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ORIGINAL ARTICLE

KIAA0319 and *ROBO1*: evidence on association with reading and pleiotropic effects on language and mathematics abilities in developmental dyslexia

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Substantial heritability has been reported for developmental dyslexia (DD), and *KIAA0319* and *ROBO1* appear as more than plausible candidate susceptibility genes for this developmental disorder. Converging evidence indicates that developmental difficulties in oral language and mathematics can predate or co-occur with DD, and substantial genetic correlations have been found between these abilities and reading traits. In this study, we explored the role of eight single-nucleotide polymorphisms spanning within *KIAA0319* and *ROBO1* genes, and DD as a dichotomic trait, related neuropsychological phenotypes and comorbid language and mathematical (dis)abilities in a large cohort of 493 Italian nuclear families ascertained through a proband with a diagnosis of DD. Marker-trait association was analyzed by implementing a general test of family-based association for quantitative traits (that is, the Quantitative Transmission Disequilibrium Test, version 2.5.1). By providing evidence for significant association with mathematics skills, our data add further result in support of *ROBO1* contributing to the deficits in DD and its correlated phenotypes. Taken together, our findings shed further light into the etiologic basis and the phenotypic complexity of this developmental disorder.

INTRODUCTION

Developmental dyslexia (DD) is a complex heritable condition typically diagnosed in the first school years characterized by an impairment in reading abilities in spite of normal intelligence and adequate educational opportunities.

Converging evidence from high-risk and longitudinal studies indicates that early developmental problems in oral language[1–7] and mathematical skills[8–10] can constitute associated features of DD. Strong genetic correlations between language, mathematical and reading traits have been consistently reported,[8,10–14] indicating that the degree of overlap in genetic influences is comparable across different regions of the liability distribution.

Even if addressing the issue of pleiotropy is among the major aims of contemporary genetic research on cognitive sciences,[15–17] the identification of elements supporting shared genetic influence is a less straightforward question. Few molecular genetic studies have been conducted for language and mathematics[18,19] compared with the extensive molecular genetic literature on DD. As substantial heritability

has been indeed reported for DD and DD-related traits,[15,20] at least nine DD loci have been mapped and specific identified genes have been consistently reported to be DD candidate susceptibility genes.[21]

A DD susceptibility locus on the short arm of chromosome 6, that is, DYX2, has been reported by at least five independent studies.[21] From these studies of DYX2, there are two genes that stand out: *KIAA0319* and *DCDC2*. Since the first significant association findings,[22] several studies tested *KIAA0319* for association with DD and DD-related traits with both positive[23–33] and negative results[34,35] in clinical samples, as well as in the general population.[36,37] Furthermore, a three-single-nucleotide polymorphism (SNP) risk haplotype has been shown to associate with neuropsychological skills in reading impaired families,[21] likewise in general population samples.[26,36] Finally, recent linkage studies reported significant findings for specific language impairment[38,39] and speech sound disorder[40] on this genomic region.

Linkage to the peri-centromeric region of chromosome 3 has been observed for DD susceptibility in three independent genome-

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wide screens.[41] Hannula-Jouppi et al.[42] provided the primary evidence supporting ROBO1 as a DD candidate gene, reporting the observation of a translocation t(3;8)(p12;q11) in an individual who also had DD, and of a 35-Mb haplotype cosegregating with DD in the large Finnish family and including ROBO1. Recently, Bates et al.[43] found associations between several SNPs of ROBO1 and reading-related measures in a general population sample, indicating that ROBO1 can be involved in language acquisition. A negative association between markers spanning within the ROBO1 and the diagnosis of DD have been newly found in a case–control study in an Indian sample.[44] Furthermore, significant linkage results have been reported for specific language impairment[38] and speech sound disorder[45] to the DD locus on chromosome 3.

Finally, and perhaps of more interest, for both the risk variants mentioned hitherto, there is initial evidence of a proper functional role. In the presence of the risk haplotype on chromosome 6p21, the expression of the KIAA0319 gene is specifically reduced.[46] This haplotype harbors the rs9461045 minor allele, which confers reduced luciferase expression in both neuronal and non-neuronal cell lines, and is implicated in a nuclear protein-binding site.[28] By knocking down the expression of this specific nuclear protein-binding site, the risk haplotype KIAA0319 expression can be almost entirely rescued.[28] Functional studies have also revealed that KIAA0319 likely involved in neuronal migration and axon growth,[47–55] which is in turn consistent with the original neuroanatomical finding of focal architectonic dysplasias and neuronal ectopias in the brains of people with DD.[56,57] Some further support is offered by a recent animal study showing that embryonic RNA interference of KIAA0319 expression resulted in rapid auditory processing and spatial learning deficits.[51,52] Regarding the ROBO1 gene, Hannula-Jouppi et al.[42] demonstrated that its expression is significantly reduced by the 35-Mb risk haplotype, which is associated with DD in the large Finnish family.

The bulk of results reviewed so far shows that KIAA0319 and ROBO1 genes appear as more than plausible candidates for DD, DD-related neuropsychological phenotypes and DD-correlated traits. We already yielded evidence about the effects of the other two well-replicated DD candidate genes (that is, DYX1C1 and DCDC2) on DD and DD-related and DD-correlated phenotypes in an Italian clinical sample.[58–60] In this study, we assessed the possible association with specific aspects of the phenotypic profile of DD and eight SNPs within KIAA0319—including those forming the three-SNP haplotype tested by Francks et al.[23]—and ROBO1, in order to further investigate the contribution of these genes to DD and DD-related phenotypes in an Italian sample. Moreover, we investigated the pleiotropic effects of the selected KIAA0319 and ROBO1 markers while using concurrent measurements of language and mathematics abilities.

MATERIALS AND METHODS

The protocol was approved by the Scientific Institute, IRCCS Eugenio Medea Bioethics and Scientific Board.

Sample

This study is part of an ongoing project on the genetics of reading disabilities at the Child Psychopathology Unit, Scientific Institute, IRCCS Eugenio Medea, Bosisio Parini, Lecco, Italy, and at the Centro Regionale di Riferimento per i Disturbi dell'Apprendimento—CRRDA ('Regional Reference Center for the Specific Learning Disability'), ULSS 20, Verona, Italy.[58–64]

The ascertainment scheme has been reported in detail elsewhere.[61] Briefly, nuclear families were recruited if probands met the criteria for DD according to the DSM-IV.[65] In particular, the inclusion criteria for the present study were (1) either accuracy or speed < -2.00 s.d. on timed text reading, single words reading or single non-word reading tests, (2) a full-scale IQ above 84 and (3) absence of neurological or sensorial disorders.

After parental informed consent, the offspring underwent an extensive medical assessment and a battery of tests, which evaluate text, word and non-word reading,[66–68] writing under dictation of word, non-word and sentences-containing homophones,[68] forward/backward digit span,[69] phonemic elision and blending,[70] language abilities,[71] mathematics abilities[72,73] and the Wechsler Intelligence Scale for Children, Revised[74] or the Wechsler Intelligence Scale for Children, third edition.[75] For all tests, standardized scores on the Italian population are provided (for an extensive description of tests, see Supplementary Information 1). Siblings were administered only two subtests of the intelligence scale, that is, vocabulary and block design, that show a high correlation (r) with, respectively, verbal IQ ($r = 0.82$)[74,75] and performance IQ ($r = 0.73$),[74,75] and were included if they were fully biological, older than 6 years and younger than 18 years, if they had no history of neurological and sensorial disorders and if the mean score of vocabulary and block design subtests was > 7, regardless of their reading performance. Blood or mouthwash samples were obtained from all offspring and their biological parents.

Phenotypes

Eighteen phenotypes were used in association analyses (for a detailed description, see Supplementary Information 1): DD as a discrete trait, READING-Accuracy, READING-Speed, SPELLING, Auditory STM, ELISION, BLENDING, five language abilities (that is, semantic comprehension, Token test, syntactic comprehension, rapid automatized naming and semantic fluency) and six mathematics abilities (that is, mental calculation, both accuracy and speed; written calculation, both accuracy and speed; number dictation and numerical facts).

Genotyping

Sample collection and DNA extraction have been described in detail elsewhere.[64] In particular, we explored the contribution of five among the most replicated markers within KIAA0319 and TTRAP (that is, rs2038137, rs114138463, rs4504469, rs9461045 and rs2143340) and of four markers within ROBO1 (that is, rs333491, rs6803202, rs9853895 and rs7644521), which have been found associated with reading-related measures in a previous study.[43] Markers rs2038136 and rs2038135 within KIAA0319 were typed as a byproduct of rs2038137 sequencing.

Portions of the region upstream of the KIAA0319 gene were amplified from genomic DNA (primer sequences and amplification protocols are available from the authors on request) and analyzed by direct sequencing. A 0.5-μl aliquot of each amplified DNA sample was labeled with a BigDye Terminator 3.1 cycle sequencing kit (Life Technologies, Monza, Italy) and sequenced on an ABI3130xl Genetic Analyzer (Life Technologies). Sequences were aligned with Autoassembler (Applied Biosystems, Monza, Italy) and scored for polymorphisms rs2038137, rs2038136, rs2038135 and rs114138463. Polymorphisms rs333491, rs6803202, rs9853895 and rs7644521 in ROBO1; rs4504469 and rs9461045 in KIAA0319, and rs2143340 in TTRAP were analyzed with quantitative PCR and were typed using TaqMan SNP Genotyping assays (Life Technologies) on a 7900HT Sequence Detection System (Life Technologies).

Genotype error checking was completed in PEDSTATS,[76] and inconsistent genotypes were zeroed out and were not considered for further analysis. Allelic frequencies and Hardy–Weinberg equilibrium for the markers under consideration are shown in Table 1. As SNPs required a minor allele frequency of at least 5% in order to be considered as a potential SNP for further analyses, rs114138463G/A was excluded (Table 1). For all the remaining genotyped SNPs, P-values for deviation from the Hardy–Weinberg equilibrium were not < 0.05, and thus no SNPs were excluded. The linkage disequilibrium structure of KIAA0319 and ROBO1 was analyzed using the parental genotypes only; linkage disequilibrium was extracted and plotted in Haploview 4.0

Table 1 Allele frequencies and HWE's *P*-values for the selected *KIAA0319* end *ROBO1* markers

Gene	Marker	Allele	Allele frequency[a]	HWE[a]
KIAA0319	rs4504469C/T	C	0.650	0.719
		T	0.350	
	rs2038137G/T	G	0.663	0.801
		T	0.337	
	rs2038136G/C	C	0.665	0.935
		G	0.335	
	rs2038135G/T	G	0.678	0.954
		T	0.322	
	rs114138463G/A	G	0.989	0.951
		A	0.011	
	TTRAP-rs2143340A/G	A	0.829	0.999
		G	0.171	
	rs9461045C/T	C	0.788	0.763
		T	0.212	
ROBO1	rs6803202C/T	T	0.515	0.992
		C	0.485	
	rs9853895C/T	C	0.618	0.163
		T	0.382	
	rs333491A/G	A	0.525	0.126
		G	0.475	
	rs7644521C/T	T	0.871	0.991
		C	0.129	

Abbreviation: HWE, Hardy–Weinberg equilibrium.
[a]Allele frequencies and HWE were calculated in parents.

(Figures 1a and b). Genotyped markers showing r^2-values $\geqslant 0.80$ were then dropped; on this basis, four markers for *KIAA0319* gene (that is, rs4504469C/T, rs2038137G/T, rs9661045C/T and *TTRAP*-rs2143340A/G) and four markers for *ROBO1* gene (that is, rs6803202C/T, rs9853895C/T, rs333491A/G and rs7644521C/T) were then chosen for further analyses.

Statistical analysis

Genetic association was investigated by the quantitative transmission disequilibrium test (version 2.5.1) as modeled by Abecasis *et al.*[77]

Discrete trait analysis. Single-marker transmission disequilibrium test analyses were carried out for DD as a discrete trait using the '-ad' option, which allows to test using only affected individuals. As the association between the *KIAA0319*-selected markers and DD as a discrete trait was already investigated in a substantially overlapping sample (nuclear families' overlap 61.7%)[78] the present study total sample, discrete trait analysis were conducted only for the *ROBO1*-selected SNPs.

Quantitative trait analysis. Quantitative measures were analyzed using the '-wega' option, which allows to adopt commonly used variance components, that is, the environmental variance (e), polygenic variance (g) and additive major locus (a), and the '-ao' option, which allows for families of any size (with or without parental genotypes), and should be used in a variance component framework, and represents a variant of the '-ap' option, which evaluates the evidence for population stratification. Performance IQ was included as covariate[26,43] with the '-c' option. We calculated the genetic association for the total sample for both the *KIAA0319* and *ROBO1* genes. Moreover, as following up association signals over different severity groups may confirm a possible true contribution of the selected SNPs to DD and DD-related performance,[79] we tested the genetic association for a subsample selected by severity, that is, by selecting only the nuclear families in which at least one offspring scored $\leqslant 2.50$ s.d. below the general population mean on either accuracy or speed in either text-, or word-, or non-word reading tasks ($n=311$). Only empirical *P*-values are reported and were computed from 1.000 Monte-Carlo permutations.
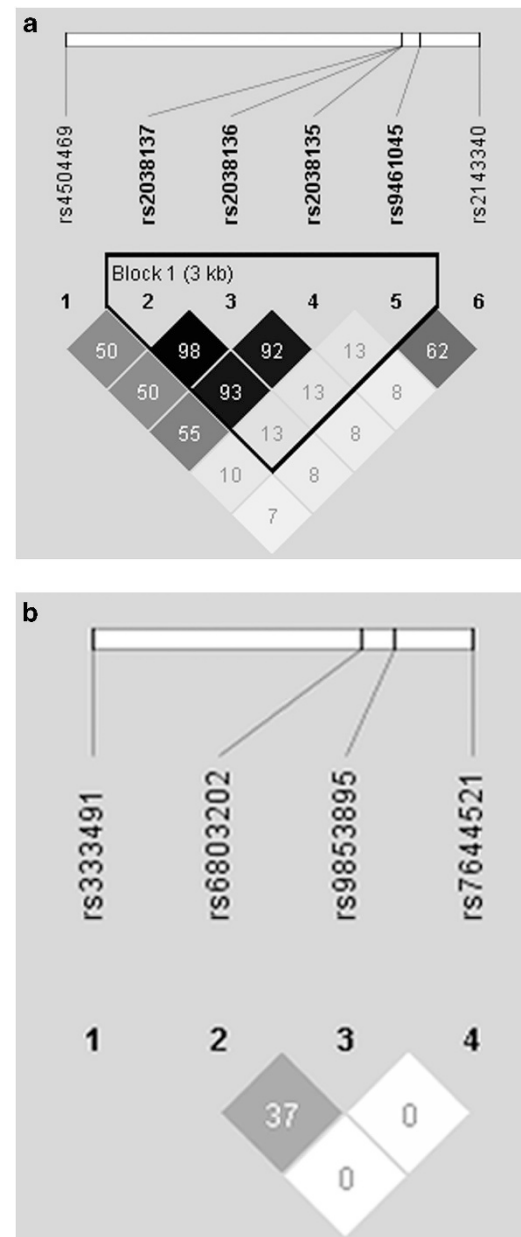

Figure 1 (**a**) Haploview plot showing pairwise linkage disequilibrium (r^2-values) for seven SNPs within *KIAA0319* based on parents' genotypes. Numbers in squares are r^2-values. (**b**) Haploview plot showing pairwise linkage disequilibrium (r^2-values) for four SNPs within *ROBO1* based on parents' genotypes. Numbers in squares are r^2-values.

The haplotype association analysis on the three-SNP haplotype[23] was performed in PBAT (http://www.hsph.harvard.edu/clange/default.htm).[80,81] To adjust for the covariate, we used Performance IQ as interaction variable,[26,43] and nominal *P*-values are reported.

For both single-marker TDT analyses and haplotype association analysis, Bonferroni correction for multiple testing was not implemented, as it would be too conservative for multiple correlated traits (Supplementary Table 1) and non-independent genetic markers.[23,24,34,60,82–84] Thus far, we decided to adjust the significance levels by the false discovery rate method[85] applied to the tests performed for each marker and haplotype (that is, 17 phenotypes), separately for each marker and haplotype.[86–89]

RESULTS

The total sample consisted of 493 unrelated nuclear families of probands with DD (804 offspring); except for 63 families that had only one parent available, all parents were represented, yielding a total sample of 1727 individuals, all of Italian ancestry. Of this total sample: (i) 352 families (599 offspring) had complete measures of READING-Accuracy, READING-Speed, SPELLING and auditory STM (group 1), and (ii) 179 families (229 offspring) had complete measures of

Table 2a Market-trait association empirical *P*-values[a] in both the group 1 of the total sample (*n* = 352) and the selected-by-severity subsample (*n* = 311) for *KIAA0319* markers

	KIAA0319 marker									TTRAP marker		
	rs4504469C/T			rs2038137G/T			rs9661045C/T			rs2143340A/G		
	Informative families	χ^2 *(df)*	*Empirical P-value*	*Informative families*	χ^2 *(df)*	*Empirical P-value*	*Informative families*	χ^2 *(df)*	*Empirical P-value*	*Informative families*	χ^2 *(df)*	*Empirical P-value*
READING-Accuracy												
Total sample	287	3.28 (420)	0.056	273	1.39 (431)	0.274	208	0.52 (407)	0.587	180	0.08 (415)	0.807
Severity sample	235	2.53 (351)	0.112	228	1.59 (360)	0.224	182	0.35 (344)	0.632	157	0.49 (349)	0.458
READING-Speed												
Total sample	287	0.13 (420)	0.749	273	0.05 (431)	0.865	208	0.14 (407)	0.812	180	1.26 (415)	0.273
Severity sample	235	0.02 (351)	0.903	228	0.10 (360)	0.787	182	0.00 (344)	0.999	157	2.10 (349)	0.145
SPELLING												
Total sample	281	2.07 (412)	0.116	269	0.02 (423)	0.890	207	0.06 (399)	0.856	180	1.76 (407)	0.247
Severity sample	229	1.64 (343)	0.191	224	0.04 (352)	0.852	181	0.01 (336)	0.953	157	2.27 (341)	0.164
Auditory STM												
Total sample	240	0.14 (341)	0.705	233	0.39 (255)	0.588	168	0.45 (331)	0.458	149	0.00 (340)	0.944
Severity sample	190	0.00 (280)	0.976	189	0.72 (290)	0.437	143	0.34 (276)	0.526	127	0.00 (280)	0.964

READING represents the average between word- and non-word-reading tasks; SPELLING is the average between word-, non-word spelling and orthographic choice tasks; Auditory STM represents the average between forward/backward number digit spans.
[a]Empirical *P*-value computed from 1000 permutations.

Table 2b Market-trait association empirical *P*-values[a] in both the group 1 of the total sample (*n* = 352) and the selected-by-severity subsample (*n* = 311) for *ROBO1* markers

	ROBO1											
	rs6803202C/T			rs9853895C/T			rs333491A/G			rs7644521C/T		
	Informative families	χ^2 *(df)*	*Empirical P-value*	*Informative families*	χ^2 *(df)*	*Empirical P-value*	*Informative families*	χ^2 *(df)*	*Empirical P-value*	*Informative families*	χ^2 *(df)*	*Empirical P-value*
READING-Accuracy												
Total sample	313	1.06 (420)	0.386	273	4.09 (402)	0.110	270	0.75 (410)	0.366	173	1.15 (407)	0.218
Severity sample	266	1.54 (350)	0.288	234	4.92 (336)	0.072	231	0.83 (343)	0.343	143	0.45 (340)	0.426
READING-Speed												
Total sample	313	0.55 (420)	0.542	273	0.00 (402)	0.995	270	2.74 (410)	0.085	173	1.22 (407)	0.205
Severity sample	266	0.66 (350)	0.528	234	0.02 (336)	0.880	231	1.87 (343)	0.142	143	0.95 (340)	0.236
SPELLING												
Total Sample	306	0.00 (412)	0.948	268	0.05 (394)	0.859	267	1.24 (402)	0.162	170	1.30 (400)	0.206
Severity sample	259	0.09 (342)	0.789	229	0.09 (328)	0.815	228	1.25 (335)	0.186	140	1.09 (333)	0.300
Auditory STM												
Total sample	262	0.23 (344)	0.607	229	0.02 (327)	0.862	226	0.58 (334)	0.464	147	0.02 (332)	0.921
Severity sample	220	0.03 (281)	0.846	195	0.01 (269)	0.911	192	0.64 (274)	0.475	120	0.03 (272)	0.876

READING represents the average between word- and non-word reading tasks; SPELLING is the average between word-, non-word spelling and orthographic choice tasks; Auditory STM represents the average between forward/backward number digit spans.
[a]Empirical *P*-value computed from 1000 permutations.

ELISION, BLENDING, language and mathematics abilities (group 2; Supplementary Tables 2 and 3).

No significant associations were found between DD as a discrete trait and any of the *ROBO1* markers in both the total sample and the selected-by-severity subsample.

After implementing the false discovery rate correction, market-trait associations for DD-related neuropsychological phenotypes (that is, READING-Accuracy, READING-Speed, SPELLING and Auditory

STM) were not-significant in both group 1 and in the selected-by-severity subsample (Tables 2a and b). Regarding the DD-correlated neuropsychological traits, significant association was found between the 'A' allele of the *ROBO1*-rs333491A/G marker and mental calculation-accuracy ($\chi^2 = 10.24$; df = 102; nominal *P*-value = 0.001; empirical *P*-value = 0.003; *q*-value = 0.030; 62 informative families; genetic effect = −0.746; Table 3b) in group 2. The same association was found in the selected-by-severity subsample; the 'A' allele of the

Table 3a Market-trait association empirical *P*-values[a] in both the group 2 of the total sample (*n* = 179) and the selected-by-severity subsample (*n* = 154) for *KIAA0319* markers

| | KIAA0319 marker | | | | | | | | | TTRAP marker | | |
| | rs4504469C/T | | | rs2038137G/T | | | rs9661045C/T | | | rs2143340A/G | | |
	Informative families	χ^2 (df)	Empirical *P*-value	Informative families	χ^2 (df)	Empirical *P*-value	Informative families	χ^2 (df)	Empirical *P*-value	Informative families	χ^2 (df)	Empirical *P*-value
ELISION												
Total sample	113	0.28 (169)	0.569	104	0.00 (176)	0.940	82	0.00 (159)	0.965	71	0.19 (163)	0.524
Severity sample	88	1.03 (135)	0.264	83	0.04 (140)	0.797	68	0.02 (130)	0.850	58	0.19 (131)	0.516
BLENDING												
Total sample	115	2.13 (170)	0.133	106	0.67 (177)	0.321	84	0.52 (160)	0.426	73	0.97 (164)	0.283
Severity sample	90	3.59 (136)	0.050 0.635[b]	85	0.46 (141)	0.406	70	1.59 (131)	0.161	60	1.49 (132)	0.172
SC												
Total sample	50	0.06 (91)	0.852	53	0.47 (90)	0.451	42	1.85 (83)	0.177	38	2.09 (86)	0.158
Severity sample	41	0.78 (76)	0.403	47	0.61 (76)	0.404	37	2.40 (72)	0.141	34	2.37 (74)	0.145
TOKEN												
Total sample	51	0.04 (90)	0.834	53	0.31 (89)	0.515	41	0.49 (81)	0.466	37	0.36 (84)	0.359
Severity sample	42	0.47 (75)	0.462	47	0.00 (75)	0.721	36	0.74 (70)	0.389	33	3.41 (72)	0.214
SYC												
Total sample	48	0.06 (91)	0.839	52	0.22 (90)	0.718	40	0.06 (82)	0.757	38	0.00 (85)	0.993
Severity sample	39	0.65 (76)	0.481	46	1.47 (76)	0.252	35	0.13 (71)	0.638	34	0.00 (73)	0.997
RAN												
Total sample	51	0.00 (91)	0.953	53	1.11 (90)	0.317	43	0.29 (83)	0.555	39	0.82 (86)	0.312
Severity sample	42	0.12 (76)	0.768	47	1.53 (76)	0.248	38	0.76 (72)	0.344	35	1.09 (74)	0.255
FLUENCY												
Total sample	51	0.25 (90)	0.668	51	0.21 (89)	0.647	41	8.27 (81)	0.013 0.221[b]	37	3.04 (84)	0.062
Severity sample	42	0.21 (75)	0.630	45	0.32 (75)	0.573	36	4.19 (70)	0.044 0.748[b]	33	1.96 (72)	0.173
MC-Accuracy												
Total sample	70	2.11 (117)	0.153	65	4.00 (124)	0.055	50	0.38 (108)	0.497	45	0.49 (109)	0.464
Severity sample	62	2.39 (101)	0.110	58	5.12 (107)	0.037 0.212[b]	47	0.51 (96)	0.509	41	0.47 (97)	0.481
MC-Speed												
Total sample	70	0.05 (117)	0.839	65	0.57 (124)	0.405	50	1.31 (108)	0.355	45	0.44 (109)	0.560
Severity sample	62	0.01 (101)	0.949	58	0.37 (107)	0.537	47	0.56 (96)	0.577	41	0.18 (97)	0.728
WC-Accuracy												
Total sample	70	0.78 (117)	0.389	65	4.04 (124)	0.089	50	0.00 (108)	0.956	45	0.05 (109)	0.866
Severity sample	62	0.33 (101)	0.554	58	7.00 (107)	0.028 0.212[b]	47	0.12 (96)	0.739	41	0.01 (97)	0.947
WC-Speed												
Total sample	70	0.22 (116)	0.538	65	0.20 (124)	0.683	50	0.12 (107)	0.690	45	0.05 (108)	0.812
Severity sample	62	0.01 (101)	0.913	58	0.25 (107)	0.625	47	0.01 (96)	0.910	41	0.00 (97)	0.961
ND												
Total sample	70	0.01 (116)	0.888	65	0.04 (123)	0.851	50	0.89 (107)	0.407	45	1.56 (108)	0.265
Severity sample	62	0.02 (100)	0.797	58	0.00 (106)	0.988	47	0.59 (95)	0.483	41	1.24 (96)	0.313
NF												
Total sample	70	0.40 (117)	0.611	65	0.43 (124)	0.567	50	1.16 (108)	0.851	45	0.17 (109)	0.688
Severity sample	62	0.01 (101)	0.949	58	0.68 (107)	0.508	47	0.12 (96)	0.767	41	0.41 (97)	0.537

Abbreviations: FDR, false discovery rate; FLUENCY, semantic fluency; MC, mental calculation; ND, number dictation; NF, numerical facts; RAN, rapid automatized naming; SC, semantic comprehension; SYC, syntactic comprehension; TOKEN, token test; WC, written calculation.
The *q*-values are bold if they are ⩽0.05.
[a]Empirical *P*-value computed from 1000 permutations.
[b]FDR correction's *q*-values.

ROBO1-rs333491A/G marker was significantly associated with mental calculation-accuracy ($\chi^2 = 11.90$; df = 89; nominal P-value ⩽ 0.001; empirical P-value ⩽ 0.001; q-value = 0.010; 56 informative families; genetic effect = −0.458; Table 3b).

No significant associations were found between KIAA0319 markers and any of the neuropsychological traits in both the total sample and the selected-by-severity subsample (Tables 2a and b and Tables 3a and b).

Finally, we investigated the KIAA0319 three SNP haplotype (rs4504469, rs2038137 and rs2143340) originally identified by Francks et al.[23] The 1-1-2 haplotype—where 1 is the major allele and 2 is the minor allele of each SNP[23,47]—showed a frequency of 14.3% in our sample. After implementing the false discovery rate correction on the nominal P-values, significant association between this haplotype and READING-Accuracy was found in the selected-by-severity subsample (informative families = 108, nominal P-value = 0.003, q-value = 0.025).

DISCUSSION

This study was primarily designed to verify the reproducibility of previous findings of an involvement of the KIAA0319 and ROBO1 genes in DD, in a sizable sample of Italian families ascertained

Table 3b Market-trait association empirical P-values[a] in both the group 2 of the total sample ($n = 179$) and the selected-by-severity subsample ($n = 154$) for ROBO1 markers

| | ROBO1 marker | | | | | | | | | | | |
| | rs6803202C/T | | | rs9853895C/T | | | rs333491A/G | | | rs7644521C/T | | |
	Informative families	χ^2 (df)	Empirical P-value	Informative families	χ^2 (df)	Empirical P-value	Informative families	χ^2 (df)	Empirical P-value	Informative Families	χ^2 (df)	Empirical P-value
ELISION												
Total sample	115	0.02 (167)	0.878	96	0.00 (150)	0.982	93	1.25 (157)	0.197	74	1.00 (154)	0.343
Severity sample	95	0.01 (132)	0.908	81	0.00 (119)	0.982	77	1.84 (124)	0.125	57	0.53 (121)	0.501
BLENDING												
Total sample	116	1.45 (168)	0.241	97	0.81 (152)	0.372	95	1.03 (159)	0.272	74	0.33 (156)	0.508
Severity sample	96	1.56 (133)	0.201	82	1.45 (121)	0.193	79	0.15 (126)	0.677	57	0.24 (123)	0.546
SC												
Total sample	72	0.10 (90)	0.781	44	0.53 (74)	0.570	59	0.02 (79)	0.886	32	0.28 (78)	0.581
Severity sample	62	0.49 (75)	0.555	38	2.40 (61)	0.175	51	0.02 (67)	0.898	27	0.40 (66)	0.551
TOKEN												
Total Sample	70	1.30 (89)	0.210	43	3.62 (72)	0.320	58	0.77 (77)	0.405	32	0.00 (76)	0.473
Severity sample	60	2.03 (74)	0.168	37	1.32 (59)	0.374	50	1.18 (65)	0.239	27	0.53 (64)	0.480
SYC												
Total sample	71	0.00 (90)	0.984	45	0.05 (73)	0.845	57	0.76 (78)	0.395	33	0.02 (77)	0.942
Severity sample	61	0.01 (75)	0.923	39	0.10 (60)	0.791	49	1.22 (66)	0.328	28	0.00 (65)	0.965
RAN												
Total sample	73	0.11 (90)	0.721	45	0.27 (74)	0.604	59	1.18 (79)	0.341	32	0.01 (78)	0.934
Severity sample	63	0.00 (75)	0.992	39	1.12 (61)	0.361	51	3.75 (67)	0.110	27	1.04 (66)	0.516
FLUENCY												
Total sample	71	0.71 (89)	0.408	44	4.08 (72)	0.059	58	0.11 (77)	0.731	31	1.95 (76)	0.173
Severity sample	61	0.36 (74)	0.542	38	0.77 (59)	0.403	50	0.14 (65)	0.691	26	3.81 (64)	0.065
MC-Accuracy												
Total sample	77	0.12 (115)	0.713	59	0.89 (99)	0.265	62	10.24 (102)	0.003 **0.030**[b]	42	0.58 (100)	0.457
Severity sample	66	0.00 (99)	0.962	53	0.70 (85)	0.303	56	11.90 (89)	<0.001 **0.010**[b]	37	1.36 (87)	0.214
MC-Speed												
Total sample	77	1.29 (115)	0.214	59	0.84 (99)	0.270	62	4.58 (102)	0.041 0.203[b]	42	0.03 (100)	0.838
Severity sample	66	0.42 (99)	0.454	53	0.42 (85)	0.385	56	4.22 (89)	0.071	37	0.10 (87)	0.663
WC-Accuracy												
Total sample	77	0.28 (115)	0.639	59	0.42 (99)	0.500	62	3.07 (102)	0.100	42	0.10 (100)	0.731
Severity sample	66	0.81 (99)	0.408	53	0.06 (85)	0.784	56	2.84 (89)	0.104	37	0.00 (87)	0.988
WC-Speed												
Total sample	77	0.51 (114)	0.450	59	1.13 (98)	0.127	62	0.99 (101)	0.147	42	0.06 (99)	0.742
Severity sample	66	0.39 (99)	0.465	53	0.84 (85)	0.331	56	0.72 (89)	0.347	37	0.59 (95)	0.483
ND												
Total Sample	77	0.62 (114)	0.288	59	0.03 (98)	0.815	62	0.01 (101)	0.913	42	0.00 (99)	0.955
Severity sample	66	0.72 (98)	0.246	53	0.10 (84)	0.669	56	0.07 (88)	0.795	37	0.03 (86)	0.828
NF												
Total sample	77	0.03 (115)	0.839	59	0.01 (99)	0.939	62	2.85 (102)	0.086	42	0.23 (100)	0.624
Severity sample	66	0.02 (99)	0.855	53	0.18 (85)	0.677	56	2.84 (89)	0.096	37	0.82 (87)	0.359

Abbreviations: FDR, false discovery rate; FLUENCY, semantic fluency; MC, mental calculation; ND, number dictation; NF, numerical facts; RAN, rapid automatized naming; SC, semantic comprehension; SYC, syntactic comprehension; TOKEN, token test; WC, written calculation.
The q-values are bold if they are ⩽0.05.
[a] Empirical P-value computed from 1000 permutations.
[b] FDR correction's q-values.

for DD. We already yielded partial initial evidence about the effects of the *KIAA0319* and *ROBO1* genes on reading-related neuropsychological phenotypes in a gene-by-environment association analysis of quantitative DD-related phenotypes.[64] We now integrate the study of these genes by exploring and confirming their contribution to DD, DD-related and DD-correlated phenotypes by association analyses. In particular, we provide evidence toward a role of one SNP in *ROBO1* (that is, rs333491A/G) in DD-related neuropsychological phenotypes, in a large Italian clinical sample. The associations were supported by the variant of the selected polymorphism, for which a positive association was found in previous works.[43]

Our data show that *ROBO1* gene is associated with the mathematics cognitive phenotypes (that is, mental calculation), suggesting that this gene can also account for part of the reduced mathematical skills that are observable in these children. To our knowledge, the only study that addressed a similar question[19] reported several SNPs associated with mathematical skills in chromosomal regions that do not pertain to the gene examined in this hypothesis-driven study. Our result is quite surprising; a significant, even if low, correlation has been indeed observed between 'Mental Calculation' and 'Reading, accuracy', 'Spelling' and 'Elision' ($r = 0.21$, $r = 0.17$ and $r = 0.21$, respectively; see Supplementary Table 1), and, more generally, *ROBO1* has been reported as a plausible candidate for DD, DD-related neuropsychological phenotypes and DD-correlated traits in previous studies.[41,42] Nevertheless, negative association between markers spanning within the *ROBO1* and DD has been also reported.[44] Similarly, we did not find any association between *ROBO1* and DD, DD-related and DD-correlated phenotypes. This lack of association may be due to the low correlation between mathematics skills and DD-related and DD-correlated neuropsychological traits (see Supplementary Table 1). These results suggest that *ROBO1* gene can account for not only part of the disabilities proper of DD, but also for part of the reduced mathematical skills that are observable in these children, thus following a pleiotropic mode of action.[15–17,90]

Turning to the results with the linguistic phenotypes, our negative results contrast with previously published studies[38–40,45] that reported pleiotropic effects of DD candidate genes for linguistic traits. Similar to what we found in a previous study,[60] this discrepancy, however, could be connected to the age of participants in our study and the type of tests that constitute adequate measures of linguistic skills at different ages. The studies that yielded positive results[38–40] were indeed assessing phenotypes in children of a much younger age than ours and were able to test articulation skills, as it is appropriate for younger children. Our measures of language abilities may have turned out to be insensitive to association with these genes because the deficits in linguistic skills measured by the previous positive studies may undergo spontaneous catch-up with time and development, so that by assessing older children we found linguistically better functioning children for crucial phenotypes, such as 'articulation'. Moreover, this discrepancy may also be due to the tests used; our linguistic tests were simply not measuring the same types of deficits as measured by previous positive studies.[45]

Finally, our findings further support the effect of the main 'risk' rs4504469–rs2038137–rs2143340;1-1-2 haplotype[23] in the etiology of reading abilities,[23,26,36] by showing that it had a detrimental effect on Performance IQ-adjusted reading measure within the most severely affected children.

Main conclusions

This study provide evidence about the association between *ROBO1* and DD-related neuropsychological phenotypes. Moreover, these findings likely indicate shared biological and cognitive processes that underlie reading and mathematics, and represent a first evidence in favor of pleiotropic effect of the *ROBO1* gene on reading-correlated neuropsychological skills in a sizable sample of Italian families ascertained for DD. Therefore, these data provide further evidence about the influence of this gene on the etiology of DD and its correlated neuropsychological traits, by exploring their effects in a non-English-speaking sample.

Nevertheless, our study should be viewed with some limitations in mind. First, even if by assuming a conservative estimate of heritability for each phenotype of 0.3, for a sample of 493 nuclear families, the conditional power is $> 95\%$ for an α-level of 0.05 (PBAT Power Calculator, http://www.biostat.harvard.edu/~fbat/pbat.htm), these data need replication in independent samples in order to evaluate the influence of these genes in non-English-speaking populations. Second, as with all analyses of complex traits, the significance of the current association findings with DD-correlated phenotypes should be interpreted cautiously until they can be replicated in other large, directly selected for specific language impairment, speech sound disorder and/or developmental dyscalculia samples.

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