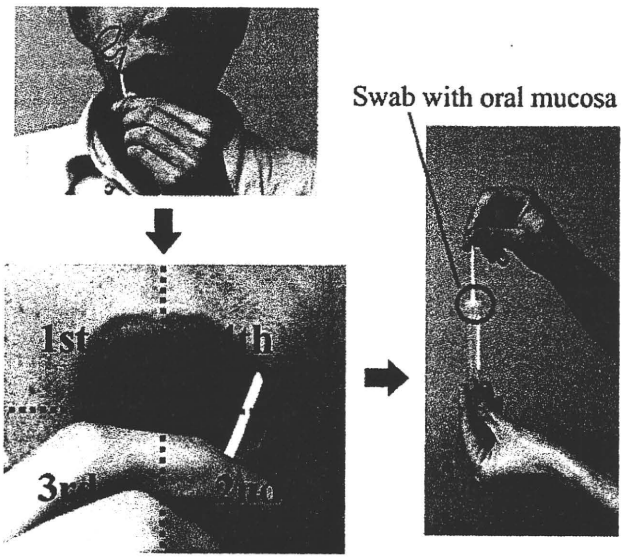

Fig. 2. Procedure for collecting oral mucosa (see Subheading 3.2.1).

5. Finally, rinse mouth with clean water or drink something.
6. Cap the centrifuge tube firmly and send it back to the researchers as soon as possible so that the researchers can store it at 4°C until DNA extraction.

3.2.2. Purification of Genomic DNA

1. After separating four swabs with oral mucosa from the sticks using scissors, if needed, and placing them in a 2 mL micro-centrifuge tube, dry them up for 2 h at room temperature, and add 500 µL PBS to the sample (see Note 5).
2. Extract total genomic DNA using the DNA purification kit according to the manufacturer's instructions. Each buffer used in each purification step is 500 µL.
3. Store eluted genomic DNA at 4°C until used. If not used for an extended length of time, storage at −20°C is recommended.

3.2.3. Whole Genome Amplification (WGA)

1. Amplify the total genomic DNA using a whole genomic DNA amplification kit. 10 ng of purified template genomic DNA is sufficient.
2. Purify the amplified genomic DNA by conventional ethanol precipitation and dissolve in 300–400 µL TE buffer.
3. Measure the concentration of the purified DNA (see Note 6) and store it at 4°C until use. If not used for an extended length of time, storage at −20°C is recommended.

3.3. Genotyping

To date, many technologies of genotyping polymorphisms, most often single nucleotide polymorphisms (SNPs), have been

developed and advanced. We do not describe all of these details here because the respective features of each of these methods have been discussed extensively elsewhere (12–16). Generally, most genotyping methods consist of forming allele-specific products v a detection procedure to identify them (15). The biochemical techniques involved or reagents and instruments required in each step differ among various genotyping methods, impacting accuracy, cost, throughput, and laboratory availability. Researchers must choose the most suitable method that meets their requirements. In the following subsections, we briefly describe the protocol for several genotyping methods.

3.3.1. PCR–RFLP

PCR–RFLP is one of the methods utilizing endonuclease (restriction enzyme) in the allelic discrimination steps. It does not require costly equipment and thus is feasible in most molecular biology laboratories. Additionally, throughput is not diminished if the numbers of samples per SNP are not extensive. The commonly recognized drawbacks of this technique are that it is labor-intensive, not suitable for large-scale clinical applications, and applicable only when the SNPs alter a restriction enzyme cutting site (12, 16).

1. Perform PCR in a total of 10 µL solution containing 5–50 ng purified genomic DNA as the template, DNase-free water, forward and reverse primers to amplify the region encompassing the polymorphic site, DNA polymerase, and reaction buffer including dNTP and $MgCl_2$. During the PCR reactions, control the temperature, such as with PROGRAM TEMP CONTROL SYSTEM PC-818-02. For example, the PCR program for amplifying the region of the A1032G SNP in the G-protein-activated inwardly rectifying potassium (GIRK) channel gene, *GIRK2*, is the following: 95°C for 2 min, followed by 35–40 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 8 min (17).
2. Digest the amplified DNA fragments with the restriction enzyme in a reaction solution containing buffer, the restriction enzyme to discriminate the genotypes, and the PCR product as the substrate.
3. Separate the digestion products by electrophoresis using 1–2% agarose gel in 1× TAE buffer and stain with ethidium bromide for visualization under ultraviolet illumination. Detect the DNA fragment size pattern specific to the genotype of the loaded sample.

3.3.2. AS–PCR

AS-PCR utilizes the difference in the extension efficacy of DNA polymerases depending on whether the 3′ ends of the primers are matched or mismatched for hybridization at the polymorphic site.

Although the allele-specific primers used in this method often bear labeling tags, such as fluorescence (18, 19), we describe here a method in which such tags are not involved, and detection of allele-specific products are carried out by gel electrophoresis, which is more labor-intensive for large-scale genotyping but has lower initial set-up costs.

1. Perform PCR in a total of 10 µl solution containing 5–50 ng purified genomic DNA as the template, DNase-free water, forward and reverse primers to amplify the region including the polymorphic site, DNA polymerase, and reaction buffer including dNTP and $MgCl_2$. During the PCR reactions, control the temperature, such as with PROGRAM TEMP CONTROL SYSTEM PC-818-02. For example, the allele-specific PCR program for the A118G SNP in the *OPRM1* is the following: 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 62°C and 64°C for 30 s for the forward primer specific for A and G, respectively, and 72°C for 1 min, with a final extension at 72°C for 8 min (20).
2. Separate the presence of allele-specific PCR products by electrophoresis using 1–2% agarose gel in 1× TAE buffer and stain with ethidium bromide for visualization under UV illumination. For example, Fig. 3 shows the detection of the allele-specific PCR products for the A118G SNP.

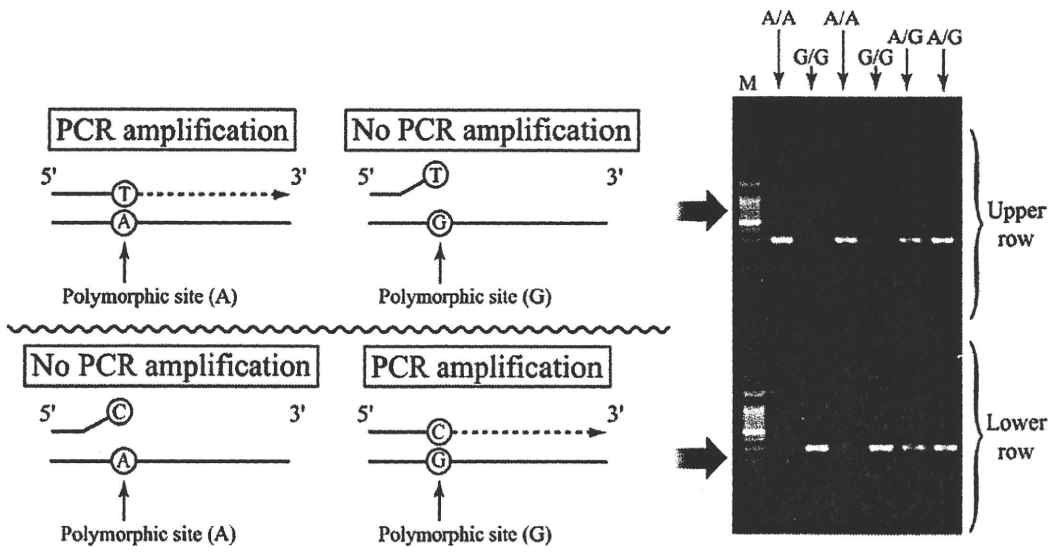

Fig. 3. Detection of allele-specific PCR products for the A118G SNP in the µ-opioid receptor gene (*OPRM1*). The principle as well as the results of AS-PCR is illustrated. Upper and lower rows in each lane indicate the A allele- and G allele-specific PCR products for each sample, respectively. The presence of only A-specific and G-specific products indicates the A/A genotype and G/G genotype, respectively, whereas the presence of both products indicates the A/G genotype. M, 100 bp DNA size marker.

3.3.3. TaqMan® SNP Genotyping Assays

TaqMan Assays are representative genotyping methods that utilize hybridization with allele-specific oligonucleotide probes at the region including the polymorphic site. It also utilizes 5′ exonucleotide activity of DNA polymerase in PCR reactions and techniques of fluorescence resonance energy transfer (FRET) in the detection step. This technique was developed by Applied Biosystems and supports ready-to-use, validated assays providing both the reagents and instrumentation for genotyping common SNPs (16, 21). Although the initial cost of this method is high, the running cost of this method is relatively low. Therefore, this method is useful for analyses of small numbers of SNPs using large-size samples, such as analyses in clinical and diagnostic settings (15).

1. Perform PCR in a total of 5 µl solution containing 5–50 ng purified genomic DNA as the template, with 40× (or 20× or 80×) SNP Genotyping Assay TaqMan® Universal PCR Master Mix. The PCR program is the following: 95°C for 10 min, followed by 40 cycles of 92°C for 15 s and 60°C for 1 min.
2. Perform the post-PCR plate read using a real-time PCR system. Genotyping is determined after generating standard curves to quantify the amount of DNA in each sample and identifying allele types.

3.3.4. MPEX

The modified MPEX is a recently developed, allele-specific extension (ASE) method (22). In the modified MPEX, hybridization and extension reactions are only performed on the substrate, a plastic S-BIO® PrimeSurface® with a biocompatible polymer whose surface chemistry offers extraordinarily stable thermal properties and chemical properties advantageous for enzymatic reactions on the surface (22). At least 50 oligonucleotides for different SNPs can be spotted onto the same surface area (22), and this method appears to be suitable for restricted SNP analysis focused on a moderate number of candidate genes that might affect human sensitivity to opiates. We demonstrate here the significance of this method combined with multiplex PCR by analyzing representative SNPs on different LD blocks of *OPRM1* (23).

3.3.4.1. Oligonucleotide Module Fabrication

1. The oligonucleotide probes are designed to hybridize allele-specific PCR products of *OPRM1* and are dissolved in spotting solution to a final concentration of 0.2 µM.
2. The oligonucleotides are spotted (approximately 600 µm in diameter, approximately 12.5 nl/spot) on the surface of S-Bio® PrimeSurface® (BS-11608) using a BioChip Arrayer® spotting robot.
3. The modules (gasket-type hybridization cassettes) are incubated overnight in a humid chamber with 250 mM sodium phosphate buffer at room temperature.

4. The excess amine-reactive group (MEONP) is inactivated for 5 min at room temperature in blocking buffer solution.
5. After the modules are washed in boiling water for 2 min, they are washed in water at room temperature for 2 min and then dried by centrifugation.
6. The oligonucleotide modules are stored in a desiccated state at 4°C until use.

3.3.4.2. Preparation of Template Multiplex PCR Products and Their Confirmation

1. PCR reactions are carried out using Multiplex PCR Mix® in a 20 μL total reaction volume containing 10 μL Multiplex PCR Mix 1, 0.1 μL Multiplex PCR Mix 2, 1 μL template genomic DNA (0.5–19.75 ng), and an appropriate concentration of each primer (G5953A, A2109G: 5.0 μM; C691G, A118G: 1.0 μM). PCR is performed for 40 cycles consisting of denaturation at 94°C for 30 s, annealing at 54°C for 90 s, and extension at 72°C for 90 s.
2. After the PCR reaction, the multiplex PCR products are purified with the Wizard® SV 96 PCR Clean-Up System according to the manufacturer's instructions (http://www.promega.com/tbs/tb311/tb311.pdf). To reduce residual primers, an optional wash protocol using 80% ethanol is also performed.
3. The amplified products are electrophoresed on 2.0% agarose gels and then visualized by ethidium bromide staining.
4. An 8/10 volume of purified PCR product is used for the further modified MPEX reaction.

3.3.4.3. Modified MPEX Reaction

1. After the template PCR products have been denatured at 95°C for 20 min on a thermal cycler, the PCR products are subjected to an annealing reaction with immobilized oligonucleotides on the modules in a 100 μL reaction volume containing 1× PCR buffer supplied by QIAGEN (information regarding the components is not available), 0.1% TritonX100, 0.04 mM dNTP, and 2.5 U HotStar TaqTM DNA polymerase (QIAGEN).
2. The modules are rinsed three times with MPEX Buffer A.
3. The samples are further incubated at 66°C for 3 h, and biotin-dUTP is incorporated during the extension of the complementary strand. This reaction is performed in a hybridization oven that should be prewarmed at least 1 h before the reaction. To avoid a decrease in temperature, the modules are covered with aluminum foil.
4. The residual reaction mixture is removed by decantation, and the modules are then agitated with 100 μL of MPEX Buffer

A for 1 min on a Double Shaker NR-3 (65 r/min; Taitec, Saitama, Japan).

5. After the removal of MPEX Buffer A, the modules are further agitated with 100 µL of MPEX Buffer B for 1 min.
6. The MPEX Buffer B is completely removed by decantation and centrifugation.

3.3.4.4. Visualization by Colorimetric Reaction

1. A working solution of Streptavidin-AP should be prepared immediately before the reaction (0.15 µL of Streptavidin-AP, 15 µL of 10× MPEX Buffer A, 75 µL of 2× MPEX Buffer B, to a volume of 150 µL with distilled water).
2. The working solution is added to the modules, and the samples are incubated at 37°C for 10 min. During this reaction, the modules are covered with aluminum foil.
3. The residual reaction mixture is removed by decantation, and the modules then are agitated with 100 µL of MPEX Buffer A for 1 min on a Double Shaker NR-3 (65 r/min; Taitec, Saitama, Japan).
4. After the removal of MPEX Buffer A, the modules are further agitated with 100 µL of MPEX Buffer B for 1 min.
5. The MPEX Buffer B is completely removed by decantation and centrifugation.
6. The colorimetric detection of the AP-labeled complementary strand is performed in BCIP/NBT substrate solution at 37°C for 30 min.
7. The BCIP/NBT substrate solution is removed, and the modules then are agitated with 100 µL of distilled water for 1 min on a Double Shaker NR-3 (65 r/min; Taitec, Saitama, Japan).
8. For the purpose of taking photographs, the modules are immediately dried by centrifugation.
9. The dark purple stains are scanned on a GT-9700F personal image scanner.
10. The scanned data are stored using the free software Epson TWAIN 5 (http://www.epson.jp/dl_soft/list/1379.htm) and then graphically manipulated using Adobe® Photoshop Elements v. 4.0. The recommended resolution of the graphics is more than 600 dpi.
11. The SNPs are assessed principally by visual inspection of the signal intensities.

3.4. Statistical Analyses

The statistical methodologies for detecting genetic polymorphisms affecting human sensitivity to opiates can be diverse, depending on how the study is designed by researchers and what variables

and covariables are incorporated into the analyses from the clinical data of the subjects. In the following subsections, we concisely describe our protocol for investigating the association between genetic polymorphisms and human sensitivity to opiates. Here we cover only population association studies for quantitative traits in which unrelated individuals without population stratification who were treated with analgesics are genotyped at a number of polymorphisms. We do not address family-based association studies and case–control studies, which have an important role in efforts to understand the effects of genes on disease but require different types of statistical analyses. For more information, recent review articles have discussed association studies using various statistical approaches (24). All of the statistical analyses we describe can be performed using one or more of the software programs listed in Table 1, all of which can be found at the Genetic Analysis Software website (http://www.nslij-genetics.org/soft/).

3.4.1. Data Validation

Before beginning an association analysis, the genotype data should be appropriately formatted to be inputted into the software.

1. Check the genotyped polymorphism data. To perform precise and unbiased haplotype estimation, remove individual data in which genotypes for most of the polymorphisms of interest were not successfully determined due to inherent problems of such DNA samples. (Some software may automatically remove such genotype data or predict such data based on the observed genotypes at neighboring SNPs.)
2. Perform a statistical test to check the genotype data for deviation from Hardy-Weinberg Equilibrium (HWE). In most cases, deviation from HWE in healthy subjects indicates a genotyping error, inbreeding, stratification, or natural selection of the population, and thus further analysis using the genotype data for that SNP may be abandoned.
3. Format the genotype data to be inputted into the statistical analysis software or genetic analysis software (Table 1). Many software programs for analyzing haplotype-based associations might require the genotype data to be in a specific format (e.g., linkage format).
4. For a haplotype-based association study, perform haplotype phasing of the samples using genotype data and one of the suitable genetic analysis software programs (Table 1).

3.4.2. Linkage Disequilibrium (LD) Analysis

Information of LD between the SNPs at the region of interest is important in several ways. Even if the causal SNP is not directly genotyped, one could capture the association between other SNPs that show a strong LD with the causal SNP and a specific phenotype.

Additionally, if two SNPs are in strong LD, and almost no recombination is assumed between them, genotyping only one of the SNPs is sufficient. The strength of LD between two SNPs is often measured by the values D' and r^2, both of which are commonly used together but calculated by different formulas. Furthermore, a subset of the SNPs that is selected based on the information of LD relationships and tags the representative haplotypes at the region appropriately (25) are termed "haplotype tagging SNPs" or "tag SNPs" and might promote the efficiency of genotyping and further analyses without reducing the power to detect the expected association.

1. Estimate the strength of LD between the SNPs by calculating the values D' and r^2 based on the genotype data of the subjects using the suitable genetic analysis software programs (Table 1).
2. Select the tag SNPs that best represent the haplotypes at the region by using the suitable genetic analysis software programs, such as "Tagger" ((36); Table 1).

3.4.3. Association Study for Distinct SNPs

To explore the association between SNPs and some quantitative traits with normal distribution, several types of statistical analysis are possible, such as analysis of variance (ANOVA) and linear regression, which assumes a linear relationship between the mean value of the trait and the genotype.

1. Perform the statistical tests by running a software program to investigate the association between each distinct SNP and the phenotypic traits of interest. In the case of ANOVA, phenotypic traits and genotypes of a SNP should be treated as dependent and independent variables, respectively. Most software programs can also accept covariable data as well as genotype and phenotype data such as age, sex, and other characteristics of the subjects.
2. Check whether the output P-value achieves the level of significance for detecting a positive association.
3. In many cases, corrections of multiple testing might be required for the number of SNPs tested to avoid a type I error. However, corrections such as Bonferroni might not be required in some cases to avoid a type II error (26, 27).

3.4.4. Association Study for Haplotypes

Relatively few software programs are available for analyzing association between haplotypes and quantitative traits such as analgesic requirements compared with software programs that are available for case–control studies. Furthermore, estimated haplotypes of individuals or haplotype frequencies of the population tend to differ among the software used, depending on the algorithms employed by the programs. Minor differences

in haplotype frequency estimates can produce very large differences in statistical tests (28). Therefore, comparing the outcomes of similar analyses carried out by different software programs is advisable to confirm the results.

1. Perform the statistical tests by running a software program to investigate the association between one or more haplotypes and the phenotypic traits of interest. In many cases, haplotype-based association studies might be completed concurrently with haplotype estimations of the populations involved.
2. Check whether the output *P*-value achieves the level of significance for detecting a positive association.
3. In some cases, corrections of multiple testing might be required for the number of haplotypes tested to avoid a type I error. However, corrections such as Bonferroni might not be required in some cases to avoid a type II error (26, 27).

3.4.5. Utilization of Databases

Numerous databases have become openly available that are helpful for designing a study and for analyzing the results. Below are a few databases that are useful for surveying genes and genetic polymorphisms and downloading data that could expedite study analyses. A recent review article discusses bioinformatics approaches for SNP analyses (29).

1. To obtain overall information of any SNP, consult the dbSNP database ((30); http://www.ncbi.nlm.nih.gov/SNP/), which contains the largest amount of data on genetic variations, including SNPs.
2. To obtain Genotype-to-Phenotype information, consult HGVbase (Human Genome Variation database; http://hgvbase.cgb.ki.se; formerly known as HGBASE), which provides a high-quality, nonredundant database of available genomic variation data of all types (31).
3. To obtain information of SNPs related to drug response, consult the PharmGED database (http://bidd.cz3.nus.edu.sg/phg/), which provides information about the effects of a particular protein polymorphism, noncoding region mutation, splicing alteration, or expression variation on the response of a particular drug.
4. To obtain overall information about the haplotype map of the human genome or to download genotype data for linkage disequilibrium analysis or other purposes, consult the HapMap database ((32); http://www.hapmap.org/index.html.en), which helps researchers find genes associated with human disease and pharmaceutical response.

4. Notes

1. Any form of clean cotton swab can be used, such as the commercially available Sterile Omni Swab (Whatman plc, Kent, United Kingdom) or a commonly used small wad of cotton wrapped around the end of a small rod made of wood, rolled paper, or plastic. To maintain cleanliness, each swab should be packaged before use.
2. The DNA polymerase used in the AS-PCR should be the one that lacks proofreading activity because the polymerase with proofreading activity could substitute a mismatched base for the correct base at the polymorphic site, owing to its exonuclease activity from 3′ to 5′.
3. The oligonucleotides used here were single-stranded 19–27 mer 5′-C6-amino-oligonucleotides. The oligonucleotides were designed and synthesized by NovusGene (Tokyo, Japan).
4. All researchers handling human genomes must observe the laws and guidelines set forth in their respective countries. Such laws and guidelines may be enacted based on the Declaration of Helsinki, World Medical Association (WMA), and Universal Declaration on the Human Genome and Human Rights adopted by the UNESCO Bioethics Programme General Conference in 1997. For example, researchers in Japan must observe the "Ethical Guidelines for Analytical Research on the Human Genome/Genes" issued by the Japanese Ministry of Education, Culture, Sports, Science and Technology, the Ministry of Health, Labour and Welfare, and the Ministry of Economy, Trade and Industry.
5. Clean the scissors used to separate the swab from the sticks with ethanol each time to avoid genomic DNA contamination from the previous sample.
6. The concentration of the purified DNA should be similar among samples because heterogeneity in the concentration of genomic DNA could increase the generation of false products in the AS-PCR, leading to misjudging the genotype of the sample.

Acknowledgments

We acknowledge Mr. Michael Arends for his assistance with editing the manuscript. This work was supported by grants from the Ministry of Health, Labour and Welfare of Japan (H17-Pharmaco-001, H19-Iyaku-023), the Ministry of Education,

Culture, Sports, Science and Technology of Japan (20602020, 19659405, 20390162), The Naito Foundation, and The Mitsubishi Foundation.

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No.37

In: Acute Pain: Causes, Effects and Treatment
Editors: Sam D'Alonso and Katherine L. Grasso

ISBN 978-1-60741-223-6

Chapter V

Association between Analgesic Requirements after Major Abdominal Surgery and Polymorphisms of the Opioid Metabolism-Related Gene *ABCB1*

***Daisuke Kobayashi*[1,2,*], *Daisuke Nishizawa*[1,*], *Shinya Kasai*[1], *Junko Hasegawa*[1], *Makoto Nagashima*[3], *Ryoji Katoh*[3], *Yasuo Satoh*[4], *Megumi Tagami*[4], *Masakazu Hayashida*[5], *Kenichi Fukuda*[2] and *Kazutaka Ikeda*[1,#]**

[1]Division of Psychobiology, Tokyo Institute of Psychiatry, Tokyo, Japan;
[2]Department of Clinical Oral Health Science, Tokyo Dental College, Suidoubashi Hospital, Tokyo, Japan;
[3]Department of Surgery, Toho University Sakura Medical Center, Sakura, Japan;
[4]Department of Anesthesiology, Toho University Sakura Medical Center, Sakura, Japan;
[5]Department of Anesthesiology, Saitama Medical University International Medical Center, Hidaka, Japan

Abstract

Analyses of opioid-related molecules using genetically modified animals have clarified the mechanisms of the analgesic action of morphine, its related side-effects, and individual variabilities in sensitivity. In the present study, we examined the relationship between the dosage of analgesics, including opioids, required after major abdominal

* These authors contributed equally to this study.
# Corresponding author: Kazutaka Ikeda, Ph.D., Director, Molecular Psychiatry Research Tokyo Institute of Psychiatry, 2-1-8 Kamikitazawa, Setagaya-ku, Tokyo 156-8585, Japan, Phone: +81-3-3304-5701; Fax: +81-3-3329-8035; E-mail: ikedak@prit.go.jp.

surgery and polymorphisms of an opioid metabolism-related gene, ATP-binding cassette, sub-family B (MDR/TAP), member 1 (*ABCB1*). We studied 129 patients (74 males, 55 females; aged 28-80 years old) who were administered analgesics, including opioids, for pain relief following major abdominal surgery and who provided informed consent. The analyses were performed using multivariate analysis, with $p < 0.05$ considered statistically significant. We found a significant association between analgesic requirements and *ABCB1* polymorphisms. Studies on gene polymorphisms associated with analgesic requirements are important for developing appropriate personalized treatments with less side-effects.

1. Introduction

Opioids are commonly used as effective analgesics for the treatment of acute and chronic pain. However, sensitivity to opioid analgesics is well known to vary widely among individual subjects [1]. Because of this variability, a dose of an opioid analgesic that can produce satisfactory pain relief without adverse effects in some patients might cause under-dosing or overdosing in other patients. Among opioid-related genes, the μ-opioid receptor gene is the major candidate gene for investigation [2] because μ-opioid receptor knockout mice display abolished morphine-induced analgesia in several tests of nociceptive responsiveness [3]. Recently, several studies in humans have revealed a relationship between genetic variations in the μ-opioid receptor gene and sensitivity to opioid analgesics [4-8].

An opioid metabolism-related gene, ATP-binding cassette, sub-family B (MDR/TAP), member 1 (*ABCB1*), encodes a blood-brain barrier transporter P-glycoprotein that plays an important role in controlling substance passage between the blood and brain. Thus, the ABCB1 protein reportedly plays a role in morphine metabolism [9]. Genetic variations in the human *ABCB1* gene have been the most extensively studied. More than 50 single nucleotide polymorphisms (SNPs) and insertion/deletion polymorphisms in the *ABCB1* gene have been reported. However, information is still limited with respect to the actual effects of these genetic polymorphisms on the function of the ABCB1 protein. In the present study, we examined the possible association of three *ABCB1* polymorphisms with sensitivity to opioid that have been reportedly associated with methadone requirements, an opioid used in heroin dependence treatment.

2. Methods

2.1. Subjects

One hundred twenty-nine patients participated in the association study. Subjects had undergone major open abdominal surgery, mostly gastrectomy for gastric cancer and colectomy for colorectal cancer, under combined general and epidural anesthesia at Research Hospital, Institute of Medical Science, The University of Tokyo, or at Toho University Sakura Medical Center. Peripheral blood or oral mucosa samples were collected from these subjects for gene analysis.

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

2.2. Clinical Data

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 through the catheter placed in the lower thoracic or upper lumbar epidural space at a constant rate of 2 ml/h. Whenever the patient complained of significant postoperative pain despite continuous epidural analgesic, appropriate doses of opioids, including buprenorphine, pentazocine, and pethidine, and/or nonsteroidal anti-inflammatory drugs (NSAIDs), including diclofenac, flurbiprofen, and indomethacin, were systemically administered as rescue analgesics at the discretion of surgeons. Also, epidural morphine was administered in some patients. The clinical data sampled included age, gender, body height, body weight, postoperative diagnosis, type of operation, duration of operation, and frequency and doses of rescue analgesics (opioids and/or NSAIDs) administered during the first 24 h postoperative period, for which analgesic therapy would be required in most patients.

To allow intersubject comparisons of rescue analgesic doses required during the first 24 h postoperative period, doses of opioids and NSAIDs administered as rescue analgesics during this period were converted to the equivalent dose of systemic pentazocine. The dose of epidural morphine required for pain relief after abdominal surgery is approximately one-fifth that of systemic morphine [10]. Systemic pentazocine is one-third as potent as systemic morphine, and systemic buprenorphine is approximately 30-times as potent as systemic morphine [11]. Systemic flurbiprofen, diclofenac, and indomethacin are equivalent [12, 13]. The analgesic effect of systemic diclofenac, 1 mg/kg, is comparable to that of systemic fentanyl, 1 μg/kg [14, 15]. Systemic fentanyl is 100-times more potent than systemic morphine [16]. Therefore, systemic buprenorphine, 400 μg was converted to the equivalent systemic pentazocine dose of 30 mg in this study. As systemic pethidine is considered to be less potent than systemic morphine, 35 mg systemic pethidine was converted to the equivalent systemic morphine dose of 10 mg and thus systemic pentazocine dose of 30 mg. Systemic flurbiprofen, 50 mg, and systemic diclofenac, 50 mg, and systemic indomethacin, 50 mg, were converted to the equivalent systemic pentazocine dose of 15 mg. The frequency of rescue analgesic administration was determined as the frequency of the use of rescue analgesics during the first 24 h postoperative period. The total dose of rescue analgesics administered was determined as the sum of systemic pentazocine-equivalent doses of all opioids and NSAIDs given to patients as rescue analgesics during the same period.

2.3. Genotyping

Total genomic DNA was extracted from peripheral blood samples by standard procedures. For genotyping rs1128503, the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was adopted. To perform PCR-RFLP, the restriction enzyme EcoO109I (Takara K.K. Japan, Shiga, Japan) and two primers of H44 and H45 were used (Table 1).

Table 1. Primers used in the analyses of *ABCB1* polymorphisms

rsID	Sequence	Forward/Reverse	Primer No.
1128503	ATCCAGCTCTCCACAAAATATCATAAAG	Forward	H44
	CTGATCACCGCAGGGTCTAGCTCGCATGG GTCCTC	Reverse	H45
1045642	ACACAAACTTTTCCTTAATCTCACAG	Forward	H51
	TTTGGTTGCTAATTTCTCTTCACTTC	Reverse	H52
2032582	ACTCTTAGCAATTGTACCCATCAT	Forward	H46
	AAACACATTCTTAGAGCATAGT	Reverse	H50

First, PCR was performed in a final volume of 10 μl containing 5× GoTaq™ reaction buffer (Promega K.K. Japan, Tokyo, Japan), 0.8 mM dioxyribonucleoside triphosphate, 0.5 μM of each primer, 0.05 U GoTaq™ DNA polymerase (Promega), and 5-50 ng extracted genomic DNA as the template. The PCR program was the following: 95°C for 1 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 5 min. The amplified DNA fragments were digested by the restriction enzyme at 37°C in a total of 10 μl reaction solution containing 10× L Buffer, 0.75 U EcoO109I (Takara K.K. Japan, Shiga, Japan), and 5 μl PCR product as the substrate. The digestion products were analyzed by electrophoresis using 1-2% agarose gel and ethidium bromide staining for visualization under ultraviolet illumination. The appearance of both the 366 bp and 134 bp DNA fragments of the loaded sample corresponded to the C/C genotype. The appearance of the 490 bp, 366 bp, and 134 bp fragments corresponded to the T/C genotype, and the appearance of the 490 bp fragment corresponded to the T/T genotype.

For genotyping rs1045642, the PCR-RFLP method was also adopted. To perform PCR-RFLP, the restriction enzyme Mbo I and two primers of H51 and H52 were used (Table 1). First, PCR was performed in a final volume of 10 μl containing 2× GoTaq™ Master Mix (Promega), 0.5 μM of each primer, and 5-50 ng extracted genomic DNA as the template. The PCR program was the following: 95°C for 1 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 5 min. The amplified DNA fragments were digested by the restriction enzyme at 37°C in a total of 10 μl reaction solution containing 10× K Buffer (Takara), 1 U MboI (Takara), and 5 μl PCR product as the substrate. The digestion products were analyzed by electrophoresis using 1-2% agarose gel and ethidium bromide staining for visualization under ultraviolet illumination. The appearance of the 172 bp, 169 bp, and 98 bp DNA fragments of the loaded sample corresponded to the C/C genotype. The appearance of the 341 bp, 172 bp, 169 bp, and 98 bp

fragments corresponded to the T/C genotype, and the appearance of the 341 bp and 98 bp fragments corresponded to the T/T genotype.

For genotyping rs2032582, direct sequencing was adopted. To perform direct sequencing, two primers of H46 and H50 were used (Table 1). First, PCR was performed in a final volume of 10 μl containing 2× GoTaq™ Master Mix (Promega), 0.5 μM of each primer, and 5-50 ng extracted genomic DNA as the template. The PCR program was the following: 95°C for 1 min, followed by 35 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 5 min. Following the cycle sequencing reaction with the BigDye® Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems Japan, Tokyo, Japan) according to the manufacturer's instructions and purification of the PCR products, DNA sequences of the fragments were determined using the automated sequencer ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems Japan) with both forward and reverse primers.

2.4. Statistical Analysis

The χ^2 test was performed for all genotype frequency data using FreeJSTAT 8.2 for Windows (free software by M. Sato, Japan; current version is available at http://www.vector.co.jp/soft/win95/business/se030917.html; accessed October 13, 2008) to investigate the deviation of the distributions from those in the theoretical Hardy-Weinberg equilibrium. Analysis of variance (ANOVA) was performed to examine the contribution of the SNPs to the frequency of 24 h analgesic requirements and postoperative 24 h analgesic requirements. ANOVA was performed using SPSS (12.0J for Windows, SPSS Japan, Tokyo, Japan). Power analyses were performed using G*Power version 3.0.5 [17]. Values of $p < 0.05$ were considered statistically significant.

3. Results

The clinical data of the 129 subjects who were included in the association study are provided in Table 2. Rescue analgesics were required in 72 patients. Doses of rescue analgesics administered to patients are shown in Table 2. The allele frequencies in these SNPs were similar to those in a previous report [18] (Table 3). The genotype distributions for the three SNPs selected were not significantly different from the theoretical values of Hardy-Weinberg equilibrium (data not shown).

Two-way ANOVA was performed to examine the effects of SNPs and gender on the frequency of rescue analgesic administration and the total dose of rescue analgesics administered. Statistical power analyses for the ANOVA revealed that the expected power (1 minus type II error probability) was 71% for the Cohen's conventional "medium" effect size 0.25 [19] when the sample size was 129 and type I error probability was set at 0.05. Significant associations were not observed between rs1045642 and rs2032582 and the frequency of rescue analgesic administration and total dose of rescue analgesics administered (data not shown). Significant associations were not observed between the three SNPs and

total dose of rescue analgesics administered after Bonferroni correction. However, a significant main effect of the *ABCB1* rs1128503 SNP on the frequency of rescue analgesic administration was found when the C/C genotype was compared with the combined C/T and T/T genotypes ($F_{1, 125} = 6.283$, $p = 0.013$; Figure 1). This difference was significant even after Bonferroni correction for the three SNPs ($p = 0.039$), indicating that the carriers of the C/C genotype required less frequent rescue analgesics compared with carriers of the C/T or T/T genotype.

Table 2. Clinical data of subjects included in the study

N		Minimum Maximum		Mean	SD
Gender					
male	74				
female	55				
Age	129	28	80	63.57	9.92
Height (cm)	129	133	175	158.21	8.34
Weight (kg)	129	35	80	56.24	10.42
Frequency of analgesic administration	129	0	8	0.95	1.18
Total dose of rescue analgesics (mg)	129	0	112.5	14.07	18.56
Epidural morphine (mg)	2	0.5	2	1.25	1.06
Systemic pentazocine (mg)	24	15	75	26.25	17.83
Systemic buprenophine (mg)	15	0.2	0.8	0.47	0.16
Systemic petidine (mg)	2	17.5	35	26.25	12.37
Systemic diclofenac (mg)	24	25	100	39.58	19.39
Systemic flurbiprofen (mg)	12	50	150	62.50	31.08
Systemic indomethacin (mg)	4	25	50	43.75	12.5

Table 3. Genotype and allele frequency of *ABCB1* polymorphisms

rs1128503(1236T/C)			rs2032582(2677G/T/A)			rs1045642(3435C/T)		
t	N	frequency	Genotype	N	frequency	Genotype	N	frequency
C/C	25	0.194	A/A	7	0.054	C/C	52	0.403
C/T	52	0.403	A/G	23	0.178	C/T	51	0.395
T/T	52	0.403	A/T	18	0.136	T/T	26	0.202
			G/G	23	0.178			
			G/T	30	0.233			
			T/T	27	0.209			
total	129	1.000	total	129	1.000	total	129	1.000
Allele frequency			Allele frequency			Allele frequency		
C	102	0.395	A	55	0.213	C	155	0.601
T	156	0.605	G	99	0.384	T	103	0.399
			T	102	0.395			
total	258	1.000	total	258	1.000	total	258	1.000