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Genetic associations with neonatal thyroid-stimulating hormone levels

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BACKGROUND: Elevations or deficits in thyroid hormone levels are responsible for a wide range of neonatal and adult phenotypes. Several genome-wide, candidate gene, and meta-analysis studies have examined thyroid hormones in adults; however, to our knowledge, no genetic association studies have been performed with neonatal thyroid levels.

METHODS: A population of Iowa neonates, term (n = 827) and preterm (n = 815), were genotyped for 45 single-nucleotide polymorphisms (SNPs). Thyroid-stimulating hormone (TSH) values were obtained from the Iowa Neonatal Metabolic Screening Program. ANOVA was performed to identify genetic associations with TSH concentrations.

RESULTS: The strongest association was rs4704397 in the *PDE8B* gene ($P = 1.3 \times 10^{-4}$), followed by rs965513 ($P = 6.4 \times 10^{-4}$) on chromosome 9 upstream of the *FOXE1* gene. Both of these SNPs met statistical significance after correction for multiple testing. Six other SNPs were marginally significant ($P < 0.05$).

CONCLUSION: We demonstrated for the first time two genetic associations with neonatal TSH levels that replicate findings with adult TSH levels. These SNPs should be considered early predictors of risk for adult diseases and conditions associated with thyroid hormone levels. Furthermore, this study provides a better understanding of the thyroid profile and potential risk for thyroid disorders in newborns.

Endocrine disorders are substantial contributors to neonatal morbidity and mortality; of these, congenital hypothyroidism (CH) is the most common (1). CH is a common and preventable cause of mental retardation, with an incidence rate of approximately 1 in 2,350 live births (2). Early treatment with thyroxine (T_4) with subsequent supplementation for life produces excellent results for both growth and development (2,3). CH is screened for at birth through the detection of T_4, thyroid-stimulating hormone (TSH), or both (3).

In the preterm infant, thyroid function undergoes postnatal changes related to an immature hypothalamic–pituitary axis, along with the interrupted exposure to maternal thyroid hormone and thyroid-releasing hormone from the placenta. Owing to immature hypothalamic–pituitary axis function, T_4 is lower in preterm infants as compared with neonates born at term, and there is a direct correlation between the serum T_4 level and the degree of prematurity (4). Preterm neonates with abnormal thyroid function may have transient hypothyroxinemia of prematurity and may be misreported as true cases of CH (2). Hence, it is essential to take gestational age and birth weight into consideration when making the differentiation between transient hypothyroidism and true cases of CH (2).

Among healthy subjects, TSH shows considerable variability between individuals, whereas this variability is much less in the same individual when TSH is measured repeatedly over an extended period of time (5). Previous studies have observed that TSH variability is under strong genetic regulation; studies have estimated heritability of up to 65% for variation in adult serum TSH (6). In addition, there have been several genome-wide association studies reporting multiple genetic variants associated with TSH levels in adults (5,7–9). Furthermore, in candidate gene studies, the Asp727Glu polymorphism in the TSH receptor gene (*TSHR*) is associated with adult serum TSH levels, which further supports a genetic contribution in assessing the variation of TSH (10). To our knowledge, no studies have examined the association of candidate single-nucleotide polymorphisms (SNPs) with neonatal TSH measurements. We genotyped term and preterm infants for 45 SNPs in 24 candidate genes that are known to play a role in TSH production or metabolism and examined these polymorphisms for associations with TSH levels measured at birth as part of the Iowa Neonatal Metabolic Screening Program. Understanding variation in TSH levels and the genes responsible may be particularly important in a population at risk for abnormal TSH levels such as preterm infants.

RESULTS

Analysis was performed on a total of 1,583 neonates (404 University of Iowa State Hygienic Laboratory (SHL) preterm neonates, 799 SHL term neonates, and 380 University of Iowa Hospitals and Clinics Neonatal Intensive Care Unit (NICU) neonates) (**Table 1**). The three cohorts were not statistically different from each other when comparing their gender (**Table 1**). Differences between mean TSH level, gestational age, and birth weight were statistically significant among all three cohorts (**Table 1**). The difference in mean for age at screening was statistically significant between SHL preterm and NICU preterm but not between SHL term and SHL preterm cohorts (**Table 1**). The difference in total parenteral nutrition was statistically

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significant between SHL term and SHL preterm but not between SHL preterm and NICU preterm cohorts (Table 1).

Association results for all genotyped SNPs in combined populations are shown in Figure 1 and **Supplementary Table S1** online. Association results for all genotyped SNPs in individual populations are shown in Figure 2 and **Supplementary Table S2** online.

Eight SNPs were nominally significantly associated with neonatal TSH levels in all study populations combined ($P < 0.05$) (Table 2, **Supplementary Table S3** online, and Figure 1).The strongest association was rs4704397 in the *PDE8B* gene ($P = 1.3 \times 10^{-4}$), followed by rs965513 ($P = 6.4 \times 10^{-4}$) on chromosome 9 upstream of the *FOXE1* gene. Both of these SNPs met the threshold for statistical significance after correction for multiple testing (corrected significance threshold set at $P < 0.001$). Although none of the associations in the individual populations met the significance threshold after correction for multiple testing, the strongest associations remained rs4704397 in the *PDE8B* and rs965513 near *FOXE1* (Figure 2). Each copy of the minor A allele for rs4704397 in *PDE8B* was associated with an increase in TSH levels in both term and preterm infants (Table 2), as well as an increase of 0.6 mIU/l in TSH levels in the combined cohort. For SNP rs965513 in *FOXE1*, each copy of the minor A allele was associated with a decrease in TSH levels in both term and preterm infants (Table 2), as well as a decrease of 0.2–0.7 mIU/l in TSH levels in the combined cohort. Two other SNPs in *FOXE1* (rs1443432 and rs3021523) showed marginally ($P < 0.05$) significant association with TSH levels. Each copy of the G allele of rs1443432 and each copy of the C allele of rs3021523 were associated with a decrease in TSH levels in both term and preterm infants (Table 2), as well as a decrease of 0.2–0.5 and 0.2–0.6 mIU/l, respectively, in TSH levels in the combined

Table 1. Demographic characteristics of cohorts

	SHL term ($N = 799$)	SHL preterm ($N = 404$)	NICU preterm ($N = 380$)	*P* value[a]	*P* value[b]
TSH level (mIU/l)	8.9 ± 4.5	7.4 ± 4.5	5.9 ± 3.8	<0.001	<0.001
Male gender	454 (56.8%)	230 (56.9%)	222 (58.4%)	0.97	0.67
Gestational age (wk)	40.0 ± 0	34.0 ± 2.6	31.2 ± 3.1	<0.0001	<0.0001
Birth weight (g)	3,489.9 ± 434.0	2,405.1 ± 695.3	1,773.4 ± 712.3	<0.0001	<0.0001
Age at screening (h)	36.4 ± 10.6	37.6 ± 12.8	29.5 ± 7.0	0.68	<0.0001
Total parenteral nutrition	2 (0.3%)	51 (12.6%)	38 (10.0%)	<0.001	0.25

NICU, neonatal intensive care unit; SHL, State Hygienic Laboratory; TSH, thyroid-stimulating hormone.

[a]*P* values for differences between SHL term and SHL preterm. [b]*P* values for differences between SHL preterm and NICU preterm.

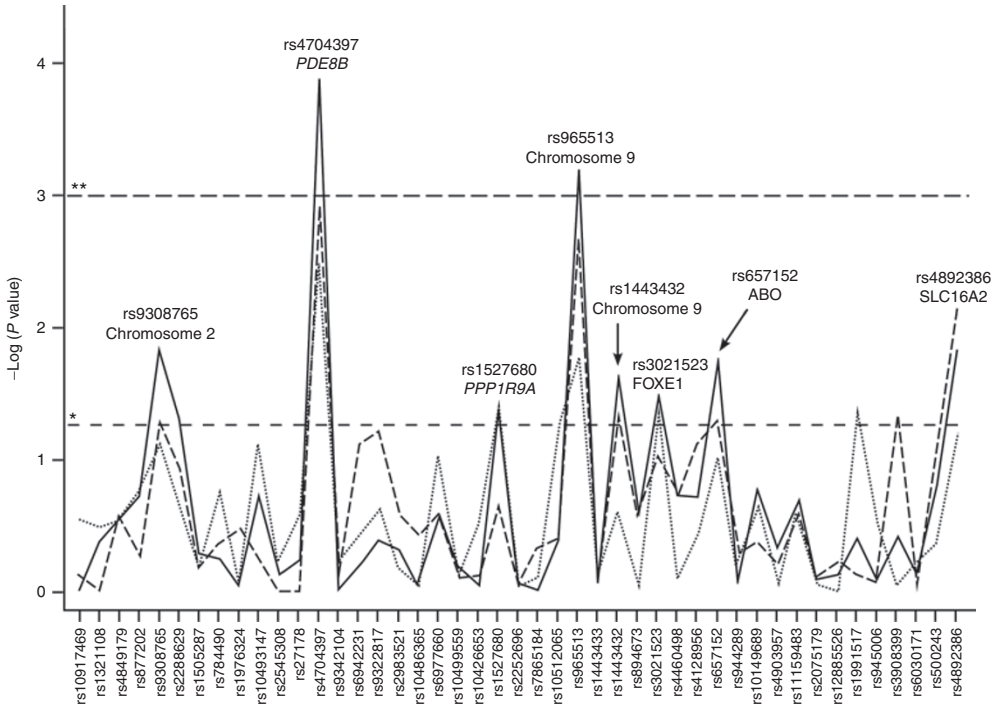

Figure 1. Association statistics for all genotyped SNPs with neonatal TSH level for combined populations. The *x*-axis is a list of all genotyped markers. The *y*-axis is the $-\log_{10}$ of the *P* value from the ANOVA analysis. The horizontal dashed lines represent the *P* value cutoffs; *$P = 0.05$, **$P = 0.001$. The solid line represents all populations combined. The dashed line represents the SHL preterm and SHL term cohorts combined. The dotted line represents the SHL preterm and the NICU preterm cohorts combined. NICU, neonatal intensive care unit; SHL, State Hygienic Laboratory; SNP, single-nucleotide polymorphism; TSH, thyroid-stimulating hormone.

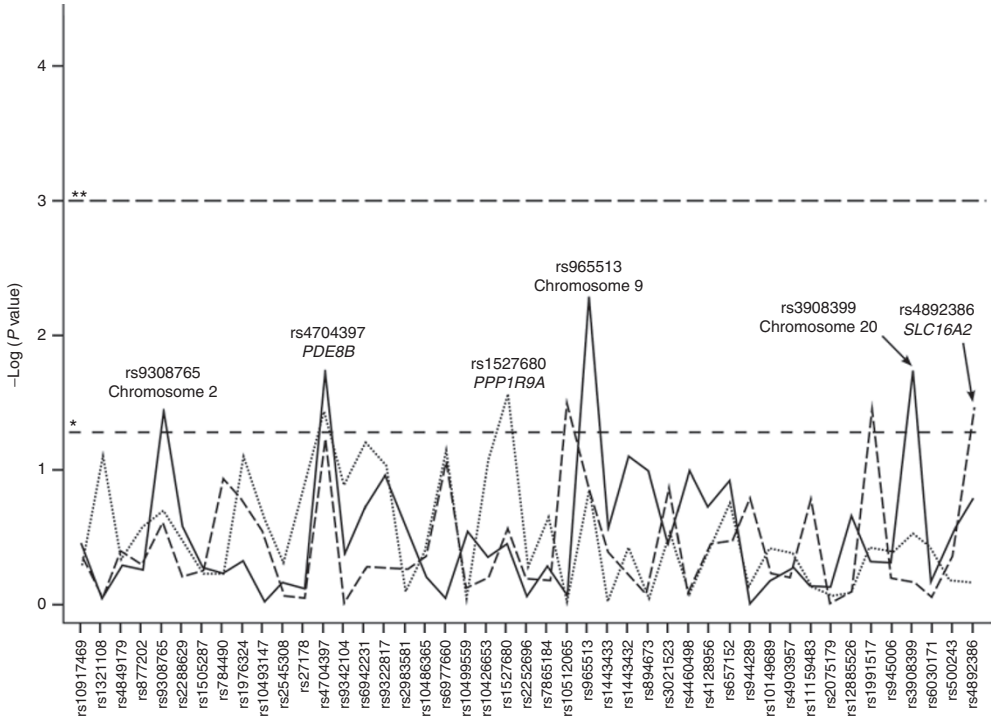

Figure 2. Association statistics for all genotyped SNPs with neonatal TSH level for individual populations. The *x*-axis is a list of all genotyped markers. The *y*-axis is the $-\log_{10}$ of the *P* value from the ANOVA analysis. The horizontal dashed lines represent the *P* value cutoffs; $*P = 0.05$, $**P = 0.001$. The solid line represents the SHL term population. The dashed line represents the SHL preterm population. The dotted line represents the NICU preterm population. NICU, neonatal intensive care unit; SHL, State Hygienic Laboratory; SNP, single-nucleotide polymorphism; TSH, thyroid-stimulating hormone.

Table 2. TSH means and SDs for *PDE8B* and *FOXE1* significant SNPs

		SHL term ($N = 799$)		SHL preterm ($N = 404$)		NICU preterm ($N = 380$)		All cohorts
Gene	SNP	Mean (SD)	*P* value	Mean (SD)	*P* value	Mean (SD)	*P* value	*P* value
PDE8B	rs4704397		0.02		0.06		0.04	1.3×10^{-4}
	AA	10.2 ± 4.5		8.0 ± 4.6		6.2 ± 3.9		
	GA	8.7 ± 4.6		7.5 ± 4.6		6.2 ± 3.9		
	GG	8.4 ± 4.3		6.7 ± 4.3		5.3 ± 3.5		
FOXE1	rs965513		5.2×10^{-3}		0.14		0.14	6.4×10^{-4}
	AA	9.0 ± 5.0		6.6 ± 3.9		5.1 ± 3.4		
	GA	8.5 ± 4.3		7.1 ± 4.3		5.8 ± 3.7		
	GG	9.3 ± 4.6		7.7 ± 4.8		6.2 ± 3.9		
	rs1443432		0.08		0.56		0.36	0.02
	AA	9.3 ± 4.5		7.6 ± 4.8		6.1 ± 3.9		
	AG	8.6 ± 4.4		7.3 ± 4.5		5.9 ± 3.8		
	GG	8.8 ± 4.9		7.1 ± 3.8		5.1 ± 3.2		
	rs3021523		0.37		0.13		0.28	0.03
	CC	8.5 ± 4.4		6.8 ± 3.2		5.6 ± 2.5		
	TC	8.7 ± 4.4		6.9 ± 4.6		5.7 ± 3.5		
	TT	9.1 ± 4.6		7.6 ± 4.6		6.1 ± 4.0		

NICU, neonatal intensive care unit; SHL, State Hygienic Laboratory; SNP, single-nucleotide polymorphism; TSH, thyroid-stimulating hormone.

cohort. Haplotype analysis revealed significant associations of specific allele combinations with TSH levels. Near the *FOXE1* gene, the presence of the AT and GT haplotypes at rs965513 and rs1443433 was significantly associated with a decrease and an increase of TSH levels, respectively ($P = 4.2 \times 10^{-4}$ and $P = 4.6 \times 10^{-4}$) (Table 3).

The four remaining SNPs showed marginally significant associations with TSH levels: rs9308765 on chromosome

Table 3. SNP haplotypes in the *FOXE1* gene that are significantly associated ($P < 0.001$) with natural log–transformed TSH levels

Gene	SNPs Haplotype	Frequency	β	*P* value
FOXE1	rs965513-rs1443433			
	AT	0.23	−0.08	4.2×10^{-4}
	GT	0.62	0.07	4.6×10^{-4}

Frequency: frequency of haplotype indicated. β: β coefficient for linear regression model; positive value indicates the haplotype is associated with an increase in TSH level and negative value indicates the haplotype is associated with a decrease in TSH level. *P* value: *P* value for association between natural log–transformed TSH and a specific haplotype composed of the alleles listed.

SNP, single-nucleotide polymorphism; TSH, thyroid-stimulating hormone.

Table 4. SNP haplotypes in the *TSHR* gene that are significantly associated ($P < 0.001$) with natural log–transformed TSH levels

Gene	SNPs Haplotype	Frequency	β	*P* value
TSHR	rs10149689–rs4903957–rs11159483			
	AGT	0.06	−0.16	7.9×10^{-4}
TSHR	rs4903957–rs11159483–rs2075179			
	GTT	0.05	−0.16	6.2×10^{-4}
TSHR	rs10149689–rs4903957–rs11159483–rs2075179			
	AGTT	0.03	−0.28	4.2×10^{-5}
TSHR	rs4903957–rs11159483–rs2075179–rs12885526			
	GTTA	0.05	−0.17	7.8×10^{-4}

Frequency: frequency of haplotype indicated. β: β coefficient for linear regression model; positive value indicates the haplotype is associated with an increase in TSH level and negative value indicates the haplotype is associated with a decrease in TSH level. *P* value: *P* value for association between natural log–transformed TSH and a specific haplotype composed of the alleles listed.

SNP, single-nucleotide polymorphism; TSH, thyroid-stimulating hormone.

2 ($P = 0.01$), rs657152 in the *ABO* gene ($P = 0.02$), rs4892386 in the *SLC16A2* gene ($P = 0.01$), and rs1527680 in the *PPP1R9A* gene ($P = 0.04$). However, the associations between these SNPs and TSH levels were not always in the same direction in the three studied cohorts, and further investigation is needed to confirm the associations (**Supplementary Table S3** online). Although there were no significant single-locus associations with TSH levels in *TSHR* in any of the cohorts, the presence of the AGT haplotype at rs10149689, rs4903957, and rs11159483, the GTT haplotype at rs4903957, rs11159483, and rs2075179, the AGTT haplotype at rs10149689, rs4903957, rs11159483, and rs2075179, and the GTTA haplotype at rs4903957, rs11159483, rs2075179, and rs12885526 was significantly associated with TSH levels ($P = 7.9\times10^{-4}$, $P = 6.2\times10^{-4}$, $P = 4.2\times10^{-5}$, and $P = 7.8\times10^{-4}$, respectively) (**Table 4**).

DISCUSSION

Elucidating the genetic basis of TSH variability is currently an area of interest to further the understanding of adult conditions related to TSH levels. For example, low serum TSH levels are associated with an increased risk of atrial fibrillation in adults older than 60 y (11). Thyroid hormone levels within the normal physiological range have also been shown to affect bone mass and density in healthy men aged 25–45 y (12), as well as in men and women older than 55 y (13). Thyroid hormones also play a key role in neonatal as well as adult normal physiology, affecting almost all tissues and maintaining healthy status of all human systems, including cognition, cardiovascular function, skeletal health, and balanced energy and metabolic status (14). This is particularly relevant in preterm infants, in whom the variability of TSH is particularly high because of postnatal changes in thyroid function due to premature interrupted exposure to maternal thyroid hormones as well as an immature hypothalamic–pituitary axis (2). Understanding the shared genetic associations with TSH levels in both the neonatal period and through adulthood will be useful for earlier prediction of risk for adult diseases that are affected by TSH levels.

In this light, we aimed to identify genetic polymorphisms that may play a role in TSH variation, especially in preterm infants. We observed genetic associations that have an effect on TSH levels in either term or preterm infants or both. However, most of our nominally significant associations were found in the term population, and these associations reached multiple-testing correction levels of significance in all populations combined. This may be due to the wide range of variability in TSH levels of preterm infants because of an immature hypothalamic–pituitary axis and the skewed nature of our preterm population, in which early preterm infants are overrepresented. Our most significant association was with SNP rs4704397 in the *PDE8B* gene ($P = 1.3\times10^{-4}$). Each copy of the minor A allele was associated with an increase of 0.6 mIU/l in TSH levels in the combined cohort. This finding is consistent with what has previously been reported in adult serum TSH levels (7).The *PDE8B* gene is expressed most abundantly in the thyroid gland, where it has threefold higher levels than in the next highest tissue (15). It is expressed at lower levels in some other tissues, including the brain, spinal cord, and placenta (16). The *PDE8B* gene encodes a high-affinity adenosine 3′,5′-cyclic monophosphate (cAMP)–specific phosphodiesterase to regulate the level of cAMP in cells and plays a vital role in signal transduction (15,16). Common genetic variants in *PDE8B* may affect steroid hormone physiology, such as levels of TSH. For example, an intronic SNP in *PDE8B* (rs4704397) that was identified in the genome-wide association study by Arnaud-Lopez *et al.* (7) as being associated with adult serum TSH levels was then reported to be associated with subclinical hypothyroidism during pregnancy (17), as well as with TSH levels in obese children (18). Of note, we found the same SNP to be associated with TSH levels in both preterm and term infants, suggesting that this gene plays an important role in regulating TSH levels at birth as well as thyroid function throughout life. All four studies (adult serum TSH level, ref. (7); pregnancy TSH level, ref. (17); obese children TSH level, ref. (18); and this newborn TSH level study) came to the same

Table 5. List of genotyped markers

Marker	Gene	Location	Chromosome	Position	MAF
rs10917469	—	Intergenic	1	19843576	0.19
rs1321108	*TSHB*	Promoter	1	115572365	0.5
rs4849179	*PAX8*	Intron	2	113985170	0.42
rs877202	*PAX8*	Intron	2	114019129	0.26
rs9308765	—	Intergenic	2	119043209	0.11
rs2288629	*EPHA4*	Intron	2	222307310	0.16
rs1505287	*THRB*	Intron	3	24412690	0.37
rs784490	*TTC21A*	Intron	3	39173530	0.24
rs1976324	—	Intergenic	3	87212806	0.31
rs10493147	*HSPA4L*	Intron	4	128737499	0.24
rs2545308	—	Intergenic	4	181637915	0.44
rs27178	*PDE4D*	Intron	5	58587025	0.46
rs4704397	*PDE8B*	Intron	5	76518442	0.48
rs9342104	*CGA*	Intron	6	87798512	0.44
rs6942231	*HACE1*	Intron	6	105191814	0.45
rs9322817	*HACE1*	Intron	6	105232233	0.4
rs2983521	*PDE10A*	Intron	6	166057203	0.2
rs10486365	*TMEM196*	Intron	7	19801364	0.1
rs6977660	*TMEM196*	Intron	7	19805480	0.14
rs10499559	—	Intergenic	7	22109459	0.16
rs10486653	*NPSR1*	Intron	7	34711663	0.2
rs1527680	*PPP1R9A*	Promoter	7	94534886	0.15
rs2252696	*SLA*	Intron	8	134063532	0.43
rs7865184	*ZDHHC21*	Intron	9	14687867	0.45
rs10512065	*GNAQ*	Intron	9	80625644	0.13
rs965513	—	Intergenic	9	100556109	0.37
rs1443433	—	Intergenic	9	100579219	0.19
rs1443432	—	Intergenic	9	100583195	0.47
rs894673	*FOXE1*	Promoter	9	100612270	0.47
rs3021523	*FOXE1*	Coding exon, synonymous	9	100616583	0.29
rs4460498	*FOXE1*	Downstream	9	100620412	0.46
rs4128956	*MED27*	Intron	9	134818509	0.38
rs657152	*ABO*	Intron	9	136139265	0.38
rs944289	—	Intergenic	14	36649246	0.41
rs10149689	*TSHR*	Promoter	14	81415800	0.46
rs4903957	*TSHR*	Intron	14	81448511	0.32
rs11159483	*TSHR*	Intron	14	81501311	0.25
rs2075179	*TSHR*	Coding exon, synonymous	14	81562998	0.1
rs12885526	*TSHR*	Intron	14	81574429	0.36
rs1991517	*TSHR*	Coding exon, missense	14	81610583	0.1
rs945006	*DIO3*	3′-UTR	14	102029277	0.12
rs3908399	—	Intergenic	20	12901275	0.27
rs6030171	*PTPRT*	Intron	20	40994094	0.31
rs500243	*SLC16A2*	Intron	X	73676685	0.37
rs4892386	*SLC16A2*	Intron	X	73718735	0.32

MAF, minor allele frequency; UTR, untranslated region.

conclusion, that each copy of the minor A allele was associated with an increase in TSH levels. In the study by Arnaud-Lopez *et al.* (7), the authors suggest that because cAMP is necessary for thyroid hormone secretion due to TSH stimulation, when *PDE8B* catalyzes the hydrolysis and inactivation of cAMP in the thyroid gland, it results in decreased generation of thyroid hormone T_4 and triiodothyronine (T_3), resulting in a negative feedback loop to produce more TSH. Hence, genetic variation in *PDE8B* may affect *PDE8B* activity, resulting in altered cAMP, TSH, and probably other downstream effects. In the study by Arnaud-Lopez *et al.* (7), all exons in the *PDE8B* gene were sequenced in 40 patients to identify a possible etiologic variant in linkage disequilibrium with the intronic rs4704397; however, no coding variants were identified. Further investigation and sequencing of this gene will be needed in adult and newborn samples to identify the regulatory regions causing the association between *PDE8B* and thyroid levels.

We also found SNPs rs965513 and rs1443432 near the *FOXE1* gene ($P = 6.4 \times 10^{-4}$ and 0.02, respectively) and SNP rs3021523 in the *FOXE1* gene ($P = 0.03$), to be associated with TSH levels in all the populations combined. Although, only rs965513 meets the multiple-testing correction level of significance, with the other two SNPs being only marginally significant, this may be secondary to the moderate, but not complete, linkage disequilibrium with each other (rs965513, rs1443432: $D' = 0.863$, $r^2 = 0.519$; rs965513, rs3021523: $D' = 0.835$, $r^2 = 0.48$) and the fact that rs965513 and rs1443433 also have strong effects as part of a haplotype associated with TSH levels. The *FOXE1* gene encodes a transcription factor that is essential for the initiation of thyroid differentiation at the embryonic stage (19). Mutations of the *FOXE1* gene may result in thyroid dysgenesis, leading to both familial cases as well as cases of syndromic CH in the Bamforth–Lazarus syndrome (OMIM no. 241850 (http://omim.org/)), a rare inherited disorder characterized by CH, cleft palate, and spiky hair (20). *FOXE1* also plays an important role in regulating the transcription of different thyroid-specific genes resulting in the regulation of thyroid hormone synthesis (21). Two SNPs, rs965513 near *FOXE1* (20) and rs1443434 in the *FOXE1* gene (22), have been previously shown to be associated with adult serum TSH levels. Our replication of this finding with newborn TSH levels further implicates the involvement of this locus in determining TSH levels in newborns, indicating that this association is present at birth.

We further identified four haplotypes in the *TSHR* gene to be significantly associated with TSH levels. The protein encoded by the *TSHR* gene is the TSH receptor (TSHR). TSHR is present on thyroid cells, and when activated by TSH secreted from the pituitary gland, intracellular cAMP is upregulated, resulting in activation of various cellular processes ending with an increased production of thyroid hormone (23). SNPs in the *TSHR* gene were previously found to be associated with adult serum TSH levels in a genome-wide association study reported by Arnaud-Lopez *et al.* (7). We did not find an association with single SNPs in the *TSHR* gene; however, we found SNP haplotypes to be associated with newborn TSH levels.

One limitation of this study is that we were not able to connect TSH measurements to medical record information to obtain more detailed information on neonatal illness or race for the SHL preterm and term cohorts. However, in 2009, 86.9% of the births in Iowa were Caucasian, suggesting that the majority of our samples are from Caucasians. Another limitation was that we do not have follow-up data on our study cohort to check for the development of conditions later in life. On the other hand, our study illustrates the utility of having institutional review board–reviewed access to stored, deidentified newborn dried bloodspot samples for genetic studies. This is also, to our knowledge, the first study to examine the genetics of TSH levels in newborns. Unraveling the genes that affect TSH levels will enhance our understanding of the genetic regulation and physiology of the thyroid and the pituitary–thyroid axis as well as the genes that may be involved in different thyroid diseases. This knowledge may be used to help individualize thyroid-related treatments according to the individual's specific genotype. Recognizing TSH variation and the genetic factors affecting it early in infancy may be a useful adjunct in population screening for the early prediction and possible treatment or prevention of adult diseases and conditions affected by TSH levels.

METHODS

Study Population

Study samples were obtained from two sources. The first was from the University of Iowa SHL, where we obtained deidentified residual dried blood spots on a population of Iowa neonates: term ($n = 827$) and preterm ($n = 413$). Approval for the use of the deidentified data and blood spot cards was granted by the Iowa Department of Public Health, and a waiver of consent was obtained from the institutional review board at the University of Iowa (IRB no. 200908793). All subjects had TSH measurements between day 1 and 3 of life and had not received a transfusion before sample collection. Preterm birth was defined as birth before 37 completed weeks of gestation. Gestational age was determined from the records obtained from the SHL. Quantification of TSH was performed at the SHL using Clinical Laboratory Improvement Amendments–certified methods. TSH was determined by solid-phase, time-resolved fluoroimmunoassay from dried newborn blood spots using PerkinElmer's AutoDELFIA platform (Waltham, MA). DNA was extracted from one dried whole-blood spot for all term samples and two dried whole-blood spots for all preterm samples using the AutoGen (Holliston, MA) QuickGene-810 nucleic acid extraction machine with the DNA Tissue Kit (AutoGen) and following manufacturer's recommendations.

The second source was from the University of Iowa Hospitals and Clinics NICU. Preterm infants admitted to this NICU were either born in-house or transferred from referring units within the first 28 d of life. Preterm birth was also defined as birth before 37 completed weeks of gestation. Gestational age was determined from the first day of the last menstrual period. Gestational age was confirmed through obstetrical judgment and if uncertain, ultrasound measurements were used for confirmation. All families provided signed informed consent (UI IRB199911068) to be included in a repository of samples designed to provide DNA and limited epidemiologic data to study the complications of newborn infants (24). Preterm infants from singleton births, without congenital anomalies, with gestational ages between 22 and 36 wk ($n = 402$) were included in this study, and early preterm infants (<34 wk gestation) were overrepresented from their population frequency due to the referral patterns of our NICU. TSH measured by the SHL as part of routine neonatal screening was linked to the sample and medical record data. DNA was extracted from cord blood, discarded venous blood, or buccal swab for all infants. All DNA was extracted using standard protocols.

Marker Selection and Genotyping

We chose a total of 45 SNPs encompassing 24 candidate genes and 8 intergenic loci. The list of genes and SNPs genotyped are in Table 5. SNPs were chosen either on the basis of their previously reported associations with adult serum TSH levels or on the basis of thyroid biology and candidate genes from the literature. In the first case, we chose the exact SNPs for which associations were reported. In the second case, we chose SNPs from the HapMap database (http://hapmap.ncbi.nlm.nih.gov/) on the basis of their ability to tag the surrounding SNPs in the candidate gene. All SNPs were genotyped using TaqMan assays (Applied Biosystems, Foster City, CA) on the EP1 SNP Genotyping System and GT48.48 Dynamic Array Integrated Fluidic Circuits (Fluidigm, San Francisco, CA). All SNP genotyping assays were available and ordered using the Assay-on-Demand service from Applied Biosystems. These genotyping assays included primers to amplify the region containing the SNP of interest and two TaqMan Minor Groove Binder probes that are specific to the polymorphic variant alleles at the site labeled with different fluorescent reporter dyes, FAM and VIC. All reactions were performed using standard conditions supplied by Fluidigm. Following thermocycling, fluorescence levels of the FAM and VIC dyes were measured using the EP1 Reader (Fluidigm), and genotypes were scored using the Fluidigm Genotyping Analysis software (Fluidigm). Genotypes were uploaded into a Progeny database (Progeny Software, South Bend, IN) containing the phenotypic data for subsequent statistical analysis. Genotyping efficiency was >98% for all markers, with the exception of rs9322817 in *HACE1* (93.5%) and rs1014968 in *TSHR* (90.8%). Forty-three individuals with genotyping efficiency <95% (i.e., missing data on >2 markers) were excluded from further analysis. In addition, only 16 individuals had an abnormal (TSH ≥ 25.0 mIU/l) TSH level on initial screen. To avoid potential confounding, these individuals were excluded, leaving 404 SHL preterm neonates, 799 term neonates, and 380 NICU neonates for analysis.

Statistical Analysis

Demographic characteristics were compared between cohorts using χ^2 tests for categorical traits and Wilcoxon rank sum tests for continuous traits. All demographic factors listed in Table 1, year of sampling, and TSH assay lot were individually associated ($P \leq 0.001$) with the natural log-transformed TSH measurement in the combined (N = 1,583) sample cohort using ANOVA. Step-wise modeling was used to determine a final list of covariates for inclusion when testing association between SNP genotype and TSH level. Gestational age ($P = 5.55 \times 10^{-19}$), age at the time of sampling ($P = 1.63 \times 10^{-42}$), gender ($P = 1.52 \times 10^{-8}$), and year of sampling ($P = 7.36 \times 10^{-4}$) were all significantly associated with TSH in the final model; birth weight (P = 0.02), assay lot (P = 0.09), and total parenteral nutrition (P = 0.25) were not included in the final model due to marginal significance. Hardy–Weinberg equilibrium was evaluated for each marker using Fisher exact tests for all cohorts. All markers were tested for association with the natural log–transformed TSH measurement adjusting for gender, gestational age, age at the time of sampling, and year of sampling using ANOVA in the combined cohort (N = 1,583). A Bonferroni significance threshold of P = 0.001 (0.05/45 markers) was used to correct for multiple testing. Subanalyses were performed in each cohort separately to determine the individual population effects. All analyses were performed in STATA software version 12.0 (Stata Corp, College Station, TX).

Haplotype Analysis

Haplotype analysis of SNPs in the same gene or region was used to evaluate regional associations with natural log–transformed TSH levels. Haplotype analysis was performed with linear regression adjusted for gender, gestational age, age at the time of sampling, and year of sampling in the combined cohort. Haplotype analysis was performed using sliding windows of 2, 3, and 4 SNPs along a gene or region of interest. Haplotype analyses were performed with PLINK software (http://pngu.mgh.harvard.edu/purcell/plink/) (25).

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/pr

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