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Genetic analysis of Hyperemesis Gravidarum reveals association with stress-induced calcium channel (RYR2)

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Running Title: RYR2 linked to Hyperemesis Gravidarum

Abstract

Hyperemesis Gravidarum (HG), severe nausea/vomiting in pregnancy (NVP), leads to electrolyte imbalance, ketonuria, and can cause neurologic, renal, and liver abnormalities. HG is also associated with an increased risk of adverse fetal outcome and neurodevelopmental delay. Evidence for a genetic predisposition suggests understanding the genetic component is essential in discovering an etiology. We performed whole-exome sequencing of 5 families followed by analysis of candidate variants in 584 cases and 431 controls. One novel and one known rare variant in RYR2 segregated with disease in 2 families. The novel variant was not found in 584 cases and 431 controls. The known rare variant, rs3766871, has a proven biological effect and was almost twice as common in cases as controls (p=0.046), and almost 4-times as common when comparing extreme ends of the spectrum, 106 cases with intravenous tube-feeding (TPN) and 141 controls with no NVP (p=0.023). Independent replication of rs3766871 using Norwegian and Australian GWAS data was supportive. Common RYR2 variants rs790899 and rs1891246 were significantly associated with HG and severity. RYR2 copy-number analysis was performed in 139 women with no NVP and 101 TPN-treated women and revealed a deletion in a severe patient. RYR2 encodes a stress-induced calcium channel biologically involved in vomiting, linked to cyclic-vomiting syndrome (CVS), and may play a role in thyroid function and fertility. Additionally, RYR2 is a drug target (Inderal) already used to treat HG and CVS. Thus, herein we provide genetic evidence for a pathway and therapy for the etiology and treatment of HG.

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Key Words: Hyperemesis Gravidarum, Nausea, Vomiting, Pregnancy, Ryanodine receptor, RYR2

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Introduction

HG occurs in approximately 0.2-2% of pregnancies and leads to significant weight loss, dehydration, electrolyte imbalance, and ketonuria (1-3). Until 60 years ago, HG was an important cause of maternal mortality with ten percent of cases ending in death (4). Although maternal mortality has since decreased, 6 deaths due to HG have been reported recently (5), as well as morbidity including Wernicke's encephalopathy (6), acute renal failure (7), liver function abnormalities (8), splenic avulsion (9), esophageal rupture (10), pneumothorax (11), and post-traumatic stress symptoms (12). HG is also associated with poor fetal/child outcomes including a 4-fold increased risk of preterm birth and a 3-fold increased risk of neurodevelopmental delay in children (13-14).

A variety of potential causative factors have been investigated, but the etiology remains unknown. Evidence for a genetic predisposition is provided by classic twin studies of Norwegian, Spanish, and Finnish cohorts that all support a genetic component to NVP (15-16). In a series of family based studies, there is evidence that female relatives of patients with HG are more likely to be affected, with a 17-fold increased risk if a sister has HG (17-20). Recently, mutations in the thyrotropin receptor gene have been linked to hyperemesis gravidarum accompanied by gestational thyrotoxicosis in an affected family and an additional case. This suggests a genetic etiology has already been identified in, at minimum, a subgroup of cases (21). Thus, understanding the genetic component is essential in discovering the

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causal pathway(s).

The objective of this study was to perform whole-exome sequencing on HG families to identify rare variants conferring susceptibility to HG and to validate these findings in a large cohort of affected and unaffected individuals from the United States, followed by replication in cohorts from Australia and Norway.

Results

Whole-exome sequencing identifies RYR2 variants linked to HG in 2 of 5 families.

We sequenced the entire exomes (~50 Mb) of 15 affected individuals and 3 unaffected individuals from 5 families with HG. The mean coverage was 54 fold. Reads were mapped to the human genome reference build UCSC hg19 using BWA (22). On average, 3223 single-nucleotide variants were detected in each individual and a total of 58006 variants were detected in all 5 families combined (Figure 1). The synonymous variants were subsequently discarded resulting in 29856 variants. The identified variants were further filtered against variants present in the HapMap, 1000 Genomes Project, and dbSNP132 databases, resulting in 13509 novel variants and known variants with minor allele frequency <5% (23,24). These variants were further filtered by selecting variants predicted to affect protein function using PolyPhen and SIFT (25,26). Filtering for missense and stop gain or stop loss variants that were shared by any of the 3 whole-exome sequenced unaffected family members resulted in 6481 variants.

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As we did not find any single variant that was shared by all the affected members across all of the families, we focused on variants within each family shared by all 3 whole-exome sequenced affected subjects. For example, 94 variants were shared by all 3 affected individuals in Family 1, and 227 variants were shared by all 3 affected individuals in Family 2. We searched for variants and/or genes that were shared by more than one family. 27 genes were identified that carried rare variants in the affected family members in more than one family. These variants were evaluated based on a functional effect, which included variants located in genes functionally relevant to reproduction (ie hormones), nausea and vomiting (ie gastric tract, vomiting center of the brain), and genes expressed in relevant tissues (ie ovary, placenta). This resulted in identification of the gene RYR2 involved in 2 of 5 families as a strong candidate based on its functional potential: RYR2 encodes a stress induced calcium channel that is part of a signaling pathway for emesis expressed in the vomiting center of the brain (27-28). It is the only gene identified with variants significantly linked to cyclic vomiting syndrome, is involved in thyroid function, and is differentially expressed in cumulus cells of the pre-ovulatory follicle (29-31).

Genotyping the novel and rare RYR2 variants in the US cohort provides confirmation.

In the largest HG family (Family 1), the novel variant in the RYR2 gene (RYR2:NM_001035:exon68:c.T9830G:p.Leu3277Arg) was confirmed by Sanger Sequencing to be shared by four affected sisters and was not shared by either of 2 unaffected sisters, the unaffected mother, nor the unaffected maternal aunt (Figure

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2A). Genotyping via Taqman showed the RYR2 variant to be unique in the sample to Family 1, as it was not identified in 584 HG cases and 431 unaffected controls (Table 1). The nucleotide at the location of this novel variant is 100% conserved across vertebrates and invertebrates. The mutation changes a hydrophobic amino acid to an electrically charged amino acid, and is predicted to be damaging and deleterious (SIFT Prediction Score=0; Provean Prediction Score=-5.38).

In Family 2, the variant RYR2:NM_001035:exon37:c.G5656A:p.Gly1886Ser rs3766871 was shared by all 3 affected sisters (Figure 2B). Genotyping via Taqman identified variant rs3766871 (Family 2) to be twice as common (p=.046) in cases than controls (in 38 out of 580 additional cases and 17 out of 431 controls) and four times more common (p=.023) when comparing the extreme ends of the clinical spectrum, 9 out of 106 cases requiring tube feeding compared to 3 out of 141 controls who reported no nausea/vomiting in pregnancy) (Table 1). The SNP rs3766871 is already known to have a biological effect. Substitution of serine for glycine causes a significant increase in cellular calcium oscillation activity compared to wild-type RYR2 in HEK293 cells (32). Interestingly, the effect is completely abolished by substitution of a neighboring SNP, which may explain why rs3766871, although significantly more common in extreme cases, is also present in a subset of unaffected individuals. The SNP rs3766871 has also been associated with ventricular arrhythmias and is an independent predictor of sudden cardiac death (33).

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Summary statistics were supportive but not statistically significant for RYR2 in both a Norwegian and an Australian GWAS.

Genotype data for rs3766871 were imputed in both Norwegian and Australian datasets. Although statistical significance was not achieved probably due to the rarity of rs3766871 and the small number of affected individuals, there is a supportive trend in both cohorts. In the Norwegian cohort there is a 1.3-fold OR for this SNP (reference allele A), and in the Australian cohort, after removal of the cases with no weight loss to better reflect the severe end of the clinical spectrum of NVP, there was a 1.2-fold OR for rs3766871 (Table 1). In addition, common RYR2 SNPs (rs790899 and rs1891246) were significantly linked to HG in both the Norwegian and Australian GWAS using the case-control phenotypes (Table 1). In the Norwegian dataset, adding the zscore for weight change until gestational week 18 as a covariate increased the odds ratio and significance for the common RYR2 variants, and suggested a strong association with weight loss (Table 2A). In the smaller Australian dataset, using the continuous severity measure, neither rs790899 nor rs1891246 reached statistical significance.

Copy-number analysis identifies a deletion in RYR2 in an extreme HG case requiring intravenous feeding (TPN).

Copy-number analysis was performed because likely pathogenic duplications in RYR2 have been reported previously in developmental delay/autism (34). A deletion in exon 16 was identified in RYR2 (Table 1) among DNA isolated from 101 cases requiring total parenteral nutrition (TPN) for severe HG. The deletion was not

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observed in any of the remaining samples including 139 extreme controls reporting no nausea and no vomiting in any of their pregnancies.

Discussion

This is the first whole-exome association study of HG and suggests RYR2 may play a role in the biology of HG. We have successfully identified two rare variants in RYR2 that are linked to HG using a whole-exome sequencing approach followed by genotyping a large validation cohort from the US. Independent replication in GWAS studies from Norway and Australia are suggestive of a role for RYR2 and revealed 2 common variants likely to be associated with HG. Copy-number screening identified a deletion in RYR2 in an extreme HG case requiring intravenous feeding.

Ryr2 encodes a stress-induced calcium channel and variants in the gene, including rs3766871 identified in this study, have been shown to cause aberrant Ca++ signaling.[31] Variants in the RYR2 gene are associated with ventricular tachychardia, so it is of particular interest that the rate of HG in postural tachychardia syndrome is as high as 59%, well above the expected 0.5-2% (35). The role RYR2 variants play in HG etiology is unknown, but there are several intriguing avenues to explore further. Firstly, RYR2 encodes a stress-induced calcium channel that is the only ryanodine receptor expressed in the vomiting center of the brain (27) and has been implicated in a signaling pathway underlying emesis in an animal model (28). Secondly, the thyroid hormone has been shown to induce RYR2 overexpression (30), while the drug Inderal (Propranolol, used to treat hyperthyroidism) blocks RYR2 phosphorylation and lowers its expression (36). Hyperthyroidism accompanies HG

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in as many as 60% of pregnancies (37) and mutations in the thyrotropin receptor have been linked to HG (21), providing additional genetic evidence that this pathway may be causal in some cases. Of note, two of 6 recent maternal deaths secondary to HG were accompanied by severe thyrotoxicosis/thyroid storm (5). Thirdly, in a GWAS study of Cyclic Vomiting Syndrome (CVS), RYR2 was the only gene identified with variants significantly linked to the disease (29), and Inderal has been used to effectively treat 92% of children with CVS (38). Likewise, in 1980 a patient presenting with severe thyrotoxicosis and hyperemesis gravidarum reportedly responded dramatically to Inderal treatment (39). As her thyroid function improved and the Inderal was discontinued, she again returned to the hospital with severe vomiting. Upon restarting medication, her vomiting ceased and she continued treatment until term.[10] Our findings provide evidence for a biological pathway, diagnostic marker, and potential targeted therapy for the etiology and treatment of HG.

Lastly, mounting evidence suggests RYR2 may play a role in fertility. It is expressed more than 50-fold in cumulus cells compared to mural granulosa cells of human pre-ovulatory follicle, and its expression correlates with amphiregulin, a key mediator of the effect of LH/hCG and a marker for oocyte competence (31). Ryanodine receptor variants are significantly associated with pig litter size (40), and women with HG produce an abnormally high number of mature oocytes when undergoing follicle stimulation (41). A genetic link that explains both the symptoms of HG and a potential increase in fertility, would provide a rationale for why severe nausea in pregnancy has not been selected out in nature despite its link to adverse outcomes.

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The strength of this study comes from the innovative approach to identifying the etiology of HG. This disease has thus far eluded both scientists and clinicians, resulting in largely ineffective treatments, significant maternal morbidity, and an increased risk in adverse fetal outcome (13). In addition, while most biological studies of HG have been small with broad definitions of the disease, this study included not only five large HG families, but also, a validation cohort of 584 clinically defined cases and 431 well-characterized controls, and two separate GWAS studies for replication.

The limitation of this study stems from the fact that full sequencing and copy number analysis of RYR2 in all cases and controls, is cost-prohibitive. We were only able to study the 2 mutations involved in the 2 HG families in this study, not the complete gene sequence, in the validation cohorts from the United States. Also, while the US cohort used intravenous fluid treatment as its clinical criteria for HG, and the Norwegian cohort used hospitalization, the Australian cohort used a less severe phenotype, which may have led to a reduced effect for that dataset. The small sizes for GWAS may also contribute to an underestimate of the effect for the validation cohorts. Finally, the copy-number analysis that identified a deletion only surveyed 90 bp of exon 16 in 240 individuals. Mutation and copy-number analysis of the full RYR2 gene in cases and controls is now warranted to determine their frequencies in affected individuals and to understand the role of RYR2 in HG pathogenesis.

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In conclusion, from exome sequencing of 5 HG families, we have identified rare variants in RYR2 in 4 affected sisters in one HG family and in 3 sisters with HG in another family. Follow-up screening in over 1,000 individuals provided further support that RYR2 variants play a role in HG. GWAS studies were supportive and resulted in identification of 2 common RYR2 variants linked to HG, and copy-number analysis identified a deletion. Mutations in genes in the ryanodine receptor-signaling pathway may account for a substantial amount of the attributable risk of HG, although just how much must be deferred to a follow up study. RYR2 is the only ryanodine channel expressed in the vomiting center of the brain (27); rs3766871 has already been shown to result in leaky calcium signaling (32); and leaky RYR2 calcium signaling has been shown to increase vomiting in an animal model (28), but causality has not been definitively established. Additional studies are required, such as functional analysis of the novel deleterious RYR2 variant and a larger GWAS. However, this novel discovery may provide the first step in understanding the etiology of HG. The identification of genes linking HG to RYR2 provides an intriguing new avenue for diagnosis, research, and therapy.

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Materials and Methods

US Population. The source population for cases included patients primarily recruited through advertising on the HER Foundation website (42). All participants gave informed consent. This study was approved by the UCLA Institutional Review Board. The whole-exome sequencing study included 15 affected individuals and 3 unaffected individuals from 5 families. Follow-up analysis to confirm segregation in Family 1 included additional family members -3 unaffected and 1 affected individual from Family 1 (Figure 2A). The follow-up case-control population from the United States included 584 HG cases and 431 unaffected controls.

The stringent study criteria were designed to exclude all cases and controls that would increase phenotypic uncertainty. Briefly, the inclusion criteria for affected individuals was a diagnosis of HG and treatment with IV fluids or total parenteral nutrition/nasogastric feeding tube. Each participant was asked to recruit a non-blood related acquaintance with at least 2 pregnancies that went beyond 27 weeks. Controls were eligible if they experienced normal or no nausea/vomiting in their pregnancy, no weight loss due to nausea/vomiting and no medical attention in their pregnancy due to nausea/vomiting. Participants were enrolled in the family study if they had 2 or more additional family members with HG. Affected family members were eligible if they reported severe NVP accompanied by > 5% weight loss, and medication or hospitalization for HG. Control family members had the same eligibility requirements as controls, and all other family members (including males) were labeled as unknown. Each family submitted saliva samples for a minimum of three affected individuals. A recent review of whole-exome sequencing shows how

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whole-exome sequencing of single individuals, siblings, or 1 to 5 small families has successfully identified causal variants for many genetically heterogeneous conditions including hearing loss, intellectual disabilities, autism spectrum disorders, and cardiovascular disease. For example, since the introduction of the whole-exome sequencing technique, genes for 12 intellectual disability syndromes were identified by whole-exome sequencing of 3 or less families, and 13 more were identified by whole-exome sequencing of 5 or less affected individuals (43). Therefore, in this study, we chose to analyze a total of 18 individuals: 3 affected individuals from each of 5 families with HG in addition to 3 unaffected controls from 3 of the five families to further limit potential causal variants by dismissing those variants identified in unaffected family members. Pedigrees of two families of Caucasian/European descent analyzed in this whole-exome sequencing study, and whose variants are described herein, are shown in Figure 2. Family 1 is of mixed Finnish, Swedish, English, and German descent. We collected DNA from 4 cases (4 sisters) and 4 controls (2 sisters, mother, and maternal aunt) from Family 1. This family consists of 9 sisters, 5 affected and 4 unaffected, but only those siblings who participated are shown in Figure 2A. Family 2 is of mixed Scottish, German, Swiss, English, and Italian descent and we collected DNA from 3 affected sisters (Figure 2B).

DNA

Each study participant was asked to submit a saliva sample for DNA analysis. A saliva collection kit (Oragene, Ottawa, Canada) was self-administered for submitting

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2 milliliters of saliva. DNA was extracted from 75% of the saliva sample according to the manufacturer's instructions (Oragene, Ottawa Canada).

Whole-Exome Sequencing

We sequenced the entire exomes (~50 Mb) of 15 affected individuals and 3 unaffected individuals from 5 HG families. Paired end reads 100 nucleotides (2 X 100 nucleotides) were generated on an Illumina HiSeq 2000. Each sample was sequenced on 3 different lanes to avoid lane bias. Qseq files were converted into Sanger-formatted FASTQ files and reads were mapped to the reference human genome build hg19 using the Burrows Wheeler Alignment algorithm (BWA) (22). Duplicated reads were marked by Picard. The Genome Analysis Toolkit (GATK) was used for local realignment around indel sites followed by a base quality recalibration (23). For reliable SNP calling we used genotype quality ≥ 10; read QUAL ≥ 30 and a minimum read depth of 4. The combined total variants from all 18 individuals were filtered as shown in Figure 1. Synonymous variants, which are unlikely to be causal, were discarded. The identified variants were further filtered against variants present in the HapMap, 1000 Genomes Project and dbSNP132 databases, selecting for novel variants and known variants with minor allele frequency <5% (23,24,). These variants were further filtered by selecting variants predicted to affect protein function using PolyPhen and SIFT (25,26). Variants were further filtered by deleting variants present in the 3 unaffected family controls. All variants were discarded that were not shared by all 3 whole- exome sequenced affected family members with each family. Finally, we identified 27 genes

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involved in more than one family, among which the most promising candidate was RYR2: among 27 genes screened for functional effect, which included genes 1) functionally relevant to reproduction (ie hormones), 2) nausea and vomiting (ie gastric tract, vomiting center of the brain), and 3) genes expressed in relevant tissues (ie ovary, placenta, vomiting center of the brain), only RYR2 fulfilled all 3 criteria (27-31).

Genotyping

The variant in RYR2 in Family 1 is a novel variant in exon68:c.T9830G:p.Leu3277Arg. Sanger Sequencing was used to confirm whole-exome sequencing results and to confirm or deny segregation with the disease in the remaining family members who were not included in exome sequencing (1 affected sister and 1 unaffected sister, the unaffected mother, and an unaffected maternal aunt). PCR primer pairs GGAAGTCATACTGCCCATGC and GGGGTACAATGTCTTCTTCCA were designed from genomic DNA to amplify and sequence the variant. PCR amplification and sequencing were carried out using standard methods. The SIFT protein prediction tool was used to determine that the novel SNP resulted in a damaging protein product, and the Provean Prediction tool was used to determine that it was deleterious. (26,44). Taqman primers were designed for both the novel variant in Family 1 and the rare variant rs3766871 identified in Family 2, and used to screen individuals from $\geq$ 573 HG cases and $\geq$ 426 controls using Applied Biosystems PRISM 7900HT Sequence Detection System (TaqMan) for large-scale screening. The call rate was > 96%. P-values were

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calculated using a 1-tailed Fisher's exact test (45) and Odds ratios were calculated using the Odds ratio calculator (46).

Norwegian GWAS

Summary statistics for RYR2 were analyzed for independent replication in the Norwegian Mother and Child Cohort Study (MoBa), a prospective population-based pregnancy cohort conducted by the Norwegian Institute of Public Health recruited from Norway during 1999-2008 (47,48). Ethical approval for the MoBa study has been approved by the Regional Committee for Medical Research Ethics and all women provided informed written consent.

Analysis and results were calculated using data obtained from self-reported questionnaires (49). The samples in this HG study included 385 affected mothers and 2280 unaffected mothers answering questionnaire 3 (Q3) question 27; "…Have you been admitted to hospital since you became pregnant…for ....prolonged nausea and vomiting"? The samples included were singleton pregnancies of Norwegian ancestry.

Maternal genome-wide data were obtained using Illumina HumanCoreExome genotyping BeadChip v1.1. Imputation was performed with reference panel HapMap phase 3 build 36 using IMPUTE2 (50). Standard association analyses were performed in PLINK 1.7 (51). Genotypes were analyzed with allelic and genotypic approach. Regression analysis was performed using a *z-score transformed gestational weight gain (GWG)* based on maternal pre-pregnancy weight and weight

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change until week 18 of gestation and showed candidate SNPs to be strongly associated with weight loss.

Australian GWAS

The Australian sample is composed of genotyped women unselected for HG who are part of the Australian Endogene Study and the QIMR Mothers of Twins Study, which are two of the cohorts participating in the NVP Genetics Consortium (52).

As part of health and wellbeing surveys, a total of 1440 women reported their experience in their pregnancy with the most severe NVP using a five-point questionnaire adapted from Zhang et al (2011). The reported severity was distributed as follows: 'I did not have any nausea or vomiting' (n = 677); 'I had some NVP for more 7 days, but I didn't see a doctor or nurse about this. It didn't disrupt my daily routine very much' (n = 163); 'It disrupted my daily routine but it didn't affect my weight and I didn't need medication to manage it' (n = 331); 'It really disrupted my daily routine and I was prescribed medication but it didn't lead to weight loss' (n = 139); 'It really disrupted my daily routine. I lost weight. I was prescribed medication or was put on a drip or feeding tube' (n = 130). For the present study, we analyzed the continuous phenotype. We also conducted case/control analyses using 946 women who were in the extremes of the severity scale. Women reporting no NVP (n=677) were used as controls and women reporting severe NVP (n=269) with disruption of their daily routine and medication prescription, including those losing weight and put on a drip or feeding tube, were used as cases. The samples were genotyped using Illumina arrays and genotype

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imputation was completed using 1000 Genome Phase 3 version 5 as reference data. For validation of the rare imputed SNP rs3766871, we also conducted case/control analysis in a more stringent subset by removing the participants who did not report weight loss from the cases, thus limiting to a more stringent case phenotype (n=130),

Copy-number analysis of RYR2

Quantitative real-time PCR analysis of RYR2 was performed in triplicate on 10 ng from 240 DNA samples (101 extreme cases requiring tube feeding and 139 extreme controls with no nausea/vomiting in ≥ 2 pregnancies) on 384-microwell optical plates using the predesigned Taqman Copy Number Assay covering 90 base pairs within a likely pathogenic duplicated region in autism (34) (Assay ID: Hs00137466_cn FAM labeled, MGB probe, Thermofisher Scientific, Waltham, MA) and the RNaseP Copy Number Reference Assay (VIC labeled, TAMRA probe). Melt-curve analysis was applied and all results were normalized to RNaseP levels and calculated using the $\Delta\Delta C_T$ method. One sample with a deletion originally identified in the above triplicate assay along with 5 normal control samples, were assayed a second time in duplicate (re-diluted to 10 ng from the original DNA sample) to verify the deletion.

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Disclosure of interests.

The authors declare no competing financial interests.

Contribution to authorship.

All authors fulfill authorship criteria as defined in the instructions for authors.

Details of ethics approval.

This study was approved by the UCLA Institutional Review Board on 5/20/2011 as IRB#11-001681 and the Queensland Institute of Medical Research Human Research Ethics Committee.

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Figure 1. Whole-exome sequencing filtering steps identifies RYR2 variants.

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Figure 2. Pedigrees and Genotypes of A) Family 1 and B) Family 2. In Family 1, A, B, C, and D all were affected with HG. Participant A reported pic line, medication and weight loss to treat HG, B reported a 10 pound weight loss and medication to treat symptoms until birth, C reported intravenous fluids (IV) and a 23 pound weight loss, and D reported IV fluids, hospitalization, weight loss, and medication to treat her HG. Among the unaffected family members, participant E reported 2 pregnancies with mild nausea, no weight loss, and no medication; F reported 5 pregnancies with no nausea and vomiting, no weight loss, and no medication in any pregnancy, G reported 13 pregnancies with normal nausea and vomiting, no weight loss, no medication, and H reported 3 pregnancies with normal nausea and vomiting, no weight loss, and no medication. For Family 2, all 3 sisters were affected and all 3 sisters required medication and IV fluids to treat their HG. Sisters A and C were both hospitalized for HG. Their mother was also affected but did not participate.

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Table 1. RYR2 variant table.

RYR2 VARIANT	SOURCE	EXON/INTRON	METHOD	SCREENED	OR	P-value
NOVEL AA*	FAMILY 1, USA	68:c.T9830G	Genotyping	584 HG, 431 C	NA	NA
rs3766871	FAMILY 2, USA	37:c.G5656A	Genotyping	584 HG, 431 C	1.29	0.046
rs3766871	FAMILY 2, USA2	37:c.G5656A	Genotyping	106 HG, 141 C	4.27	0.023
rs3766871	NORWEGIAN	37:c.G5656A	GWAS	318 HG, 1823 C	1.32	0.661
rs3766871	AUSTRALIAN	37:c.G5656A	GWAS	269 HG, 677 C	0.89	0.693
rs3766871	AUSTRALIAN2	37:c.G5656A	GWAS	130 HG, 677 C	1.17	0.665
rs790899	NORWEGIAN	intron 95	GWAS	385 HG, 2280 C	1.19	0.033
rs790899	AUSTRALIAN	intron 95	GWAS	269 HG, 677 C	1.33	0.013
rs1891246	NORWEGIAN	intron 100	GWAS	385 HG, 2280 C	1.23	0.009
rs1891246	AUSTRALIAN	intron 100	GWAS	269 HG, 677 C	1.3	0.014
NOVEL DEL*	USA	16:237619976	Copy Number	101 HG, 139C	NA	NA

HG=Hyperemesis Gravidarum, C=Unaffected Control, TPN=Severe HG requiring tube feeding
*AA=deleterious amino acid change, *DEL=deletion in exon 16 of unknown size
NOVEL AA* (c.9830T>G, p.Leu3277Arg)
rs3766871 (NM_001035.2:c.5656G>A, NP_001026.2:p.Gly1886Ser)
rs790899 (NM_001035.2:c.13913+381G>A; XM_006711804.2:c.13943+381G>A)
rs1891246 (NM_001035.2:c.14434-490T>G; XM_005273224.1:c.14491-490T>G)
USA2 and AUSTRALIAN2 are datasets with more stringent criteria.
USA2 (HG=requiring iv feeding, C=no NVP)
AUSTRALIAN2 (HG=weight loss, C=no NVP)

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Table 2. Adding zscore weight change until gestational week 18 as covariate shows rs790899 and rs1891246 associated with weight.

CHR	SNP	BP	A1	TEST	NMISS	OR	STAT	P-VALUE
1	rs790899	237957678	C	ADD	2499	1.267	2.649	0.00808
1	rs790899	237957678	C	COV1	2499	0.2447	-11.66	2.12E-31
1	rs1891246	237981846	G	ADD	2499	1.292	2.968	0.002993
1	rs1891246	237981846	G	COV1	2499	0.2424	-11.71	1.19E-31

rs790899 (NM_001035.2:c.13913+381G>A; XM_006711804.2:c.13943+381G>A)
rs1891246 (NM_001035.2:c.14434-490T>G; XM_005273224.1:c.14491-490T>G)

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Figure 1. Whole-exome sequencing filtering steps

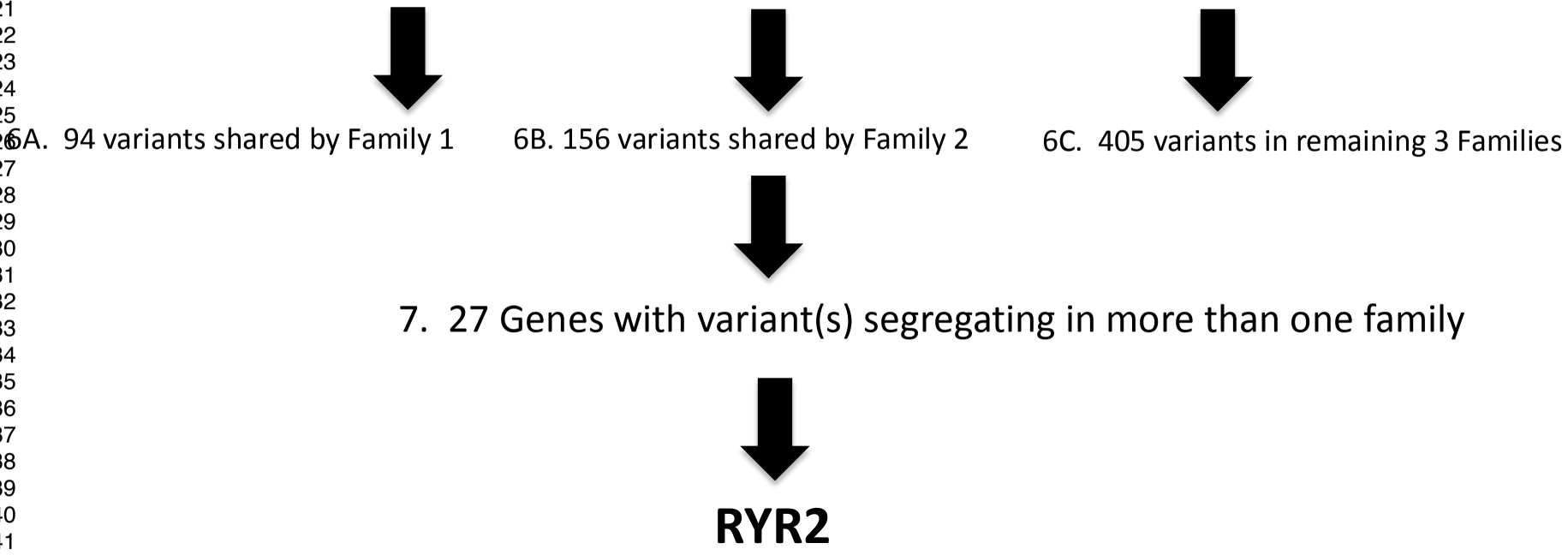

Figure 2A.

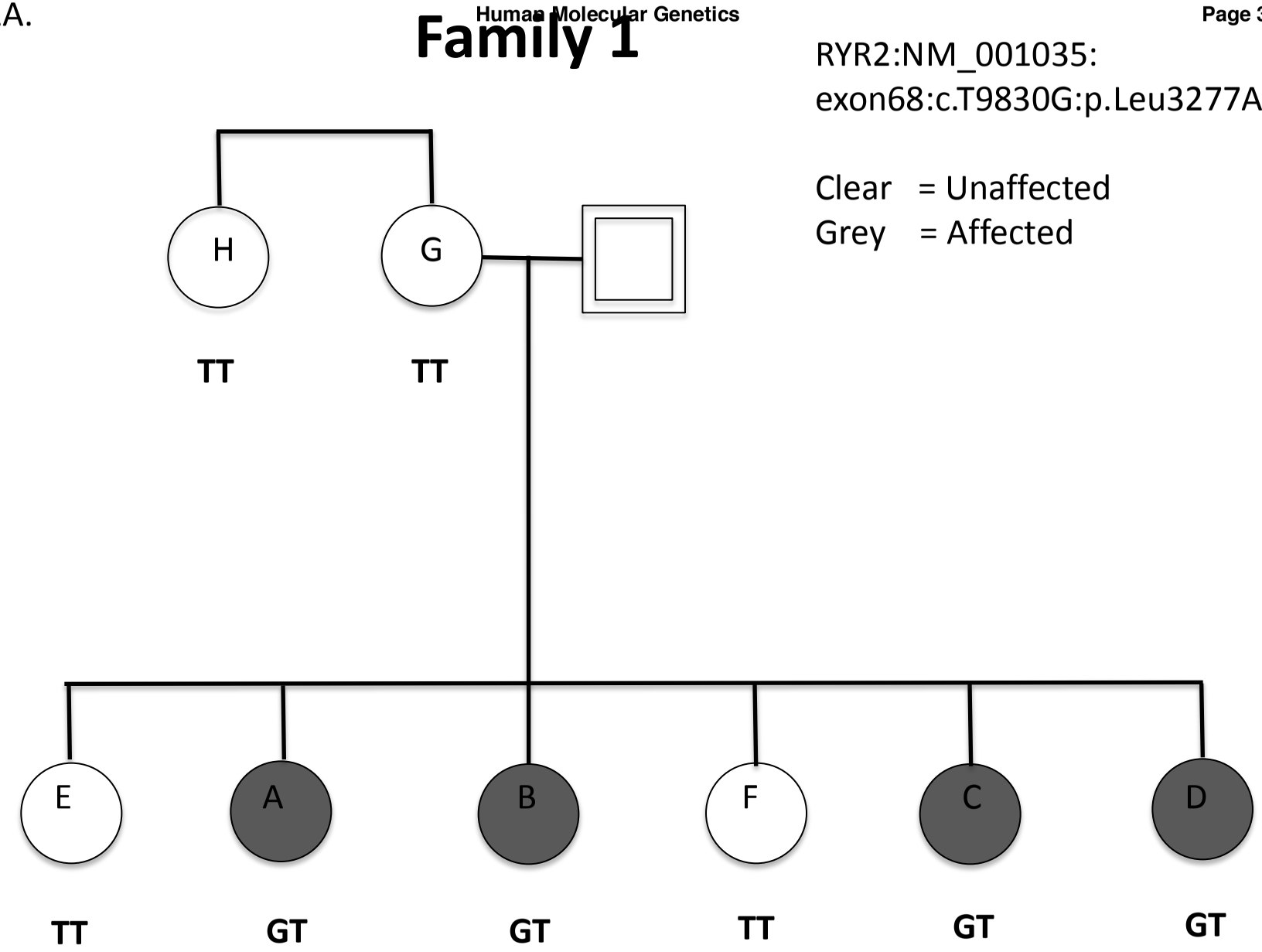
Family 1
RYR2:NM_001035:
exon68:c.T9830G:p.Leu3277Arg
Clear = Unaffected
Grey = Affected
H
G
TT
TT
E
A
B
F
C
D
TT
GT
GT
TT
GT
GT

Figure 2B.

Family 2

RYR2:NM_001035: rs3766871
exon37:c.G5656A:p.Gly1886Ser

Clear = Unaffected
Grey = Affected