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Associations Between *KCNJ6* (GIRK2) Gene Polymorphisms and Pain-Related Phenotypes

Stephen Bruehl[1], **Jerod S. Denton**[1], **Daniel Lonergan**[1], **Mary Ellen Koran**[2], **Melissa Chont**[1], **Christopher Sobey**[1], **Shanik Fernando**[1], **William S. Bush**[2], **Puneet Mishra**[1], and **Tricia A. Thornton-Wells**[2]

[1]Department of Anesthesiology, Vanderbilt University School of Medicine, Nashville, TN, USA

[2]Center for Human Genetics Research, Department of Molecular Physiology & Biophysics, Vanderbilt University School of Medicine, Nashville, TN, USA

Summary

Variations in the KCNJ6 gene appear to influence both acute and chronic pain phenotypes.

G-protein coupled inwardly rectifying potassium (GIRK) channels are effectors determining degree of analgesia experienced upon opioid receptor activation by endogenous and exogenous opioids. The impact of GIRK-related genetic variation on human pain responses has received little research attention. We used a tag SNP approach to comprehensively examine pain-related effects of *KCNJ3* (GIRK1) and *KCNJ6* (GIRK2) gene variation. Forty-one *KCNJ3* and 69 *KCNJ6* tag SNPs were selected, capturing the known variability in each gene. The primary sample included 311 Caucasian patients undergoing total knee arthroplasty in whom post-surgical oral opioid analgesic medication order data were available. Primary sample findings were then replicated in an independent Caucasian sample of 63 healthy pain-free individuals and 75 individuals with chronic low back pain (CLBP) who provided data regarding laboratory acute pain responsiveness (ischemic task) and chronic pain intensity and unpleasantness (CLBP Only). Univariate quantitative trait analyses in the primary sample revealed that 8 *KCNJ6* SNPs were significantly associated with the medication order phenotype ($p < 0.05$); overall effects of the *KCNJ6* gene (gene set-based analysis) just failed to reach significance ($p=.054$). No significant *KCNJ3* effects were observed. A continuous GIRK Related Risk Score (GRRS) was derived in the primary sample to summarize each individual's number of *KCNJ6* "pain risk" alleles. This GRRS was applied to the replication sample, which revealed significant associations ($p<.05$) between higher GRRS values and lower acute pain tolerance and higher CLBP intensity and unpleasantness. Results suggest further exploration of the impact of *KCNJ6* genetic variation on pain outcomes is warranted.

Keywords

Pain; Chronic Pain; Genetic; GIRK; Potassium Channel; Polymorphism; Post-Surgical Pain; *KCNJ3*; *KCNJ6*

Corresponding Author: Stephen Bruehl, Ph.D., Vanderbilt University Medical Center, 701 Medical Arts Building, 1211 Twenty-First Avenue South, Nashville, TN 37212. Phone: (615) 936-1821, Fax: (615) 936-8983, Stephen.Bruehl@vanderbilt.edu.

Introduction

Genetic influences on human pain perception and risk for chronic pain are likely to be polygenic[8]. Numerous single nucleotide polymorphisms (SNPs) have been identified in human studies as potential contributors. SNPs in genes encoding for the mu opioid receptor *(OPRM1)*, the beta-2 adrenergic receptor *(ADRB2)*, and catechol-O-methyltransferase *(COMT)* have all been demonstrated to influence acute pain sensitivity[7,9,10,13,16,38,49], chronic pain intensity[11,19,28,34], and risk for development of chronic pain conditions[6,9,12,15,19,29,39,43]. Prior work also suggests that pain-related SNPs (e.g., A118G SNP [rs1799971] of the *OPRM1* gene) may influence responses to opioid analgesics, although the degree of this influence remains debatable[45].

One commonality between *OPRM1* and *COMT* SNPs targeted in prior work is that each can potentially influence the magnitude of opioid inhibition upon activation of opioid receptors by endogenous or exogenous opioid agonists[1,20,49]. The degree of opioid inhibition upon receptor activation is also influenced by numerous effectors, including G-protein coupled inwardly rectifying potassium (GIRK) channels of the Kir3.X family[25-27]. GIRK channels are activated by the and subunits of heterotrimeric $G_{i/o}$ proteins following stimulation of opioid, receptors by endogenous or exogenous opioids. The ensuing efflux of potassium ions hyperpolarizes the membrane potential, dampens neuronal excitability, and limits nociceptive transmission[14]. Several studies in animals document that both the *KCNJ3* (GIRK1) and *KCNJ6* (GIRK2) genes can influence pain and opioid analgesic responses[17,25,27,42]. Indeed, the possibility of direct pharmacological manipulation of GIRK channel activity has been suggested as one avenue for developing novel analgesic medications[2,21,32,44].

Surprisingly, human work examining whether GIRK-related genetic variation influences pain responses has been sparse. Only two studies have explored this topic, both examining the pain-related impact of a small number of SNPs in the *KCNJ6* gene. In patients undergoing major abdominal surgery, homozygous carriers of the A allele of the A1032G SNP (rs2070995) required rescue pain medication more frequently than those with the G allele, although no associations with post-surgical acute pain ratings were observed[33]. Other work found that compared to individuals with the G allele, homozygous carriers of the A allele required more methadone yet had fewer withdrawal symptoms in methadone substitution therapy patients, and required marginally higher opioid doses for pain control in chronic pain patients[24]. No human studies to date have examined the potential influence of *KCNJ3* gene variants on pain-related outcomes, although such influence is suggested by animal work. For example, genetic deletion or pharmacological inhibition of *KCNJ3*-containing channels increases thermal nociception and blunts the analgesic response to opioids[26,27].

The current study used a tag SNP approach to explore possible associations between a comprehensive array of SNPs in the *KCNJ3* and *KCNJ6* genes and a post-surgical pain phenotype (oral opioid analgesic medication orders) in a large informatics-based sample. Findings were then replicated in an independent sample combining data from three previously published studies using similar entry criteria[3-5] with regard to measures reflecting acute laboratory pain responsiveness and chronic low back pain intensity phenotypes.

Methods

Design

This study used a correlational design to examine the impact of a comprehensive array of *KCNJ3* and *KCNJ6* SNPs on oral analgesic medication orders in a large clinical post-surgical primary sample, with replication of the resulting pain-relevant SNPs on acute laboratory pain and chronic back pain phenotypes in an independent sample.

Subjects

Primary Sample—The primary sample used to initially identify pain-relevant *KCNJ3* and *KCNJ6* SNPs was a large clinical post-surgical sample with electronic medical record data available in whom an informatics approach could be applied. To focus on patients with a comparable degree of tissue injury, the primary sample was drawn from a pool of 881 patients seen at Vanderbilt University Medical Center since 2002 who displayed a CPT code of 27447 (total knee arthroplasty; TKA), who had undergone a unilateral TKA, and who had DNA samples available in BioVU, the Vanderbilt biobank of de-identified DNA samples obtained for research purposes from discarded blood[36,37]. For this study, the selected BioVU DNA samples were linked in a de-identified manner to pain-relevant phenotypes via matching to the electronic inpatient medication order database at Vanderbilt (Wizorder). Routine DNA sampling and electronic medication records were implemented over differing time periods resulting in only a subset of patients in the potential subject pool with information available from both sources. The key phenotype targeted in the primary informatics sample was total number of oral opioid analgesic medication orders entered during each given patient's inpatient hospital stay following TKA. For this portion of the study, patients included in the primary sample were limited to Caucasian patients with BioVU DNA samples who had the necessary medication order information available in Wizorder to permit characterization of this phenotype (n=311). The decision to restrict the final sample to Caucasian patients (the largest single racial group) was made to reduce potential confounds related to population substructure. To validate the oral analgesic medication order phenotype, post-surgical pain intensity data available in a subset of 82 patients from this larger pool were manually extracted from the Synthetic Derivative database, the Vanderbilt de-identified electronic medical records database.

Replication Sample—To maximize statistical power in the replication sample, the current study combined data from three similar studies previously conducted in our lab in which DNA samples were obtained in chronic low back pain (CLBP) subjects and healthy pain-free subjects[3-5]. Both groups contributed data regarding laboratory acute pain response phenotype (ischemic pain threshold and tolerance), with the CLBP group also providing data regarding chronic pain phenotype (chronic back pain intensity and unpleasantness). For the acute pain phenotype, only those subjects experiencing the ischemic task in the absence of study drugs or other experimental manipulations that might alter pain responses were included in replication analyses. The current sample was restricted to Caucasian subjects for comparability with the primary sample and to minimize the potential influence of population substructure. All subjects met basic study medical eligibility criteria which were similar across the three studies. These criteria were: age between 18-55 years, current normotensive status (resting blood pressure < 140/90), not pregnant, no history of cardiovascular disease, hypertension, liver or kidney disorders, or opiate dependence; no current daily use of opioid analgesics, and no current use of anti-hypertensive medications. Absence of recent opiate use was confirmed via urine opioid screen in 66 of the subjects (all subjects participating in Bruehl et al.[3,4]). Additional inclusion criteria for the CLBP group were chronic daily low back pain of at least 3 months duration with an average past month severity of at least 3/10. The final replication sample size was n=112, including 46 subjects from Bruehl et al.[5], 11

subjects from Bruehl et al.[4], and 55 subjects from Bruehl et al.[3]. Of the final replication sample, 63 (56.3%) were healthy pain-free controls (Pain-Free) and 49 (43.7%) were individuals with CLBP. Characteristics of both the primary and replication samples are summarized in Table 1.

Procedures

The Vanderbilt University Institutional Review Board (IRB) approved all procedures in this study. Patients providing data in the primary post-surgical sample were all given the opportunity to opt out of DNA collection in accordance with IRB guidelines. All laboratory study subjects (replication sample) were volunteers who provided written informed consent prior to study participation.

Primary Sample Procedures—Data on inpatient oral opioid analgesic medication orders entered post-TKA into the Wizorder electronic database were used to define the oral analgesic medication order phenotype. For each patient, an automated total count of any oral opioid analgesic medication orders entered was derived using SPSS syntax language (96.4% of orders were for oral immediate release oxycodone). Data on post-TKA intravenous analgesic orders were also available, but were deemed inappropriate for analysis due to inadequate variability (more than 50% of patients had only a single intravenous analgesic order entered).

To validate the oral analgesic order phenotype, standardized post-surgical pain ratings (0 − 10 scale, anchored with “No Pain” and “Worst Possible Pain”) obtained during inpatient physical therapy in the 3 days following the TKA procedure were extracted in a subset of 82 patients with available data. Pain ratings at rest and during activity were averaged over the three days for use as the overall post-surgical pain intensity measure.

Replication Sample Procedures—Detailed procedures for each laboratory study are provided elsewhere[3-5]. In brief, after providing informed consent, laboratory study subjects completed a packet of demographic and psychometric questionnaires. For CLBP subjects, this packet included a visual analog scale measure of past month overall chronic back pain intensity (VAS Intensity; anchored with “No, Pain” and “Worst Possible Pain”), as well as a parallel scale assessing the affective component of chronic pain (VAS Unpleasantness; anchored with “Not Unpleasant at All” and “The Most Unpleasant Possible”). These measure were used to define the chronic pain phenotype for replication analyses. Both CLBP and Healthy subjects also participated in a standardized ischemic forearm acute pain task, a laboratory measure of acute pain sensitivity. Ischemic task procedures in all three laboratory studies were based on those described by Maurset et al.[30]. In brief, subjects were first asked to raise their dominant forearm over their head for 30 seconds followed by two minutes of dominant forearm muscle exercise using a hand dynamometer at 50% of his or her maximal grip strength (as determined prior to beginning the laboratory procedures). Immediately following this, a BP cuff was inflated on the participant's dominant bicep to 200 mmHg. The cuff remained inflated until participants indicated that their pain tolerance had been reached, up to a maximum of 5 minutes (due to ethical requirements). Pain threshold was defined as the number of seconds elapsed between task onset and the subject's report that the task had become “painful.” Pain tolerance was defined as the number of seconds elapsed between task onset and the subject's expressed desire to terminate the task. These measures comprised the acute laboratory pain responsiveness phenotype.

Genetic Assays

Genetic samples were obtained via blood drawn from an indwelling venous cannula [3,5] or via buccal sampling[4]. DNA was extracted using the Gentra Systems AutoPure automated DNA, extraction system in the Vanderbilt University DNA Resources Core.

We used a tag SNP approach to avoid redundancy in genotyping of variants that were expected to be in high linkage disequilibrium with each other. We selected tag SNPs from, candidate genes *KCNJ3* and *KCNJ6* based on the HapMap CEU reference population with the, goal of capturing at least 80% of the variation in each gene while reducing the need for genotyping every variant. For *KCNJ3*, 41 tag SNPs were selected to capture 100% of the allelic variation in 181 SNPs across the gene with a mean r^2 of 0.949. For *KCNJ6*, 69 tag SNPs were selected to capture 100% of the allelic variation in 301 SNPs across the gene with a mean r^2 of 0.952. See Supplementary Tables 1 and 2 for the full list of tag SNPs for each gene and the alleles they capture.

Genotyping was performed using Sequenom MassARRAY (Sequenom, Inc., San Diego, CA) and TaqMan OpenArray (Applied Biosystems, Foster City, CA) platforms. Four Sequenom pools were designed that incorporated all but three of the selected tag SNPs (one that needed to be in a pool by itself and two that failed assay design; all from *KCNJ6)*. Direct genotyping of three remaining *KCNJ6* tag SNPs was conducted using pre-made TaqMan SNP genotyping assays.

Negative controls (no template) and positive controls (DNA samples with known genotypes from Coriell Institute for Medical Research, Camden, NJ) were included for assay validation. Inter- and intra-plate experimental duplicates and HapMap controls were run on each assay plate to serve as positive controls for examining genotyping accuracy. Individuals who were blinded to clinical study data and hypotheses conducted semi-automated genotype calling with manual inspection of intensity clusters. Genotyping call rates and tests of Hardy Weinberg Equilibrium (HWE) were calculated for all genotyped SNPs.

Statistical Analysis

All genetic association analyses in the primary sample were conducted using PLINK, Version 1.07 (http://pngu.mgh.harvard.edu/purcell/plink/)[35]. Demographic and replication sample analyses were conducted using the IBM SPSS Statistics Version 20 statistical package (IBM SPSS Statistics, Inc., Chicago, IL). All analyses used the maximum number of cases available for each phenotype.

Univariate analyses were conducted assuming an additive model for each SNP, in which having two copies of the coded allele was expected to modify risk by twice as much as having a single copy. For the oral analgesic medication order phenotype, a quantitative trait (QT) analysis was conducted using linear regression. A two-tailed probability value of $p<.05$ was used as the criterion for statistical significance in univariate analyses in the primary sample. Probability values were not corrected for multiple comparisons in univariate analyses due to the exploratory nature of this study. However, to provide a means of addressing potentially elevated family-wise error rate due to examination of multiple SNPs within each gene, we also conducted gene set-based analyses for each gene using PLINK. For these analyses, all tagged SNPs within each gene were considered in the gene set, and the average of the single-marker (QT) test statistics was computed as the gene-set test statistic. Permutation testing was then used to determine the empirical p-value for the experimental gene-set statistic[31]. In the current study, results of these set-based analyses reflected the overall influence of the given gene on the oral analgesic medication order phenotype.

Replication sample analyses examined associations between the GIRK-Related Risk Score derived in the same manner as in the primary post-TKA informatics sample (GRRS; detailed below) and the acute and chronic pain phenotypes. Associations with the chronic pain intensity and unpleasantness measures were examined using Pearson correlational analyses. Because the distribution of ischemic pain task tolerance times was truncated due to 61.9% of subjects reaching the maximum permitted task duration (300 seconds), analyses of the acute pain phenotype used two complementary approaches. Pearson correlations were used to examine associations between GRRS values and the continuous pain threshold and pain tolerance values, and t-tests were used to compare GRRS values between subjects who tolerated the full 5-minute ischemic task and those who did not. Due to the directional nature of the confirmatory, hypotheses in the replication sample, a one-tailed $p<.05$ value was used as the criterion for significance in replication analyses to maximize statistical power.

Results

Preliminary Analyses

Inspection of genotyping results from positive controls and experimental duplicates confirmed assay validity and concordance of genotype calls. Genotyping efficiency exceeded 91% for all SNPs, with a median efficiency of 99%. Five SNPs were flagged as being out of Hardy-Weinberg equilibrium ($p < 0.01$) in the complete BioVU pool of 881 patients but were not removed from the analysis.

KCNJ3 and *KCNJ6* SNPs and the Analgesic Medication Order Phenotype

Mean and standard deviation of the oral analgesic medication order count in the TKA sample are reported in Table 1. Validity of this key study phenotype was supported by the fact that it was correlated significantly with pain ratings obtained during post-surgical rehabilitation that were available in a subset of 82 patients ($r = 0.26$, $p=.01$), in a direction indicating that more oral analgesic medication orders were entered for patients reporting greater post-TKA pain intensity. Table 2 summarizes the significant univariate associations between GIRK-related SNPs and the oral analgesic medication order phenotype. Eight *KCNJ6* SNPs exhibited significant effects, with no significant effects for *KCNJ3*. Figure 1 portrays the chromosomal position of the 8 significant *KCNJ6* SNPs. In the set-based analysis which addressed possible family-wise error rate inflation due to testing multiple SNPs in univariate analyses, the overall influence of the *KCNJ6* gene on the oral analgesic medication order phenotype just failed to reach the criterion for statistical significance (empirical $p = 0.054$). The gene-set based analysis of the overall influence of the *KCNJ3* gene was not significant (empirical $p = 1.0$).

Derivation of the GIRK-Related Risk Score

To provide a simple means of summarizing the univariate results, a GIRK-Related Risk Score (GRRS) was derived based on the oral analgesic medication order phenotype in the primary sample. This GRRS included the 8 *KCNJ6* SNPs showing significant univariate. associations with the oral medication order phenotype (rs1543754, rs1787337, rs2211843, rs2835925, rs2835930, rs858035, rs928723, rs9981629). SNPs were coded for number of risk alleles present (0,1,2), such that more copies of the risk allele were associated with a greater number of oral analgesic medication orders. Mean number of oral medication orders by risk allele status for these 8 *KCNJ6* SNPs are presented in Table 3. Values were then summed across all 8 SNPs for a given individual, yielding a continuous GRRS ranging from 0-15 in the primary sample (see Table 1). Within the post-TKA sample in which it was derived, this GRRS was correlated positively with number of oral analgesic orders entered into the medical record [$r = 0.25$, $p<.001$]

Replication of the GRRS in the Laboratory Study Sample

Application of the same GRRS scoring method to the combined replication samples resulted in GRRS values ranging from 2-12 (see Table 1). Associations between GRRS values and the two measures of acute laboratory pain responses were examined in the combined replication subsamples. In line with the direction of effects in the primary sample, subjects with longer ischemic pain tolerance times (i.e., relatively less pain sensitive) were found to have significantly lower GRRS values [$r(109) = -0.21$, $p=.01$]. Consistent with these correlational findings, subjects reaching the maximum allowable pain tolerance on the ischemic pain task were found to have significantly lower GRRS values (i.e., fewer risk alleles) than those not reaching maximum tolerance [Less than Maximum Tolerance: 8.1 ± 1.80; Maximum Tolerance:, 7.4 ± 1.96; $t(109) = 1.80$, $p=.04$]. The association between ischemic pain threshold and GRRS values was not significant ($p = .45$).

Replication regarding the chronic pain phenotype was conducted within the CLBP replication sample only. Subjects with higher GRRS values were found to report significantly greater past month chronic low back pain intensity [$r(46) = 0.29$, $p=.02$]. Association between GRRS values and the affective component of chronic pain (i.e., past month chronic low back pain unpleasantness) was of similar magnitude [$r(46) = 0.29$, $p=.02$]. Overall, results for both acute laboratory pain tolerance and the chronic back pain phenotype in the replication sample are in a direction supporting the validity of the *KCNJ6* effects noted in the primary post-TKA sample regarding the oral analgesic medication order phenotype. Comparison of GRSS scores between the pain-free and CLBP replication samples did not reveal significant differences ($p>.10$; see Table 1).

Discussion

Genetic influences on pain are polygenic[8], with SNPs in the *OPRM1*, *COMT*, and *ADRB2* genes previously shown to influence acute and chronic pain intensity[7,9,10,11,13,16,19,28,34,38,49] or risk for development of chronic pain[6,9,12,15,19,29,39,43]. Both *OPRM1*- and *COMT*-related genetic influences on pain may involve opioid mechanisms[1,20,49]. GIRK channels are important effectors that can determine the degree of opioid inhibition occurring upon opioid receptor activation[14], and therefore, variations in GIRK-related genes provide another potential opioid-related pathway by which pain responses may be genetically influenced. Animal studies confirm the relevance of both *KCNJ3* and *KCNJ6* genes to pain outcomes[17,25,27,42]. However, to date, only two human studies have explored this issue[24,33], with both limited to testing a relatively small number of *KCNJ6* SNPs. The current study employed a tag SNP approach to examine a comprehensive array of polymorphisms capturing known variability in the *KCNJ3* and *KCNJ6* genes as they, relate to an informatics-based post-surgical pain phenotype (oral opioid analgesic medication orders following TKA), with subsequent replication of significant pain-related effects regarding acute and chronic pain phenotypes in an independent laboratory-based sample.

Univariate quantitative trait analyses revealed that 8 *KCNJ6* SNPs were significantly associated with the oral analgesic medication order phenotype. Gene set-based analysis indicated that the effect of variation in the *KCNJ6* gene overall on this post-surgical pain phenotype just failed to reach statistical significance ($p= .054$). A pain-related influence of *KCNJ6* was not unexpected, given that the only two prior human studies examining GIRK-related genetic variation on pain outcomes showed effects for *KCNJ6*. Both previous studies reported that the A1032G SNP (rs2070995) of the *KCNJ6* gene showed significant effects on opioid analgesic responses, although Nishizawa et al.[33] did not find statistically significant effects on acute (post-surgical) pain responses. In contrast to the latter study which found A1032G SNP effects on post-surgical rescue medication requirements, the

current study did not find significant effects of the A1032G SNP (tagged by rs858003 in this study; r^2=1.0 based on HapMap CEU population) on the post-surgical medication order phenotype examined. Nonetheless, a number of other *KCNJ6* SNPs not examined in prior work were associated in the current study with the post-surgical oral medication order phenotype. Whether the *KCNJ6* SNPs showing pain-related effects in the current study influence opioid analgesic *responses*, as in Nishizawa et al.[33] and Lotsch et al.[24], could not be directly tested due to limitations of the informatics data available. This possibility remains to be examined in future work.

Findings in the primary sample documenting pain-related effects of several *KCNJ6* SNPs are strengthened by results of cross-validation in an independent sample. A continuous GIRK-related risk score (GRRS) derived for each individual to summarize *KCNJ6* SNPs that exhibited significant pain-related effects in the primary sample was found to be associated in the same, direction with both acute and chronic pain phenotypes in the laboratory-based replication sample. Specifically, higher GRRS values were associated with lower pain tolerance to a standardized acute laboratory pain task and higher chronic low back pain intensity and unpleasantness. Taken together, these findings underscore the likely pain-relevance of variation in the *KCNJ6* gene.

Although prior work had examined pain-related *KCNJ6* influences in a limited way, no previous human study had examined variation in the *KCNJ3* gene as it relates to pain phenotypes. Results of the current work did not reveal any significant *KCNJ3* effects on the post-surgical analgesic medication order phenotype in the large primary sample. Nonetheless, positive findings in past animal studies[26,27] suggest that it may yet be worthwhile investigating possible impact of *KCNJ3* SNPs as they relate to other pain-relevant phenotypes.

GRRS values that captured significant pain-related *KCNJ6* influences in the primary sample, and were replicated vis-à-vis acute and chronic pain-related phenotypes in the laboratory sample, nonetheless did not display significant differences between the CLBP and pain-free groups in the replication sample. The effect size for observed GRRS differences across CLBP and pain-free groups was very small (eta squared = 0.003), suggesting that it is unlikely that inadequate power alone can explain the absence of significant GIRK-related chronic pain risk differences in this study. However, given the limited pain phenotype examined in the primary sample used to derive the GRRS and that this is the first study examining a comprehensive array of *KCNJ3* and *KCNJ6* polymorphisms, further investigation may be warranted. Previous cross-sectional studies document that variability in the alpha-1 adrenergic receptor, *ADRB2*, and *COMT* genes may all be associated with risk for chronic pain conditions such as chronic orofacial pain, fibromyalgia, and chronic low back pain[6,9,12,15,19,29,43]. Future studies should, consider the possibility that variations in these genes might interact with *KCNJ6* genetic variation to modify chronic pain-risk phenotypes.

The present study used a tag SNP approach to capture the known variation represented in the CEU HapMap population in *KCNJ3* and *KCNJ6* genes, using 41 and 69 SNPs, respectively. The magnitude of the associations between the continuous GRRS (reflecting multiple SNPs) and all three acute and chronic pain-related phenotypes tested uniformly indicated small effect sizes in the range of $\underline{r}$ = 0.21 − 0.29. This is consistent with the idea of there being many SNPs with relatively small effects influencing pain phenotypes[23]. A more complete understanding of these multiple genetic inputs into pain outcome variability will require genome wide association studies, although prospects for such studies are hampered by the very large sample sizes required. Targeted deep sequencing approaches might yield additional rare variant findings in candidate genes, and whole genome sequencing holds the

potential for identifying rare variants in novel genes as well. However, these approaches are most powerful when applied to families segregating a pain phenotype or individuals exhibiting an extreme phenotype, suggesting the presence of a deleterious mutation.

The pathways through which the *KCNJ6* SNPs identified in this study influence pain-related phenotypes are not immediately clear. Annotation using the Genome-Wide Annotation Repository indicated that all *KCNJ6* tag SNPs demonstrating significant effects in this study were in noncoding regions. This does not imply that they are functionally irrelevant; introns are known in some cases to influence gene transcription[22] and gene splicing, which could in turn affect the relative frequency of different GIRK channel isoforms[18,40,46,47]. Two of the intronic SNPs exerting significant pain-related effects in the current study, rs1543754 and rs2835930, have been shown in prior work to influence *KCNJ6* expression in the brain[48]. Another *KCNJ6* SNP in the current study has demonstrated links indicating it might potentially exert pain-related, 17 effects via non-GIRK pathways. RS9981629, despite its location in the *KCNJ6* gene, may alter, expression of a nearby gene, *DYRK1A*[48]. *DYRK1A* is a dual-specificity tyrosine, phosphorylation-regulated kinase, and plays a role in signaling pathways relating to brain, development[41]. Whether and how *DYRK1A* might affect pain-relevant phenotypes is unknown.

Several potential study limitations are acknowledged. The impact of race/ancestry on the results must be considered. Tag SNPs examined in this study were all selected based on Caucasian HAPMAP samples, and thus the study cannot address the possibility that these tag SNPs may not have captured all variation in *KCNJ3* and *KCNJ6* genes for non-Caucasians. Due to concerns about possible confounding related to population substructure and the fact that the available samples were primarily Caucasian, the current analyses were restricted to Caucasian individuals only. Whether results would be similar in other ancestral groups remains to be tested.

A second limitation relates to the oral medication order phenotype examined in the primary sample. Due to limitations of the informatics data available for analysis, it was not possible to examine the number of individual analgesic medication doses actually administered or directly assess their efficacy. The total count of inpatient oral analgesic medication orders entered provided a simple, indirect proxy for ongoing difficulties with pain control necessitating additional orders. The fact that this medication order measure correlated significantly and in the expected positive direction with ratings of post-surgical pain that were available in a subset of patients does provides convergent support for the validity of the medication order phenotype.

A final potential limitation is the fact that the univariate analyses did not correct for family-wise error rate, a potentially relevant issue given the number of tag SNPs being examined. However, as an exploratory study testing for the pain-related effects of multiple *KCNJ3* and *KCNJ6* SNPs not previously examined in humans, we felt that this relatively liberal, approach was justified as a means of guiding future more definitive research. The gene set-based analysis, which did address family-wise error rate (testing all SNPs in a single analysis), indicated that *KCNJ6* gene influences on the oral medication order phenotype just failed to reach statistical significance (p=.054). More importantly, replication of the GRRS in an independent laboratory-based sample provided converging evidence supporting an association between *KCNJ6* SNPs and pain-related phenotypes.

In summary, results of this study indicate that variation in the *KCNJ6* gene is associated with both acute and chronic pain phenotypes. Although for mechanistic reasons it is likely that *KCNJ6* gene variation influences pain in part via its effects on opioid receptor function,

a more complete understanding of pathways underlying these associations must await future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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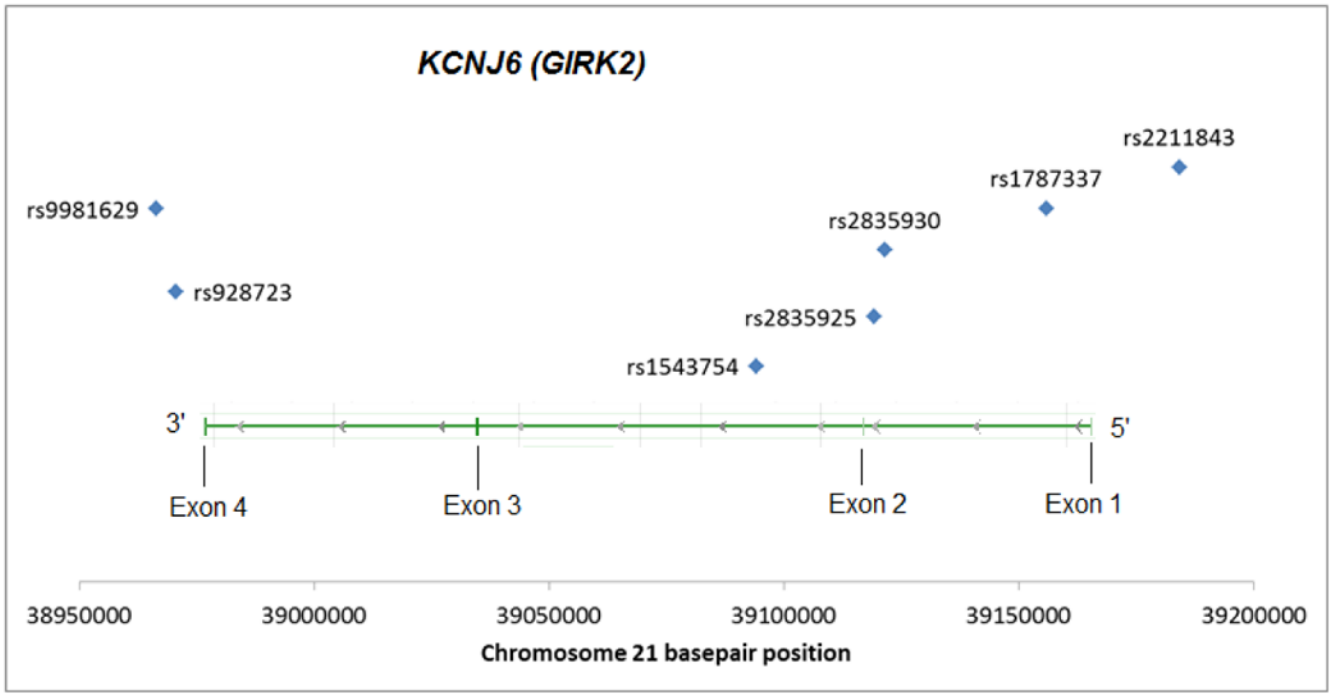

Figure 1.
Location of *KCNJ6* SNPs exhibiting significant associations with the oral analgesic medication order phenotype. Gene diagram and basepair positions are from NCBI build 37.p10 primary assembly.

Table 1

Characteristics of each study sample.

	Primary Sample	Replication Sample	
Variable	**TKA Medication Order Phenotype (n=311)**	**CLBP (n=49)**	**Healthy (n=63)**
Gender (Female %)	63.3	53.1	57.1
Age (years)	62.8 ± 10.48	36.4 ± 10.37 *	31.9 ± 9.25
Total Oral Analgesic Medication Orders	4.6 ±2.96	--	--
Ischemic Acute Pain Threshold (sec.)	--	48.5 ± 68.47	39.9 ± 60.63
Ischemic Acute Pain Tolerance (sec.)	--	260.0 ±76.40	250.9 ± 78.04
Chronic Pain Duration (median, mo.)	--	70.0	--
VAS Chronic Pain Intensity (0-100)	--	43.5 ± 15.43	--
VAS Chronic Pain Unpleasantness (0-100)	--	48.1 ± 17.28	--
GIRK Related Risk Score (GRRS)	8.0 ± 2.64	7.5 ± 2.01	7.7 ± 1.86

* $p<.05$ for age difference between CLBP and Healthy replication subsamples.

Note: Summary statistics are presented as percentages or mean ± SD. TKA = Total Knee Arthroplasty.

Table 2

SNPs in the *KCNJ6* gene significantly associated with the oral opioid analgesic medication order phenotype in quantitative trait analyses.

			Association with Oral Medication Orders		
SNP	**Risk Allele (RA)**	**RA Frequency**	**Beta**	**R^2**	**p-value**
rs1543754	C	0.466	0.754	0.034	<0.001
rs858035	T	0.701	0.728	0.027	0.002
rs9981629	C	0.421	0.573	0.020	0.009
rs928723	A	0.481	0.550	0.018	0.013
rs2835925	A	0.820	0.648	0.017	0.016
rs2211843	T	0.219	0.556	0.013	0.032
rs1787337	G	0.516	0.458	0.013	0.037
rs2835930	A	0.232	0.510	0.012	0.040
KCNJ6 Set-Based Analysis (All Tag SNPs)					0.054

Table 3

Number of oral medication orders in the primary TKA sample by risk allele status for *KCNJ6* SNPs exhibiting significant effects.

	Number of Risk Alleles		
SNP	**0**	**1**	**2**
rs1543754	3.95±2.61	4.18±2.72	5.29±3.10
rs858035	3.50±1.95	4.31±2.85	4.78±2.97
rs9981629	3.94±2.53	4.74±2.78	4.82±3.51
rs928723	4.04±2.37	4.56±2.83	4.95±3.34
rs2835925	3.41±2.09	4.24±2.72	4.61±2.94
rs2211843	4.17±2.65	4.81±2.93	5.62±4.19
rs1787337	3.99±2.61	4.46±3.01	4.92±2.77
rs2835930	4.31±2.93	4.60±2.56	4.91±3.48