

Genome-wide Association Study Using Pooled DNA to Identify Candidate Markers Mediating Susceptibility to Postoperative Nausea and Vomiting

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ABSTRACT

Background: A family history has been established as a risk factor for postoperative nausea and vomiting (PONV), but the identities of susceptibility genes remain unknown. The goal of this study was to identify the genetic loci that may contribute to PONV susceptibility in an adult population.

Methods: The authors performed a genome-wide association study involving pooling of DNA obtained from 122 patients with severe PONV and 129 matched controls. Each pool was hybridized to a single nucleotide polymorphism (SNP) microarray, and probe intensity was used to predict allele frequency. Differences in allele frequency between SNP in the PONV and control groups were ranked after accounting for the pooling error. The highest ranking SNPs were selected for individual genotyping in the subjects from whom the DNA pool was comprised and in the new verification cohort consisting of 208 subjects (104 PONV patients and 104 controls).

Results: The authors identified 41 SNP targets showing substantial difference in allelic frequency between pools. These markers were first genotyped in the individual DNA samples from which the pools were comprised. The authors observed evidence for an association between PONV and 19 different loci in the genome. In the separate verification cohort, the association with PONV was observed for four SNPs. This association remained significant after correcting

What We Already Know about This Topic

- Genetic susceptibility to postoperative nausea and vomiting (PONV) has not been studied

What This Article Tells Us That Is New

- Using genome-wide association and an independent validation cohort, one single nucleotide polymorphism (rs2165870) in the promoter region of the M3 muscarinic acetylcholine receptor (CHRM3) gene was found to be significantly associated with PONV

for multiple testing ($P < 0.0023$) for one SNP (rs2165870), which is located upstream of the promoter for the muscarinic acetylcholine receptor 3 subtype (CHRM3) gene.

Conclusions: The authors performed the genome-wide association study for PONV using pooled DNA samples. Through individual genotyping, they confirmed association of at least one SNP that is predictive of PONV susceptibility.

POSTOPERATIVE nausea and vomiting (PONV) is the most common complication of anesthesia administration. Although, typically short-lived and nonfatal, PONV remains a common cause of postoperative morbidity. Well-established major PONV risk factors include female gender (postpuberty), use of volatile anesthetics, previous history of PONV or motion sickness, and the use of intra- or postoperative opioids.¹ In recent studies, a family history of PONV also has been implicated as a risk factor.^{2,3} It is believed that PONV resulting from the physiologic stress of surgery and/or the administration of anesthetic drugs might be modulated by a variety of small genomic differences between individuals. Thus, a specific pattern of genetic variation seen in select patients may be associated with an increased susceptibility to PONV. It is this hereditary component to PONV that is the focus of this study.

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To date, much of the research related to genomics and PONV has focused on pharmacogenomics. Recent work investigating the efficacy of 5-hydroxytryptamine type 3 (5-HT₃) receptor antagonists in patients with a polymorphic CYP2D6 genotype has shown that ultrarapid metabolism of these antiemetics exhibited in patients with multiple functioning copies of the CYP2D6 allele decreases the efficacy of these drugs.^{4–7} It was reported recently that rare genetic variations in the 5-HT_{3A} and 5-HT_{3B} serotonin-receptor genes (HTR_{3A} and HTR_{3B}) are associated with an individual's risk of developing PONV and chemotherapy-induced nausea and vomiting.^{8–11} Dopamine type 2 receptor (DRD2) Taq1A polymorphism has a moderate strength of association (relative risk of PONV was 1.27 for homozygotes of minor A2 allele) with the occurrence of early PONV, albeit exclusively in a Japanese population.¹² No published research is available regarding the genetic “background” responsible for the susceptibility to PONV that is observed in a significant proportion of patients in the Caucasian population.

Our study design incorporates a genome-wide association study (GWAS) approach to rapidly scan markers across the entire human genome. Historically, linkage analysis and candidate gene investigation have been used to identify genes that increase susceptibility to complex phenotypes. Current technology has enabled researchers to perform GWAS using DNA microarrays that contain hundreds of thousands of single nucleotide polymorphisms (SNP) that capture most of the variation in the human genome. By simultaneously genotyping each sample of thousands of SNPs, risk alleles and loci can be identified. Unfortunately, the price of GWAS can be prohibitively high, especially when genotyping a large number of samples. As an economically feasible alternative, we used a DNA pooling approach. Pooling allows for equal amounts of DNA to be combined to form pools of both cases and controls, which are then genotyped to estimate allele frequency differences for each SNP. This information is then used for confirmatory association studies for a limited number of identified candidate SNPs at a fraction of the cost of individual genotyping. Many recently published DNA pooling studies have confirmed or identified loci responsible for several illnesses, providing substantial support for the notion that DNA pooling is a valid alternative to the more expensive traditional GWAS.^{13–25}

It is our hypothesis that severe, intractable PONV represents a hereditary trait resulting from inheritance of several common genetic polymorphisms. The aim of this study was to combine the emerging technology of high-density SNP microarrays with a pooled genomic DNA design to identify novel loci for genes predisposing an individual to PONV in a Caucasian population during the perioperative period *via* GWAS. The results of this

investigation will be useful in developing potential preoperative testing panels for patients for PONV risk and future research developing targeted pharmacotherapy.

Materials and Methods

Study Participants

The study protocol was approved by the local Institutional Review Board at Penn State College of Medicine, Hershey, Pennsylvania, and written informed consent was obtained from each subject before participation in the study. The study participants were selected from patients (men and women, 18 yr or older) during their preoperative evaluation before elective surgery with general, inhalational anesthesia. Study participants were characterized by a history of general anesthesia for a surgical procedure on at least three separate occasions (including the current one) during the 20 yr preceding their enrollment.

Recruitment criteria for case participants (PONV group) included an extensive history of PONV on each occasion the subject underwent surgery with general anesthesia. Control subjects were selected based on history of at least three separate surgical procedures (including the current one) performed with general anesthesia with volatile anesthetics and absence of a history of PONV. In addition to the routine preoperative evaluation, patients were asked about a family history of PONV, motion sickness, migraine headaches, or any additional adverse side effects associated with anesthesia. To avoid confusion caused by the population stratification, subjects were self-declared Caucasian, and potential subjects were prospectively excluded from enrollment if they self-reported having a parent of non-European descent. Whole blood samples (0.2 ml) were collected from each participant into EDTA tubes either before or during the surgical procedure and stored at -80°C until analysis.

An initial power calculation was based on published results from several groups that successfully used a pooled DNA strategy for discovery of robust associations in GWAS.^{17,22} These studies demonstrated that even with relatively small samples, pooled methods as described can approach almost 70% sensitivity with 100 subjects in each group.²⁶ There currently are no formal statistical methods that would allow the calculation of specific numbers of patients needed for pool-based GWAS. Thus, the calculations of the approximate power statistics and sample size were performed based on the method described by Skol *et al.* (2006) and using their CaTS—Power Calculator for Two Stage Association Studies.^{27**} Based on the population incidence of PONV (35%), we calculated that the power to detect an association at $P < 10^{-7}$ (genetic risk 2.0 for an allele with frequency 0.5) is 75% with the minimum of 240 enrolled subjects (120 controls and 120 cases). Results from Power and Sample Size Calculator v.03.14†† provided a secondary power assessment, as described previously.^{28,29}

A total of 292 subjects initially were enrolled into the source cohort during the study period (2008–2009). A total

** www.sph.umich.edu/csg/abecasis/cats. Accessed January 22, 2011.

†† biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize. Accessed January 22, 2011.

of 41 patients were prospectively excluded from genotyping because of inadequate adherence to the inclusion criteria. In the PONV group of 149 patients who provided consent, 9 signed a consent but a sample was not obtained in the operating room for a variety of reasons (scheduling change, surgeon did not allow blood draw for research, and so forth). A total of 140 samples were obtained and DNA extracted; 18 samples were eliminated because the subject had fewer than two episodes of PONV or fewer than three surgeries with general anesthesia. This resulted in data of 122 patients with PONV being used in the analysis. In the control group, 143 patients provided consent on a subsequent chart review. A sample was not obtained for four who provided consent for reasons similar to those stated for the other group. Of the remaining 139 samples, 10 were eliminated because of administration of multiple doses of antiemetic in the first 24 h after surgery. Thus, 129 control samples were used for analysis. Categorical and continuous patient and demographic characteristics were analyzed and compared with Fisher exact test and *t* test, as indicated using SPSS software (version 17, SPSS Inc., Chicago, IL).

Pool Construction and Allelotyping

DNA was obtained from each blood sample by a membrane ultrafiltration method using Fuji MiniGene 80 extractor (Autogene, Holliston, MA) equipped with DNA whole blood cartridges according to the manufacturer recommendations and stored in the elution buffer. Initial DNA concentrations were determined by spectrofluorometric screening using a PicoGreen dsDNA Quantitation Reagent Kit (Molecular Probes Inc., Eugene, OR). Extracted DNA was serially diluted to a concentration of 15 ng/ μ l and then reconfirmed with the PicoGreen method. DNA pools were constructed by taking equimolar amounts of DNA from each individual. The final pools were concentrated to 50 ng/ μ l using Microcon YM-100 Centrifugal Filter Units (Millipore Corp., Bedford, MA), as required for hybridization with Illumina arrays (Illumina Inc., San Diego, CA). The final concentration of case and control pools was again confirmed with the PicoGreen method.

Genome-wide genotyping was performed using Illumina Human-610 Quad microarrays according to the manufacturer's protocol. Chips were scanned in standard mode on an Illumina BeadStation 500GX in the Functional Genomics Core Facility at Penn State Hershey Medical Center, and raw data were extracted for statistical analysis with Illumina BeadStudio v3.2 software. Because of the expected interexperiment variation, 16 replicate arrays were genotyped for each pool: 8 for controls and 8 for patients with PONV. To further minimize variability, two replicates of each pool (total of four samples) were analyzed on each Illumina Quad DNA chip.

Approximation of allele frequencies for each replicate was produced on the basis of raw data by the Illumina BeadStudio

software and based on the internal calibration and normalization procedures (without prior individual genotyping), and averaged over the number of replicates in each pool (for details see Janicki *et al.*, 2009).³⁰ The generation of b-allele frequency was performed automatically using the self-normalization algorithm of Illumina BeadStudio software (Genotyping Module v3.2).

Analysis of Pooled Data

The analyses of the pooled frequency data were performed in three stages: (1) quality control filtering of the raw allele frequency data for all SNPs on the microarray, (2) SNP ranking by statistical test, and (3) multimarker statistics. More specific information about the statistical analysis of the pooled data is available in Supplemental Digital Content 1, <http://links.lww.com/ALN/A731>, which is a detailed review of the methods used for statistical calculations. Briefly, we excluded rare SNPs (true allele frequencies of less than 5% in Caucasian population of the HapMap^{††} predicted in the control pool sample) and the 5% of SNPs showing the highest variability as indicated by the size of the SD among measures from the replicate assays. The test statistic was based on *P* values estimated using the modified chi-square statistics (T comb test),^{19,20,25} which combines experimental and sampling errors; the test has been extensively validated previously.^{13,18,21} In addition, a multimarker statistic provided for smoothing of measurement noise and identified loci with decreased odds ratio (OR) by leveraging linkage disequilibrium between adjacent SNPs. A sliding-window approach of mean test statistic values (as delineated in stage 2) was implemented for four different window sizes (5, 10, 20, and 30 consecutive SNPs) across all windows throughout the genome.^{15–17,22}

Individual Single Nucleotide Polymorphism Genotyping

Genotyping for selected markers in individual samples from DNA pools was performed at Children's Hospital Boston (Boston, Massachusetts) using a custom Sequenom iPLEX assay in conjunction with the Mass ARRAY platform (Sequenom Inc., La Jolla, CA). Two panels of SNP markers were designed using Sequenom Assay Design 3.2 software. All assays were first optimized and validated in 30 reference Caucasian samples. Details about primers for all genotyped markers are available in Supplemental Digital Content 2, <http://links.lww.com/ALN/A732>, which is a table listing all 41 SNPs of interest and the sequence of primers used in the iPLEX assay. Because of problems associated with the assay for the selected SNPs within the muscarinic acetylcholine receptor 3 subtype (CHRM3) locus, the individual genotyping for rs2355230 (highest ranking SNP in pool assay) was performed for its perfect proxy rs2165870 ($r^2 = 1$) using the TaqMan real-time polymerase chain reaction method with the validated On-Demand assay from Applied Biosystems (Foster City, CA), as described previously.⁶

^{††} HapMap Public Release 27, hapmap.ncbi.nlm.nih.gov. Accessed January 22, 2011.

Table 1. Demographic Characteristics of Study Participants

	PONV, n = 121 (n = 104)	Control, n = 129 (n = 104)	P Value
Age (yr)	52 ± 12 (51 ± 13)	54 ± 13 (58 ± 16)	0.2* (0.2*)
Female gender	88% (93%)	86% (94%)	0.6† (0.6†)
Weight (kg)	92 ± 28 (85 ± 22)	91 ± 24 (84 ± 19)	0.8* (0.9*)
Smokers (within last 20 yr)	21% (18%)	29% (26%)	0.1† (0.1†)
Family history of PONV	78% (48%)	0.8% (0%)	< 0.001† (< 0.001†)
History of motion sickness	54% (50%)	6% (5%)	< 0.001† (< 0.001†)
History of migraines with nausea and vomiting	41% (41%)	7% (3%)	< 0.001† (< 0.001†)

Values in brackets indicate the demographic results observed in verification cohort. All values are presented as mean ± SD (continuous data) or percentage (for frequency data).

* Statistical significance calculated by *t* test. † Statistical significance calculated by Fisher exact test.

PONV = postoperative nausea and vomiting.

Individual Single Nucleotide Polymorphism Genotyping in the Validation Cohort

Markers selected for validation from the pooled genotyping results were genotyped in an additional 104 individuals with PONV and 104 individuals without any evidence of PONV (selected using a method similar to that described in Materials and Methods) and collected during the additional enrollment period in 2010. Characteristics of the study sample participants are shown in table 1. Most demographic characteristics of these separate individuals were similar to those comprising the pooled study; all were Caucasian, and potential subjects were prospectively excluded from enrollment if they self-reported having a parent of non-European descent. All patients had a history of at least three surgical procedures with general inhalational anesthesia. All samples in the verification study were processed and genotyped using Sequenom iPLEX assay in conjunction with the Mass ARRAY platform (Sequenom Inc.) in a manner similar to that described in Materials and Methods.

Statistical Analyses of Individual Genotype Data

All statistical calculations for the individual genotyping data were performed using SNP and Variation Suite Version 7 (SVS7; Golden Helix, Inc., Bozeman, MT). The extent to which observed genotype frequencies for each SNP deviated from the expected under Hardy-Weinberg equilibrium was assessed with a Fisher exact test. SNPs showing deviation from Hardy-Weinberg equilibrium ($P < 0.01$ in controls) were excluded from the analysis.

Individual SNP associations with PONV were estimated using logistic regression for (1) additive (each copy of the minor allele has an equivalent additional additive value, *i.e.*, 0, 1, 2); (2) dominant (1 or 2 copies of the minor allele *vs.* 0 copies of the minor allele), and (3) recessive (2 copies of the minor allele *vs.* 0 or 1 copies of the minor allele) models. Corresponding OR and 95% CI subsequently were calculated for each SNP according to the role of the SNP's minor allele in each genetic model.

Multiple Testing Corrections

Because many SNPs were analyzed, it may have been possible to obtain a significant test statistic by chance alone (false-positive results). Therefore, based on previous recommendations,³¹ the following multiple testing corrections to help reduce this possibility were selected:

(i) *Bonferroni Adjustment.* The Bonferroni adjustment multiplies each individual *P* value by the number of times a test was performed. This value, which is considered conservative, seeks to estimate the probability of this test obtaining the same value by chance at least once from all the times the test was performed. The number of times the test was performed will be equal to the number of bi-allelic markers processed.

(ii) *False Discovery Rate.* The false discovery rate is calculated for each statistical test selected. This test is based on the *P* values from the original test. A general interpretation of the false discovery rate is that it represents the expected proportion of false-positive test results among all positive results.

(iii) *Single-value Permutation Testing.* With single-value permutations, the dependent variable is permuted and the given statistical test using the given model on the given marker was performed. This process was repeated 10,000 times in our analysis. The permuted *P* value represents the fraction of permutations in which the test appears significant or more significant than it did with the nonpermuted dependent variable.

(iv) *Full-scan Permutation Testing.* The full-scan permutation technique differs from the single-value technique in that it addresses multiple testing problems. It does this by comparing the original test result from an individual marker with the most significant permuted results from all tested markers. The specified number of permutations (10,000) was done on the dependent variable, and these permutations were tested with each marker. For each permutation, only the most significant result statistic of all markers tested with that permutation was saved. The obtained *P* value represents the fraction of permutations in which this best saved value of the test statistic was more significant than the original statistical test on the given marker.

Results

In the current study, we genotyped the whole genome of pooled DNA samples from individuals with at least three surgical procedures performed with general anesthesia within the last 20 yr. The case pool contained 122 individuals with severe PONV, as defined by the occurrence of multiple episodes of PONV during a 48-h period after each of the surgical procedures with general anesthesia. The control pool had 129 matched individuals with no current or past (on at least three different occasions) history of any PONV associated with surgery involving general anesthesia. All study participants were self-reported Caucasians, and other reported demographic factors were closely matched between the two groups. Patient population and demographics are detailed in table 1. There were no statistically significant differences ($P > 0.05$) between the two groups with respect to age, weight, height, gender distribution, and cigarette smoking history. The subjects with a history of intractable PONV were more likely than controls (Fisher exact test, $P < 0.001$) to have a family history (first-degree relatives) of PONV, experience symptoms of motion sickness, and have a history of migraine-type headaches associated with nausea and vomiting (table 1).

The pooling approach used in our study provides evidence for good assessment of allele frequency differences and variation in these estimates. The average SD for eight replicates of each DNA pool was ± 0.018 (0.0183 and 0.018 in control and PONV samples, respectively). These estimates of variability allowed us to estimate 0.9 power to detect 10% allele frequency differences in investigated pooled samples. For both control and PONV samples, approximately 80% and 65% of SNP displayed SD of eight replicates of less than 10% and less than 5% of mean allele frequency values, respectively.

The results of microarray analysis indicate that the proportion of SNPs with $P < 0.01$ from pooling was 0.018 and consistent with what would be expected by chance if there were no true associations. Conversely, at smaller P value thresholds, there were substantially more significant SNPs than expected by chance. For example, at the $P = 0.00001$ we would expect to see approximately 6 SNPs under the null hypothesis of no associations, but we observed 175 SNPs, indicating that there were a number of true associations (see table in Supplemental Digital Content 3, <http://links.lww.com/ALN/A733>, which lists the proportions of SNPs exceeding different P value thresholds).

With the selected threshold of significance ($P < 10^{-7}$ representing $P < 0.05$ with Bonferroni correction for approximately $5 \cdot 10^5$ comparisons), we observed a total of nine SNPs equal to or exceeding the study threshold. Furthermore, 42 SNPs exceeded the $P < 10^{-6}$ threshold. Figure 1 shows the detailed P values for all markers interrogated on the microarray, and the table in Supplemental Digital Content 4, <http://links.lww.com/ALN/A734>, lists other top

SNPs with the corresponding allele differences at a level of significance of $P < 0.0001$.

The highest level of statistical significance ($P < 10^{-9}$) for an individual SNP and sixth greatest difference in allele frequency between groups (0.235) was observed in the pooled DNA assayed for the SNP rs2355230, which maps in the locus of the muscarinic acetylcholine receptor 3 subtype (M3 mAChR) gene (CHRM3) on chromosome 1. The physical position of CHMR3 locus spans approximately 285 kb on chromosome 1, from position 237,850 to 238,168 (build 36.3).³² Because of the relatively small numbers of other SNPs in linkage disequilibrium with this significant SNP in the CHRM3 region and present on Human 610-Quad microarray (fig. 2), the sliding window produced relatively small peak of mean statistics for this region (for details see Supplemental Digital Content 5, <http://links.lww.com/ALN/A735>, a graph depicting the block structure of linkage disequilibrium for the 5'-untranslated fragment and beginning of the coding sequence of the CHRM3), despite the presence of one SNP with the highest level of significance.

The results of the entire sliding window analysis for the GWAS in the pools are shown in Supplemental Digital Content 6, <http://links.lww.com/ALN/A736>, which illustrates the results of sliding window analysis of the entire genome at different resolution levels. The best two windows were found at 4q31.23 and 6q21, which were primarily driven by several SNPs with ranking near the top of all markers. Both windows consistently displayed the highest level of mean T comb statistics for all widths of windows used (5–30 SNPs).

To assess whether differences in allele frequency between case and control pools were artifactual and limit the costs of the validation experiments, we further selected a subset of 41 SNPs. These SNPs were selected based on the highest test statistic (T comb) score and/or the results of the sliding-window analysis. Genotyping analysis of these SNPs in the individual samples was carried out by Mass ARRAY. Ten SNPs failed in various stages of genotyping and data cleaning, and 12 showed significant deviation from the Hardy-Weinberg equilibrium. The remaining 19 SNPs are listed in table 2. We observed evidence (at nominal significance level of $P < 0.05$) for association (expressed as OR) between PONV and 19 different SNPs identified. The minor allele status of 9 SNPs was associated with an increased PONV risk (OR: 1.7–3.1), and in the remaining 10 SNPs, the minor allele status was associated with a decreased PONV risk (OR: 0.2–0.62). Six SNPs remained significantly associated after correction for multiple testing ($P < 0.0012$). The highest level of significance was found again for the perfect proxy of rs2355239 ($r^2 = 1$)—rs2165870 in the locus upstream of the putative promoter region of the CHRM3 gene. Another close proxy, rs7527924 ($r^2 = 0.92$) displayed nominal statistical significance ($P < 0.05$) in the individually genotyped samples. The physical position of these three SNPs spanned ~ 10 kb, overlapping the 5' end of the CHRM3

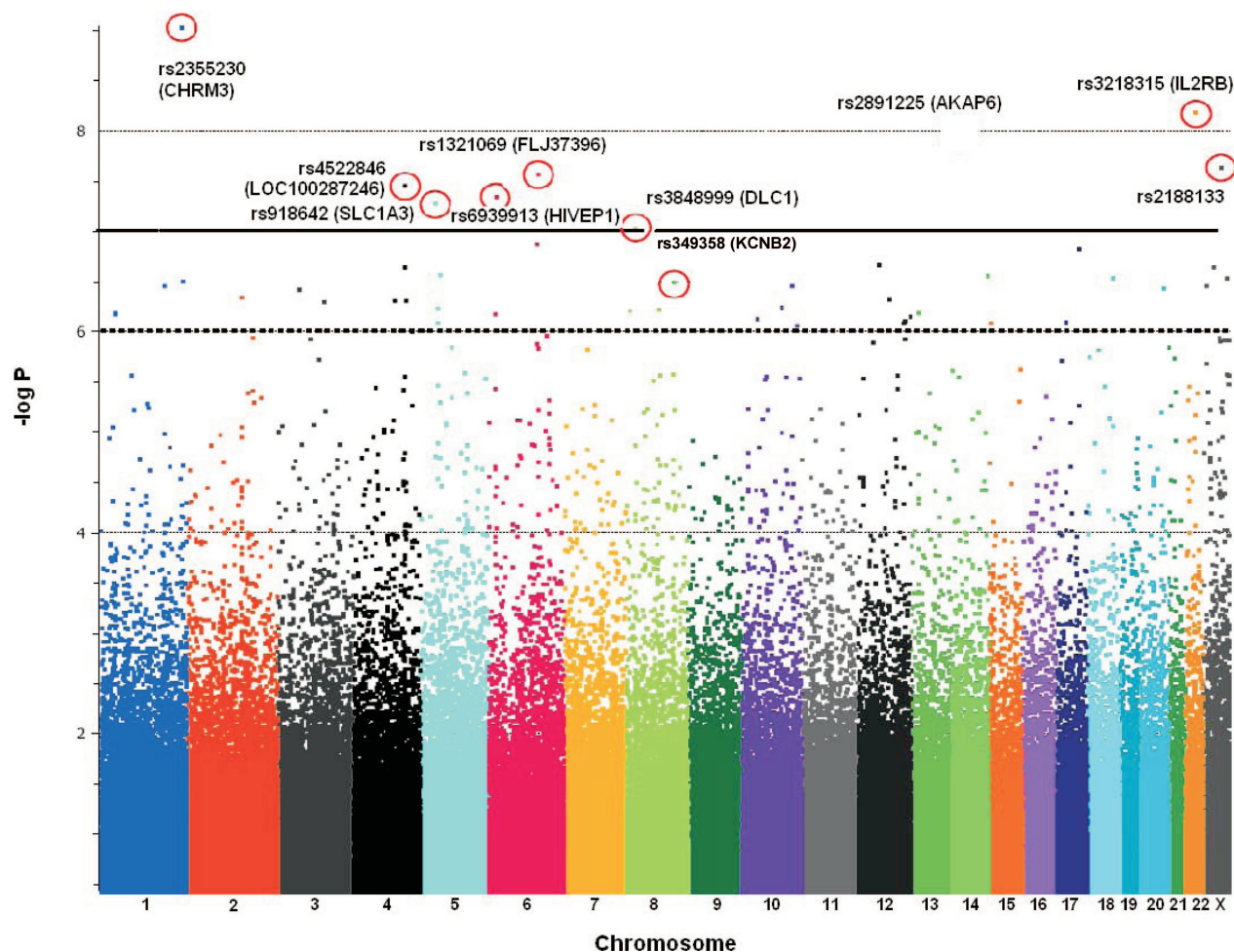


Fig. 1. Plot of combined Z-test P values against chromosomal location for pooled data. For a genome-wide association study (GWAS) on roughly 500,000 markers remained after quality control filtering (assuming the markers are independent), the significance level is 10^{-7} . Therefore, the solid line of genome-wide significance is plotted at this level, although the combined Z-statistics provides only an approximation of the P values produced from individual genotyping. The single nucleotide polymorphisms (SNPs) exceeding the 10 significance threshold are circled in red. The second horizontal line (dashed) provides the approximate level of significance at 10^{-6} level because all SNPs selected for individual genotyping had at least this level of statistical significance.

gene. The detailed relation of the SNPs in this region is shown in figure 2.

In the separate verification cohort of 208 subjects (104 with PONV and 104 controls) who were enrolled subsequently to the pool DNA part of the study, we analyzed individual genotyping for 28 SNPs with the highest score observed in the previous discovery part. Of the 28 SNPs in the validation cohort for which genotyping was done, the genotypes of 24 were considered genotyping failures because they genotyped poorly (cutoff of less than 85%) or failed Hardy-Weinberg equilibrium ($P < 0.001$) (rs2118109, rs12470772, rs2188133, rs7690646, rs8127178, rs351739, rs10176346, rs2256853) or for the remaining 16 SNPs, the difference between investigated groups was lacking significant association with PONV at nominal significance level ($P > 0.05$; rs17662523, rs918642, rs9597332, rs7718298, rs13210693, rs2453568, rs4522846, rs7679928, rs2891226, rs5979668, rs4920706, rs11654663, rs11802794, rs7730111,

rs11663884, rs349363). For the remaining four SNPs, the highest level of significance ($P < 0.0023$) (table 3), which remained significantly associated after correction for multiple testing, was confirmed for the rs216870, which is the perfect surrogate of rs2355239 in the locus of the promoter of the CHRM3 gene. Another one SNPs in the locus of KCNB2 and IL2RB, as well as one SNP in the intergenic loci, displayed nominal level of significance ($P < 0.05$) in the individually genotyped samples.

Discussion

Our GWAS was aimed at identifying common genetic determinants of increased susceptibility to intractable PONV. From the initial pooled DNA scan, we identified 41 SNPs putatively associated with PONV risk. Genotyping in individual samples provided statistical confirmation (at nominal $P < 0.05$) with 19 of a total of 41 SNPs. Six SNPs remained

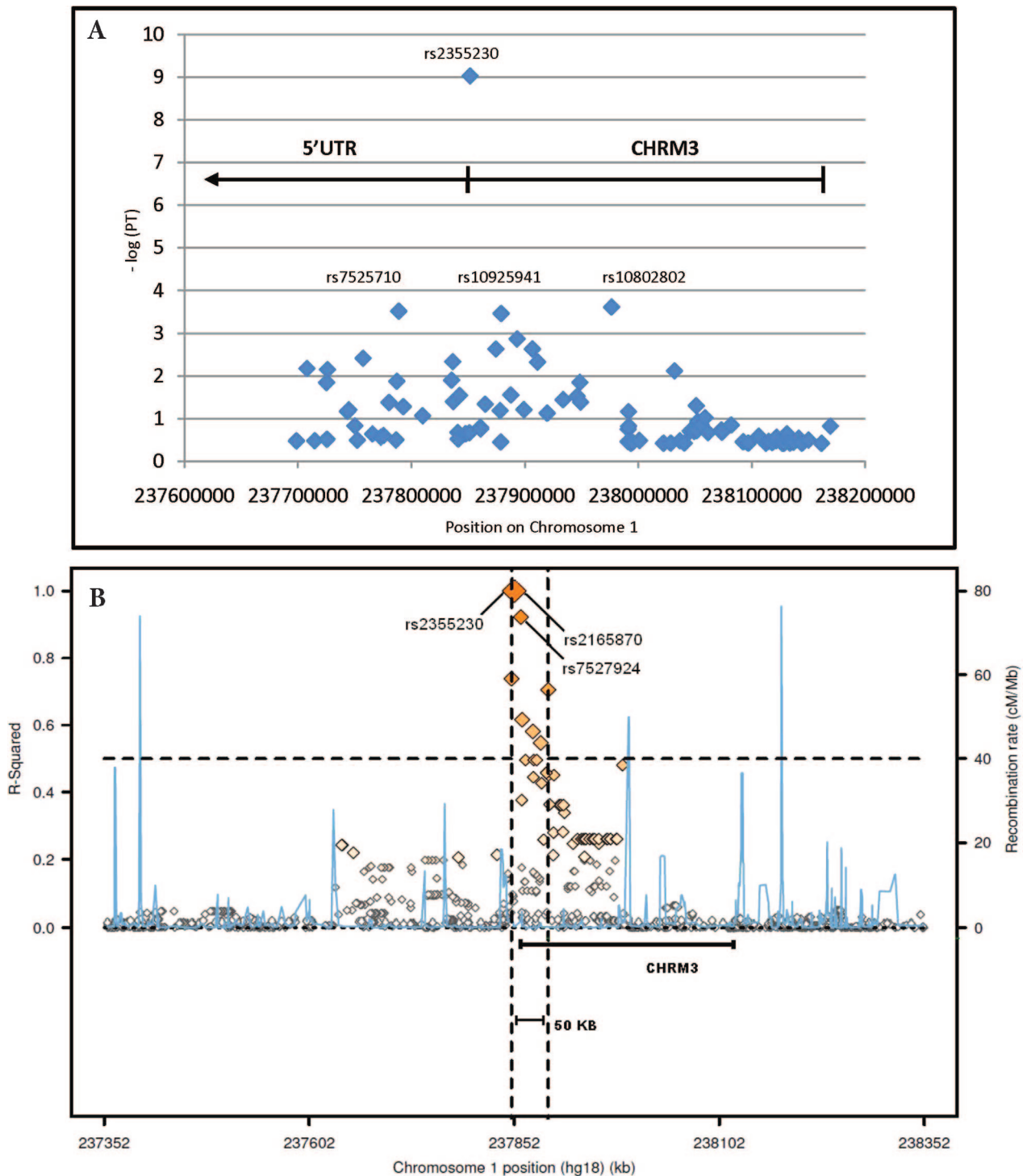


Fig. 2. Identification of single nucleotide polymorphisms (SNPs) located in the 5'-untranslated fragment and beginning of the coding sequence of the CHRM3 gene on chromosome 1. Plot of combined $-\log(P)$ values for allelic frequency difference (vertical axis) versus position on chromosome 1 (in kb, horizontal axis) for SNPs analyzed by the Illumina Human 610-Quad microarray within the locus of CHRM3 gene (A). SNPs in linkage disequilibrium (according to HapMap Release 27) with rs2355230 (measured as R^2 on left vertical axis) in relation to the position on chromosome 1 (expressed in kb on horizontal axis), and approximate position of the coding sequence of the CHRM3 gene. The right side vertical axis indicates the recombination rate (in cM/Mb) along the nucleotide sequence on chromosome 1 fragment surrounding rs2355230 (B).

Table 2. Results of Individual Genotyping of Samples from the DNA Pools

SNP	Chr	Chromosomal Position	Gene Region	Genetic Model	Minor/Major Allele	Odds Ratio	Lower 95% CI	Upper 95% CI	P Value Observed	FDR	Permutation P Values (Single/Multiple)
rs2165870	1	237852043	CHRM3	Dominant	A/G	3.1	1.78	5.3	0.000055	0.0001	0.00007/0.001
rs7527924	1	237859839	CHRM3	Dominant	G/A	2.4	1.01	5.7	0.049	0.084	0.049/0.83
rs12470772	2	161483735	TANK	Additive	T/C	2.34	1.26	4.34	0.0001	0.002	0.0002/0.0025
rs7679928	4	150287864	LOC100287246	Recessive	G/A	0.2	0.09	0.46	0.00003	0.0006	0.0002/0.0004
rs918642	5	36739410	SLC1A3	Dominant	A/G	1.95	1.11	3.4	0.0185	0.02	0.0073/0.25
rs7730111	5	44168034	FGF10	Dominant	C/T	0.39	0.16	0.94	0.0345	0.067	0.036/0.69
rs7718298	5	85303137	NBPF22P	Dominant	A/C	0.26	0.12	0.58	0.0006	0.008	0.0008/0.0171
rs9374013	6	108488040	OSTM1	Additive	A/G	2.05	1.1	3.79	0.0021	0.02	0.0017/0.0606
rs13210693	6	109705657	FLJ37396	Additive	A/G	0.45	0.22	0.93	0.0001	0.001	0.0004/0.0027
rs349358	8	73614029	KCNB2	Additive	C/T	0.39	0.21	0.93	0.0005	0.003	0.0003/0.0139
rs532967	12	116216722	NOS1	Additive	A/G	1.7	0.96	3.1	0.0327	0.07	0.033/0.69
rs2891225	14	32415383	AKAP6	Additive	A/G	0.49	0.27	0.89	0.0022	0.02	0.0024/0.0712
rs915364	14	100668695	LOC730237	Additive	A/C	0.39	0.19	0.82	0.0050	0.02	0.0042/0.15
rs2453568	17	19398101	SLC47A1	Dominant	T/C	2.35	1.3	4.2	0.0059	0.02	0.0063/0.19
rs7226214	17	62813775	PITPNC1	Recessive	T/C	0.31	0.09	1.03	0.0460	0.11	0.047/0.75
rs11663884	18	61353988	CDH7	Additive	T/C	0.48	0.22	1.04	0.0189	0.04	0.016/0.46
rs625704	22	18565457	LOC388849	Additive	A/G	2.37	1.25	4.49	0.0035	0.019	0.0033/0.11
rs3218315	22	35859670	IL2RB	Additive	A/G	2.14	1.18	3.88	0.0141	0.04	0.0134/0.39
rs12156831	X	113274437	LOC286528	Dominant	T/C	0.62	0.33	1.15	0.0461	0.08	0.0431/0.81

Boldface type signifies single nucleotide polymorphisms (SNPs) that remain significantly associated with postoperative nausea and vomiting (PONV) phenotype after all types of adjustments for multiple comparisons (Bonferroni correction at $P = 0.0012$ threshold, false discovery rate (FDR) < 0.05 , single and multiple value permutation ($n = 10,000$) adjustment ($P < 0.05$).

AKAP6 = A-kinase anchor protein 6; CDH7 = cadherin 7, type 2; CHRM3 = cholinergic receptor, muscarinic 3; FGF10 = fibroblast growth factor 10; IL2RB = interleukin-2 receptor subunit beta; KCNB2 = potassium voltage-gated channel subfamily B member 2; LOC (with number) = location in the chromosome which is not associated with any known gene; NBPF22P = neuroblastoma breakpoint family, member 22 (pseudogene); NOS1 = nitric oxide synthase 1 (neuronal); OSTM1 = osteopetrosis-associated transmembrane protein; PITPNC1 = phosphatidylinositol transfer protein, cytoplasmic 1; PONV = postoperative nausea and vomiting; SLC47A1 = solute carrier family 47, member 1; SNP = single nucleotide polymorphism; TANK = TRAF family member-associated NF- κ -B activator.

significantly associated after correction for multiple testing ($P < 0.0012$). The highest level of statistical significance, confirmed in the independent verification cohort, was observed for SNP rs2165870, which maps in the 5'-untranslated fragment of the CHRM3 gene on chromosome 1q43.³² It is important to note that the SNP with the highest significance level in the pooled DNA assay was rs2355230, which also maps within the locus of the 5'-untranslated fragment of CHRM3. Because we were unable to construct primers for

this SNP in the iPLEX assay, we instead genotyped its perfect proxy, rs2165870 ($r^2 = 1$), positioned 80 bases upstream from the CHRM3 coding sequence, as well as its close proxy rs7522924 ($r^2 = 0.92$), which maps within the first exon of the CHRM3 (fig. 2) in individual samples. All three SNPs are located between two neighboring recombination sites on chromosome 1 (fig. 2), implying that all three are part of a single linkage disequilibrium block with several other putative markers within the CHRM3 locus and may correlate

Table 3. Significant Single Nucleotide Polymorphisms Confirmed by Individual Genotyping in the Independent Verification Cohort

SNP	Chr	Chromosomal Position	Gene Region*	Genetic Model	Minor/Major Allele	Odds Ratio	Lower 95% CI	Upper 95% CI	P Value Observed
rs2165870	1	237852043	CHRM3	Dominant	A/G	2.3	1.36	4	0.0023
rs349358	8	73614029	KCNB2	Dominant	C/T	1.8	1	3.3	0.048
rs3218315	22	35859670q	IL2RB	Dominant	A/G	1.87	1.1	3.3	0.04
rs703363	10	119333474	Intergenic additive	A/C	1.8	1	3.3	0.05	

From a total number of attempted genotyping of 28 single nucleotide polymorphisms (SNPs) in the validation cohort, the genotypes not listed in the table ($N = 24$) were considered genotyping failures ($N = 8$) because they genotyped poorly (cutoff of less than 85%) or they failed Hardy-Weinberg equilibrium ($P < 0.001$) (rs2118109, rs12470772, rs2188133, rs7690646, rs8127178, rs351739, rs10176346, rs2256853), or for the remaining 16 SNPs, the difference between investigated groups was observed at nominal significance level ($P > 0.05$; rs17662523, rs918642, rs9597332, rs7718298, rs13210693, rs2453568, rs4522846, rs7679928, rs2891226, rs5979668, rs4920706, rs11654663, rs11802794, rs7730111, rs11663884, rs349363). For the remaining five SNPs (listed in the table), boldface type signifies the SNP that remains significantly associated with PONV phenotype after adjustment for multiple comparisons (Bonferroni correction at $P = 0.0025$ threshold).

Chr = chromosome; CHRM3 = cholinergic receptor, muscarinic 3; IL2RB = interleukin-2 receptor subunit beta; KCNB2 = potassium voltage-gated channel subfamily B member 2; PONV = postoperative nausea and vomiting; SNP = single nucleotide polymorphism.

closely with the PONV status. These data also suggest, albeit indirectly, that the muscarinic acetylcholine receptor 3 subtype may play a role in the pathogenesis observed with the increased susceptibility to PONV. Five muscarinic receptor subtypes (M1–M5) have been demonstrated to be distinct gene products on the basis of molecular characterization. These receptors were shown to share a high level of sequence homology and belong to the super family of G protein-coupled receptors possessing seven transmembrane-spanning domains. In most neurons, M1, M3, and M5 receptors appear to be positively coupled to phospholipase C, resulting in the formation of diacylglycerol and the release of inositol trisphosphate, which in turn liberates free, intracellular Ca^{2+} . It has been suggested that the unwanted effects (*e.g.*, salivation, nausea and emesis, and atrial arrhythmia) obtained with muscarinic agonists result primarily through muscarinic acetylcholine receptor 3 subtype activation (and to some degree M2), and not M1 muscarinic acetylcholine receptor stimulation.³³ The connection of muscarinic acetylcholine receptor 3 subtype to PONV could be further reinforced by the nonspecific muscarinic receptor antagonists (*e.g.*, scopolamine) being effective in the attenuation of motion sickness, a syndrome that seems to be closely related to PONV (*i.e.*, previous history of either PONV or motion sickness are both major risk factors for PONV).^{34,35} Previously published studies demonstrated that sequence variations in the promoter region of the CHRM3 gene may be associated with asthma, atopy, and early-onset type 2 diabetes.^{36,37}

The initial findings from the pooled assays were successfully replicated for only 4 of 24 SNPs in the validation cohort. It appears that the other 16 SNPs (from the 24 that appeared valid in the initial group) were found to lack statistical significance. However, the number of enrolled subjects was rather limited in the verification cohort and based on the statistical power calculations targeting one most significant SNP observed in the initial group. Thus, it is entirely possible that with the significant increase in the number of the study subjects, more SNPs could become significant after correction for multiple testing.

There are several potential limitations to this study. First, the current anesthesiology literature does not include a standardized definition of severe, intractable PONV or familial-type PONV. Many patients received PONV prophylaxis, either as a routine medication during the perioperative period or during their stay in the postanesthesia care unit, often in response to an initial, often very mild PONV episode. We elected to define “severe PONV” as repeated, severe episodes of PONV within 48 h presenting on at least three different occasions associated with the use of general anesthesia with volatile anesthetics and opioids (with or without prior use of different antiemetics) to best ensure that subjects with mild PONV symptoms on a single occasion would be excluded. In most cases, intractable PONV in a patient was associated with similar symptoms in an individual from the immediate family (parents, siblings, including two pairs of monozygotic

twins). A reported lack of family history does not preclude the possibility of familial occurrence of PONV because the information about immediate family may not always be readily available or immediate family may not know of family members’ exposure to general anesthesia. A history of intractable PONV often was associated with symptoms of motion sickness and migraine-type headaches (often with concomitant nausea and vomiting). Thus, it is possible that observed associations between genomic markers and PONV are not exclusively related to intractable PONV but may represent an association with the accompanying syndromes of motion sickness, migraine, and family history of PONV. Second, we limited our analysis exclusively to individuals of European descent because our patient population has an insufficient number of non-European subjects to allow for statistical accommodation of genetic admixture. Thus, additional studies are needed to compare our findings with those involving non-European patients with PONV symptoms. It is also important to caution that our results are most highly applicable to one gender because most of the subjects in the PONV and control groups were women. This is consistent with the well-known predominance of PONV symptoms in female patients. Third, the statistical power in this study was limited by the available sample size.

However, because the current study sample was based upon selection of extremely discordant individuals, the power to detect genetic determinants of PONV is expected to be increased. For example, control subjects were selected based on multiple surgical procedures in the past with general anesthesia and no evidence of PONV; as such, these individuals are presumably resistant to developing PONV. Based on the population incidence of PONV (35%), we calculate that the power to detect an association is 75%. Therefore, we expect that the sample size used here is adequate for detecting loci with major effects, such as the putative one found on CHRM3, KCNB2, and IL2RB. Even with relatively small sample size, our study and similar ones are necessary to develop the framework for larger trials capable of determining the important genetic variations and calculating the required patient number to achieve an adequately powered trial.

As with any genetic associations, our findings will require additional replications and functional validation. To facilitate this process, we provide Supplemental Digital Content 4, <http://links.lww.com/ALN/A734>, which lists the top SNPs, with the corresponding allele differences at a level of significance $P < 0.0001$ (as a resource for investigators with the requisite samples to advance this line of research). The current study shows the potential of GWAS to discover genes and pathways that are involved in PONV. In a recent editorial about anesthesia pharmacogenomics of PONV, Candiotti³⁸ suggests that in the translation of genetic studies such as ours to the clinical practice of anesthesiology, the exact location of a particular variant may be essentially irrelevant to clinicians at this time. What is relevant is that genetic studies

potentially help to clarify observed clinical phenomena (*e.g.*, intractable PONV). By understanding the mechanisms of medication and treatment failures (*e.g.*, resistance of PONV to standard antiemetic medication), alternative practices can be investigated and eventually put into clinical practice. In this respect, the results of our work might suggest that in patients with certain types of intractable PONV the routine antiemetic prophylaxis with 5HT₃ antagonists may not be optimal because of the presence of putative casual genetic mutations outside of the 5HT₃ system.

In summary, the findings of the current study support the use of a pooling-based approach for GWAS and provide evidence of potential association with susceptibility to PONV. Although our analysis does not provide exhaustive coverage of the genetic components affecting propensity for PONV, replication studies in large series and different ethnic populations that confirm our results, as well as identification of potential functional variants for further characterization, could represent a significant step toward defining a genetic profile for the estimation of an individual's risk for PONV.

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