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Common Genetic Variants in *NEFL* Influence Gene Expression and Neuroblastoma Risk

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Abstract

The genetic etiology of sporadic neuroblastoma is still largely obscure. In a genome-wide association study, we identified single-nucleotide polymorphisms (SNP) associated with neuroblastoma at the *CASC15*, *BARD1*, *LMO1*, *DUSP12*, *HSD17B12*, *HACE1*, and *LIN28B* gene loci, but these explain only a small fraction of neuroblastoma heritability. Other neuroblastoma susceptibility genes are likely hidden among signals discarded by the multiple testing corrections. In this study, we evaluated eight additional genes selected as candidates for further study based on proven involvement in neuroblastoma differentiation. SNPs at these candidate genes were tested for association with disease susceptibility in 2,101 cases and 4,202 controls, with the associations found replicated in an independent cohort of 459 cases and 809 controls. Replicated associations were further studied for *cis*-effect using gene expression, transient overexpression, silencing, and cellular differentiation assays. The neurofilament gene *NEFL* harbored three SNPs associated with neuroblastoma (rs11994014: $P_{\text{combined}} = 0.0050$; OR, 0.88; rs2979704: $P_{\text{combined}} = 0.0072$; OR, 0.87; rs1059111: $P_{\text{combined}} = 0.0049$; OR, 0.86). The protective allele of rs1059111 correlated with increased NEFL expression. Biologic investigations showed that ectopic overexpression of *NEFL* inhibited cell growth specifically in neuroblastoma cells carrying the protective allele. *NEFL* overexpression also enhanced differentiation and impaired the proliferation and anchorage-independent growth of cells with protective allele and basal *NEFL* expression, while impairing invasiveness and proliferation of cells homozygous for the risk genotype. Clinically, high levels of *NEFL* expression in primary neuroblastoma specimens were associated with better overall survival ($P = 0.03$; HR, 0.68). Our results show that common variants of *NEFL* influence neuroblastoma susceptibility and they establish that *NEFL* expression influences disease initiation and progression. *Cancer Res; 74(23); 6913–24. ©2014 AACR.*

Introduction

Neuroblastoma is a cancer of the sympathetic nervous system. It is the most frequent solid tumor of early childhood with a remarkable variation in clinical presentation ranging from favorable localized tumors that can spontaneously regress to metastatic disease that shows relentless progression (1). Despite intensive therapies, the survival rate for the patient subgroup with the most aggressive form remains approximately 40%. While familial neuroblastoma, which accounts for approximately 1% of cases, is in large proportion due to mutations in the *ALK* gene (2), the genetic bases of sporadic neuroblastoma remain largely unknown (3). Our ongoing genome-wide association study (GWAS) has demonstrated that genetic variants within the *CASC15, BARD1, LMO1, DUSP12, HSD17B12, HACE1*, and *LIN28B* genes (4–8) are strongly associated with neuroblastoma in North American patients of European descent, and each of these associations have been replicated in an independent Italian population (9). However, these risk variants only explain a small proportion of neuroblastoma heritability and additional predisposing variants to neuroblastoma remain to be discovered.

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The remaining genetic risk is probably made up by a combination of common variants with very modest effect sizes and by rare variants with stronger effects. Some of the common genetic risk variants are probably hidden among signals discarded by the multiple testing correction needed in the analysis of GWAS data. This multiple testing correction is necessary to exclude false-positive loci, but simultaneously it discards many true-positive risk loci. Different research strategies have been proposed (10) for extracting these hidden true-positive loci, such as increasing the GWAS sample size, performing a meta-analysis of GWAS datasets, replicating hundreds to thousands of GWAS signals in a larger cohort, performing imputation and epistasis analysis, using pathway-based and single nucleotide polymorphism (SNP)-set enrichment approaches, and others.

Here, to discover new common variants associated with neuroblastoma, we adopted a strategy based on the selection of genes with high relevance to neuroblastoma differentiation, which were identified in our previous proteomic study (11). Arrested differentiation of neuroblasts at various stages is a crucial early event in neuroblastoma pathogenesis, especially in the more aggressive cases. Indeed, a particular characteristic of neuroblastic tumors is their broad spectrum of cellular differentiation, ranging from undifferentiated cells that indicate a poor prognosis to those showing greater differentiation and predicting a generally favorable outcome (12). This heterogeneity suggests that dysregulated differentiation of sympathetic progenitor cells plays a key role in neuroblastoma pathogenesis. We thus hypothesized that variation in genes involved in neuroblastic differentiation might predispose to neuroblastoma development. This hypothesis is supported by recent research showing that functional alterations of key regulators of neuron development can induce neuroblastic malignant transformation (13, 14).

In the present study, we performed a gene-based association analysis of eight genes recently shown to be major regulators of neuroblastoma differentiation (11). A large cohort of 2,101 patients and 4,202 control subjects was used as discovery set, whereas an independent population of 459 cases and 809 controls was used as replication set. The results showed a significant association of SNPs within *NEFL* with neuroblastoma risk. Additional *in silico* and *in vitro* analyses demonstrated a functional role of the neuroblastoma-associated SNPs and indicated that *NEFL* likely has a role in disease development and progression.

Materials and Methods

Study subjects

This study was approved by the Ethics Committee of the Medical University of Naples and the Children's Hospital of Philadelphia.

This study included a GWAS dataset of 2,101 neuroblastoma patients registered through the North American-based Children's Oncology Group and 4,202 cancer-free children of self-reported Caucasian ancestry who were recruited and genotyped by the Center for Applied Genomics at the Children's Hospital of Philadelphia. European American cases and controls have been described in detail in a previous publication (8). In addition, this study also included 459 neuroblastoma patients and 809 cancer-free controls of Italian origin. Additional details and eligibility criteria for genotyping of both populations are reported in Supplementary Information. This study was approved by the Ethics Committee of the Medical University of Naples and the Children's Hospital of Philadelphia.

Selection of genes involved in neuroblastoma differentiation

Our previous study has demonstrated that eight proteins are differentially expressed during neuroblastoma differentiation by two-dimensional differential in-gel electrophoresis analysis of the cytosolic and nuclear protein expression patterns of LAN-5 cells following neuronal differentiating agent *all-trans*-retinoic acid treatment (11). The retinoic acid differential expression patterns of these eight proteins were further validated in three cell lines (LAN-5, SH-SY5Y, and SK-N-BE) by Western blotting and gene expression (11). This motivated us to select the genes (*EEF1A*, *EEF2, GNB2, NEFL*, *PCNA*, *PRDX2*, *SCG2*, and *VBP1*) that encode the eight proteins to be tested for association with neuroblastoma development (Table 1).

Table 1. Gene-based association analysis in neuroblastoma GWAS dataset (2,101 cases and 4,202 controls)

Gene sets	NSNP	NSIG	ISIG	EMP	FDR	SNPs
EEF1A1	4	0	0	1	1	NS
EEF2	6	0	0	1	1	NS
GNB2	3	0	0	1	1	NS
NEFL	8	4	2	0.0076	0.0304	rs118727|rs169061
PCNA	6	0	0	1	1	NS
PRDX2	2	0	0	1	1	NS
SCG2	7	1	1	0.0037	0.0296	rs4673067
VBP1	0	0	0	1	1	NS

Abbreviations: EMP, empirical gene-based *P* value; FDR, false discovery rate correction method applied to EMP; ISIG, number of significant also passing LD-criterion; NS, not significant; NSIG, total number of SNPs below *P* value threshold; NSNP, number of SNPs in gene and surrounding genomic region (±20 kb).

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SNP genotyping

The European American DNA samples were genotyped using the Illumina Infinium II BeadChip HumanHap550 v1, v3, and Quad610 arrays according to methods detailed elsewhere (8). The quality control analyses for this GWAS dataset are described in detail elsewhere (8). We analyzed six SNPs of *NEFL* (rs196830, rs169061, rs11994014, rs2979704, rs1059111, and rs3761) in the Italian cohort. The DNA samples were genotyped using SNP Genotyping Assay on 7900HT Real-time PCR system (Applied Biosystems). More details on SNP genotyping are reported in Supplementary Information.

Gene-based association analysis

The SNP genotypes of each selected gene and surrounding genomic region (±20 kb) were extracted from the GWAS dataset. The analysis was carried out by using the set-based test implemented in the PLINK software (15). Briefly, for each gene, a standard single SNP analysis (case–control association) is performed. SNPs with P value > 0.05 and/or a Linkage Disequilibrium (LD) coefficient $r^2 > 0.5$ with a significant SNP (defined as a P value < 0.05) are filtered out. From the remaining independent SNPs, the statistic for each gene is calculated as the mean of the single SNP statistics. To correct for testing multiple SNPs within a gene, an empirical gene-based P value is calculated on the basis of 10,000 replicates of this procedure after random permutation of case–control status, as the proportion of replicates with a statistic larger than the one observed. To correct for testing multiple genes, we applied the false discovery rate method to the empirical gene-based P values.

Genotype imputation of putative functional SNPs at *NEFL* locus

As SNPs rs2979704, rs1059111, rs3761 were not included in the Illumina HumanHap550 array, genotype imputation was performed in the European American GWAS. Pre-phasing was performed first using SHAPEIT (16), followed by imputation using 1000 Genomes data (phase I integrated release) and IMPUTE2 (17). SNPs with minor allele frequency (MAF) <1% and/or IMPUTE2-info quality score <0.8 were removed. To account for imputation uncertainty, the remaining SNPs were tested for association with neuroblastoma using the frequentist test under the additive model with the "score" method implemented in SNPTEST (18). A detailed description of genotype imputation is reported in Supplementary Information.

SNP-gene expression correlation analysis in tumor tissues and neuroblastoma cell lines

The influence of SNPs on *NEFL* gene expression was evaluated using data from genome-wide mRNA expression profiling (GSE3960) and SNP array genotyping (pha002845) in 51 neuroblastoma patients, and from qRT-PCR analysis in eight neuroblastoma cell lines. A detailed description of the analysis is reported in Supplementary Information.

In vitro functional analysis

A detailed description of the luciferase assay and other experiments performed to evaluate the *NEFL* effect on neuroblastoma cell line phenotype is reported in Supplementary Information.

Cell lines

The human SH-SY5Y, SK-N-AS, IMR-32, SK-N-BE2c, and HEK293T/17 cell lines were obtained from the American Type Culture Collection (respectively ATCC #CRL-2266, #CRL-2137, #CCL-127, #CRL-2268, and #CRL-11268). SH-SY5Y, SK-N-AS, and HEK293T/17 cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM; Sigma); the IMR32 cell line was grown in Minimum Essential Medium Eagle (MEM; Sigma) and the SK-N-BE2c cell line was grown in DMEM/F12 medium (Sigma). The medium was supplemented with 10% heat-inactivated FBS (Sigma), 1 mmol/L L-glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL; Invitrogen). The cells were cultured at 37°C, 5% CO_2 in a humidified atmosphere. The cumulative culture length of the cells was fewer than 6 months after resuscitation. Early-passage cells were used for all experiments and they were not reauthenticated.

Gene expression data for survival analysis and association with neuroblastoma stages

NEFL normalized gene expression array data of three independent sets of neuroblastoma patients (399 total) were downloaded from the website Oncogenomics (http://home.ccr.cancer.gov/oncology/oncogenomics/): (i) "Kahn dataset" composed of 46 samples (Affymetrix Human Exon 1.0 ST Array, GSE27608); (ii) "Oberthuer dataset" composed of 251 samples (Agilent array Chip_10k_v1, E-TABM-38); and (iii) "Seeger dataset" composed of 102 samples (Affymetrix HG-U133A and HG-U133B array, GSE16254). Median centered normalization was used for all gene expression data. This allowed us to perform survival analysis on the three datasets combined. However, to overcome any biases due to the different platforms used in each dataset, we also performed a meta-analysis on the summary statistics (see below). Analysis of association between *NEFL* expression and neuroblastoma stages was performed using normalized microarray data freely available at the GEO database (GSE45547, GSE14880). Data for tumor stages were available.

Statistical analysis

Hardy–Weinberg equilibrium was evaluated using the goodness-of-fit χ^2 test in control subjects. For genotyped SNPs, two-sided χ^2 tests were used to evaluate differences in the distributions of allele frequencies between all patients and controls. ORs and 95% CIs were calculated to assess the relative disease risk conferred by a specific allele. Combined analysis was performed using the weighted Z-score method in METAL based on sample size, P value, and direction of effect in each study (19). The Student t test was used to compare the differences in the mRNA expression levels. LD and haplotype analyses were performed using the website tool SNAP v2.2 (http://www.broadinstitute.org/mpg/snap/index.php; ref. 20) and Haploview v4.2 software (21). qRT-PCR data were analyzed by the $2^{-\Delta Ct}$ method as described in our previous article (22). To test the association of gene expression levels with overall survival, individual gene expression profiles were dichotomized by median split into

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"high" or "low" expression groups, and Kaplan–Meier survival curves were plotted for each group. Cox regression analysis was applied to calculate HR for meta-analysis. A test of heterogeneity of combined HRs was carried out using Cochran Q test (significant at $P < 0.05$) and Higgins I^2 statistic. The I^2 of >50% were considered to represent significant heterogeneity. Given the absence of heterogeneity among studies ($P > 0.1$), a fixed effects generic inverse variance model was used. Meta-analysis was performed using Review manager 5.0 (http://www.cochrane.org).

Results

Biologically driven gene-based association analysis

Eight genes shown to be associated with neuroblastoma differentiation (11) were tested for genetic associations with neuroblastoma risk. The gene-based test performed on the discovery set (2,101 cases and 4,202 controls) showed that the two genes *NEFL* and *SCG2* were significantly associated with neuroblastoma susceptibility (Table 1). However, *NEFL* had eight SNPs, five of which were significant, but only two of which were independently significant based on an r^2 threshold of 0.5; whereas *SCG2* had seven SNPs, but only one was significant.

Prioritization of SNPs in *SCG2* and *NEFL*

To select putative functional SNPs to be further validated in the Italian cohort, we examined the genetic association and the predicted biologic role of SNPs at both loci. At the *SCG2* locus (surrounding region ± 20 kb), only SNP rs4673067 resulted to be significant (data not shown). SNP function prediction analysis using the Web tool SNPinfo Web Server (23) showed no relevant function for this SNP and those in LD with it ($r^2 >$ 0.60; Supplementary Table S1). At the *NEFL* locus, three intergenic and two flanking (in the 3′ downstream and promoter regions) polymorphisms were significantly associated with neuroblastoma (Table 2, Fig. 1A). SNP function prediction analysis (23) showed that none of these five SNPs had putative biologic function (data not shown). Thus, we carried out a prioritization functional analysis on SNPs in LD ($r^2 > 0.60$) with the five typed SNPs (Supplementary Tables S2–S6). The results indicated that three polymorphisms within the *NEFL* 3′UTR (rs3761, rs2979704, and rs1059111) had a potential biologic effect (Table 2, Fig. 1A). Each of these SNPs was located in evolutionary conserved regions and predicted to influence the binding of diverse transcription factors and microRNAs (Supplementary Tables S7–S9). In contrast, the analysis by UTRscan (http://itbtools.ba.itb.cnr.it/utrscan) showed that none of the abovementioned SNPs altered sequence characteristics of the specific UTR motifs (such as: polyadenylation signal, AU rich element, Selenocysteine Insertion Sequence and others reported in the Supplementary Table S10). Taken together, *in silico* data suggest that the functional role of SNPs located in the 3′ UTR of *NEFL* are likely due to alteration of miRNA binding sites, enhancer elements, or both. Notably, rs3761 was in LD with three significant typed tag-SNPs (rs118727, rs196830, and rs17830286), whereas rs2979704 and rs1059111 (in strong LD with each other; $r^2 = 1$; Supplementary Fig. S1) were in LD with only one typed tag-SNP (rs11994014). To further verify if other functional SNPs could be associated

Table 2. Associations and functional prioritization of SNPs in *NEFL* and its surrounding region (±20 kb)

	SNP association in GWAS dataset									SNP functional prioritization				
SNP	Role	A1	F_A	F_U	A2	P	OR	L95	U95	Proxy SNP ID	Proxy SNP function	r^{2a}	RegPotential score	Conservation score
rs118727	Intergenic	C	0.14	0.12	T	**0.0026**	1.18	1.06	1.32	rs3761	*NEFL*/3′-UTR	1	0.21	0.132
rs196830	Intergenic	C	0.15	0.12	A	**0.0012**	1.19	1.07	1.33	rs3761	*NEFL*/3′-UTR	1	0.21	0.132
rs169061	Intergenic	C	0.40	0.43	T	**0.0008**	0.88	0.81	0.95	no-LD[b]	—	—	—	—
rs11994014	Downstream	A	0.20	0.22	G	**0.0368**	0.91	0.83	0.99	rs2979704[c] and rs1059111	*NEFL*/3′-UTR	0.63	0.07 and 0.36	0.96 and 1.00
rs2979685	Promoter	T	0.36	0.36	G	0.8723	1.01	0.93	1.09	—	—	—	—	—
rs17830286	Promoter	T	0.16	0.15	C	**0.0085**	1.15	1.04	1.27	rs3761	*NEFL*/3′-UTR	0.64	0.21	0.132
rs13254844	Promoter	T	0.05	0.05	C	0.5270	0.95	0.80	1.12	—	—	—	—	—
rs17830392	Promoter	G	0.08	0.07	A	0.2101	1.09	0.95	1.26	—	—	—	—	—

Abbreviations: A1, minor allele; A2, major allele; F_A, minor allele frequency in cases; F_U, minor allele frequency in controls L95, lower bound of 95% CI for OR; U95, upper bound of 95% CI for OR; RegPotential, regulatory potential score (ESPERR regulatory potential based on 7 species) downloaded from UCSC genome bioinformatics website; conservation, vertebrate Multiz Alignment and Conservation score (17 Species) downloaded from UCSC genome website. Bold indicates the significant P-values.

[a]Cutoff = 0.60.

[b]No SNP was found in LD with $r^2 > 0.60$.

[c]rs2979704 and rs1059111 are in strong LD ($r^2 = 1$).

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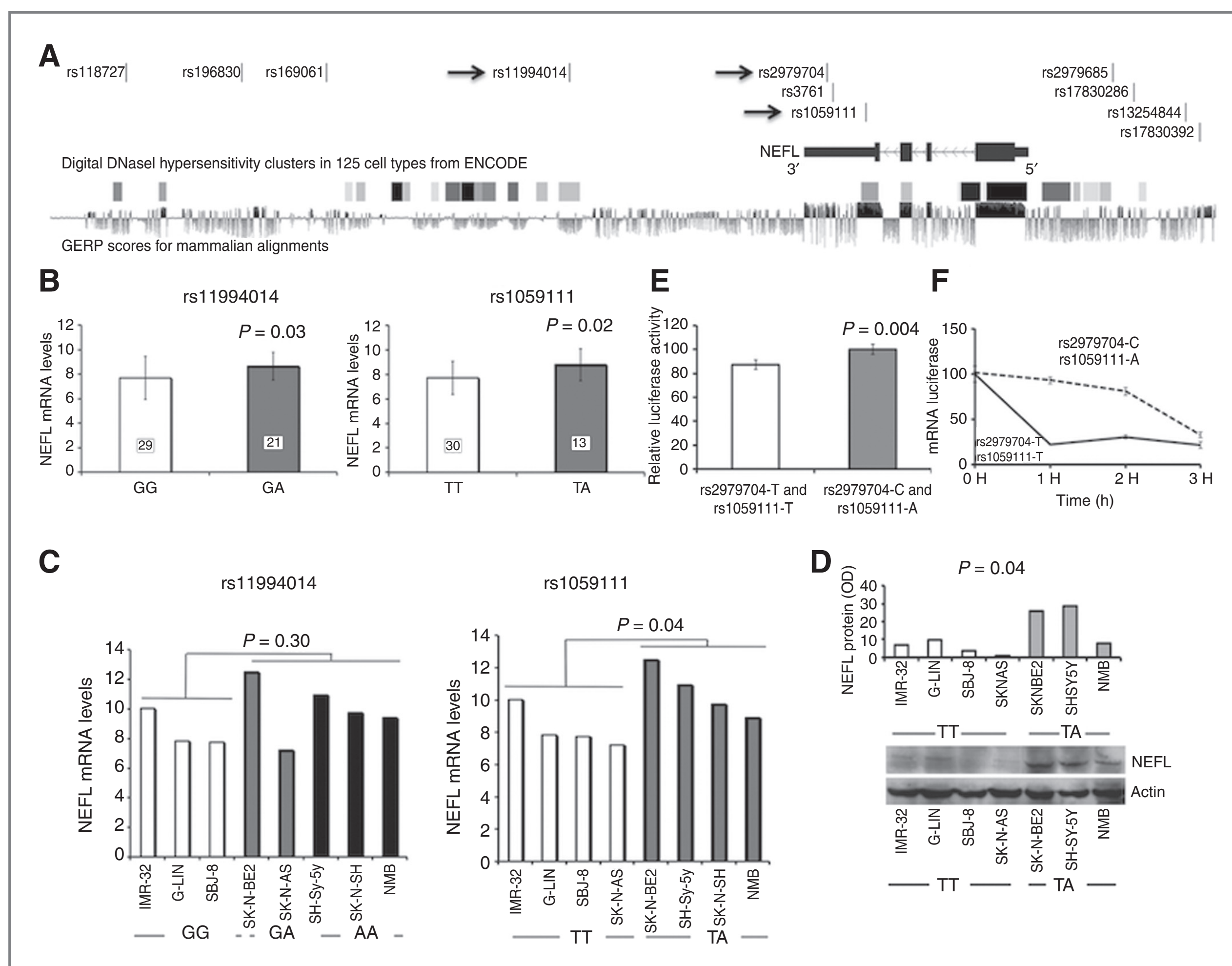

Figure 1. *NEFL* genotype and gene expression association. A, plot showing *NEFL* gene and analyzed SNPs. Arrows, SNPs validated in the Italian population. DNase hypersensitive areas in 125 cell types from ENCODE data and GERP score for mammalian alignment (data retrieved from UCSC genome browser, https://genome.ucsc.edu/) are also plotted. B, microarray-based expression profiling on primary tumors demonstrates that *NEFL* expression correlates with rs11994014 (GG, 7.7 ± 1.7; and GA, 8.6 ± 1.1) and rs1059111 genotype (TT, 7.7±1.3; and TA, 8.8 ± 1.3). The genotypes of rs1059111 have been imputed (see Supplementary Information). C and D, qRT-PCR (rs11994014 GG, 8.5 ± 1.3; rs11994014 GG/GA, 10.0 ± 1.9; rs1059111 TT, 8.2 ± 1.2; rs1059111 TA, 10.6 ± 1.4; C) and Western blotting (rs1059111 TT, 5.3 ± 3.7; rs1059111 TA, 20.8 ± 11.2; D) analysis on neuroblastoma cell lines confirms the association between rs1059111 protective genotype and *NEFL* expression. Data are shown as mean ± standard deviation. E, luciferase report gene assay carried out in HEK293 cells confirms the same association (rs1059111 T, 87.1 ± 3.8; rs1059111 A, 100 ± 4.4). Data shown in percentage are the mean ± standard deviation from nine independent transfection experiments, each done in triplicate and compared with promoter less control F, analysis of 3′UTR-allelic mRNA stability in HEK293 cells. The data are presented as the means value ± standard deviation from three independent experiments ($P < 0.05$).

with neuroblastoma, we used the 1000 Genomes data to impute all of the common variants (MAF > 0.05) across *SCG2* and *NEFL*. No significant association was found at the *SCG2* locus (Supplementary Table S11). The rs3761 SNP in *NEFL* resulted to be the strongest significantly associated with neuroblastoma. Other two SNPs (rs62503767 and rs62503769) were significant (Supplementary Table S11) but no relevant biologic function was predicted (data not shown). Together, these data led us to exclude *SCG2* SNPs from further analyses and to focus our attention on *NEFL* SNPs.

Replication study

We genotyped and tested for association in 459 neuroblastoma patients and 809 controls of Italian origin two tag-SNPs (rs196830, the most significant among the three tag-SNPs, and rs11994014), the three prioritized SNPs (rs3761, rs2979704, and rs1059111), in addition to the significant typed SNP not in LD with other SNPs (rs169061; Table 3). Only the tag-SNP rs11994014 and the linked functional SNPs rs2979704 and rs1059111 confirmed the associations with neuroblastoma (Table 3). Specifically, the minor alleles rs11994014-A, rs2979704-C, and rs1059111-A were associated with a decreased risk of neuroblastoma ($P_{\text{combined}} = 0.0050$; OR, 0.88; 95% CI, 0.81–0.96; $P_{\text{combined}} = 0.0072$; OR, 0.87; 95% CI, 0.78–0.96; $P_{\text{combined}} = 0.0049$; OR, 0.86; 95% CI, 0.77–0.95). All of these SNP alleles were associated with the favorable clinical parameter age at diagnosis < 18 months in the European American cohort (Supplementary Table S12) but not in the

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Table 3. SNP associations at the *NEFL* locus

SNP	Role	European Americans					Italians						Combined						
		A1	F_A	F_U	A2	*P*	OR	L95	U95	F_A	F_U	*P*	OR	L95	U95	Meta *P*	OR	L95	U95
rs196830	Intergenic	C	0.146	0.125	A	0.0012	1.19	1.07	1.33	0.11	0.12	0.5189	0.92	0.70	1.20				
rs169061	Intergenic	C	0.396	0.427	T	0.0008	0.88	0.81	0.95	0.46	0.50	0.0351	1.20	1.01	1.41				
rs11994014	Downstream	A	0.200	0.216	G	**0.0368**	**0.91**	0.83	0.99	0.26	0.30	**0.0230**	**0.78**	0.64	0.95	**0.0050**	0.88	0.81	0.96
rs2979704[a]	3′UTR	C	0.122	0.132	T	**0.0877**	**0.91**	0.81	1.02	0.15	0.19	**0.0049**	**0.72**	0.57	0.90	**0.0072**	0.87	0.78	0.96
rs1059111[a]	3′UTR	A	0.122	0.132	T	**0.0876**	**0.91**	0.81	1.02	0.14	0.19	**0.0016**	**0.69**	0.55	0.87	**0.0049**	0.86	0.77	0.95
rs3761[a]	3′UTR	A	0.139	0.121	G	0.0043	1.17	1.04	1.30	0.11	0.13	0.3286	0.87	0.67	1.15				

NOTE: Meta-analysis *P* value calculated using METAL.

Abbreviations: A1, minor allele; A2, major allele; F_A, minor allele frequency in cases; F_U, minor allele frequency in controls; L95, lower bound of 95% CI for OR; U95, upper bound of 95% CI for OR.

[a]Imputed SNPs in the European American GWAS dataset.

Italian cohort (Supplementary Table S13). This may be due to the relatively low number of Italian cases in the clinical subgroups. We found no significant correlation between the associated SNPs and other clinical covariates such as risk group, INSS stage, and *MYCN* status.

Functional analysis of significant SNPs

To investigate if the neuroblastoma-associated SNPs have a *cis*-effect on *NEFL*, we tested for SNP-gene expression associations at tag-SNP rs11994014 and prioritized SNP rs1059111 using different approaches. For these analyses, we chose the putative functional polymorphism rs1059111 because it showed the highest scores for potential regulation and conservation and it is predicted to alter miRNA binding sites (Table 2, Fig. 1A and Supplementary Table S8). Moreover, an additional analysis using HaploReg v2 (24) showed that rs1059111 resides within a DNase hypersensitive area in the SK-N-BE2c neuroblastoma cell line, the highest conserved region among the regions of other SNPs (Fig. 1A) and is predicted to alter the regulatory motifs of the transcriptional factor neuron restrictive silencer factor (NRSF; Supplementary Table S9). Interestingly, the *NEFL* gene expression increased when we silenced NRSF in two neuroblastoma cell lines (SH-SY5Y and SK-N-BE2c; Supplementary Fig. S2A–S2D). These findings strengthen the role of NRSF as oncogene and repressor of differentiation in neuroblastoma (25, 26) and provide evidences to support the role of *NEFL* in neuroblastoma differentiation. The analysis of gene expression variation using genome-wide expression and SNP arrays of neuroblastoma tumors demonstrated that both SNPs affect expression of *NEFL*. In particular, the presence of the protective alleles (A and A) for SNPs rs11994014 and rs1059111 significantly correlated with increased *NEFL* mRNA expression (Fig. 1B). A qRT-PCR gene expression analysis was performed in neuroblastoma cell lines. The mRNA expression of *NEFL* was significantly higher in neuroblastoma cell lines heterozygous at rs1059111 (TA; Fig. 1C). Only a trend toward association between high mRNA levels and presence of the protective allele A was observed for rs11994014 without reaching the threshold for statistical significance (Fig. 1C). These results were confirmed using freely available data (http://www.broadinstitute.org/ccle) on gene expression and SNP arrays for 16 neuroblastoma cell lines (Supplementary Fig. S3A) and for 198 lymphoblastoid cell lines (Supplementary Fig. S3B). The correlation of SNP rs1059111 with *NEFL* expression was validated by Western blotting (Fig. 1D). Induction of 3′UTR activity of the construct containing rs2979704-C and rs1059111-A alleles was higher than that of the construct containing T alleles as assessed by luciferase report gene assay (Fig. 1E). Finally, *NEFL* mRNA abundance from clones containing the rs2979704-C and rs1059111-A alleles after actinomycin-D addition was significantly higher than expression from clones containing the T alleles (Fig. 1F). Together, these data indicate that the A allele of SNP rs1059111 in 3′UTR of *NEFL* confers a decreased neuroblastoma risk and induces gene overexpression in neuroblastoma tumors compared with the alternative allele T. This is probably due to the alteration of miRNA binding site, enhancer elements, or both. Further investigations are needed to address

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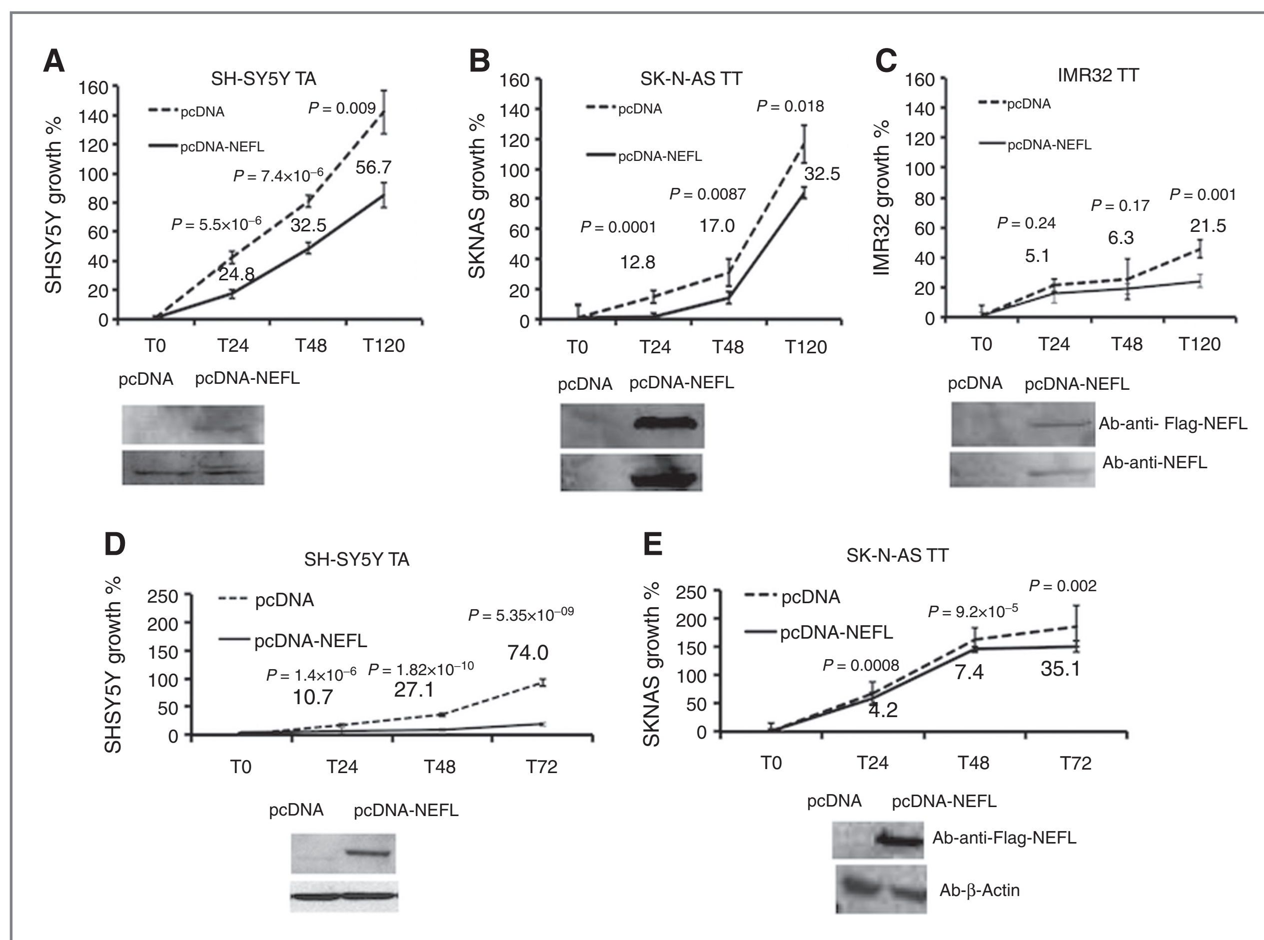

Figure 2. The protective allele A of the SNP rs1059111 is associated with cell growth disadvantage through increased *NEFL* expression. A, in the cells with neuroblastoma protective allele and higher *NEFL* expression levels, *NEFL* transient overexpression leads to inhibition of cellular growth. B and C, in cells homozygous for the risk allele that have low *NEFL* expression, the growth inhibition is less marked. Detection of NEFL protein by using antibody against Flag and NEFL assessed the *NEFL* transiently overexpression in SH-SY5Y, SK-N-AS, IMR32 cell lines. The variability of the forced NEFL expression may to be due to the different transfection efficiency of the three different cell lines. D and E, we thus produced a stable overexpression of *NEFL* in SH-SY5Y and SK-N-AS cells to obtain a further validation. The experiments were performed in three diverse clones for each experimental point and the shown results are representative of the mean among the different clones. The numbers represent the cell number difference between control and *NEFL* overexpression. Times (*T*) represent the hours.

this issue. Moreover, transient overexpression of *NEFL* resulted in significant growth inhibition in neuroblastoma cell heterozygous for the rs1059111 protective A allele that had high *NEFL* expression (Fig. 2A). In the cell lines homozygous for the risk T allele that had low *NEFL* expression, the growth inhibition was less marked (Fig. 2B and C). The same results were confirmed in cell lines with stable expression of *NEFL* (Fig. 2E and D). These data suggest that the protective alleles are associated with growth disadvantage through increased *NEFL* expression. We have also evaluated cell growth and invasion after *NEFL* silencing in two TA genotype cell lines (SH-SY5Y and SK-N-BE2c). We observed no effect on cell growth, but increased cell invasion was evident in both the cell lines (Supplementary Fig. S4A–S4C). We hypothesize that decrease of cell growth observed in cell lines with enforced expression of *NEFL* (Fig. 2A) is probably due to the effect of cell conversion into more differentiated status.

Functional analysis of *NEFL* overexpression

To unravel *NEFL* contribution to neuroblastoma tumorigenesis and progression, we generated *NEFL* stable clones in two cell lines heterozygous for the protective minor allele (SH-SY5Y) and homozygous for the risk major allele (SK-N-AS). SH-SY5Y *NEFL* stable clones showed an enhanced differentiated phenotype as assessed by increased neurites length, whereas SK-N-AS *NEFL* stable clones became flatter and larger than control pcDNA clones (Fig. 3A and B). Moreover, the expression of neuronal differentiation markers is also enhanced by *NEFL* overexpression in SH-SY5Y cells but not in SK-N-AS cells (Fig. 3C). The analysis using freely available data (http://www.broadinstitute.org/ccle) showed that three of these neuronal markers were upregulated in neuroblastoma cell lines with the protective allele and high level of *NEFL* basal expression (Supplementary Fig. S5). In both *NEFL* overexpressing cell lines, we observed a reduced proliferation as assessed by the

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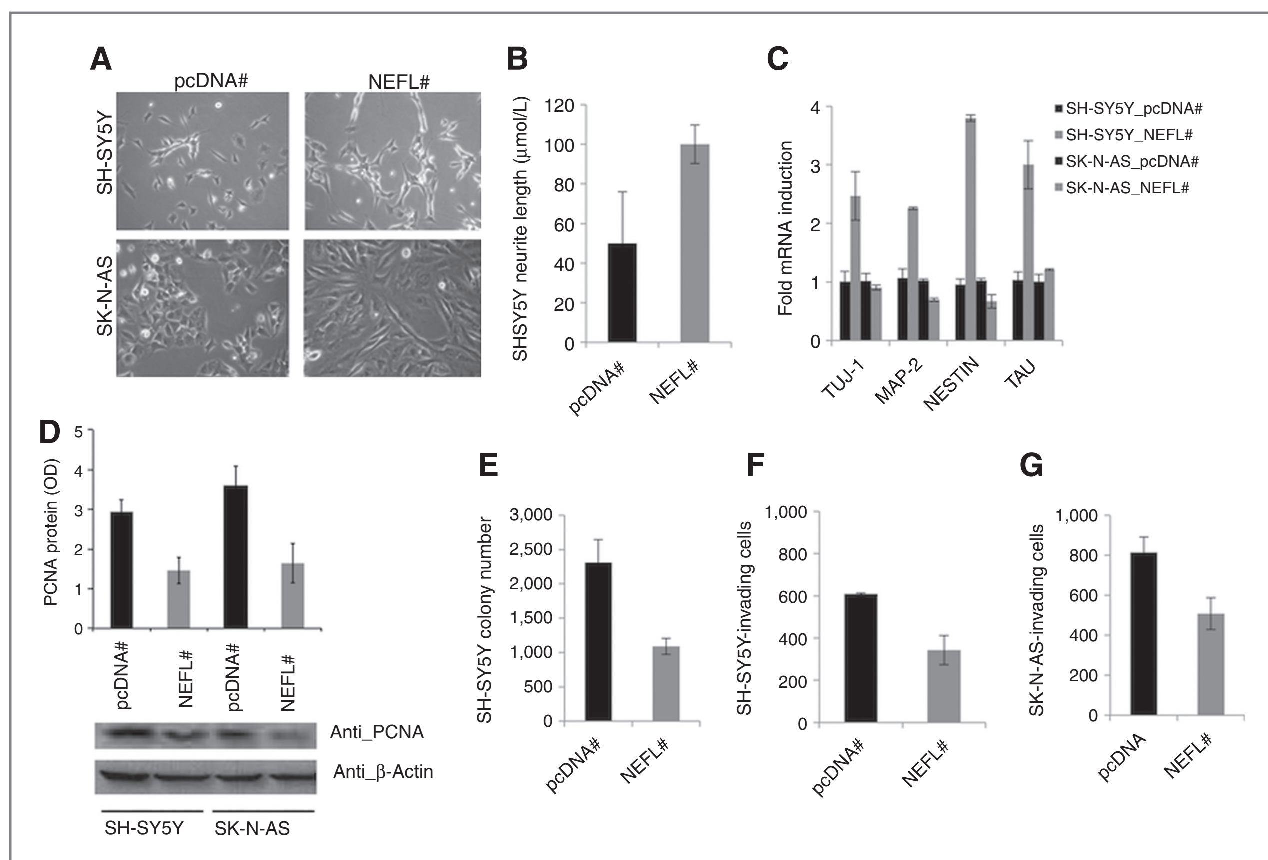

Figure 3. Different phenotypic effect of the *NEFL* overexpression in two neuroblastoma cells with protective and risk genotype for the SNP rs1059111. A, SH-SY5Y (TA genotype) *NEFL*-overexpressing stable clones show longer neurites processes than pcDNA control cells, whereas SK-N-AS (TT genotype) *NEFL*-overexpressing stable clones become flatter. B, neurites length in SH-SY5Y cell clones were measured from the cell body to the furthest tip of the process using LAS/AF tool (Leica Microsystems). C, the expression of neuronal differentiating markers is enhanced in SH-SY5Y *NEFL* stable clones, whereas it does not change in SK-N-AS *NEFL* stable clones, as shown by qRT-PCR. D, the expression of PCNA is decreased in *NEFL* stable clones in both cell lines. E, *NEFL* overexpression impairs the growth in soft agar of SH-SY5Y stable clones as shown by a decreased number of colonies with respect to pcDNA cells. *NEFL* impairs the number of invading cells in two-dimensional invasion assay in SH-SY5Y (F) and SK-N-AS (G) stable clones. The experiments were performed in three diverse clones for each experimental point and the shown results are representative of the mean among the different clones. OD, optical density.

PCNA Western blotting analysis (Fig. 3D) but not an increased caspase activity (data not shown). In an experiment of anchorage-independent growth in soft agar, we observed that *NEFL* overexpression in SH-SY5Y cells impaired the growth ability (Fig. 3E). In contrast, SK-N-AS cells showed no or very little anchorage-independent growth in soft agar (data not shown). *NEFL* overexpression in SH-SY5Y (Fig. 3F) and SK-N-AS (Fig. 3G) cells impaired the migratory ability in invasion assay. These findings suggest that different phenotypic effect of the overexpression might be due to underlying genotype and basal *NEFL* expression in the two neuroblastoma cell lines.

Correlation analysis of *NEFL* gene expression with clinical outcome

To examine the relevance of *NEFL* in patients, we investigated if gene expression was associated with clinical outcome. Analysis of three publicly available gene expression array data of neuroblastoma showed that high *NEFL* expression is associated with better survival (Fig. 4A–D, $P_{\text{combined}} = 0.04$; Fig. 4E, $P_{\text{meta-analysis}} = 0.03$; HR, 0.68; 95% CI, 0.48–0.97), well-differentiated tumors (Fig. 4F, $P = 0.002$), and favorable stages (Fig. 4G, stage I vs. stage IV; $P = 2.3 \times 10^{-4}$). No significant association was found between *NEFL* expression and the prognostic variables age at diagnosis, *MYCN* status, and risk group (data not shown).

Discussion

Further investigation of eight genes that we previously identified as being essential mediators of neuroblastoma differentiation has identified *NEFL* as a susceptibility gene. Particularly, the minor allele of SNP rs1059111 correlates with increased levels of *NEFL* and confers protection against neuroblastoma development, and is associated with a more benign phenotype in those that do develop. *In silico* analyses suggest that the SNP rs1059111 may alter the binding site of microRNAs and transcription factors. However, *in vitro* validation experiments of these data need to be performed in future

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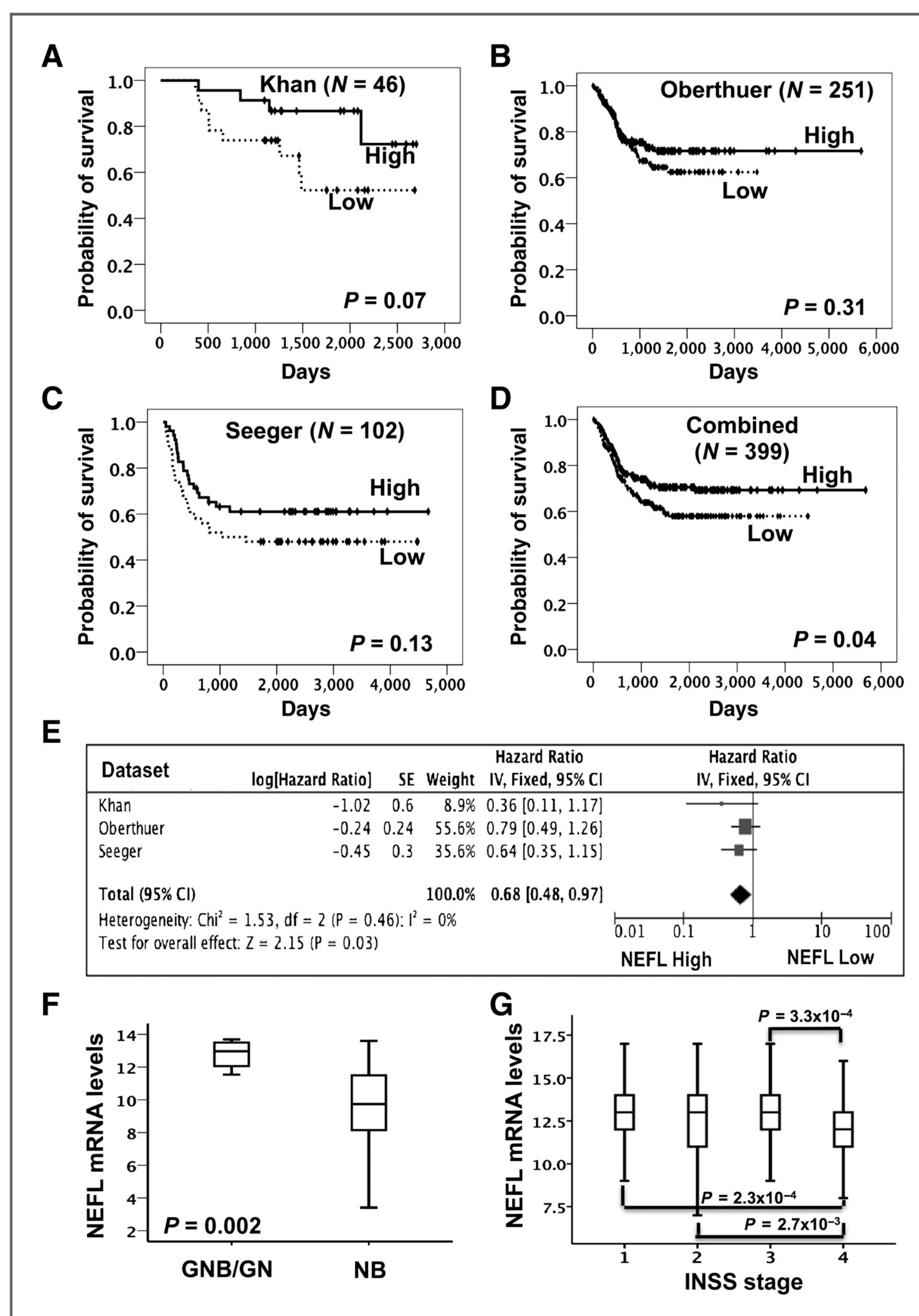

Figure 4. *NEFL* expression is associated with good outcome and favorable neuroblastoma stages. A–C, Kaplan–Meier analysis using published array data (Oncogenomics laboratory Web tool). D, Kaplan–Meier analysis using combined array data from the three independent sets. E, meta-analysis of HRs from the Cox regression analysis of each dataset. F and G, changes in expression for *NEFL* in ganglioneuroblastoma (GNB) and ganglioneuroma (GN; F) and in favorable stage using published array data (GEO database; G).

studies. Forced overexpression of *NEFL* enhanced cellular differentiation in cells with the protective allele and basal *NEFL* expression, but had little effect on cells with the risk genotype and undetectable basal *NEFL* expression. Silencing of *NEFL* in cells with the protective allele did not affect cell proliferation but induced cell invasion. Here, we present preliminary data providing evidence that *NEFL* expression sustains differentiated phenotype, impairs soft agar growth and proliferation in cells with the protective allele, whereas impairs invasiveness and proliferation in cells homozygous for the risk major allele. Accordingly, high *NEFL* mRNA expression levels correlate with well-differentiated tumors and show association with a better clinical outcome. This suggests a dual function for *NEFL*: suppressing cancer initiation and also cancer progression. Taken together, these data provide direct evidence that a functional DNA variant in the 3′UTR region of *NEFL* influences neuroblastoma susceptibility and that low expression of *NEFL* plays a role in malignant neuroblastic transformation and disease progression, likely by disrupting a normal neuronal differentiation program.

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The *NEFL* gene encodes a type IV intermediate filament heteropolymer that functionally maintains the neuronal caliber and plays an important role in the intracellular transport of neurotransmitters to axons and dendrites (27). Mutations in *NEFL* cause one of the most severe Charcot–Marie–Tooth disease phenotypes (28). Recently, several studies have proposed *NEFL* as a tumor-suppressor gene. Indeed, deletions of the chromosomal region 8p21, where *NEFL* is located, have been found in different cancer diseases such as prostate cancer (29–32), breast cancer (33–35), colorectal cancer (36, 37), hepatocellular carcinoma (38), and squamous cell carcinoma of the head and neck (HNSCC; refs. 39, 40). Very recently, Chen and colleagues have demonstrated that in HNSCC, NEFL protein is physically associated with tuberous sclerosis 1 (TSC1), and *NEFL* downregulation by methylation process leads to functional activation of mTOR pathway and consequentially confers cisplatin resistance (41). The same research group also showed that *NEFL* expression induces cancer cell apoptosis and inhibits invasion in HNSCC cell lines (42). In agreement with our findings, literature data strongly suggest that *NEFL* might play a critical role in suppressing cancer initiation and/or progression.

Neuroblastoma can be considered a malignancy due, at least in part, to a loss of normal differentiation pathways (12). Here, we suggest that downregulation of *NEFL* expression through constitutional DNA variation can predispose to neuroblastoma development because low levels of *NEFL* can affect the normal differentiation of sympathetic neurons. As a consequence, the derivate immature cells may be susceptible to secondary mutations that could ultimately lead to neuroblastoma. In agreement with our hypothesis, two research groups have already reported that genetic events affecting the dosage of neuronal prodifferentiation genes might lead to the accumulation of a cell population that is unable to differentiate and can thus acquire the necessary transforming genetic or epigenetic aberrations (43, 44). Wu and colleagues have demonstrated that genes involved in brain development and neuronal differentiation, such *as BMP4, POU4F3, GDNF, OTX2, NEFM, CNTN4, OTP, SIM1, FYN, EN1, CHAT, GSX2, NKX6-1, PAX6, RAX*, and *DLX2*, are strongly enriched among genes frequently methylated in astrocytomas (44). Pei and colleagues showed that a reduced dosage of *PHOX2B* during development, through either a heterozygous deletion or dominant-negative mutation, imposes a block in the differentiation of sympathetic neuronal precursors, resulting in a cell population that is likely to be susceptible to secondary transforming events (43). Even if our findings suggest that low *NEFL* expression can play a role in tumor initiation by affecting neuronal differentiation, we do not exclude that *NEFL* can be involved in different molecular mechanisms whose alteration can contribute to tumor initiation and progression in neuroblastoma. Indeed, NEFL has been found to interact with proteins such as TRIM2 (45) and TSC1 (41) with high relevance in cancer and that participate in various important cellular processes.

Interestingly, another gene mutated in Charcot–Marie–Tooth disease, *KIF1B* (28), seems to act as a haploinsufficient tumor suppressor, and its downregulation might potentially contribute to tumorigenesis of cancers, including neuroblastoma (46, 47). These data strengthen the hypothesis that alteration in normal expression of genes involved in neuronal development can induce neuroblastoma carcinogenesis.

In summary, we have identified *NEFL* as a novel susceptibility gene for neuroblastoma starting from the analysis of a set of genes involved in neuronal differentiation and using data from a neuroblastoma GWAS. Our results support the hypothesis that downregulation of *NEFL* gene expression through functional hereditable DNA variation can contribute to malignant transformation of sympathetic progenitor cells. Moreover, we provide evidence that *NEFL* expression enhances differentiation and impairs cancer progression in neuroblastoma cell lines. This study has demonstrated that a hypothesis-driven GWAS follow-up study is a useful strategy for identifying novel disease susceptibility genes and that genetic and functional datasets can be merged to maximize discovery efforts. GWASs so far have led to substantial advances in our understanding of the role of common variation in complex disease and have identified previously unknown pathogenic pathways in neuroblastoma (4–8, 48) and other disorders (49). However, many disease-associated variants remain unknown or functionally uncharacterized and biologic implications of risk-associated variants in pathogenesis are largely unknown (49). The methodologic approach here presented might be a useful tool to overcome some of the limits emerged from GWAS studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Capasso, H. Hakonarson, A. Iolascon, J.M. Maris
Development of methodology: F. Cimmino, F. Totaro, L. Pezone, J.M. Maris
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Hakonarson, J.M. Maris
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Capasso, S. Diskin, G. Acierno, G. Petrosino, L. McDaniel, H. Hakonarson, M. Devoto, J.M. Maris
Writing, review, and/or revision of the manuscript: M. Capasso, S. Diskin, H. Hakonarson, A. Iolascon, M. Devoto, J.M. Maris
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Diamond, J.M. Maris
Study supervision: J.M. Maris

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Common Genetic Variants in *NEFL* Influence Gene Expression and Neuroblastoma Risk

Mario Capasso, Sharon Diskin, Flora Cimmino, et al.

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