Genetic analysis of dyslexia candidate genes in the European cross-linguistic NeuroDys cohort

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[#] We would like to express our deepest condolences on the loss of our colleague and friend Leo Blomert, who passed away in 2012.

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Running title: Association study of dyslexia candidate genes

ABSTRACT

Dyslexia is one of the most common childhood disorders with a prevalence of around 5-10% in school age children. While an important genetic component is known to play a role in the aetiology of dyslexia we are far from understanding the molecular mechanisms leading to the disorder. Several candidate genes have been implicated in dyslexia, including *DYX1C1*, *DCDC2*, *KIAA0319*, and the *MRPL19*/*C2ORF3* locus, each with reports of both positive and no replications. We generated a European cross-linguistic sample of school-age children – the NeuroDys cohort – that includes more than 900 individuals with dyslexia, sampled with homogenous inclusion criteria across eight European countries, and a comparable number of controls. Here, we describe association analysis of the dyslexia candidate genes/locus in the NeuroDys cohort. We performed both case-control and quantitative association analyses of single markers and haplotypes previously reported to be dyslexia-associated. While we observed association signals in samples from single countries, we did not find any marker or haplotype which was significantly associated with either case-control status or quantitative measurements of word-reading or spelling in the meta-analysis of all eight countries combined. Like in other neurocognitive disorders, our findings underline the need for larger sample sizes in order to validate possibly weak genetic effects.

Keywords: dyslexia, word-reading, spelling, association study, candidate genes

INTRODUCTION

Developmental dyslexia is a specific developmental disorder that affects about 5-10% of school-aged children.[1,2] It is characterized by a severe reading disorder (RD) and spelling problems, which interferes with academic achievement or activities of daily living that require reading skills.[3] These difficulties cannot be attributed to unimpaired general intelligence, gross neurological deficits, or uncorrected visual or auditory problems.[4,5] A multifactorial aetiology is most likely, caused by interactions between genetic and environmental factors.[6] Studies have repeatedly indicated that first degree relatives of affected individuals have a 30-50% risk of developing the disorder.[6,7]

Genetic linkage studies of dyslexia have identified several loci which may contribute to the disorder.[8,9] In addition, at some of these loci, association studies or translocation breakpoint mapping have led to the identification of genetic variants associated with disease risk.[10]

DYX1C1 (dyslexia susceptibility 1 candidate 1, MIM 608706) on chromosome 15q21.3 was identified as a candidate gene by breakpoint mapping of a translocation co-segregating with dyslexia in one Finnish family.[11] Furthermore, two putative functional variants in *DYX1C1* were found to be dyslexia-associated in a population sample of Finnish origin.[11] Other groups also found *DYX1C1* associations in their dyslexia sample[12], but also reported an opposite allelic trend with their association findings.[13,14] It has been speculated that this may be due to a different haplotype structure between samples and populations. *DYX1C1* has also been associated with reading and spelling ability in a large unselected group of adolescents from Australia.[15] Furthermore, it has been shown that dyslexia-associated variants within the promoter region of *DYX1C1*[16] influence the binding affinity of transcription factor complexes.[17]

Two genes have been reported to be associated with dyslexia within the linkage region on chromosome 6p22.2: *DCDC2* (Doublecortin domain-containing protein 2, MIM 605755)[18-20] and *KIAA0319* (MIM 609269).[21,22] Independent replications have been reported for both genes: *DCDC2*[23-27] and *KIAA0319*.[27-31] The role of *KIAA0319* in dyslexia was also supported by the identification of a single variant associated with dyslexia and affecting the gene expression of *KIAA0319*.[30,32] In addition, two independent studies have identified an interaction between single nucleotide polymorphisms (SNPs) within *DCDC2* and *KIAA0319*.[31,33] A recent brain imaging study found support for effects on white matter structure in overlapping regions of human brains for the three dyslexia candidate genes *DYX1C1*, *DCDC2*, and *KIAA0319*.[34]

On chromosome 2p12, a locus close to the genes *MRPL19* and *C2ORF3* (also named *GCFC2*) has been shown to be associated with dyslexia in two independent samples of Finnish and German origin.[35] However, until now these associations have not been replicated in independent dyslexia samples[24] but the same genetic variants have been found to be associated with measures of general cognitive abilities.[36]

Conducting association studies of cognitive phenotypes is plagued with challenges, such as the variability in both the initial ascertainment and subsequent phenotypical assessment of the samples.[37,38] To address this issue the NeuroDys Consortium embarked in a large sample collection across eight different European countries applying the same inclusion and exclusion criteria for phenotypic characterisation[39] and collected 958 cases and 1,150 controls. In the present study, this sample was used to explore the contribution of the dyslexia candidate genes in such a cross-linguistic cohort. On the basis of existing replication studies, we chose 19 SNPs within the

dyslexia candidate genes *DYX1C1*, *DCDC2*, *KIAA0319*, and within the *MRPL19*/*C2ORF3* locus (Table 1) and performed case-control and quantitative (*i.e.* word-reading and spelling) association analyses of single markers and haplotypes.

SUBJECTS AND METHODS

Subjects

All parents of children participating in this study gave their written informed consent for participation. The same inclusion and exclusion criteria were applied in all partner countries.

Inclusion and exclusion criteria for all participants:

- Age between 8 and 12 years.
- At least 1 ½ years of formal reading instruction.
- An age-appropriate scaled score of at least 7 on WISC Block Design, and of at least 6 on WISC Similarities (standardized tests of non-verbal and verbal intelligence respectively with a population mean=10 and SD=3[40]).
- An attention scale score within the 95th percentile of the age-appropriate norm, either from the Child Behavior Check-List[41] or from the Conners questionnaire[42] from the parents.
- The following exclusion criteria from the parental questionnaire: hearing loss; uncorrected sight problems; language of the test not spoken by at least one parent since birth; test language not being the child's school language; child missed school for any period of 3 months or more;

formal diagnosis of ADHD (attention deficit-hyperactivity disorder); medication for epilepsy or behavioural problems.

Inclusion criterion for the dyslexia cases:

- More than 1.25 SD below grade level on a standardized word-reading test.

Inclusion criterion for the controls:

- Less than 0.85 SD below grade level on a standardised word-reading test.

The NeuroDys cohort is composed of 958 dyslexia cases and 1,150 controls from eight different European countries: Austria, France, Germany, The Netherlands, Switzerland, Finland, Hungary, and the United Kingdom (Table 2).

Phenotypes

Dyslexia: On top of common inclusion and exclusion criteria (see above), children were classified according to word-reading ability; dyslexic (case) if below -1.25 SD or control if above -0.85 SD.

Word-reading: With the exception of English, word-reading accuracy and word-reading speed were assessed by presenting word lists under a speeded instruction ("Read as quickly as possible without making mistakes"). Both accuracy and speed were recorded, and converted into a composite word-reading fluency measure (number of words correctly read per minute), then into Z-scores based on age or grade-appropriate norms for each language. In English, reading was not timed and therefore this measure reflects word-reading accuracy only.

Spelling: Standardized spelling tests were given by each contributor. All tests required the spelling of single words dictated in sentence frames and the number of spelling errors were counted. Grade specific Z-scores were calculated based on age or grade-appropriate norms for each language.

Genotyping

Samples were genotyped for 19 SNPs using the Sequenom MassARRAY system (Sequenom, San Diego, USA) in one of three laboratories. The United Kingdom (UK) samples were genotyped at the Wellcome Trust Centre for Human Genetics (Oxford, UK), the Finnish samples were genotyped at the mutation analysis facility (MAF) of the Karolinska Institutet (Stockholm, Sweden) while the remaining six sample sets (from Austria, France, Germany, Hungary, Switzerland, and The Netherlands) were genotyped at the Life & Brain Center (Bonn, Germany). For all sample sets independently, SNPs with a minor allele frequency (MAF) <1% and a call rate <95% were excluded. All SNPs were in Hardy-Weinberg-Equilibrium (HWE, $p>0.01$) and individuals with a call rate <85% were excluded. After these quality control measures, 15 of the 19 SNPs genotyped remained in common for all eight sample sets (Supplementary Table 1 and Supplementary Table 2).

Statistical analyses

Tests for heterogeneity were conducted using Genepop (http://genepop.curtin.edu.au/). Association analyses for single markers as well as for haplotypes were performed using PLINK (http://pngu.mgh.harvard.edu/~purcell/plink/). Z-score based meta-analysis was

calculated in R (http://www.r-project.org/). Haplotypes were selected based on previously published positive associations, *i.e.* rs917235-rs714939 (G-G), rs1000585-rs917235-rs714939 (G-G-G), and rs917235-rs714939-rs6732511 (G-G-C) for the *MRPL19/C2ORF3* locus[35] and rs793862-rs807701 (A-C) for the *DCDC2* locus.[19] Correction for multiple testing was performed using the Bonferroni method. The correction based on 19 single markers and four haplotypes – analysed for three traits (case-control, word-reading, spelling) – results in a significance threshold of p=0.00072 (= 0.05/69 tests).

RESULTS

We performed a genetic heterogeneity analysis of all sample sets included in the study, in order to assess whether we could analyse the whole data set as a single sample or as a meta-analysis. For this, we tested at each locus if alleles were drawn from the same distribution in all eight populations. This analysis revealed significant inter-population differences between the eight sample sets but with no significant differences in allele frequencies for the sample sets from Central Europe ("CE" sample, Supplementary Table 3). We therefore performed a case-control analysis in each of the eight sample sets separately, followed by a meta-analysis across the "CE" samples (580 cases and 625 controls from Austria, France, Germany, Switzerland, and The Netherlands) and a meta-analysis across all samples from the NeuroDys cohort ("All" sample: 958 cases and 1,150 controls, Table 2).

Case-control association study

SNPs: In the single marker case-control analysis of each separate sample set, several SNPs reached nominal significance (p<0.05). These included two SNPs from *DYX1C1*

tested in the Dutch sample and one SNP from *DCDC2* tested in the Hungarian sample (Supplementary Table 4). However, none of these SNPs withstood correction for multiple testing. In the meta-analysis of the "CE" and "All" samples, no single SNP reached nominal association (Table 3).

Haplotypes: Furthermore, we tested if any previously reported haplotypes showed association using the case-control status. Only the rs793862-rs807701 haplotype from the *DCDC2* locus showed nominal association in the Hungarian sample set (Supplementary Table 5). However, this association did not withstand correction for multiple testing. In the "CE" and "All" sample, none of the tested haplotypes showed association with dyslexia (Table 4).

Quantitative trait association study

In a second step, we performed a quantitative trait analysis using two measurements – word-reading and spelling – for all cases of the eight single samples sets separately. Subsequently, we performed a meta-analysis for the quantitative traits across the cases from the "CE" (N=580) and the "All" (N=958) samples.

SNPs: For some of the genotyped SNPs, we observed nominal associations with word-reading or spelling in single sample sets (Supplementary Table 6 and Supplementary Table 8). However, only one marker within *DYX1C1* – associated with spelling – withstood correction for multiple testing (rs3743205, $p=2.98 \times 10^{-04}$, $p_{corrected}=0.0206$; Supplementary Table 8) in the Switzerland sample set. The meta-analysis across the "CE" cases resulted in one nominal association between a *DYX1C1* SNP and the quantitative trait word-reading (Table 3). For spelling, four markers within *KIAA0319* showed nominal association. However, none of these associations withstood correction

for multiple testing (Table 3). In the "All" sample, we did not observe association for the trait word-reading and spelling (Table 3).

Haplotypes: The haplotype association analysis using the quantitative trait word-reading in each sample set separately revealed four nominally significant haplotypes - three of them in the German sample and one in the Hungarian sample. However, none of the haplotypes withstood correction for multiple testing (Supplementary Table 7). Furthermore, we observed three nominally significant associations with haplotypes in the spelling analysis: two haplotypes in the German set and the third haplotype in the set from The Netherlands. Again, none of them remained significant after Bonferroni correction (Supplementary Table 9). The haplotype analysis using the quantitative traits revealed no significant association in the "CE" or "All" samples (Table 4).

DISCUSSION

In the present study we conducted a candidate gene association analysis in the NeuroDys cohort which is composed of 958 individuals with dyslexia and 1,150 controls from Austria, Finland, France, Germany, Hungary, Switzerland, The Netherlands, and the UK. Participants to the study were recruited using consistent ascertainment criteria across all countries.[39] To our knowledge, this study represents the first cross-linguistic genetic association analysis in dyslexia. We tested 19 SNPs and four haplotypes previously reported to be associated with dyslexia. The markers were located in the dyslexia candidate genes *DYX1C1*, *DCDC2*, *KIAA0319*, and the *MRPL19*/*C2ORF3* locus. Although we observed several nominal associations in samples from individual countries (Supplementary Table 4-9), none of them were

significantly associated with dyslexia or any quantitative phenotypes (*i.e.* word-reading and spelling) in the whole NeuroDys cohort ("All" sample, Table 3 and Table 4).

Different reasons may be causing this lack of association. Firstly, the samples included were of different ethnic origin and different SNPs or haplotypes may contribute to disease or trait risk in divergent populations. This may be particularly true for the Finnish sample, where differences in the genomic architecture compared to other European populations have been previously reported.[43,44] Even for samples from Central Europe, population-specific haplotypes may exist.[45,46] Secondly, it is possible that the genetic risk associated with dyslexia is language-dependent. However, this hypothesis seems rather unlikely for the samples from Austria, Germany, and Switzerland as these populations are using the same language (*i.e.* German) and we failed to find any association withstanding multiple testing correction restricting our analyses to these samples (data not shown).

Nevertheless, even if the susceptibility to dyslexia is not language-dependent, the necessary adaptation of the common ascertainment scheme and of the test battery to each language's properties and to each local environment may have introduced some heterogeneity. In addition, environmental factors – in particular pre-school (nursery/kindergarden) education and teaching methods applied in schools – are different between countries. Thirdly, one limitation of this study is that we have not included measures which cover the whole spectrum of dyslexia related traits.[38,47] Previous association studies have reported an association between some of the herein reported genes and phonological processing, orthographic awareness, auditory memory, and rapid naming.[38] The missing analysis of relevant subtypes, quantitative measures, or the severity of dyslexia could be a further factor for the lack of association in this study.

Fourthly, it is quite possible that the samples used in this study were underpowered to replicate the associations that have been observed previously. It is a known phenomenon that the genetic effect of SNP associations is often overestimated in initial studies (winner's curse). If *DYX1C1*, *DCDC2*, *KIAA0319*, or the *MRPL19*/*C2ORF3* locus harbour common risk variants contributing to dyslexia, the use of an underpowered case-control sample seems to be the most likely explanation for our replication failure.

Despite all the above mentioned general causes to our failure in replicating the associations previously reported, gene-specific factors might also be a cause. For example, studies have shown that *KIAA0319* appears to be more relevant in controlling general reading[27,28] abilities and association with this phenotype is more likely to be detected by quantitative trait analysis. However, we failed to detect any association using quantitative trait analysis but it has to be noted that our sample was selected for representing the lower tail of the reading distribution and therefore is not optimal for testing quantitative traits such as general reading skills. Another example concerns *DYX1C1*, which was originally implicated in the aetiology of dyslexia in a Finnish dyslexia family by breakpoint mapping. It is possible that this gene represents a genuine dyslexia risk gene and that common risk variants in *DYX1C1* are contributing to the phenotype, as supported also by associations with reading and spelling in an unselected adolescent cohort from Australia.[15] However, it might be also possible that high-penetrance mutations in *DYX1C1* or in the other dyslexia candidate genes are only present in some familial cases. In this case, a deep sequencing approach in families with dyslexia would be more appropriate in order to find an enrichment of such high-penetrance private mutations.

Genome-wide association studies (GWAS) have been successful in mapping risk genes for many complex traits including neuropsychiatric disorders. It has become clear that the success of these studies largely depends on sample sizes, for example a sample size of several thousand individuals seems to be the requirement for achieving significant associations.[48,49] A GWAS on such a large dyslexia sample would provide an appropriate approach to identify the still unknown dyslexia risk variants. Therefore we conclude that efforts should focus in collecting samples of adequate size by applying similar ascertainment criteria across different countries as we have done with the NeuroDys Consortium.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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