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## Identification of rare noncoding sequence variants in gamma-aminobutyric acid A receptor, alpha 4 subunit in autism spectrum disorder

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### Abstract

Alterations of the gamma-aminobutyric acid (GABA) signaling system has been strongly linked to the pathophysiology of autism spectrum disorder (ASD). Genetic associations of common variants in GABA-receptor subunits, in particular *GABRA4* on chromosome 4p12, with ASD have been replicated by several studies. Moreover, molecular investigations have identified altered transcriptional and translational levels of this gene and protein in brains of ASD individuals. Since the genotyped common variants are likely not the functional variants contributing to the molecular consequences or underlying ASD phenotype, this study aims to examine rare sequence variants in *GABRA4*, including those outside the protein coding regions of the gene. We comprehensively re-sequenced the entire protein coding and noncoding portions of the gene and putative regulatory sequences in 82 ASD individuals and 55 developmentally typical pediatric controls, all homozygous for the most significant previously associated ASD risk allele (G/G at rs1912960). We identified only a single common, coding variant and no association of any single marker or set of variants with ASD. Functional annotation of non-coding variants identified several rare variants in putative regulatory sites. Finally, a rare variant unique to ASD cases, in an evolutionary conserved site of the 3' UTR, shows a trend toward decreasing gene expression. Hence, *GABRA4* rare variants in noncoding DNA may be variants of modest physiological effects in ASD etiology.

### Keywords

Autism; GABRA4; sequencing; noncoding

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## Introduction

Autism spectrum disorder (ASD) comprises a range of neurodevelopmental conditions characterized by difficulties in social relatedness, communication, and behavior [1] and are diagnosed at a rate of approximately 1 in 68 children over eight years old in the United States[2]. There is a strong genetic component to risk for ASD. Among identified genetic contributors to ASD are large chromosomal irregularities, [3], copy number variations (CNVs), [4, 5], and recurrent *de novo* protein altering variants[6–9].

Genome-wide attempts to identify common genetic variants contributing to ASD risk have been moderately successful implicating a number of genes and genomic regions. These include the region between Cadherin 9 (*CDH9*) and Cadherin 10 (*CDH10*) on chromosome 5p14.1 [10, 11], *MACROD2* on 20p12.1 [12], and the intergenic region between *SEMA5A* and *TAS2R1* on 5p15.31 [13]. Though these studies have identified several positive associations, they have not been widely replicated. One possibility is that hundreds of factors impact ASD risk [14] and a complex architecture of many rare disease causing variants make it difficult to replicate single association signals in studies using current sample sizes [15].

Though genome-wide common variant association results have not widely replicated, there have been several candidate gene studies that have implicated the GABAergic system in ASD. GABA is the primary inhibitory neurotransmitter in the adult brain and directs neuronal development, a process which is altered in ASD [16–18]. Cytogenetic [19–21] and genotyping based linkage and association studies [22–24] have repeatedly implicated GABA receptors in ASD. Furthermore, candidate gene association tests for GABA receptor genes have revealed common polymorphisms in *GABRA4* on chromosome 4p12 with association to ASD in multiple datasets [25–27]. Beyond these genetic findings, functional evidence for the role of *GABRA4* in ASD is supported by biochemical studies which have shown reduced protein and mRNA levels in the cerebella of patients with autism [28–30].

Since the common SNPs associated with ASD in *GABRA4* may not be the functional variants contributing to ASD phenotypes, it is necessary to follow-up association with comprehensive re-sequencing to uncover the complete catalogue of potentially functional rare genetic variants. To identify rare potentially functional variants we sequenced an 89kb region of chromosome 4 including the entire *GABRA4* transcriptional region, 5kb 5' and 10kb 3' of the gene, in 82 White European ASD individuals and 55 age and ethnicity matched controls. We identify novel variants in evolutionarily conserved and predicted functional regions of *GABRA4* and demonstrate that these can function by altering transcription of the gene.

## Materials and Methods

### Sample Ascertainment

The 82 ASD individuals sequenced in this study were recruited and enrolled as part of a previous study of GABA receptor genes [25]. The 55 controls consisted of white individuals aged 4 to 21 years sampled through the John. P. Hussman Institute for Human Genomics

(Miami, FL). Control individuals were screened to determine whether they have been diagnosed with or have a parent or sibling with a developmental, behavioral, neurological or other disability. Parents of young children or the participants were informed and signed the informed consent, completed the Social Communication Questionnaire [31] to screen for potential ASDs, and donated saliva. In order to enrich for rare variants in possible linkage disequilibrium (LD) with a previously identified risk allele we chose only homozygous risk ASD individuals and controls (G/G at rs1912960). Genomic DNA was purified from whole blood from ASD individuals and saliva from controls using Puregene chemistry on the Qiagen Autopure LS according to standard automated Qiagen protocols (Qiagen, Valencia, CA, USA).

### Targeted Region Amplification

We targeted chr4.hg19:46918163-47005187 encompassing 74kb of *GABRA4* (NM\_000809) and 10kb upstream and 5kb downstream of the gene with long-range PCR. Ten tiled primer pairs each amplifying ~8.7kb were designed using Primer3 software (<http://frodo.wi.mit.edu>) [32, 33] (Online Resource 1) and synthesized by IDT (Integrated DNA Technologies, Coralville, IA, USA). PCR was performed using the Expand Long Range dNTPack PCR kit (Roche Applied Science, Indianapolis, IN, USA) on Veriti 96-Well Fast Thermal Cyclers (Life Technologies, Carlsbad, CA, USA). PCR products were visualized on 0.75% agarose-ethidium bromide gel and primer dimer and nonspecific products removed with the NucleoFast 96 PCR kit (Machery-Nagel, Bethlehem, PA, USA) following standard manufacturer protocol for a vacuum manifold.

### Massively Parallel Sequencing

Each of the 10 PCR products for each individual were quantified in a high-throughput microplate format on the SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA) using the Quant-iT PicoGreen dsDNA assay kit (Life Technologies, Carlsbad, CA, USA). The products were equimolar pooled, sequencing libraries prepared using reagents from New England Biolabs (NEB, Ipswich, Massachusetts, USA) according to Illumina manufacturer instructions, and 12 libraries pooled together into a single multiplexed sequencing library. These used for paired end cluster generation on the Illumina v2 flow cell (Illumina, San Diego, CA, USA) and sequenced on the Illumina GAIIx with a paired-end run of 52 cycles, with a 7-cycle index read.

### Sequence Analysis

Raw image analysis and base calling were performed with Illumina Pipeline v1.5 and Sequence Control Software v2.5, and individual sequencing reads binned using a `split.on.index.pl` script supplied by Illumina. Subsequent data processing was done through an in-house pipeline including: alignment to the hg19 human reference genome with the Burrows-Wheeler Aligner (BWA) [34], generation of coverage metrics for quality control with PICARD, and genotype calling with the Genome Analysis Toolkit (GATK) Universal Genotyper [35]. As these reads result from PCR and coverage was extremely high, duplicates were not removed before variant calling. Only variant positions with a read depth greater than 50×, a call-rate of at least 95% across all samples, normalized Phred-scaled likelihood (PL) of a reference genotype = 100, and at least 30% alternate allele reads were

analyzed. Variant annotation was performed with ANNOVAR software [36, 37] and an in house non-coding variant annotation pipeline utilizing available ENCODE data [38]. For this pipeline, DNase hypersensitivity and transcription factor binding sites identified in any neuronal lineage cell line (SK-N-SH, BE2\_C, Gliobla, HA-sp, HAc, NH-A, PFSK-1, SH-SY5Y, SK-N-MC, SK-N-SH\_RA, and U87) were acquired from the UCSC table browser and compared against variant positions with BEDtools[39].

### Rare variant association analyses

Single variant association was determined using the Fisher's exact test implemented in PLINK [40] with power calculations performed with the Genetic Association Study (GAS) Power Calculator[41, 42]. To identify sets of variants associated with ASD, the optimized Sequence Kernel Association Test (SKAT-O) [43, 44] was performed with resampling with 10,000 iterations. This analysis was performed for all variants, for minor allele frequency cutoffs of 0.1, 0.05, 0.01, and 0.005, and for variants within discrete functional units. We also implemented a sliding window approach in each of the minor allele cut offs in groups of ten markers, overlapping by five, and used SKAT-O to determine association of variants within any window.

### Functional assessment of 3'UTR variants by luciferase assay

The 9.3kb 3'UTR of *GABRA4* was amplified from a control individual with Q5 Hot Start High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA, USA) and cloned into 5' PmeI and 3' XhoI sites of the pmirGLO vector (Promega, Madison, WI, USA) using Blunt/TA Ligase Master Mix (New England BioLabs, Ipswich, MA, USA). Mutations were induced with the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA, USA). Confirmation of correct orientation, presence of the mutagenized base, and absence of off-target variants in all clones were determined by tiled PCR and Sanger sequencing of the entire insert on the ABI3730xl. Luciferase activity was measured in Human embryonic kidney (HEK293) transfected with 4ug of plasmid with Lipofectamine (Life Technologies, Carlsbad, CA, USA). We utilized the Dual-Glo® Luciferase Assay (Promega, Madison, WI, USA) in seven independent transfections for biological replication and two technical replicates for each experiment. We performed two-tailed, unequal variance Student's t-tests across the seven biological replicates to compare luciferase expression from constructs with induced mutations compared to the control 3'UTR sequence.

## Results

### PCR Amplification

PCR optimization resulted in ideal thermocycling conditions for each primer set to yield a specific, single product represented by a band at the expected ~8.7kb size when visualized on an agarose gel (Figure 1). We successfully amplified the entire targeted region of *GABRA4* in 82 ASD cases and 55 controls using ten tiled long range PCR reactions. Quantitation of the products after PCR cleanup showed DNA concentrations of ~35ng/μl and total yields of ~1.75μg of purified PCR products. We pooled  $2 \times 10^{-15}$  moles (~100ng) of each product into a single library per individual to be used in the sequencing sample preparation procedure.

## Sequence Alignment and Variant Detection

Sequencing of the 137 samples in 2×52 paired end sequencing runs of the Illumina GAIIX and subsequent splitting of reads based on indexes, resulted in 1.8million ± 0.3million reads passing Illumina quality filters per sample. Of these, BWA aligned 97.8 ± 0.8% to the 87kb targeted region. This resulted in an average depth of coverage of 872 ± 180× at each targeted base position. Coverage demonstrated fluctuation across the targeted region but more than 97% of all targeted bases across all samples were covered at least 50× to allow for confident variant calling (Figure 2).

We identified 387 single nucleotide variants (SNVs) (Online Resource 2) and 52 short insertion-deletions (indels) (Online Resource 3) across all individuals. All individuals sequenced were genotyped in a previous GWAS study [10] and 36 markers from that study are in targeted region. Overall concordance of genotype calls across all samples was 98.6% suggesting highly accurate genotype calling at common variant positions as well as sample identity integrity. Of the total variants, 87 of 387 SNVs (22.4%) and 18 of 52 indels (34.6%) were not present in dbSNP138 or 1000 Genomes release 3. The average minor allele frequency of these novel variants was 0.004% while those in dbSNP were 14.1% in our sequenced population suggesting that these novel variants are novel or very rare in the overall and sequenced population. There was no difference in the average number of variants between cases and controls t-test  $p = 0.48$  suggesting both not an overall increase in variation based on status nor by sample source (blood or saliva).

## Variant Annotation

In order to assess potential functional effects of the variants identified, we annotated each variant using genomic features databases with ANNOVAR and publically available ENCODE databases. Only one variant was identified within the 1,665bp open reading frame of the gene at chr4.hg19:46995366G>T (rs2229940). It is a common variant with a MAF of 43.8% in our sequenced cases, 40% of sequenced controls, and 36.7% in the Exome Variant Server suggesting this coding variant is unlikely to be a risk allele for ASD. Therefore, we sought to determine the functional potential of the remaining noncoding variants using fetal CNS or neuronal cell-line data from the ENCODE Consortium. Overall, 25 total variants (14 rare variants with 1000 Genomes frequency < 0.05) overlapped with any ENCODE DNase Hypersensitivity, transcription factor binding site (all were CTCF sites), or putative enhancer region as defined by H3K4me1 or H3K9ac (Online Resource 4).

## Association analysis

Though at this sample size we are only powered to detect variants of large effects, odds ratio >4 at minor allele frequency of 0.2 and odds ratio > 6 at minor allele frequency 0.01, we analyzed each individual variant for association with ASD via a Fisher's Exact test of the number of variant alleles, but none survived multiple testing correction for overall significance across all variants tested (threshold  $p < 1.2 \times 10^{-4}$ ) with a lowest p-value of 0.03.

Because aggregation of rare variants could be associated with ASD, we performed analyses with SKAT-O on sets of rare variants. First, we ran a series of analyses including all 387 SNVs and subsequently only SNVs with MAF cut-offs of 0.1 (272 SNVs), 0.05 (266 SNVs),

0.01 (198 SNVs), and 0.005 (150 SNVs). No sets demonstrated significance, with the lowest p-value being 0.118 in the set of rare SNVs with a MAF cut-off of 0.005. Second, we adapted this approach to identify potential regions within *GABRA4* with sets of rare variants associated with ASD, by dividing our targeted region into windows consisting of ten SNVs overlapping by five positions. We created these windows at the same MAF cut-offs for a total of 80 (all variants), 56 (0.1 MAF cut-off), 54 (0.05 MAF), 39 (0.01MAF) and 30 (0.005 MAF) windows, but found no region of significance. Finally, because rare variants within specific functional units rather than spatially distributed could be important, we performed SKAT-O across the eight introns, the 3'UTR, 5'UTR, the proximal promoter (5kb from the transcription start site), and the 3' regulatory region (5kb downstream of the gene). Again, no regions achieved significance.

### Functional analysis of 3'UTR variants

In order to assess potential functional individual variants, we focused on those in the 3'UTR. First we utilized the most recent version of TargetScan[45] and the software MicroSNiPer[46] to identify potential disruptions or creation of miRNA binding sites, but none were found. Therefore, we identified four variants in the 3'UTR with GERP scores greater than one, suggesting evolutionary and potential conservation, and unique to our ASD cases at chr4.hg19:46929991A>G (GERP = 2.59), chr4.hg19:46926840T>C (GERP = 1.06), chr4.hg19:46922930G>T (GERP = 1.06) and chr4.hg19:46929433G>T (GERP = 1.16). Since our hypothesis is that rare variants can have a functional effect, we tested whether these variants could alter the expression of genes by cloning the *GABRA4* 3'UTR to a luciferase reporter. We created five constructs one containing the wild type 3'UTR and one each with the four case unique mutations. Transfection of the wild type into HEK293 cells demonstrated a significant decrease in overall firefly luminescence in comparison with transfection of pmirGLO alone. However, none of the other four constructs showed a significant difference from the wild-type in expression with only the A to C variant at chr4:46922930 showing a slight trend toward significance ( $p < 0.16$  and  $p < 0.2$  in two biological replicates, each of four technical replicates) (Figure 3).

### Discussion

The technological advances of massively parallel sequencing allow for comprehensive variant discovery across large and targeted genomic regions [47]. Sequencing of candidate regions has been touted as a logical follow-up to common variant association studies by GWAS or candidate gene association approaches in complex diseases [48]. Identification and characterization of all genetic polymorphisms within an associated gene or genomic region, as done in this study for *GABRA4*, has the potential to identify all common genetic variants that might explain a greater proportion of contributable risk than previously genotyped markers and reveal rare variants which are not sufficiently covered by GWAS. Several post-association analysis sequencing studies have been performed on candidate genes and genomic regions for complex disorders ranging from cancer [49] to sick sinus syndrome [50] to schizophrenia [51]. Association of the G/G genotype of rs1912960 in *GABRA4* with ASD by the genotype-pedigree disequilibrium test (geno-PDT) has been demonstrated in two separate family based studies with  $p=0.003$ [25] and  $p=0.0046$ [27]. As

such, sequencing of individuals homozygous at chr4.hg19:g.46953881C>G (G/G at rs1912960) should enrich for discovery or rare ASD risk variants in *GABRA4*.

The most recent sequencing efforts in ASD have focused on exome and whole genome sequencing to identify rare and *de novo* mutations in several genes which can contribute to autism etiology [6–9, 52–56]. *GABRA4* was not implicated by these studies as having a *de novo* gene disrupting mutation, nor did the whole genome studies particularly investigate the gene. As in the whole genome studies, our targeted approach offers the benefit of uncovering genetic variation beyond the exome by identifying the complete compendium of variants from exons, introns, untranslated regions, and potential up and downstream regulatory elements. In addition, this experimental design allows focus on a single gene with evidence for genetic association [25, 27], a function that could potentially modulate autism behaviors [57], and biochemical evidence for variability in expression and function in autistic patients [28–30, 58]. Moreover, this candidate gene approach allows for greater depths of coverage, greater sequencing completion rates, and a lower cost when compared to larger scale sequencing studies [59].

We identified variants at 439 sites (387 SNVs and 52 indels) across the *GABRA4* region with about 22% of those variants not previously reported in large scale sequencing efforts such as the 1000 Genomes Project. These variants do not occur disproportionately in cases or controls nor are there any regions of the gene that have an association of rare variants with ASD. Therefore, we attempted a single variant analysis to identify rare functional variation in ASD cases. As only a single of these variants, and a common one, was in the coding portion of the gene, we annotated all of the variants using available genomic feature databases to identify potential noncoding functional variants. Because ASD is likely to alter neuronal development or function, we annotated our variants against functional regions identified by the ENCODE project in neuronal cell lines. These included regions of open chromatin (DNase hypersensitivity sites) where variation could alter chromatin conformation and therefore gene expression, and transcription factor binding sites, where variation could alter gene regulatory mechanisms. While 14 rare variants occurred in at least one predicted ENCODE functional domain, again there was no trend for elements specific to ASD. We identify novel variants in evolutionarily conserved and potentially functional regions of *GABRA4* including those overlapping transcription factor binding sites and in the 3' UTR. We also identified four variants unique to cases with high evolutionary conservation, but none showed statistical difference in affecting gene transcription in a luciferase assay. Candidate gene analysis of genes similarly associated with ASD, *NLGN3* and *NLGN4X* [60], have arrived at similar conclusions as those seen in this study.

In summary, we have comprehensively re-sequenced the entire autism candidate gene *GABRA4* gene region, including all exons, intron, UTRs, and up and downstream potential regulatory regions, in 82 autism cases and 55 controls. While we identify novel and rare variation in the gene, there is no statistical association with any individual variant or any cluster of rare variants in the gene. While association with *GABRA4* and ASD has been replicated and functional evidence points toward a role, these data suggest that individual rare variants in the gene may not play a role. Rather, there is a possibility that combinations of rare variants in this gene with others may induce risk. Alternatively, the effect of each rare

variant may be undetectable in the assays used here, and future research is warranted. Overall, it is important to sequence candidate genes in larger cohorts to identify the entire spectrum of variation, including in noncoding portions of genes, to point toward the subtle functional effects each of these rare risk variants might play in a complex disease etiology of ASD.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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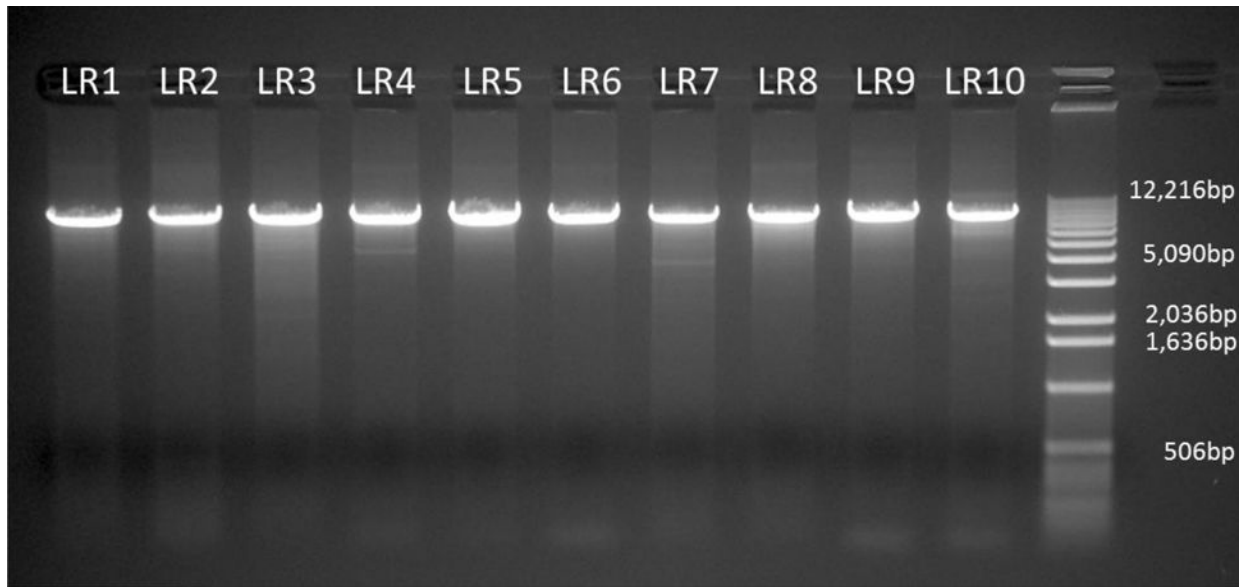
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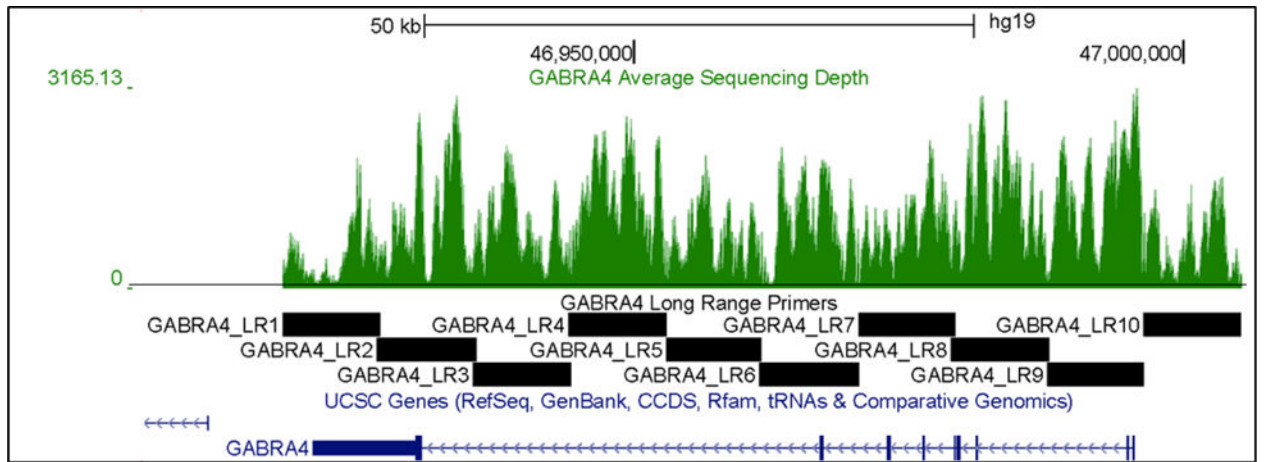
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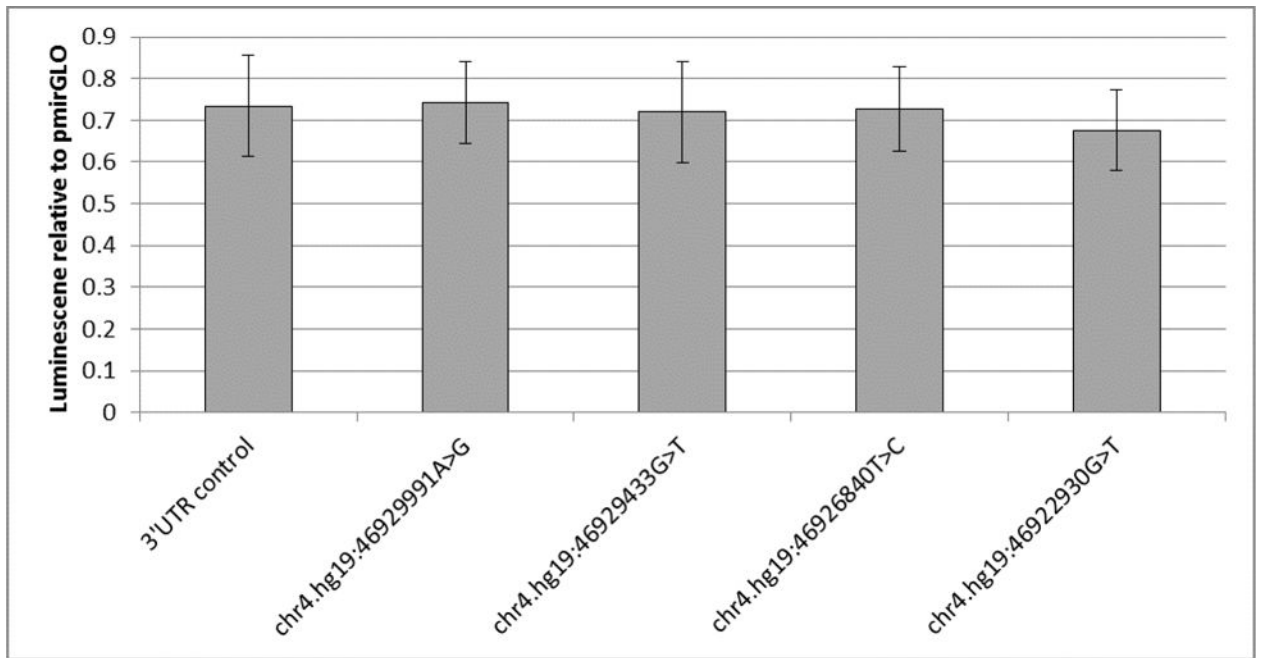
**Figure 1.**

Agarose gel electrophoresis of PCR amplicons. 50ng of PCR products prior to NuceloFast clean-up were run on a 0.75% agarose gel for 1 hour at 90V, stained with ethidium bromide, and imaged under ultraviolet light. At the right is a 1kb ladder with bands at indicated sizes to estimate amplicon size.



**Figure 2.** Sequence Depth Coverage Across Targeted Region. The average depth of coverage at each position across all samples is indicated in a green histogram with a black line representing 50× coverage on a UCSC Genome Browser track. The black bars represent the 10 amplicons used to target the region.





**Figure 3.**

Luciferase assay for four case unique conserved variants in the 3'UTR. Four variants unique to cases and with GERP>1 were cloned into pMirGLO and their luciferase in HEK293 cells measured. Columns represent percentage of luciferase fluorescent units compared to pmirGLO transfected alone. 3'UTR control is a clone of the wild type 3'UTR and each position label is the induced variation tested. Error bars represent SEM across 8 technical replicates.