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Identification of *KCNJ11* as a functional candidate gene for bovine meat tenderness

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Tizioto PC, Gasparin G, Souza MM, Mudadu MA, Coutinho LL, Mourão GB, Tholon P, Meirelles SL, Tullio RR, Rosa AN, Alencar MM, Medeiros SR, Siqueira F, Feijó GL, Nassu RT, Regitano LCA. Identification of *KCNJ11* as a functional candidate gene for bovine meat tenderness. *Physiol Genomics* 45: 1215–1221, 2013. First published October 22, 2013; doi:10.1152/physiolgenomics.00137.2012.—The potassium inwardly rectifying channel, subfamily J, member 11 (*KCNJ11*) gene was investigated as a candidate for meat tenderness based on the effects reported on muscle for *KCNJ11* gene knockout in rat models and its position in a quantitative trait locus (QTL) for meat tenderness in the bovine genome. Sequence variations in the *KCNJ11* gene were described by sequencing six amplified fragments, covering almost the entire gene. We identified single nucleotide polymorphisms (SNP) and validated them by different approaches, taking advantage of simultaneous projects that are being developed with the same Nelore population. By sequencing the *KCNJ11* in Nelore steers representing extreme phenotypes for Warner-Bratzler shear force (WBSF), it was possible to identify 22 SNPs. We validated two of the identified markers by genotyping the whole population ($n = 460$). Analysis of association between genotypes and WBSF values revealed a significant additive effect of a SNP at different meat aging times ($P \leq 0.05$). In addition, an association between the expression levels of *KCNJ11* and WBSF was found, with lower expression levels of *KCNJ11* associated with more tender meat ($P \leq 0.05$). The results showed that the *KCNJ11* gene is a candidate mapped to a QTL for meat tenderness previously identified on BTA15 and may be useful to identify animals with genetic potential to produce tender meat. The effect of *KCNJ11* observed on muscle is potentially due to changes in activity of KATP channels, which in turn influence the flow of potassium in the intracellular space, allowing establishment of the membrane potential necessary for muscle contraction.

beef; differential expression; molecular markers; Warner-Bratzler shear force

MEAT TENDERNESS IS ONE OF the major issues for the beef cattle market (30). With this in mind, it is important to develop procedures to accurately predict this trait. However, despite its importance and known moderate to high heritability (16), restricted genetic improvements in beef cattle breeding programs have been obtained, probably due to the cost involved in its evaluation and because it is measured only after slaughter. An alternative to circumvent these limitations is the identification of molecular markers that may be useful to more accurately identify superior genotypes earlier in life.

Traditional breeding strategies select animals based on estimated breeding values calculated from phenotypic and pedigree records; however, these methods involve no effort to identify the genes and pathways controlling target traits (12). The candidate gene strategy has been used to identify molecular markers associated with traits of economic importance in cattle. In addition, the identification of genes underlying variation in production traits would enhance understanding of the biology of phenotypic profiles. Generally, candidate genes are elected on the basis of their known physiological or biochemical function and position.

Calpain and calpastatin genotypes have been associated with meat tenderness in different cattle populations (2, 7, 8, 17, 24) because of their key role in proteolysis. However, meat tenderness is under polygenic control, and the genes identified so far explain only part of the genetic variation for this trait. Identifying more gene markers related to meat quality traits would improve carcass quality prediction. The inclusion of validated trait-associated markers in dense single nucleotide polymorphism (SNP) panels would also benefit genomic selection (12).

The potassium inwardly rectifying channel, subfamily J, member 11 (*KCNJ11*, Gene Bank ID: 532060), is involved in the insulin secretion pathway (1), and mutations are related to muscle weakness (11). It is located on bovine chromosome 15, near a quantitative trait locus (QTL) for meat tenderness (21). Transgenic mice with a *KCNJ11* gene knockout have reduced glycogen storage, lean phenotype, lower body fat, and muscle weakness (1).

Consideration of this gene as a positional candidate for controlling meat tenderness was based first on its involvement in muscle strength (1, 11). Further investigation of the *KCNJ11* function revealed a major role in glycogen storage, a key component of the postmortem biochemical process that takes place during transformation of muscle into meat.

The aim of this study was to evaluate *KCNJ11* as a candidate gene for a QTL affecting meat tenderness. To accomplish this objective, we identified polymorphisms and, using both nucleotide and mRNA variation, performed association studies in a Nelore population.

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Table 1. *Primer sequences used to amplify and sequence KCNJ11 (GenBank ID: 532060)*

Primer	Sequences 3′-5′	Primer Position, bp	Amplicon Size, bp
1F	TGTGTGAGGTCTGGTCAGCC	39	582
1R	ACACGTCCTGTAGGAAGCGG	621	
2F	TATGTGCTCACGCGACTAGC	476	707
2R	ACCCACACCATTCTCCATGG	1183	
3F	AGTGCCACCATCCACATGCA	1076	644
3R	AGCCTCACACAAGGTCCTGG	1720	
4F	GCCCAAGTTCAGCATCTCTC	1573	556
4R	CTCATCACCTGCCCTTCCTA	2129	
5F	GAAGAACAGGCCCCACATT	1982	686
5R	GCAAGAACAGCCCAGAGTTT	2668	
6F	AGGCCATTGATTGTGACCGG	2533	533
6R	GATGAGGCAACTGAAGCACA	3066	

KCNJ11, potassium inwardly rectifying channel, subfamily J, member 11; F, forward; R, reverse; bp, base pairs; Primer position (bp), position where the primer F starts and the primer R finishes.

MATERIALS AND METHODS

Animals and phenotypic data. A total of 460 Nelore steers, offspring of 32 sires chosen to represent the main breeding lineages in Brazil, were used to obtain genotypic and phenotypic data. Of these steers, 144 were used for tissue quantitative expression analysis. Half-sibling families were produced by artificial insemination in commercial and purebred Nelore dams. The animals were raised and allocated to two feedlots, as previously described (27). They were slaughtered when 5 mm of back fat thickness was achieved. This study was approved by Embrapa Pecuária Sudeste's ethics committee.

After slaughter, 2.5 cm thick steaks, corresponding to a cross section of the longissimus dorsi muscle, were collected between the 12th and 13th ribs. The steaks were identified, vacuum packed, and used to measure meat tenderness through Warner-Bratzler shear force (WBSF). Values were obtained in a TA XT2i texture analyzer coupled to a Warner-Bratzler blade with 1.016 mm thickness. WBSF analyses were performed at different aging times: 24 h (WBSF0), 7 days (WBSF7), and 14 days (WBSF14) of aging at 2°C in a cold chamber manufactured by Macquay Heatcraft do Brasil, São José dos Campos, São Paulo, Brazil.

DNA extraction. Straws of frozen semen obtained from Brazilian artificial insemination centers were used to extract DNA from bulls by a standard phenol-chloroform method (22). For the steers, 5 ml blood samples were used, and DNA extractions were performed by a salting-out method. DNA concentration was measured by spectrophotometry, and the quality was verified by the 260:280 ratio, followed by inspection of integrity through agarose gel electrophoresis.

Selection of extreme phenotypes. To identify polymorphisms in the *KCNJ11* gene, samples from 14 animals characterized as belonging to the extremes of the WBSF0 data distribution were selected for sequencing. These animals were chosen based on the residual values obtained from a statistical model (see *Statistical analysis of tissue expression data*) used to correct WBSF0 for environmental variations, considering the fixed effects of contemporary groups (CG), in which variations due to place of birth, feedlot, year of birth, and slaughter date were taken into account. We also included the covariates animal age at time of measurement and pH. After ordering the residual values, we selected seven animals, classified respectively among the 5% highest and lowest values, for sequencing. Care was taken to represent different half-sibling families.

KCNJ11 DNA sequencing primers (Table 1) were designed to cover almost the entire *KCNJ11* gene sequence deposited in the National Center for Biotechnology Information database, using the Primer3Plus software (29). Primer quality was verified with the NetPrimer software (http://www.premierbiosoft.com/netprimer).

Reactions to amplify all regions of the *KCNJ11* gene were carried out with 1× reaction buffer, 1.5 μM $MgCl_2$, 0.2 mM of each dNTP, 0.165 μM of each primer, 0.65 unit of Taq DNA polymerase, and 200 ng DNA, in a final volume of 15 μl. Amplification steps consisted of 5 min of initial denaturation at 94°C, followed by 35 cycles of 94°C, 59°C, and 72°C for 45 s each, and a final extension step at 72°C for 10 min, in an Eppendorf Mastercycler (Hauppauge, NY).

Polymerase chain reaction (PCR) products (5 μl) were purified using 2 μl of ExoSAP-IT enzyme (Invitrogen, Foster City, CA). Sequencing reactions were performed using the ABI Prism BigDye Terminator v. 3.1 cycle sequencing kit, purified by alcohol precipitation, and analyzed in an ABI Prism 3100 Avant sequencer (Applied Biosystems, Foster City, CA).

SNP description. The Phred Phrap Consed package (9, 13) was used to identify SNPs in the 14 extreme phenotype steers sequenced by the Sanger method. The SNP locations were determined through alignment to sequences of the gene in public databases and were analyzed for amino acid changes with the Gene Runner program (http://www.generunner.net/).

The Haploview program (3) was used to determine linkage disequilibrium and to identify Tag SNPs. Fisher's exact test was applied to determine if allele frequencies differ between extremes for WBSF0, by comparing genotypes at each extreme.

Quantitative expression association analysis. Two SNPs were genotyped in the remaining animals ($n = 484$) to verify association with WBSF. Two different criteria were used to choose these SNPs. One SNP was chosen because it is a TagSNP for a region with high linkage disequilibrium, and the other was chosen based on the significance of the allele frequency differences between the two WBSF extreme sample groups. Genotypes were determined by real-time PCR, using TaqMan assays (Applied Biosystems).

RNA extraction and expression data. The longissimus dorsi samples collected at slaughter from 137 animals were immediately frozen in liquid nitrogen and kept at −80°C until RNA extraction. Total RNA was extracted from 50–100 mg of muscle tissue macerated in liquid nitrogen and immediately immersed in 1 ml of Trizol (Invitrogen), following the manufacturer's protocol. The total RNA concentration was measured by spectrophotometry (Eppendorf Biophotometer, Orlando, FL), and quality was verified by the 260:280 ratio, followed by assessment of their integrity through agarose gel electrophoresis. Total RNA (1 μg) from each muscle sample was reverse transcribed to cDNA in 20 μl reactions using the ImProm-II Reverse Transcription System kit (Promega, Madison, WI), according to the manufacturer's protocol and conditions.

KCNJ11 expression was analyzed with the LightCycler 480 detection system (Roche Applied Science, Indianapolis, IN). Reactions were prepared in a final volume of 10 μl containing 1 μl of cDNA diluted 10×, 5 μl of SYBR Green I Master (Roche Applied Science) 2×, and 0.4 μM of each primer. Forward and reverse primers were designed in different exons or exon-exon junctions, with at least 50% GC, a maximum of 2°C difference in the melting temperature between primers of the same gene, a minimum score of 90 in the *NetPrimer* (http://www.premierbiosoft.com/netprimer/index.html), and amplicon size between 80 and 150 base pairs (Table 2). The constitutive genes

Table 2. *Primers used for gene expression analysis in muscle samples of Nelore steers*

Gene (GenBank ID)	Sequence (5′-3′)
KCNJ11 (532060)	F: AGTGCCACCATCCACATGCA
	R: ACCCACACCATTCTCCATGG
EEF1A (AJ238405.1)	F: GCAGCCATTGTGGAGATG
	R: ACTTGCCCGCCTTCTGTG
RPL-19 (510615)	F: GAAATCGCCAATGCCAAC
	R: GAGCCTTGTCTGCCTTCA

EEF1A, eukaryotic elongation factor 1A; RPL-19, ribosomal protein L19.

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ribosomal protein L19 (*RPL-19*) and eukaryotic elongation factor 1A (*EEF1A*) were used as reference genes in relative quantification after being tested for constant levels of expressions. These reference genes were used to normalize the relative quantitative real-time PCR data for obtaining reliable conclusions regardless of variation in total RNA concentration or cDNA synthesis efficiency among samples.

Individual efficiency of all samples was calculated with LinRegPCR software (18), using at least four points of the amplification curve during the exponential phase of PCR. A procedure was adopted from the individual efficiencies obtained through LinRegPCR to correct values for the ideal efficiency of two.

$Ct_{ij} = \log_2[E\_gene_{ij}^{ct/-gene(ij)}]$, In which Ct_{ij} is the threshold cycle (Ct) of the *j*th sample from the *i*th gene corrected for efficiency of two; and $E\_gene_{ij}^{Ct\_gene(ij)}$ is the actual efficiency calculated by the program.

Statistical analysis of genotypic data. To evaluate the influence of markers on WBSF, we used a mixed model with fixed effects of CG, genotypes, animal age at measurement, and pH as covariates (linear effects), in addition to the random effect of sire. CG included the effects of birth and feedlot place, month of birth, breeding season, and slaughter date.

Analyses were performed by the REML by the MIXED procedure in the Statistical Analysis System (SAS Institute, 2000) using the model: $Y_{ijkl} = \mu + CG_i + M_j + S_k + b_1(A_{ijkl} - a) + b_2(P_{ijkl} - p) + e_{ijkl}$, where Y_{ijkl} = observation of *l*th individual, of age *A*, sib of *k*th sire, of jth genotype for the marker, belonging to the *i*th contemporary group; μ = overall mean; CG_i = fixed effect of the *i*th contemporary group; M_j = fixed effect of *j*th genotype for the marker; *Sk* = random effect associated with the *k*th sire [$\sim N(0,\sigma_t^2)$]; b_1 = regression coefficient associated with animal's age; b_2 = regression coefficient associated with sample pH; A_{ijkl} = animal age on the date of measurement, a is the mean age at measurement; P_{ijkl} = value for each observation of pH, *p* is the mean pH of the samples; e_{ijkl} = random error associated with each observation [$\sim N(0,\sigma_e^2)$].

The mixed procedure of SAS (proc mixed) was used to test the additive-dominance model. As described in the literature (23), assuming estimated allele frequencies for the *KCNJ11* marker and using the estimated additive (a = 1/2 *homozygous genotype 1* − 1/2 *homozygous genotype 2*) and dominance deviation [d = heterozygous genotype − (1/2 *homozygous genotype 1* + 1/2 *homozygous genotype 2*)] effects for the alleles, the percentage of phenotypic variation explained by a marker was calculated using the standard formula: $\%V = 100 \times \{2pq[a + d(q - p)]^2 + (2pqd)^2\}/\sigma^2 p$, where %V is the percentage of phenotypic variation explained by the SNP, and $\sigma^2 p$ is the phenotypic variance of the trait (10). Probability values were not corrected for multiple testing.

Statistical analysis of tissue expression data. Different methods have been proposed for the selection of appropriate reference genes, including methods that take into account the possibility that the resulting variation among samples (19, 20) might be due to fixed effects (treatment, animal age, sex, breed, management, and genetic background), random effects (animal sample), their interactions, and heterogeneous variances of reference genes. A general linear mixed model that considers the fixed and random effects originated from the experimental design was used, according to the following equation: $Y_{ij} = \mu + G_i + S_j + e_{ij}$, in which Y_{ij} is the Ct for ith gene of the jth sample; μ is the average of Ct; G_i is the fixed effect for the ith gene; S_j is the random effect associated with the sample considering $s_j \sim \text{NID}(0,\sigma_a^2)$; e_{ij} is the random residual effect, with $e_{ij} \sim \text{NID}(0,\sigma_e^2)$.

On the basis of this model, we obtained best linear unbiased predictor values for random sample effect, and these were used to obtain and adjust the quantification cycle (Ct) value (CtA). After the adjustment of the Ct values, a general linear mixed model that included effects of slaughterer, parentage, age, and pH was applied to each meat aging time to verify the association of the *KCNJ11* expression level with meat tenderness.

To test whether the genotyped SNPs altered expression levels of the *KCNJ11* gene, CtA was included as a dependent variable in the statistical model, and the SNP genotype was included as a fixed effect. A similar general linear mixed model, described previously, was used to verify the association between expression level and meat tenderness, except this time, the effect of age was not included.

RESULTS

Phenotype scoring. From the 461 slaughtered steers, data on WBSF 24 h after slaughter were available for all animals, but only 423 and 434 were available for WBSF7 and WBSF14, respectively, because of losses during the aging process. As expected, a decrease in WBSF was observed from 24 h to 14 days after slaughter (Table 3).

SNP identification in the extreme phenotype animals and validation in the whole population. KCNJ11 gene sequencing revealed 22 SNPs (Table 4). Minor allele frequencies for the SNPs identified in extreme animals ranged from 0.07 to 0.46.

Only one SNP found in the coding region was a nonsynonymous mutation. SNP 1186G>A causes the substitution of an asparagine by a serine amino acid. The remaining five SNPs found in the coding region are synonymous mutations. Additionally, two SNPs were found in the 5′-untranslated region (UTR), 12 SNPs in the 3′-UTR region, and two SNPs in the intergenic region.

The disequilibrium pattern between the 22 SNPs identified in the *KCNJ11* gene showed that SNP 1526C>T is a principal TagSNP representative in a region with high linkage disequilibrium. Through the TagSNP 1526C>T it is possible to infer the genetic variation of the SNPs 189G>A, 1424T>C, 1933C>T, 1934G>A, and 1938C>T.

To evaluate the influence of *KCNJ11* on meat tenderness, as a first approach, we compared the allele frequencies of the SNPs identified between groups of steers with extreme WBSF phenotypes.

As shown in Table 4, the SNP 2342T>C presented a significant association ($P \leq 0.05$) with WBSF0. In that comparison, a prevalence of T allele in the animals grouped in the lowest residuals for WBSF0 was observed. On the basis of the above criteria, we chose SNPs 1526C>T and 2342T>C for validation in the whole population. The selection of SNP 2342T>C was reinforced by the results from a parallel experiment of RNA-sequencing in a sample of this Nelore population with extreme phenotype for WBSF7.

SNP identification from that RNA-sequencing data revealed 13 of the 20 SNPs identified in *KCNJ11* gene as located in the mRNA, including the two SNPs chosen to be validated in the whole population. In the Fisher exact test using the SNP data

Table 3. *Raw means and SD for WBSF measured at different aging times*

Trait	*n*	Means ± SD, kg/f
WBSF0	461	8.82 ± 2.02
WBSF7	423	5.09 ± 1.62
WBSF14	434	4.05 ± 1.40

WBSF0, Warner-Bratzler shear force measured 24 h after slaughter; WBSF7, Warner-Bratzler shear force measured after 7 days of cold-chamber aging; WBSF14, Warner-Bratzler shear force measured after 14 days of cold-chamber aging.

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Table 4. *Identified SNPs in KCNJ11 gene in Nelore steers with extreme phenotypes (n = 14) for WBSF measured 24 h after slaughter, P values for the Fisher exact test for comparison between extremes and MAFs*

SNP	NCBI_ss#	Gene Location	Gene Region	Flanking Sequences	*P* Value	MAFs
189G>A	537718959	189pb	5′-UTR	GGTGGC(G/A)AACAGG	0.269	0.11
241T>C	537718961	241pb	5′-UTR	CTGAGG(T/C)GTGAGG	0.049*	0.46
1059C>A	537718963	1059pb	CDs	GACCTC(C/A)GGAAGA	0.403	0.07
1186G>A	537718965	1186pb	CDs	GCAATA(G/A)CATCTT	0.272	0.40
1220C>T	537718967	1220pb	CDs	CTACCA(C/T)GTCATT	0.545	0.41
1241G>A	537718969	1241pb	CDS	CAGCCC(G/A)CTCTAT	0.227	0.22
1424T>C	537718971	1424pb	CDs	CTACTC(C/T)GTGGAC	0.254	0.13
1526C>T	537718973	1526pb	CDs	GACCCT(T/C)GTCCGC	0.254	0.13
1629C>T	537718975	1629pb	3′-UTR	TGGGCT(C/T)CCTTCT	0.195	0.34
1693C>T	537718977	1693pb	3′-UTR	GCCCCT(C/T)GGCCAG	0.195	0.15
1869C>T	537718979	1869pb	3′-UTR	TCGTCC(C/T)GCTCCC	0.176	0.46
1893A>G	537718981	1893pb	3′-UTR	TGACAG(A/G)GCTGTG	0.021*	0.42
1933C>T	537718983	1933pb	3′-UTR	TCCATC(T/C)GATCTTG	0.269	0.11
1934G>A	537718985	1934pb	3′-UTR	CCATCT(G/A)ATCTTG	0.265	0.12
1938C>T	537718987	1938pb	3′-UTR	CTGATC(T/C)TGGGCC	0.269	0.11
2009C>T	537718989	2009pb	3′-UTR	GAGGCC(C/T)GCTGCC	0.326	0.30
2291A>G	537718991	2291pb	3′-UTR	TTAATG(A/G)GGGCTC	0.333	0.40
2307A>G	537718993	2307pb	3′-UTR	GGCCCA(G/A)GGCTGC	0.833	0.10
2342T>C	537718995	2342pb	3′-UTR	GTCCCC(C/T)ACCCTG	0.018*	0.39
2621G>A	537718997	2621pb	3′-UTR	TTTGTC(G/A)TCAATA	0.004†	0.42
2952C>T	537718999	2952pb	intergenic region	ATCACA(C/T)GTCCAG	0.049*	0.46
3037G>A	537719001	3037pb	intergenic region	TCTAAC(G/A)TAACCT	0.060	0.36

SNP, single nucleotide polymorphism; CD, coding region. Gene location is based on the National Center for Biotechnology Information (NCBI) public database; gene region is based on the reference sequence deposited in the NCBI public database; minor allele frequency (MAF) of SNPs in extreme animals for shear force; *$P \leq 0.05$; †$P \leq 0.01$.

from 28 RNA sequenced animals, only SNP 2342T>C was found to be associated with WBSF measures ($P \leq 0.0351$).

The minor allele frequency observed for the two SNPs in the population was higher than in extreme samples (Table 5).

SNP 2342T>C is in Hardy-Weinberg equilibrium and had an additive effect on WBSF0 and WBSF7 ($P \leq 0.05$, Table 6). The estimated allele substitution effects of SNP 2342T>C were 0.39 and 0.38 kg for WBSF0 and WBSF7, respectively. This polymorphism accounted for 7.46 and 7.06% of the total additive variance and 8.87 and 7.94% of total genetic variance of WBSF0 and WBSF7, respectively, and dominance deviations were not significant (Table 6). The T allele is associated with reduced WBSF, as suggested by frequency comparison of extreme phenotypes. The effect of the haplotypes composed by the two SNPs 1526C>T and 2342T>C was not significant ($P \leq 0.05$) for any measure of WBSF.

Previous studies (Souza MM, Niciura SCM, Donatoni FAB, Tizioto PC, Regitano LCA, unpublished results) performed by our research group with a sample of the Nelore population used in this work ($n = 184$) did not find significant effects of polymorphisms in μ-calpain (SNP c.3379G>A: rs17872099) and calpastatin (SNP c.2959A>G: AF159246) genes on WBSF at any aging time. The effects of the *KCNJ11* gene SNP 2342T>C on WBSF0 and WBSF7 remained significant ($P \leq 0.05$) even when the SNPs on μ-calpain and calpastatin genes were included as fixed effects in the model (data not shown).

Table 5. *Allelic and genotypic frequencies of SNPs 1526C>T and 2342T>C of KCNJ11 gene in a Nelore half-sib reference population, including animals without phenotypic records*

		Frequency, %				
		Genotypic			Allelic	
SNP *KCNJ11*	*n*	CC	CT	TT	C	T
1526C>T	614	71.17	26.38	2.45	84.36	15.64
2342T>C	660	39.52	34.24	26.67	56.21	43.79

n, Number of observations.

Gene expression study. Gene expression profiling in muscle was also used to evaluate whether *KCNJ11* could be functional candidate gene affecting meat tenderness. To better characterize the involvement of *KCNJ11* in the regulation of meat tenderness, its expression level was associated with individual phenotypes in 137 animals. The influence of polymorphisms in mRNA abundance was also investigated to test the expressed QTL hypothesis.

KCNJ11 gene expression level was associated with WBSF7 ($P \leq 0.05$) (Table 7). When the expression level decreased by half, WBSF7 decreased 0.1627 (± 0.06786) kg. In other words, lower *KCNJ11* expression levels were related to more tender meat at 7 days of aging. Genotypes for SNPs 1526C>T and 2342T>C, however, did not influence the expression levels of this gene ($P \leq 0.54$ and $P \leq 0.97$, respectively).

DISCUSSION

The WBSF values observed here (Table 3) are in agreement with a previous study (25), in which a mean WBSF value of 7.14 kg was reported for Nelore-sired steers. The WBSF value is higher than that normally observed for *Bos taurus* breeds, but this is expected and in agreement with the observation of an increase in WBSF for crossbred animals with higher degrees of *Bos indicus* contribution (14). The presence of higher concentrations of calpastatin in *B. indicus* (Zebu) muscles compared with that in *B. taurus* animals has been associated with lower meat tenderness (31).

Most genes affecting a polygenic trait such as meat tenderness are unknown. Livestock genetic research seeks better

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Table 6. *Summary of the results of the mixed-model analysis of association among WBSF measures in Nelore and two polymorphisms in KCNJ11 gene*

		SNP1526 C>T				SNP2342T>C			
WBSF	*n*	Gen.	LS Means ±SE, kg/f	Add.	Dom.	Gen.	LS Means ± SE, kg/f	Add.	Dom.
0	461			ns	ns			0.3885	ns
		CC	8.86 ± 0.15			CC	9.14 ± 0.14		
		CT	8.72 ± 0.14			CT	8.70 ± 0.14		
		TT	9.40 ± 0.51			TT	8.75 ± 0.16		
7	423			ns	ns			0.3778	ns
		CC	5.38 ± 0.13			CC	5.50 ± 0.13		
		CT	5.46 ± 0.13			CT	5.51 ± 0.13		
		TT	5.69 ± 0.50			TT	5.12 ± 0.15		
14	434			ns	ns			ns	ns
		CC	4.33 ± 0.11			CC	4.35 ± 0.11		
		CT	4.49 ± 0.11			CT	4.54 ± 0.11		
		TT	4.57 ± 0.43			TT	4.26 ± 0.13		

WBSF measured at 24 h after slaughter = 0; measured at 7 days of aging = 7; measured at 14 days of aging = 14) *n*, Number of observations; Gen., genotype; LS Means ± SE, least square means adjusted by the model ± SE; Add., additive effect (kg/f); Dom., dominance deviation effect; ns, not significant ($P \leq 0.05$).

understanding of the basis of the genetic variation of target traits (12). Positional and functional candidate genes related to economically important traits have been identifed; however, the genes identified so far explain a small portion of the genetic variation. Moreover, most of candidate genes and molecular marker descriptions have been conducted in *B. taurus* cattle. Differences between taurine and indicine cattle for allele frequencies at causal mutations or the extent of linkage disequilibrium of markers could result in different marker effects being detected in different breeds. Considering this, identifying candidate genes and molecular markers in a sample of a reference Nelore population would benefit breeding programs of this breed.

In this study, two different approaches were used to evaluate whether *KCNJ11* gene affects meat tenderness. We first used *KCNJ11* sequencing to discover specific allelic variation responsible for genetic effects in an attempt to fine-map a previously described QTL (21) mapped to BTA15, and then we also evaluated mRNA abundance effects. These analyses allowed the identification of 22 SNPs in *KCNJ11*. Fisher's exact test analysis of extreme phenotype animals suggested a target marker (SNP 2342T>C) for validation in the whole population. In addition, another SNP identified as tag SNP was genotyped. The SNP 2342T>C located in the 3′-UTR region of *KCNJ11* had an additive effect on the WBSF measured at different times of meat aging (Table 6). From those results, we can suggest that selection in favor of T allele of this SNP would lead to a decrease in the mean value of WBSF0 and WBSF7 in this Nelore population, improving meat tenderness. However, it is not possible from our data to state whether the SNP 2342 T>C is a causal mutation. In the case that SNP 2342 T>C is in linkage disequilibrium with the casual mutation, recombination could break the association of the functional allele with *KCNJ11* markers. Another implication is that validation of this marker is necessary before it can be used in other cattle breeds.

Table 7. *Effect of the muscular expression level (Ct) of KCNJ11 gene on the WBSF measures in 137 Nelore steers*

WBSF	Estimated Effect, kg ± SE	*P* Value
0	−0.07868 ± 0.066	0.2403
7	−0.1627 ± 0.068	0.0187*
14	−0.03891 ± 0.056	0.4877

*$P \leq 0.05$.

Genetic variants have been explored in explaining differences in meat quality traits, but the underlying mechanisms affecting them are still poorly understood. A gene expression profiling approach was also used to evaluate whether the *KCNJ11* mRNA levels also affect the meat tenderness and to better characterize the involvement of this gene in bovine meat tenderness. It was observed that lower *KCNJ11* expression levels were associated with more tender meat after 7 days of aging, which also suggests that this gene may have an important role in meat tenderness.

Since SNP 2342 T>C was shown to have no influence on *KCNJ11* transcript abundance, these two effects of *KCNJ11* polymorphism and expression level on WBSF7 seem to be independent. The effect of *KCNJ11* expression level on WBSF7 could be explained by mutations in regulatory regions or other mechanisms, such as microRNA gene regulation, codon bias or even interaction with additional genes encoding for transcription factors.

KCNJ11 encodes a membrane protein that controls potassium flow into the cell. Potassium is required for muscle contraction and nerve impulses, and along with sodium, it helps maintain the proper balance of fluids in cells (15). A study showed that inactivation of *KCNJ11* gene in mice causes lower body weight and higher energy expenditure and muscle weakness (1). Muscle weakness associated with *KCNJ11* mutations was also observed in humans, in whom mutations in the *KCNJ11* gene are associated to developmental delay, muscle weakness, and epilepsy, in addition to neonatal diabetes (11). Studies in skeletal muscle have shown the role of KATP channels in the physiological performances of different muscle fiber types. Molecular composition and properties of muscle-specific KATP channels subunits may determine the differences among muscle types in regulation of the extracellular potassium concentration through vasodilation, modulation of glucose uptake, and drug responses (28). The effect of *KCNJ11* observed on meat tenderness in the present work is potentially due to changes in activity of KATP channels. Although the role of this gene in muscle contraction may underlie the effect on meat tenderness, this study is the first to investigate such relationship and more studies are needed to better elucidate it.

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A transcriptome study identified nine genes, including *KCNJ11,* whose expression profiles explain up to 60% of the variability in muscle mass in young Charolais bull calves. In that study, *KCNJ11* presented a significant positive correlation with muscle mass (5).

Since the *KCNJ11* gene is mapped to bovine chromosome 15, near a QTL for meat tenderness (21), and based on metabolic pathways and function on muscle previously reported (1, 5, 11), we had suggested the *KCNJ11* gene as a possible candidate to influence carcass traits in cattle, which the present study confirmed (Tables 6 and 7). Other candidate genes for meat tenderness are present in this region. Some examples are calcitonin-related polypeptide alpha (*CALCA*), which is involved in regulating calcium levels in the blood, and myogenic differentiation 1 (*MYOD1*), which has effects on muscle development (21). Although the *CALCA* gene was already suggested as a candidate for meat tenderness (21), this has not been confirmed in the literature. However, this gene could affect meat tenderness because of the importance of calcium on the calpain and capastatin proteolysis system, as well on muscle contraction. The MYOD1 effect on carcass traits has been already reported (6), but despite its known function on muscle development, its association with meat tenderness remains poorly explored.

Since several genes at different loci can affect production traits like meat tenderness, candidate gene studies are not expected to explain all the variation for the studied trait (4). The results presented here reveal that genetic variation and expression of the *KCNJ11* gene are associated with meat tenderness in a Nelore population, suggesting that *KCNJ11* is a potential positional, physiological, and functional candidate gene for meat quality traits in cattle. A SNP identified in the *KCNJ11* gene contributes to the phenotypic variance of meat tenderness and, in association with other markers, can be used to improve genetic gain for meat tenderness. The magnitude of the effects of the *KCNJ11* gene found in this work could be under- or overestimated due to the small sample size used. Before being efficiently used within breeding programs, the genetic information presented here should be extended to more breeds and populations to accurately describe this marker association and its phase relationships. Once confirmed, inclusion of the *KCNJ11*-associated SNPs in dense panels may improve accuracy of genomic selection for meat tenderness, since these SNPs are not represented in the commercially available SNP chips.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: P.C.T., G.G., M.M.S., R.R.T., S.R.M., F.S., G.L.D.F., and R.T.N. performed experiments; P.C.T., M.A.M., G.B.M., P.T., and S.L.C.M. analyzed data; P.C.T., G.G., M.A.M., L.L.C., and L.C.A.R. interpreted results of experiments; P.C.T., G.G., M.A.M., L.L.C., and L.C.A.R. drafted manuscript; P.C.T. and L.C.A.R. edited and revised manuscript; A.N.R., M.M.A., and L.C.A.R. conception and design of research; L.C.A.R. approved final version of manuscript.

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