Chapter 29

Genetic Polymorphisms and Human Sensitivity to Opioid Analgesics

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Abstract

Opioid analgesics are commonly used for the treatment of acute as well as chronic, moderate to severe pain. Well-known, however, is the wide interindividual variability in sensitivity to opioids that exists, which has often been a critical problem in pain treatment. To date, only a limited number of studies have addressed the relationship between human genetic variations and sensitivity to opioids, and such studies are still in their early stages. Therefore, revealing the relationship between genetic variations in many candidate genes and individual differences in sensitivity to opioids will provide valuable information for appropriate individualization of opioid doses required for adequate pain control. Although the methodologies for such association studies can be diverse, here we summarize protocols for investigating the association between genetic polymorphisms and sensitivity to opioids in human volunteers and patients undergoing painful surgery.

Key words: Analgesics, Genetic polymorphisms, Single nucleotide polymorphism (SNP), Genotype–phenotype association, Haplotype, Opioids, Opiates, Pain relief, Personalized medicine, Pharmacogenomics

1. Introduction

Opioid analgesics are commonly used for the treatment of acute or chronic, moderate to severe pain. However, wide interindividual variability exists in sensitivity to opioid analgesics (1). Because of this variability, a dose of an opioid that can produce satisfactory pain relief without significant adverse effects in some patients might cause underdosing or overdosing in others. Individual differences can be attributed to both genetic and environmental factors,

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although the relative influence of each of these factors is diverse (2). Genetic variations in many genes involved in opioid pharmacokinetics and pharmacodynamics might be responsible, at least partially, for the individual differences in phenotypes related to analgesic efficacy of opioids.

Numerous molecules are known to be involved in the pharmacological effects of opioids. The genes encoding these molecules are candidates for exploring the relationships between genetic variations and individual differences in phenotype traits related to opioid actions. Recently, several studies in humans have investigated the relationships between the individual genetic variations in the μ-opioid receptor gene (*OPRM1*) and sensitivity to opioids (2–8). However, such studies are still in their early stages and await future meta-analyses for clarification of the precise phenotype-to-genotype relationships.

Therefore, revealing the relationships between genetic variations in many candidate genes and interindividual differences in sensitivity to opioids will facilitate a better understanding of how human genetic polymorphisms can cause differences in sensitivity to opioids. Data from such studies will provide valuable information for appropriate individualization of opioid doses to achieve adequate pain control and open new avenues for personalized pain treatment.

2. Materials

2.1. Collecting Clinical Data

1. A form describing the design of the study approved by each Institutional Review Board (IRB) at the respective institutions.
2. Letters to the candidate subjects explaining the outline or details of the study and reply cards on which the candidates indicate whether they are interested in participating in the study.
3. Packages to be sent to participating subjects that include explanatory leaflets describing the detailed study protocol, written informed consent forms, instructions for collecting oral mucosa samples (see section 3.2.1.), cotton swabs to collect oral mucosa samples, test tubes to enclose the samples, and stamped return-mail envelopes.
4. In the case of retrospective studies, lists of ex-patient or patient candidates who had previously undergone surgery (e.g., major open abdominal surgery) and received opioids (e.g., fentanyl or morphine) postoperatively during a specific period (e.g., the first 24 h postoperative period) at the hospitals where clinical data are collected.

5. Several references of papers or books describing the method of properly converting the dose of an opioid analgesic to the equivalent dose of another opioid analgesic. For example, to allow for intersubject comparisons of opioid doses required during the specific period, doses of opioid analgesics used during this period were converted to an equivalent dose of systemic fentanyl in our study.

2.2. Preparation of Genomic DNA

1. Four cotton swabs for each subject (see Note 1).
2. Screw-cap centrifuge tube for each subject in which to enclose swabs (e.g., Corning® 15 mL PP Centrifuge Tubes; Corning Inc., Corning, NY).
3. Cup of water to rinse out subject's mouth.
4. DNA extraction kit (e.g., QIAamp DNA Mini Kit; QIAGEN, Hilden, Germany).
5. Phosphate buffered saline (PBS): 1 tablet of Phosphate Buffered Salts Tablets (Takara Bio, Otsu, Japan) is dissolved in 100 mL of distilled water.
6. Whole genomic DNA amplification kit (e.g., illustra GenomiPhi V2 Kit, 100rxns; GE Healthcare UK, Buckinghamshire, United Kingdom).
7. TE buffer: 300 μL of 1 M tris base, 60 μL of 0.5 M EDTA (pH 8.0), and distilled water to a total volume of 30 mL.
8. Spectrophotometer for measurement of the concentration of genomic DNA (e.g., NanoDrop ND-1000 Spectrophotometer; NanoDrop Technologies, Wilmington, DE).

2.3. Genotyping

2.3.1. Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR–RFLP)

1. Purified 5–50 ng genomic DNA.
2. Forward and reverse oligonucleotide primer set encompassing the specific region, including the polymorphic site for PCR amplification.
3. Reaction buffer for PCR including DNA polymerase, dioxyribonucleoside triphosphate (dNTP), and $MgCl_2$ (e.g., GoTaq® Master Mix; Promega, Madison, WI).
4. Thermal cycler (e.g., PROGRAM TEMP CONTROL SYSTEM PC-818-02; Astec, Fukuoka, Japan).
5. Agarose (e.g., Agarose ME, Classic Type; Nacalai Tesque, Kyoto, Japan), stored at room temperature.
6. TAE buffer (50×): 242 g of tris base, 57.1 mL of glacial acetic acid, 100 mL of 0.5 M EDTA (pH 8.0), and water to a total volume of 1,000 mL, stored at room temperature.
7. DNA size marker (e.g., Loading Quick® 100 bp DNA Ladder; Toyobo, Osaka, Japan), stored at −20°C.

8. Ethidium bromide solution (Sigma-Aldrich, St. Louis, MO), stored at 4°C.
9. Appropriate restriction enzymes for digestion of PCR products.

2.3.2. Allele–Specific PCR (AS–PCR)

1. Purified 5–50 ng genomic DNA.
2. Two forward oligonucleotide primers, whose 3′ ends are specific for detecting each of the two alleles at the polymorphic site, and a reverse oligonucleotide primer.
3. DNA polymerase attached with reaction buffer, dNTP, and $MgCl_2$ (e.g., GoTaq® Master Mix; Promega) (see Note 2).
4. Thermal cycler (e.g., PROGRAM TEMP CONTROL SYSTEM PC-818-02; Astec, Fukuoka, Japan).
5. Agarose (e.g., Agarose ME, Classic Type; nacalai tesque), stored at room temperature.
6. TAE buffer (50×): 242 g of tris base, 57.1 mL of glacial acetic acid, 100 mL of 0.5 M EDTA (pH 8.0), and water to a total volume of 1,000 mL, stored at room temperature.
7. DNA size marker (e.g., Loading Quick® 100 bp DNA Ladder; Toyobo), stored at −20°C.
8. Ethidium bromide solution (Sigma-Aldrich), stored at 4°C.

2.3.3. TaqMan® SNP Genotyping Assays

1. Purified 5–50 ng genomic DNA.
2. 40× (or 20× or 80×) SNP Genotyping Assay containing sequence-specific forward and reverse primers to amplify the polymorphic sequence of interest and two TaqMan® MGB probes labeled with VIC® dye to detect the sequence of one allele and with FAM™ dye to detect the sequence of another allele.
3. TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA).
4. Real-time PCR system (e.g., 7300 Real-Time PCR System; Applied Biosystems).

2.3.4. Multiple Primer Extension (MPEX)

2.3.4.1. Oligonucleotide Module Fabrication

1. S-Bio® PrimeSurface® (BS-11608) consisting of COC grafted with an original biocompatible phospholipid polymer, poly [2-methacryloyloxyethyl phosphorylcholine (MPC)-co-n-butyl methacrylate (BMA)-co-p-nitrophenyloxycarbonyl polyethyleneglycol methacrylate (MEONP)] (PMBN) hydrophilic polymer (Sumitomo Bakelite, Tokyo, Japan).
2. Oligonucleotide probes (see Note 3) designed to hybridize allele-specific PCR products of the arbitrary gene (e.g., *OPRM1*).

3. Spotting solution (250 mM sodium carbonate buffer, pH 9.0), stored at room temperature.
4. BioChip Arrayer® spotting robot (Filgen, Nagoya, Japan).
5. Oligonucleotide modules (gasket-type hybridization cassettes; one module consisting of 16 [8 × 2 lanes] hybridization wells; Sumitomo Bakelite).
6. Blocking buffer solution (0.5 N NaOH), stored at room temperature.

2.3.4.2. Preparation of Template Multiplex PCR Products and Their Confirmation

1. Multiplex PCR Mix® (TaKaRa Bio), stored at −30°C.
2. Primer pairs designed to amplify allele-specific PCR products of the arbitrary gene (e.g., *OPRM1*).
3. Thermal cycler (e.g., TaKaRa PCR Thermal Cycler Dice® Model TP600; TaKaRa Bio).
4. Wizard® SV 96 PCR Clean-Up System (Promega), stored at room temperature (22–25°C).
5. 80% ethanol, stored at room temperature.
6. Agarose (e.g., Agarose S; Nippon Gene, Tokyo, Japan), stored at room temperature.
7. TAE buffer (50×): 242 g of tris base, 57.1 mL of glacial acetic acid, 100 mL of 0.5 M EDTA (pH 8.0), and water to a total volume of 1,000 mL, stored at room temperature.
8. DNA size marker (e.g., 100 bp DNA Ladder; New England Biolabs, Ipswich, MA), stored at −20°C.
9. Ethidium bromide (Nippon Gene), stored at 4°C.

2.3.4.3. Modified MPEX Reaction

1. dNTP Set: 100 mM Solutions (GE Healthcare UK; working solution 1 mM for each dNTP), stored at −30°C.
2. HotStar Taq™ DNA polymerase (QIAGEN), stored at −30°C.
3. Biotin-11-dUTP (PerkinElmer, Wellesley, MA; working solution 1 mM), stored at −30°C and protected from prolonged exposure to light, with minimal freeze-thaw cycles.
4. 10× MPEX Buffer A: 1% TritonX100, stored at room temperature.
5. 2× MPEX Buffer B: 0.1 M phosphate buffer, pH 7.0, stored at room temperature.
6. Hybridization oven (e.g., Hybaid Midi Dual-14; Hybaid, Middlesex, United Kingdom).

2.3.4.4. Visualization by Colorimetric Reaction

1. Washing Buffer A: 10 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20, stored at room temperature.
2. Washing Buffer B: 0.1% Tween 20, stored at room temperature.

3. Streptavidin-AP (PerkinElmer), stored at −20°C, and then stored at 4°C after thawing, without refreezing.
4. BCIP/NBT substrate solution (PerkinElmer), stored at 2–8°C.
5. Scanner (e.g., GT-9700F personal image scanner; Epson, Tokyo, Japan).

2.4. Statistical Analyses

1. Suitable commonly used statistical software such as SPSS (SPSS Inc., Chicago, IL), SAS (SAS Institute, Cary, NC), JMP (SAS Institute, Cary, NC), R (freely available; http://www.r-project.org/), and/or software programs for genetic analyses (Table 1) to perform tests of Hardy–Weinberg equilibrium and linkage disequilibrium (LD) and association analyses.
2. Computer environment capable of accessing any websites of the databases of interest.

3. Methods

There are many ways of designing studies to explore the relationship between polymorphisms in some candidate genes and human sensitivity to opiates. In study designs, research subjects can be human volunteers undergoing standardized pain tests before and after administration of a given opioid, or patients undergoing standardized surgery and receiving opioids for postoperative pain control. Endpoint data that may represent the phenotypic traits related to analgesic efficacy of opioids can include the analgesic effect of the opioid evaluated by a standardized pain test or opioid requirements during and/or after standardized surgery as well as postoperative pain scores. Below are examples of an experimental study enrolling human volunteers, a prospective clinical study enrolling patients who are scheduled to have elective surgery of a given type, and a retrospective clinical study enrolling patients or ex-patients who previously underwent surgery of a given type. Research subjects are human volunteers or patients with American Society of Anesthesiologists Physical Status I or II ((9); Table 2) who do not have serious coexisting disease or a history of using opioids or other psychoneurotic agents.

3.1. Clinical Data Collection

The quality of clinical data is critical for accurately detecting polymorphisms associated with human sensitivity to opiates. Researchers may design many ways of collecting clinical data. We describe examples of our procedures and some points or issues that should be noted.

Table 1
Useful software programs for genetic analyses

Name	Application (main characteristics)	Platform	URL	Reference
Haplotype/Linkage disequilibrium analysis				
GENEPOP	A population genetics software package	–	http://genepop.curtin.edu.au	(33)
PHASE	Reconstructing haplotypes from population genotype data	–	http://www.stat.washington.edu/stephens/software.html	(34)
HAPLOVIEW	Haplotype analysis; single SNP haplotype association tests	Win/Mac/Unix	http://www.broad.mit.edu/mpg/haploview/index.php	(35)
LDSELECT	Analyses on patterns of linkage LD between polymorphic sites in a locus	–	http://droog.gs.washington.edu/ldSelect.html	(25)
GOLD	A software package that provides a graphical summary of LD in human genetic data	–	http://www.sph.umich.edu/csg/abecasis/GOLD	(36)
HAPLOTYPER	Estimation of haplotypes by MCMC	–	http://www.people.fas.harvard.edu/~junliu/Haplo/docMain.htm	(37)
Tagger	A tool for the selection and evaluation of tag SNPs from genotype data	web-based	http://www.broad.mit.edu/mpg/tagger	(38)
HAPLOBLOCKFINDER	A package for haplotype block identification, visualization and htSNP selection	UNIX/Win	http://cgi.uc.edu/cgi-bin/kzbang/haploBlockFinder.cgi	(39)
GENECOUNTING	Haplotype analysis with permutation tests for global association and specific haplotypes	Win/UNIX (Solaris)/Linux	http://www.mrc-epid.cam.ac.uk/Personal/jinghua.zhao/software.htm http://www.mrc-epid.cam.ac.uk/Personal/jinghua.zhao/software/	(40)

(continued)

Table 1 (continued)

Name	Application (main characteristics)	Platform	URL	Reference
HAPBLOCK	Dynamic programming algorithms for haplotype block partitioning and tag SNPs selection	Win/Linux/ UNIX(Solaris)	http://www-hto.usc.edu/msms/ HapBlock/	(41)
HAPLOBLOCK	Haplotype block identification, haplotype resolution and linkage disequilibrium mapping	UNIX(Solaris)/Linux/ MacOS X	http://bioinfo.cs.technion.ac.il/ haploblock/	(42)
SNPAlyze	A SNP and disease association analysis software	Win(98Me/NT4.0/ 2000/XP)	http://www.dynacom.co.jp/e/ products/package/snpalyze/index. html	(43)
EH (EHPLUS, EH+)	Estimation of haplotypes and case–control study based on estimated haplotypes	MS-DOS/UNIX	http://www.genemapping.cn/eh.htm http://linkage.rockefeller.edu/ software/eh	(44)
SNPHAP	A program for estimating frequencies of haplotypes of large numbers of diallelic markers	–	http://www-gene.cimr.cam.ac.uk/ clayton/software	–
Arlequin	An exploratory population genetics software environment able to handle large samples of molecular data	Win	http://cmpg.unibe.ch/software/ arlequin3/ http://lgb.unige.ch/arlequin/	(45)
HelixTree	Comprehensive toolset for population-based association studies	Win/Linux/MacOS X	http://www.goldenhelix.com/SNP_ Variation/HelixTree/index.html	(46)
Association study				
HARDY	MCMC program for association in two-dimensional contingency tables	UNIX (DEC-UNIX/..)	http://www.stat.washington.edu/ thompson/Genepi/Hardy.shtml	(47)

haplo.stats (formerly haplo.score)	A suite of routines for the analysis of indirectly measured haplotypes	UNIX	http://mayoresearch.mayo.edu/mayo/research/biostat/schaid.cfm	(48)
UNPHASED	A suite of programs for association analysis of multilocus haplotypes from unphased genotype data	UNIX(Solaris)/Linux/Win	http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/	(49)
HTR	Haplotype association mapping using unrelated individuals; "fixed" and "sliding" window analysis	Win/UNIX(Solaris)	http://statgen.ncsu.edu/zaykin/htr.html http://statgen.ncsu.edu/pub/zaykin/htr/	(46)
GENETIC POWER CALCULATOR	Automated power analysis for VC QTL linkage and other common tests	–	http://pngu.mgh.harvard.edu/~purcell/gpc/	(50)
CHAPLIN	Identifying specific haplotypes or haplotype features that are associated with disease	Win(2000/XP)	http://www.genetics.emory.edu/labs/epstein/software/chaplin/index.html	(51)
PAWE	Power and sample size calculations for genetic case–control association studies allowing for errors	–	http://linkage.rockefeller.edu/pawe/	(52)
Quanto	A program that computes sample size or power for association studies	Win (98/NT 2000/..)	http://hydra.usc.edu/GxE/	(53)
Hplus	Performing haplotype estimation on genetic markers and handling datasets that include case–control status	MS-Windows/Linux	http://cougar.fhcrc.org/hplus/	(54)
PLINK	A whole-genome association analysis toolset focusing purely on analysis of genotype/phenotype data	–	http://pngu.mgh.harvard.edu/purcell/plink/	(55)

SNP single nucleotide polymorphism, *LD* linkage disequilibrium, *MCMC* Markov chain Monte Carlo, *htSNP* haplotype tagging SNP, *VC* variance components, *QTL* quantitative trait locus

Table 2
American Society of Anesthesiologists Physical Status Classification System

P1:	A normal healthy patient
P2:	A patient with mild systemic disease
P3:	A patient with severe systemic disease
P4:	A patient with severe systemic disease that is a constant threat to life
P5:	A moribund patient who is not expected to survive without the operation
P6:	A declared brain-dead patient whose organs are being removed for donor purposes

3.1.1. Ethical Issues and Study Designs

1. Plan study protocol such that it meets all requirements imposed by the laws and guidelines regarding studies that handle human genomes (see Note 4).
2. Obtain approval from each respective institutional IRB for the study protocol.
3. Obtain written informed consent from each human subject after appropriately explaining, in written form, the clinical data sampling and DNA analysis.
4. A personal information manager responsible for managing personal information of the research subjects and making such information unidentifiable based on instructions from the head of the respective research institution should anonymize the collected samples before and after researchers handle the data to ensure the protection of personal information. Fig. 1 shows an example of the procedure in which personal information is protected.

3.1.2. Collecting Data in an Experimental Study Enrolling Human Volunteers

1. A given dose of a given opioid analgesic (e.g., intravenous [i.v.] injection of fentanyl, 2 μg/kg; i.v. injection of morphine, 0.2 mg/kg; i.v. infusion of remifentanil, 0.2 μg/kg/min over hours) is administered to human volunteers.
2. A standardized pain test, such as for thermal, mechanical, or electrical pain, is performed before, during, and after opioid administration (10).
3. An appropriate cutoff point is set to avoid tissue damage.
4. The pain test can be performed repeatedly, at a given interval, during and/or after opioid administration (e.g., every hour during and after i.v. infusion of remifentanil) over 4 h (10).
5. End-points can be latency to pain perception and/or pain tolerance, or pain perception and/or pain tolerance thresholds.

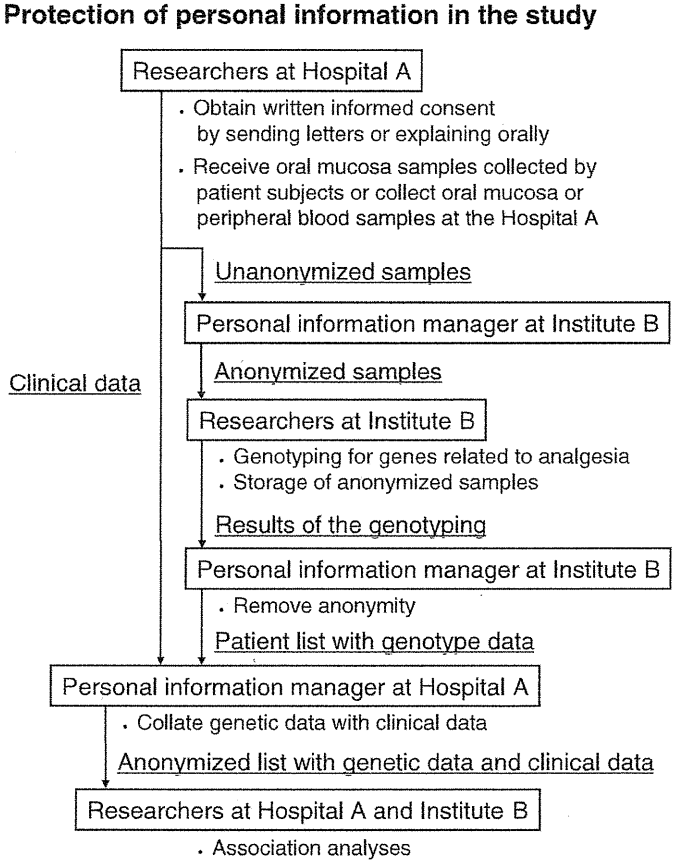

Fig. 1. Example of the procedure in which the personal information of research subjects is properly preserved. In this case, clinical data are collected at Hospital A, and genetic data are collected at Institute B.

6. The percent maximum possible effect (%MPE) can be calculated as an easy-to-interpret indicator of analgesic efficacy of the opioid: *%MPE = (cutoff value − maximum value during or after opioid administration)/(cutoff value − value before opioid administration) × 100.*
7. Whole blood (10 ml) or oral mucosa is sampled for genomic DNA analysis.

3.1.3. Collecting Clinical Data in a Prospective Study

1. Subjects are patients who are scheduled to have surgery of a given type that involves well-standardized procedures (e.g., distal gastrectomy for gastric cancer) under standardized anesthesia (e.g., sevoflurane–remifentanil anesthesia with or without epidural anesthesia).
2. Postoperative pain is managed with a single opioid analgesic according to a standardized protocol using a patient-controlled analgesia (PCA) pump (e.g., fentanyl, 20 μg per

demand dose with a lockout interval of 5 min; morphine, 2 mg per demand dose with a lockout interval of 10 min) (11).

3. Rescue analgesics (e.g., nonsteroidal antiinflammatory drugs) should be prescribed whenever the analgesic effect of the opioid is inadequate or the use of the opioid is discontinued because of significant adverse effects.
4. Postoperative pain scores are recorded at given postoperative time-points (e.g., 3, 6, 12, and 24 h after surgery) using an appropriate pain scale (e.g., visual analog scale, verbal pain rating scale, numerical pain rating scale) (11).
5. Presence/absence and severity (if present) of adverse effects of the opioid (e.g., nausea/vomiting and respiratory depression) are recorded.
6. Clinical data that may relate to analgesic efficacy of the opioid are recorded, including age, gender, type of surgery, duration of surgery, type of anesthesia, intraoperative opioid requirements, postoperative opioid requirements, rescue analgesic requirements during a given postoperative period (e.g., during the first 24 h after surgery), and postoperative pain scores.
7. Whole blood (10 ml) or oral mucosa is sampled for genomic DNA analysis.

3.1.4. Collecting Clinical Data in a Retrospective Study

1. A researcher in charge of clinical data collection (Researcher C) lists ex-patient or patient candidates who previously underwent surgery of a given type and received opioids for postoperative pain control at a particular hospital.
2. Researcher C mails letters to these candidates explaining the outline of the study protocol and reply cards on which the candidates can indicate their interest in participating in the study.
3. A researcher in charge of genomic DNA analysis (Researcher D) receives the reply cards from the candidates who are willing to participate in the study.
4. Researcher D sends packages to these candidates that include explanatory leaflets describing the detailed study protocol, written informed consent forms, instructions to collect oral mucosa samples, cotton swabs to collect oral mucosa samples, test tubes to enclose the samples, and stamped return-mail envelopes.
5. Researcher D receives signed informed consent forms and oral mucosa samples from the candidates who have been determined to be research subjects.
6. Researcher C collects clinical data from the hospital records of the research subjects that may relate to analgesic efficacy of opioids (see Subheading 3.1.3).

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.

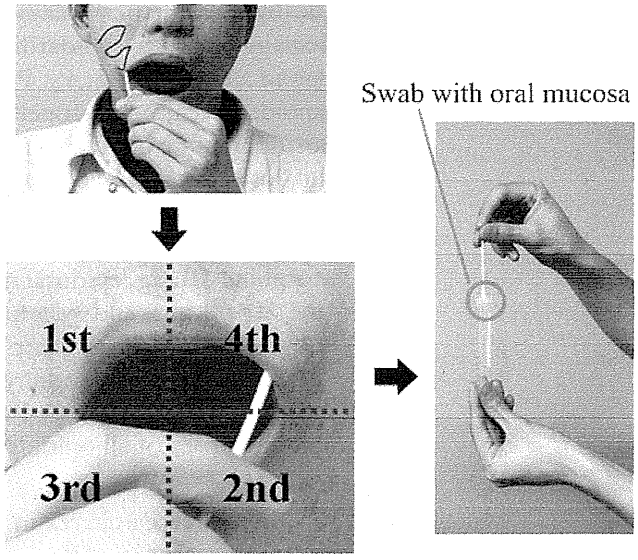

Fig. 2. Procedure for collecting oral mucosa (see Subheading 3.2.1).

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 microcentrifuge 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.

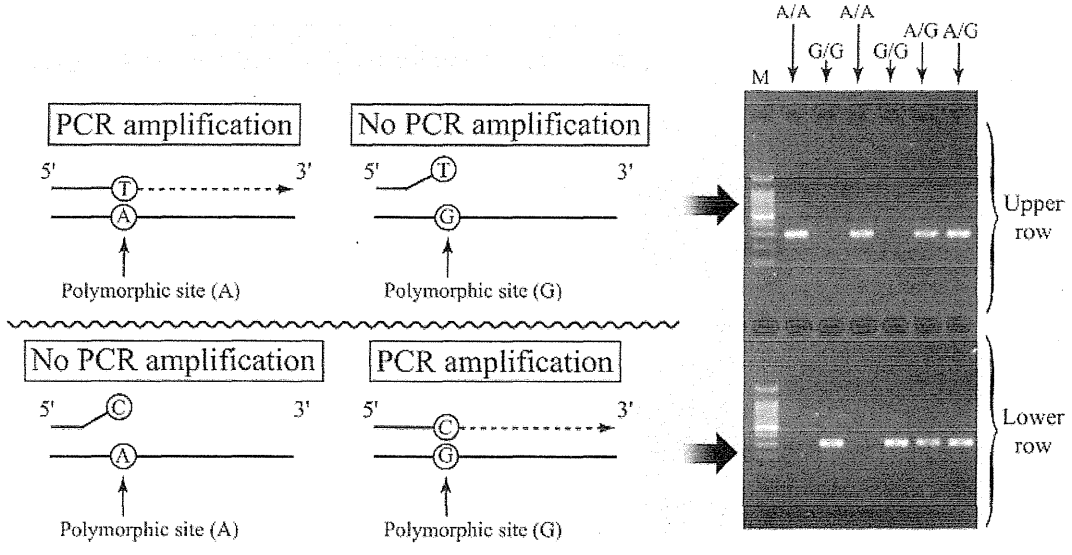

Fig. 3. Detection of allele-specific PCR products for the A118G SNP in the µ-opioid receptor gene (*OPRM1*). The principle as well as the results of AS-PCR is illustrated. Upper and lower rows in each lane indicate the A allele- and G allele-specific PCR products for each sample, respectively. The presence 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.