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Common variation at 6q16 within *HACE1* and *LIN28B* influences susceptibility to neuroblastoma

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AUTHOR CONTRIBUTIONS S.J.D and J.M.M. designed the experiment and drafted the manuscript. S.J.D. analyzed SNP data, performed SNP association study and analyzed mRNA and miRNA expression data. M.C. and A.I. replicated SNP associations in Italian cohort. V.L. and M.D. replicated SNP associations in African American cohort. E.L.C. and H.L. confirmed LIN28B protein expression by western blot. R.S. and C.W. performed siRNA knockdown experiments of *LIN28B*. E.F.A. generated miRNA expression array data including low-level summary values. K.A.C. performed RT-PCR in primary tumors. M.D. and C.H. organized samples and genotyped cases. H.H. generated and provided all control data for GWAS. M.D. and H.H. contributed to overall study design. All authors commented on or contributed to the current manuscript.

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EdSumm (same for AOP and issue): John Maris and colleagues identify common variants at 6q16 associated with neuroblastoma susceptibility. The risk variants are located near the *HACE1* and *LIN28B* genes, both of which show altered expression in advanced neuroblastomas.

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Abstract

Neuroblastoma is a cancer of the sympathetic nervous system that accounts for approximately 10% of all pediatric oncology deaths[1]. Here we report on a genome-wide association study of 2,817 neuroblastoma cases and 7,473 controls. We identified two new associations at 6q16, the first within *HACE1* (rs4336470; combined $P = 2.7 \times 10^{-11}$, odds ratio 1.26, 95% CI: 1.18–1.35) and the second within *LIN28B* (rs17065417; combined $P = 1.2 \times 10^{-8}$, odds ratio 1.38, 95% CI: 1.23–1.54). Expression of *LIN28B* and *let-7* miRNA correlated with rs17065417 genotype in neuroblastoma cell lines, and we observed significant growth inhibition upon depletion of *LIN28B* specifically in neuroblastoma cells homozygous for the risk allele. Low *HACE1* and high *LIN28B* expression in diagnostic primary neuroblastomas were associated with worse overall survival (P = 0.008 and 0.014, respectively). Taken together, we show that common variants in *HACE1* and *LIN28B* influence neuroblastoma susceptibility and that both genes likely play a role in disease progression.

Neuroblastoma is a malignancy derived from the developing sympathetic nervous system. The median age at diagnosis is 17 months, and the survival rate for the most aggressive subset remains approximately 50% despite intensive multi-modal cytotoxic therapy[1] and recent advances in immunotherapy[2]. The genetic etiology of familial neuroblastoma, which accounts for approximately 1% of cases, has recently come into focus[3-6]; however, the genetic and environmental factors that cause sporadic neuroblastoma remain largely unknown. We have recently reported common SNPs within or upstream of *LINC00340* and *FLJ44180*, *BARD1*, *LMO1*, *DUSP12*, *HSD17B12* and *DDX4-IL31RA* and a common CNV within *NBPF23* as each being highly associated with neuroblastoma[7-11]. Collectively, however, these variants still account for only a small portion of neuroblastoma heritability, and it is likely that additional predisposition loci remain to be discovered.

To identify additional variants associated with neuroblastoma, we expanded our previous genome-wide association study (GWAS) discovery cohort and analyzed 2,101 neuroblastoma cases accrued through the North American-based Children's Oncology Group (Supplementary Table 1) with 4,202 control subjects of European ancestry matched genetically and by genotyping array version to minimize genomic inflation (see Online Methods and Supplementary Figs. 1 and 2). All subjects were genotyped using the Illumina HumanHap550 or Quad610 Beadchip. We restricted our analysis to the SNPs present on both platforms that passed our quality control metrics; the genomic control inflation factor was 1.14 (Supplementary Fig. 3). Evaluation of the first three principal components in cases and controls confirmed that the slightly high inflation factor was not due to gross population stratification (Supplementary Fig. 4). Clusters of SNPs from six genomic loci reached genome-wide significance (P-values ranged from 7.8×10^{-16} to 4.8×10^{-8}; Supplementary Fig. 1 and Supplementary Table 2), including three SNPs within *LINC00340* and *FLJ44180*

at 6p22 (*P*-values ranged from 7.8×10^{-16} to 1.7×0^{-14}), ten SNPs within or near *BARD1* at 2q35 (*P*-values ranged from 4.1×10^{-14} to 3.7×10^{-8}), two SNPs within *LMO1* at 11p15 (*P*-values ranged from 1.2×10^{-13} to 3.8×10^{-10}) and one SNP within *HSD17B12* at 11p11 ($P = 4.8 \times 10^{-8}$), further confirming our previous reports[7-10]. In addition, we identified one SNP (rs4696715) at chromosome 4p16 and two SNPs (rs4336470 and rs9404576) at 6q16 that have not been reported previously (*P*-values ranged from 1.8×10^{-8} to 3.4×10^{-8}; Table 1). Several genotyped SNPs in strong linkage disequilibrium (LD) with rs4696715 at 4p16 did not show evidence for association with neuroblastoma; therefore, this SNP was not considered further here.

Closer examination of the 6q16 locus identified four additional SNPs that showed association ($P < 1 \times 10^{-4}$) with neuroblastoma (Table 1 and Supplementary Table 3). Three of these SNPs mapped to introns of the HECT domain and ankyrin repeat containing, E3 ubiquitin protein ligase 1 gene (*HACE1*) and exhibited a moderate degree of LD with rs4336470. The fourth SNP (rs17065417; $P = 1.8 \times 10^{-7}$) mapped to an intron of the lin-28 homolog B gene (*LIN28B*) and showed very little evidence for LD with rs4336470 in multiple HapMap populations (Supplementary Fig. 5). To ensure these results were not influenced by subtle substructure in the discovery phase, we included the first 20 principal components as covariates in a logistic regression analysis. This reduced the inflation factor to 1.04 but did not alter our conclusions regarding regions reaching genome-wide significance, and the *P*-values for SNPs at the newly identified 6q16 locus were essentially unchanged (Supplementary Table 4). Finally, we conditioned the analysis of the 6q16 SNPs on rs4336470 to investigate if there might be more than one independent association signal at 6q16. As expected, SNPs in modest LD with rs4336470 were no longer statistically significant after conditioning and clearly represent one signal (Supplementary Table 5). In contrast, while the signal at rs17065417 was attenuated, it remained significant ($P = 2.5 \times 10^{-4}$; Supplementary Table 5), suggesting that rs4336470 and rs17065417 may contribute independently to neuroblastoma risk. No association was observed between rs4336470 or rs17065417 genotypes and clinical or biological covariates (Supplementary Tables 6 and 7), and we observed only weak evidence for epistasis at the other significant loci (Supplementary Tables 8 and 9), suggesting that each may act independently to confer risk.

We next sought to replicate the rs4336470 and rs17065417 associations in an Italian cohort of 351 cases and 780 controls using PCR-based genotyping. Both SNPs showed evidence for association in the same direction seen in the discovery effort (Table 1 and Supplementary Table 3). To assess whether these variants influence susceptibility in other ethnic groups, and to seek additional replication, we analyzed a third independent case series comprised of 365 African American neuroblastoma cases and 2,491 genetically matched controls, all genotyped on the Illumina HumanHap550 or Quad610 BeadChip. After accounting for African admixture in logistic regression analysis, the genomic inflation factor was 1.02, as described previously[12] (Online Methods and Supplementary Fig. 6). Consistent with the lower incidence rate of neuroblastoma in African Americans[13], the rs4336470 protective allele (T) is actually the major allele in African Americans, whereas it is the minor allele in individuals of European ancestry. Despite these observed differences in allele frequencies, all SNPs mapped to *HACE1* replicated robustly in the African American cohort

(*P*-values ranged from 1.3×10^{-4} to 1.4×10^{-3}; Table 1 and Supplementary Table 3), and all associations were in the same direction. Allele frequencies for the *LIN28B* SNP rs17065417 were comparable across ethnic populations and showed a trend toward association in African Americans in the same direction as the European American discovery and Italian replication cohorts (Table 1 and Supplementary Table 3). Combined analysis using the weighted inverse-variance method in METAL[14] demonstrated that all six genotyped SNPs had *P* values beyond or approaching the conservative Bonferroni adjusted genome-wide significance threshold (*P*-values ranged from 2.7×10^{-11} to 1.6×10^{-7}; Table 1).

To identify variants at the 6q16 locus not assayed directly on the Illumina SNP arrays, we performed genotype imputation in our discovery cohort using data from the 1000 Genomes project. This analysis identified ten additional genome-wide significant SNPs (*P*-values ranged from 4.4×10^{-9} to 5.7×10^{-8}; Fig. 1). Five of these imputed SNPs were in strong LD with rs4336470 ($r^2 > 0.8$ in 1000 Genomes EUR population); three mapped to introns of *HACE1* and the other two were located just downstream of *HACE1* (Fig. 1a). The remaining five imputed SNPs reaching genome-wide significance were in strong LD with rs17065417 ($r^2 > 0.8$ in 1000 Genomes EUR population) and were located within introns of *LIN28B* (Fig. 1b). In total, we identified 46 imputed SNPs that showed strong evidence for association ($P < 1.0 \times 10^{-6}$, Supplementary Table 10). To further evaluate whether two independent association signals exist at 6q16, we conditioned the regional association analysis on either rs4336470 or rs17065417 and found that neither could fully account for the observed associations (Supplementary Fig. 7). The signal at 6q16 was only abolished across the entire region after conditioning on both rs4336470 and rs17065417 (Supplementary Fig. 8). While we cannot rule out the possibility that both SNPs may be tagging the same underlying risk variant, these data are consistent with the presence of two independent association signals at 6q16, one implicating *HACE1* and the other *LIN28B*.

The *HACE1* gene encodes an E3 ubiquitin-protein ligase that was first identified in a sporadic Wilms tumor that harbored a t(6:15)(q21;q21) translocation[15]. The rearrangement was associated with decreased *HACE1* expression, and further study showed that *HACE1* is silenced in the majority of Wilms tumors via hypermethylation of two CpG islands upstream of the transcriptional start site[15]. Similar epigenetic silencing has been reported in advanced colorectal cancer[16] and gastric carcinoma[17]. Indeed *HACE1* is down-regulated in multiple human tumors and maps to a region of common deletion or LOH, consistent with a tumor suppressor function. *Hace1* null mice form spontaneous tumors in a wide array of tissues and are susceptible to additional cancer triggers, both genetic and environmental[18]. Through its E3 ubiquitin ligase function, HACE1 has been shown to suppress cell growth and anchorage independence of human tumor cells, including the neuroblastoma cell line IMR32 (ref. 18). Studies suggest that HACE1 inhibits cell cycle progression during stress via regulation of cyclin D1 degradation[18] and that HACE1 also regulates retinoic acid receptor (RAR) activity[19]. In addition to multiple somatic alterations involving the *HACE1* locus, a constitutional t(5;6)(q21;q21) translocation that disrupts *HACE1* was recently identified, making it a putative Wilms tumor susceptibility gene[20].

The *LIN28B* gene encodes a developmentally regulated RNA binding protein and is a key repressor of the let-7 family of microRNAs[21]. Both *LIN28A* and *LIN28B* function as oncogenes that promote cellular transformation when ectopically over-expressed[22-24], and high levels of expression of *LIN28A* or *LIN28B* have been observed in several human cancers and correlate with low let-7 levels[22,24]. Post-transcriptional regulation of let-7 by *LIN28A* is required for normal development and contributes to the pluripotent state by preventing let-7–mediated differentiation of embryonic stem cells[21,25,26]. Over-expressing LIN28 or inhibiting let-7 with antisense RNA promotes reprogramming of human and mouse fibroblast to pluripotent stem cells[26,27]. In a panel of 60 pediatric cancer cell lines[28], *LIN28B* was consistently expressed at high levels in neuroblastoma (Supplementary Fig. 9). Recently, *LIN28B* and *let-7* were also identified as key regulators of glucose homeostatis[29] and hematopoiesis[30]. To date, GWAS studies have identified variants in *LIN28B* associated with human height[31] and the age of onset of puberty[32] and menarche[33]. In addition, a recent candidate gene study identified a putative association with epithelial ovarian cancer[34].

To investigate the functional relevance of neuroblastoma-associated SNPs within *HACE1* and *LIN28B*, we first analyzed a set of 12 neuroblastoma cell lines with matched genome-wide SNP genotyping and mRNA expression data. No correlation between rs4336470 and *HACE1* mRNA expression was observed ($P = 0.30$), but *LIN28B* expression was significantly higher in cell lines homozygous for the rs17065417 risk allele (A) compared to heterozygous cell lines ($P = 0.02$; Fig. 2a). No cell lines tested were homozygous for the rs17065417 protective allele (C). *LIN28A* was not expressed in neuroblastoma cell lines, consistent with recent reports that *LIN28A* and *LIN28B* may be mutually exclusive in terms of their expression in cancer cells[21]. In addition to a correlation between rs17065417 genotype and *LIN28B* expression, we observed a strong positive correlation with *MYCN* expression ($P = 0.0003$; Fig. 2b). We next confirmed *LIN28B* protein levels by western blot in four cell lines at the extremes of *LIN28B* mRNA expression (Fig. 2c). Cell lines expressing high *LIN28B* showed lower let-7 expression across the entire miRNA family (Fig. 2d). Transient knockdown of *LIN28B* resulted in significant growth inhibition in neuroblastoma cells homozygous for the rs17065417 risk allele and with high *LIN28B* expression (Fig. 3a-d), but not in the heterozygous cell line SKNAS with low *LIN28B* expression (Fig. 3e,f). Taken together, these data are consistent with the hypothesis that *LIN28B* promotes neuroblastoma tumorigenesis in part through repression of let-7 miRNAs, and that the risk alleles are associated with growth advantage through increased *LIN28B* expression; however, additional studies with larger sample sizes are needed to confirm this.

To examine the relevance of *HACE1* and *LIN28B* in tumor samples, we assayed expression of both genes by real-time quantitative RT-PCR in a representative set of 87 primary tumors obtained at time of diagnosis (Supplementary Table 1). *HACE1* expression was significantly lower ($P = 0.002$; Fig. 4a) and *LIN28B* expression was significantly higher ($P = 0.032$; Fig. 4b) in the high-risk tumors. Accordingly, low *HACE1* expression was associated with a worse overall survival ($P = 0.008$; Fig. 4c), as was high *LIN28B* expression ($P = 0.015$; Fig. 4d). Analysis of four independent mRNA expression array datasets comprising 517 neuroblastoma tumors provided robust replication of these observations (Fig. 4e-h and

Supplementary Figs. 10-12). These data support the hypothesis that *HACE1* may function as a tumor suppressor, and *LIN28B* as an oncogene, in advanced neuroblastomas.

In conclusion, here we have identified common variants within *HACE1* and *LIN28B* that are associated with neuroblastoma. Similar to other genes identified in our ongoing GWAS efforts[8,9,35], it is likely that the germline susceptibility variants identified here play an important role not only in tumor initiation but also in disease progression via *cis*-effects on major cancer genes. Further study of *HACE1* and *LIN28B* in neuroblastoma is warranted and may lead to new insights into the genetic and epigenetic mechanisms underlying an aggressive clinical phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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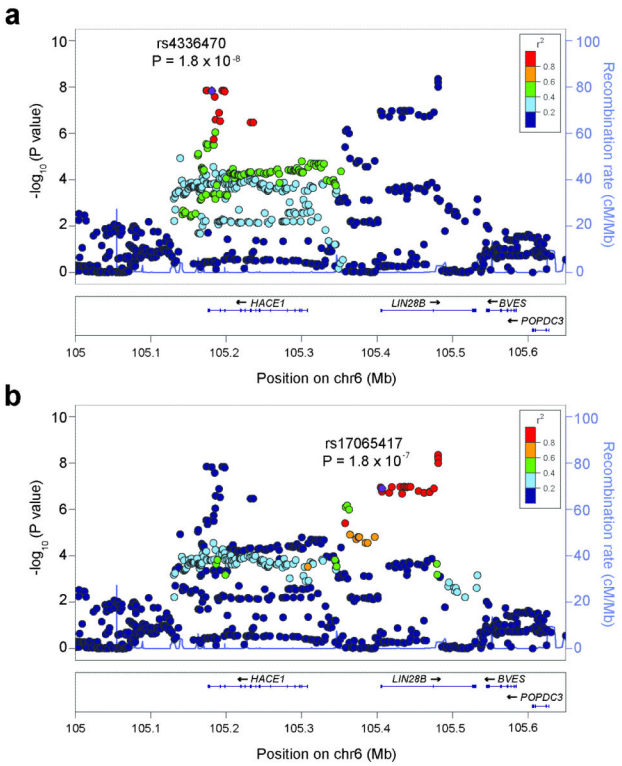

Figure 1.

Regional association plots at the *HACE1* and *LIN28B* loci. Regional association plots including both genotyped and imputed SNPs for the *HACE1* and *LIN28B* loci generated by LocusZoom[36]. Plotted are the significance of association (-$\log_{10}$ transformed *P* values) and the recombination rate. SNPs are color-coded based on pair-wise linkage disequilibrium (r^2) with the most significant genotyped SNP in the EUR (European) 1000 Genomes Interim Phase I release genotypes. The most significant genotyped SNP and associated *P*-value are labeled, and the SNP is shown in purple. (**a**) *HACE1* locus (**b**) *LIN28B* locus.

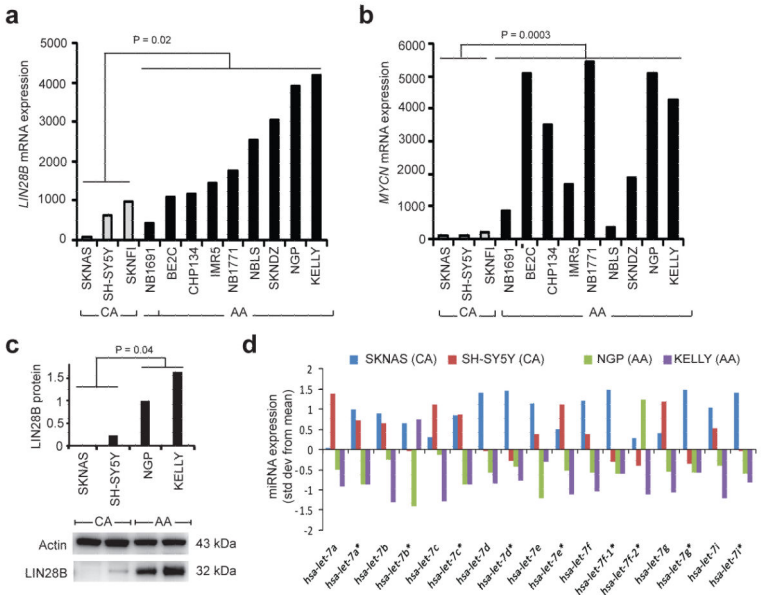

Figure 2.

LIN28B risk alleles correlate with increased *LIN28B* expression and decreased *let-7* miRNA expression. (**a**) *LIN28B* mRNA expression is significantly higher in neuroblastoma cell lines homozygous for the rs17065417 risk allele (AA) compared to neuroblastoma cell lines heterozygous for the risk allele (CA). Given the MAF of rs17065417, we did not identify any cell lines homozygous for the protective allele (CC). (**b**) Neuroblastoma cell lines homozygous for the rs17065417 risk allele show high expression of *MYCN*. (**c**) Western blot confirms increased protein expression of LIN28B in neuroblastoma cell lines homozygous for the rs17065417 risk allele. (**d**) Neuroblastoma cell lines homozygous for the rs17065417 risk allele and with high *LIN28B* expression show decreased or absent expression of the *let-7* miRNAs.

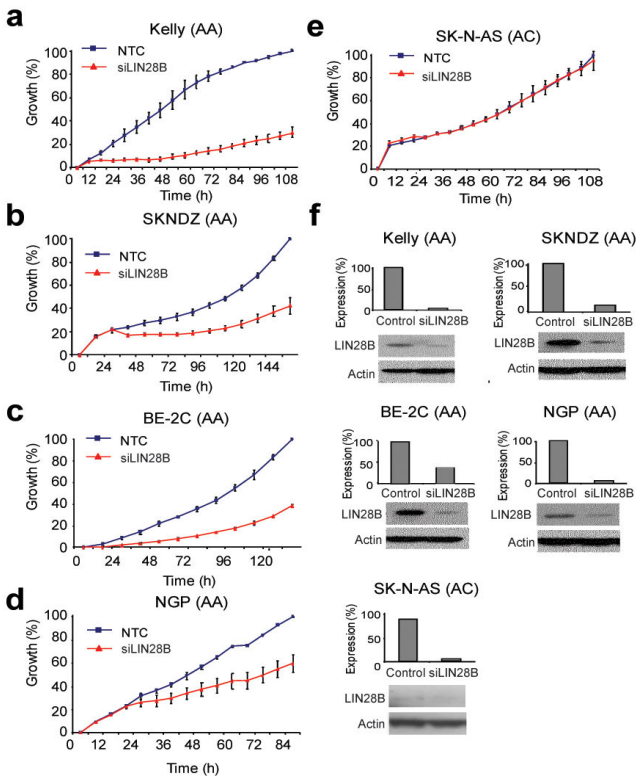

Figure 3.

Transient knockdown of *LIN28B* influences neuroblastoma cell growth in an expression-specific manner. (**a**-**d**) In cells homozygous for neuroblastoma risk alleles and with higher *LIN28B* expression levels, *LIN28B* knockdown leads to significant growth inhibition. (**e**) In cells heterozygous for the risk allele (carrying one protective allele) and with low *LIN28B* expression, *LIN28B* knockdown does not affect cell growth. (**f**) *LIN28B* knockdown as measured by quantitative reverse transcription PCR and western blot for experiments **a**–**e**.

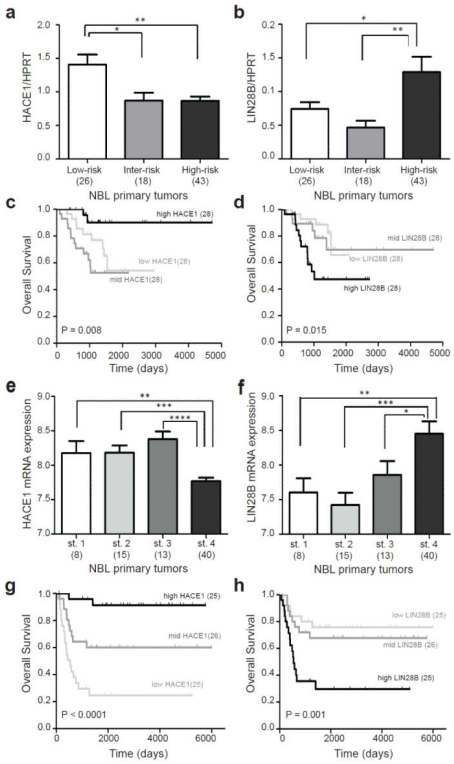

Figure 4.

HACE1 and *LIN28B* expression are associated with advanced disease and poor outcome in neuroblastoma. (**a**) *HACE1* mRNA expression is significantly lower in high-risk neuroblastoma tumors. Bar graph is shown for Children's Oncology Group (COG) risk groups. (error bars: s.e.m.). (**b**) *LIN28B* mRNA expression is significantly increased in high-risk neuroblastoma tumors. Bar graph is shown for Children's Oncology Group (COG) risk groups. (error bars: s.e.m.). (**c**) Decreased *HACE1* expression in primary tumors obtained at diagnosis is associated with worse overall survival. Kaplan-Meier analysis is shown, with patients grouped by tertiles of *HACE1* expression. Log rank *P*-value is shown. (**d**) Increased *LIN28B* expression in primary tumors obtained at diagnosis is associated with worse overall survival. Kaplan-Meier analysis is shown, with patients grouped by tertiles of *LIN28B* expression. Log rank *P*-value is shown. (**e**) Replication of decreased *HACE1* in advanced stage neuroblastoma using published Affymetrix U133 plus v2 array data (R2 bioinformatics tool). Bar graph is shown for INSS stage 1-4 (error bars: s.e.m.). (**f**) Replication of increased *LIN28B* in advanced stage neuroblastoma (R2 bioinformatics tool). Bar graph is shown for INSS stage 1-4 (error bars: s.e.m.). (**g**) Decreased expression of *HACE1* is associated with worse outcome in dataset from **e**. Kaplan-Meier is analysis shown, with patients grouped by tertiles of *HACE1* expression. Log rank *P*-value is shown. (**h**) Increased expression of *LIN28B* is associated with worse outcome in dataset from **f**. Kaplan-Meier analysis is shown, with patients grouped by tertiles of *LIN28B* expression. Log rank *P*-value is shown. ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

Table 1

Significantly associated genotyped SNPs at the *HACE1* and *LIN28B* loci at 6q16

SNP	A1/A2 (protective/ risk allele)	Discovery cohort[a] European ancestry			Italian replication[a] (TaqMan)			CHOP replication[a] African American			Combined	
		Freq A1 cases (*n* = 2,101)	Freq A1 controls (*n* = 4,202)	*P* [b]	Freq A1 cases (*n* = 351)	Freq A1 controls (*n* = 780)	*P* [b]	Freq A1 cases (*n* = 365)	Freq A1 controls (*n* = 2,491)	*P* [c]	Meta *P*[d]	OR[e] (95% CI[f])
HACE1												
rs4336470	T/C	0.30	0.35	1.8×10^{-8}	0.30	0.34	0.060	0.62	0.70	1.4×10^{-3}	2.7×10^{-11}	1.26 (1.18–1.35)
rs9404576	G/T	0.30	0.35	3.4×10^{-8}	-	-	-	0.62	0.70	1.3×10^{-3}	1.8×10^{-10}	1.27 (1.18-1.36)
rs4079063	G/A	0.43	0.47	4.0×10^{-5}	-	-	-	0.70	0.78	1.3×10^{-4}	1.3×10^{-7}	1.20 (1.12-1.29)
rs2499663	C/T	0.43	0.47	4.5×10^{-5}	-	-	-	0.70	0.78	1.5×10^{-4}	1.6×10^{-7}	1.21 (1.13-1.29)
rs2499667	G/A	0.43	0.47	2.6×10^{-5}	-	-	-	0.71	0.78	2.6×10^{-4}	1.2×10^{-7}	1.21 (1.13-1.29)
LIN28B												
rs17065417	C/A	0.08	0.11	1.8×10^{-7}	0.08	0.11	0.033	0.09	0.11	0.129	1.2×10^{-8}	1.38 (1.23-1.54)

[a] No deviations from Hardy-Weinberg equilibrium were observed ($P > 0.001$) in all cohorts.

[b] *P*-values were calculated by allelic test.

[c] *P*-values were calculated by logistic regression with percent African admixture as covariate[12].

[d] Meta-analysis *P*-value calculated using METAL[14].

[e] OR, odds ratio of risk allele based on meta-analysis.

[f] CI, confidence interval.