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Integrative genomics identifies *LMO1* as a neuroblastoma oncogene

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Author Contributions H.H. and J.M.M. conceived the study, guided interpretation of results and helped preparation of the manuscript. K.W., H.Z. and C.H. performed SNP association analysis. K.W., S.J.D., E.F.A. and J.J. performed gene expression and copy number analysis. C.W. and K.B. performed PCR validation of gene expression data. C.W., R.W.S., K.B., P.A.M., S.J.D. and K.A.C. performed and/or analysed shRNA transfection and *LMO1* overexpression experiments. N.S. and H.S. generated viral construct for human *LMO1* complementary DNA. M.C. and A.I. performed the replication study on the Italian case series, and N.R. performed the replication study on the UK case series. P.W.M. and W.B.L. performed outcome and clinical covariate analyses on the Children's Oncology Group samples. C.H., C.K., E.F., M.G., W.G. and R.C. generated the genotyping data. L.N. and M.D. helped with data analysis. S.F.A.G., Y.P.M., H.L. and M.D. advised on data interpretation. K.W. drafted the manuscript; H.H., J.M.M. and other authors edited it.

Author Information Microarray data are deposited in the GEO database under accession number GSE3960. The genotypic and phenotypic information from this study is deposited in dbGaP (www.ncbi.nlm.gov/gap) under accession number phs000124.v2.p1. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature.

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Abstract

Neuroblastoma is a childhood cancer of the sympathetic nervous system that accounts for approximately 10% of all paediatric oncology deaths[1,2]. To identify genetic risk factors for neuroblastoma, we performed a genome-wide association study (GWAS) on 2,251 patients and 6,097 control subjects of European ancestry from four case series. Here we report a significant association within LIM domain only 1 (*LMO1*) at 11p15.4 (rs110419, combined $P = 5.2 \times 10^{-16}$, odds ratio of risk allele = 1.34 (95% confidence interval 1.25–1.44)). The signal was enriched in the subset of patients with the most aggressive form of the disease. *LMO1* encodes a cysteine-rich transcriptional regulator, and its paralogues (*LMO2*, *LMO3* and *LMO4*) have each been previously implicated in cancer. In parallel, we analysed genome-wide DNA copy number alterations in 701 primary tumours. We found that the *LMO1* locus was aberrant in 12.4% through a duplication event, and that this event was associated with more advanced disease ($P < 0.0001$) and survival ($P = 0.041$). The germline single nucleotide polymorphism (SNP) risk alleles and somatic copy number gains were associated with increased *LMO1* expression in neuroblastoma cell lines and primary tumours, consistent with a gain-of-function role in tumorigenesis. Short hairpin RNA (shRNA)-mediated depletion of *LMO1* inhibited growth of neuroblastoma cells with high *LMO1* expression, whereas forced expression of *LMO1* in neuroblastoma cells with low *LMO1* expression enhanced proliferation. These data show that common polymorphisms at the *LMO1* locus are strongly associated with susceptibility to developing neuroblastoma, but also may influence the likelihood of further somatic alterations at this locus, leading to malignant progression.

Multiple somatically acquired chromosomal rearrangements, such as focal amplification of the *MYCN* oncogene or deletions at chromosome arms 1p or 11q, are each associated with an aggressive neuroblastoma phenotype[2]. Although these somatically acquired genomic alterations are of clinical use as prognostic biomarkers, until recently little was known about the constitutional genetic events that initiate tumorigenesis. Highly penetrant gain-of-function mutations in the anaplastic lymphoma kinase (*ALK*) tyrosine kinase domain were recently identified as the major cause of familial neuroblastoma, and somatic mutations in this gene implicate it as a target for therapeutic intervention[3–6]. In addition, a neuroblastoma GWAS identified common SNPs at 6p22 as being associated with susceptibility to aggressive neuroblastoma in sporadic cases[7]; follow-up association analysis on the clinically relevant group of patients with an aggressive tumour phenotype indicated that common SNPs within *BARD1* also function as susceptibility variants[8]. Finally, our GWAS has also identified a common copy number variation at 1q21.1 being highly associated with neuroblastoma and probably playing a role in early tumorigenesis through disruption of a novel neuroblastoma breakpoint family gene (*NBPF23*)[9]. Taken together, it has become clear that the embryonal cancer neuroblastoma is genetically heterogeneous, and initiation of sporadically occurring disease requires multiple interacting genetic factors, including both sequence and copy number variants.

To identify additional genetic risk factors, we expanded our previous GWAS and analysed 1,627 neuroblastoma patients accrued through the North American-based Children's Oncology Group with 3,254 genetically matched control subjects of European ancestry (see Supplementary Methods). All subjects were genotyped using the Illumina HumanHap550

BeadChip with over 550,000 SNP markers; the genomic control inflation factor was 1.08 (Supplementary Fig. 1). Clusters of SNPs from three genomic loci reached genome-wide significance ($P < 5 \times 10^{-8}$; Fig. 1a), including two SNPs within *FLJ22536/FLJ44180* at the 6p22 locus (*P* values range from 2.46×10^{-14} to 3.25×10^{-13}; Supplementary Table 1), nine SNPs within or nearby *BARD1* at the 2q35 locus (*P* values range from 3.05×10^{-13} to 9.69×10^{-9}; Supplementary Table 2), each previously reported, and two SNPs within *LMO1* (LIM domain only 1), a newly identified neuroblastoma susceptibility locus at 11p15.4 (*P* values range from 5.12×10^{-10} to 2.83×10^{-8}; Table 1 and Fig. 1b). Closer examination of the *LMO1* locus identified a total of four SNPs that show strong association signals ($P < 1 \times 10^{-4}$) with neuroblastoma (Table 1), which are in a moderate degree of linkage disequilibrium (Supplementary Fig. 2). We then examined each of the most significant SNPs from the 2q35, 6p22, 11p15.4 susceptibility loci and the 1q21.1 copy number variation. However, we did not find evidence for epistasis (Supplementary Tables 3 and 4), indicating that these susceptibility loci increase disease risk independently.

To replicate our findings, we examined the association results from an independent case series of 190 patients from the Children's Oncology Group and 1,507 control subjects, all of whom were genotyped on the Human610-Quad arrays. All four *LMO1* SNPs identified in the discovery effort showed the same direction of association in this replication cohort, with *P* values ranging from 1.01×10^{-5} to 0.058. To seek additional evidence of replication, we performed quantitative PCR-based genotyping of these four SNPs in a third independent case series from UK, as well as the two most significant SNPs in a fourth independent case series from Italy. Combined analysis by the Cochran–Mantel–Haenszel method demonstrated that two of the four SNPs had *P* values that extend well beyond the genome-wide significance threshold (Table 1). Additionally, using the two cohorts with whole-genome genotype data (discovery cohort and US replication cohort), we performed genotype imputation at 11p15.4 and identified six additional genome-wide significant markers, the most significant being rs110420 ($P = 1.17 \times 10^{-13}$), which is in complete linkage disequilibrium ($r^2 = 1$ in HapMap CEU subjects (Utah residents with ancestry from northern and western Europe) with the genotyped marker rs110419 (Fig. 1c and Supplementary Table 5).

We next determined if the *LMO1* genotypes were associated with a particular clinical phenotype and/or patient survival. Similar to the association pattern observed for the 6p22 and 2q35 (*BARD1*) loci[7,8], the risk alleles of *LMO1* were significantly associated with metastatic disease ($P = 0.0040$), advanced age (greater than 1 year, $P < 0.0001$) and a high-risk status by Children's Oncology Group criteria for treatment stratification[2] ($P = 0.0010$; Supplementary Tables 6 and 7). Consistent with this observation, the rs110419 risk allele was associated with decreased event-free survival ($P = 0.0085$; Supplementary Table 8 and Supplementary Fig. 3) and overall survival ($P = 0.0217$; Supplementary Fig. 4). Taken together, these data suggest that common germline variants at *LMO1* are associated not only with predisposition to develop neuroblastoma, but also with a predilection to develop the more aggressive form of the disease. They emphasize that *LMO1* genetic variations are associated with a particular neuroblastoma phenotype; however, this does not indicate that these variants have prognostic significance for an individual with neuroblastoma.

The *LMO1* gene encodes a cysteine-rich transcriptional regulator with two LIM zinc-binding domains that is mainly expressed in the nervous system[10]. LMO1 belongs to a protein superfamily encoded by four genes, including *LMO1*, *LMO2*, *LMO3* and *LMO4*. Multiple lines of evidence, including chromosomal translocation events and mouse models, strongly implicate this gene family in the aetiology of human cancer[11–14]. Most provocatively, retroviral insertion of the corrective gene for X-linked severe combined immunodeficiency into the *LMO2* locus resulted in T-cell leukaemias in several participants in gene therapy

trials[15]. *LMO4* represses the transcription of *BRCA1*, and dys-regulation of *LMO4* expression has been implicated in the breast car-cinogenesis[16,17]. Finally, *LMO3* has been shown to act as an oncogene in neuroblastoma through the neuronal transcription factor *HEN2*[18]. We therefore postulated that the common variants at the 11p15.4 locus discovered here may increase disease risk through a *cis*-acting effect on the regulation of expression or function of *LMO1*, but we cannot exclude the potential for *trans*-acting influences on loci distant from the discovered common variants.

We next examined tumour DNA genotyped on the Illumina SNP arrays for 701 neuroblastomas using a detection algorithm for copy number designed for tumour samples[19]. We detected relative segmental gain (copy number changes at a given locus relative to whole-genome copy number changes) at *LMO1* in 87 out of 701 tumours (12.4%); this was particularly enriched in the high-risk group where the GWAS signal was most robust (Supplementary Fig. 5a). Most tumours with 11p gain showed a duplication of the entire chromosome p arm, but four tumours (approximately 5%) showed focal gain restricted to 11p15 including the *LMO1* locus (Supplementary Fig 5b). These data demonstrate that *LMO1* is one of many genes showing somatic copy number gain on 11p, and here we used the GWAS data to prioritize it as a potential target of this somatically acquired chromosomal rearrangement.

We next examined whether somatic *LMO1* alterations were associated with neuroblastoma clinical phenotype and survival of patients (Supplementary Table 9). Gain of *LMO1* was significantly more common in tumours from patients with metastatic disease ($P < 0.0001$), advanced age (greater than 1 year, $P < 0.0001$), unfavourable pathological grade ($P = 0.0013$) and Children's Oncology Group high-risk classification ($P < 0.0001$). Gain of 11p was rarely observed in the *MYCN* amplified cases (Supplementary Table 9). Despite the strong association of 11p gain in cases without *MYCN* amplification, a known powerful adverse prognostic factor[1], *LMO1* gain was associated with decreased overall survival of patients ($P = 0.041$) (Supplementary Table 10 and Supplementary Figs 6 and 7).

To investigate how the neuroblastoma-associated *LMO1* alleles may contribute to tumour initiation and/or clinical phenotype, we next genotyped a set of human neuroblastoma-derived cell lines with Illumina SNP arrays, and measured messenger RNA (mRNA) and protein expression levels on the subset of lines without copy number changes at 11p to avoid the influence of somatic DNA alterations on gene expression. Cell lines with diploid 11p status and harbouring homozygous risk alleles showed significantly higher *LMO1* mRNA and protein expression than those with homozygous non-risk alleles (Fig. 2a and Supplementary Table 11). This trend held in an expanded set of 25 neuroblastoma cell lines with variable 11p status (Supplementary Fig. 8). To determine if this correlation existed in diagnostic tumour tissues, we next examined mRNA expression levels on a whole-genome Affymetrix expression microarray[20] in a subset of 61 neuroblastoma primary tumours from patients whose blood samples and primary tumours had both been genotyped on the Illumina SNP arrays. Among these 61 tumours, 13 harboured somatic gain of 11p. Considering both somatic and germline genotypes in the same linear regression model, we detected an association between *LMO1* copy number gains and increased *LMO1* expression ($P = 0.02$; Fig. 2b and Supplementary Table 12), as well as an association between rs110419 risk alleles and increased *LMO1* expression ($P = 0.022$; Fig. 2b). To refine the genotype-expression relationships further, we subsequently used quantitative PCR to measure *LMO1* expression in an additional set of 23 tumours without *LMO1* gain. We confirmed that the rs110419 risk allele is associated with *LMO1* expression ($P = 0.01$), independent of copy number changes (Fig. 2c). To determine whether a regulatory variant exists at a narrow promoter region of *LMO1*, we performed Sanger sequencing in 20 neuroblastoma cell lines but did not detect any potential causal variant (Supplementary Table 13). Examination of the

1000 Genomes Project data identified over 300 SNPs within or surrounding *LMO1* that are in moderate to strong linkage disequilibrium ($D' > 0.5$) with rs110419 (Supplementary Table 14); however, fine mapping of this region through resequencing will be required to identify whether any are causal *cis*-regulatory variants. Subsequent experimentation will be required to determine if causal DNA variations directly impact *LMO1* expression, and if somatic copy-number gain indeed is targeting *LMO1* for further increased expression in tumour cells.

As our germline and somatic genomic analyses implicated *LMO1* as a neuroblastoma oncogene, we next sought to determine the functional consequences of *LMO1* depletion or overexpression in a genotype- and expression-specific manner. First, after lentiviral-based shRNA infection of neuroblastoma cell lines, we were able to recover stable clones with 45–63% depletion of *LMO1* mRNA and protein (Fig. 3e). Cells with the homozygous neuroblastoma-associated genotype and high *LMO1* expression showed significantly decreased proliferation compared with mock-infected controls (Fig. 3a, b), whereas cells with homozygous non-risk alleles showed little phenotypic effect (Fig. 3c, d). Finally, to determine the cellular phenotypes of forced overexpression of *LMO1*, we stably overexpressed *LMO1* with approximately fourfold higher levels in the SK-N-BE2C cell line with low *de novo LMO1* expression, and detected significantly enhanced proliferation (Fig. 3f). Therefore it appears that inhibition of *LMO1* in cells expressing high levels of *LMO1* or activation of *LMO1* in cells with low levels of *LMO1* leads to pronounced phenotypes. Taken together, these data suggest that *LMO1* may function as an oncogene in a subset of human neuroblastomas.

In conclusion, here we have identified germline sequence variants at the *LMO1* locus that are robustly associated with neuroblastoma. We have applied an integrative genomics approach to demonstrate that common genetic polymorphisms associated with cancer predisposition may also mark regions of the genome prone to somatic alterations influencing tumour progression. Our data suggest that GWAS studies can identify previously undiscovered oncogenic drivers of a malignant phenotype, especially when they occur in a region of the genome involved in large segmental rearrangements impacting hundreds of genes. In paediatric cancers such as neuroblastoma, the real translational potential of GWAS efforts may be in discovering therapeutic targets and predictive biomarkers of tumour aggressiveness.

METHODS SUMMARY

All genome-wide SNP genotyping for the discovery cohorts was performed using the Illumina HumanHap550 BeadChip at the Center for Applied Genomics at the Children's Hospital of Philadelphia. Multi-dimensional scaling was performed using PLINK version 1.06 on a subset of SNPs not in linkage disequilibrium to identify subjects of European ancestry, and all control subjects were genetically matched to patients. The first replication case series was genotyped by Illumina Human610 BeadChip, yet two additional replication case series were genotyped by TaqMan. Genotype imputation was performed by MACH (http://www.sph.umich.edu/csg/abecasis/MaCH/) on discovery and replication case series with whole-genome genotypes. Alteration calls in tumour copy number were generated from data of SNP signal intensity by the OverUnder[19]. Survival analyses used the methods of Kaplan and Meier, with standard errors following the methods of Peto *et al.*[21]. For gene expression profiling by Affymetrix U95Av2 microarrays, the expression measures for each probe set was extracted and normalized using robust multi-array average protocols from raw CEL files. Association tests on genotype and expression were performed on log-transformed expression values by linear regression or *t*-test. For quantitative PCR on *LMO1*, TaqMan probes were purchased from Applied Biosystems with assay identity Hs00231133_m1.

Relative expression of the target gene was determined by normalization to *HPRT1* using a standard curve method with ten serial dilutions according to the manufacturer's instructions. All quantitative PCR reactions were performed in triplicate with an ABI PrismTM 7900HT Sequence Detection System (Applied Biosystems). For the *LMO1* knockdown experiments, the lentiviral particles for shRNA knockdown were purchased from Santa Cruz, including copGFP Control Lentiviral Particles (catalogue number sc-108084) and LMO1 shRNA(h) Lentiviral Particles (catalogue number sc-38025-v). Pooled clones of SK-N-BE2C cells with *LMO1* overexpression were created through stable transfection of full-length *LMO1* complementary DNA in pCDNA3.1 as previously described[22].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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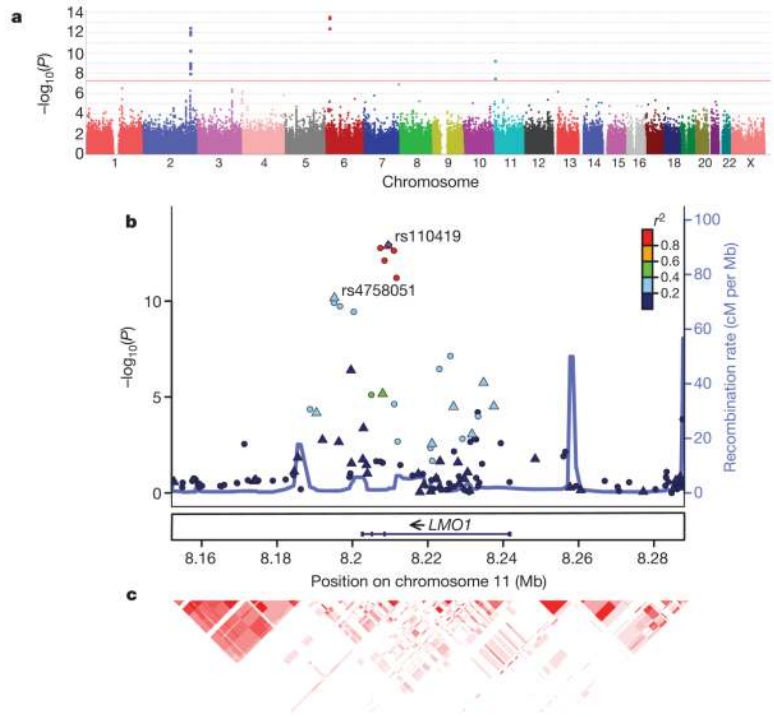

Figure 1. Discovery of *LMO1* at 11p15.4 as a neuroblastoma susceptibility locus

a, Manhattan plot of GWAS results from the discovery cases series, with the red horizontal line representing genome-wide significance threshold ($P < 5 \times 10^{-8}$). **b**, Genomic position (National Center for Biotechnology Information build 36) of genotyped (triangles) and imputed (circles) SNPs. The *P* values are calculated by combining discovery and replication case series with whole-genome genotypes, and SNPs are coloured based on their correlations with rs110419 (purple diamond). Estimated recombination rates from the HapMap data are overlaid. **c**, Degree of linkage disequilibrium between SNPs (as r^2 values) is represented by red colour intensity in the corresponding cells.

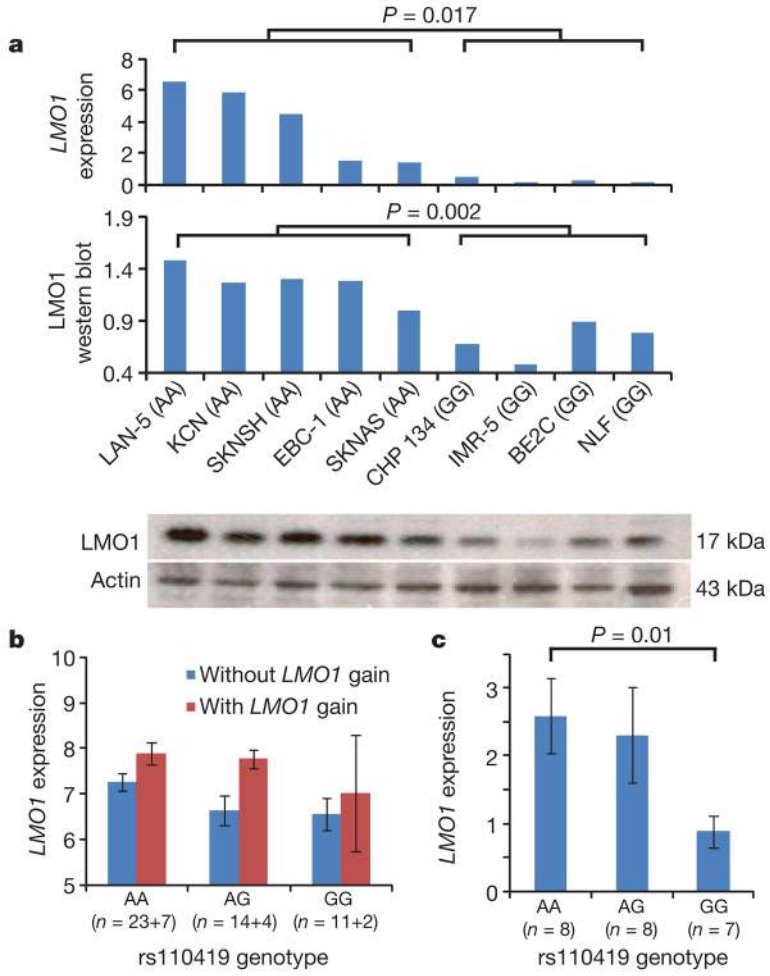

Figure 2. *LMO1* germline genotypes and somatic copy number gains are associated with mRNA and protein expression

a, *LMO1* mRNA and protein expression in nine human neuroblastoma-derived cell lines are highly correlated with rs110419 genotype. **b**, Microarray-based expression profiling on 61 primary tumours confirms that *LMO1* gene expression is associated with both *LMO1* gain ($P = 0.02$, *t*-test) and risk genotypes ($P = 0.022$, linear regression). **c**, Quantitative PCR-based expression profiling of an independent set of primary neuroblastomas without *LMO1* gain confirms the same association. Error bars, s.e.m.

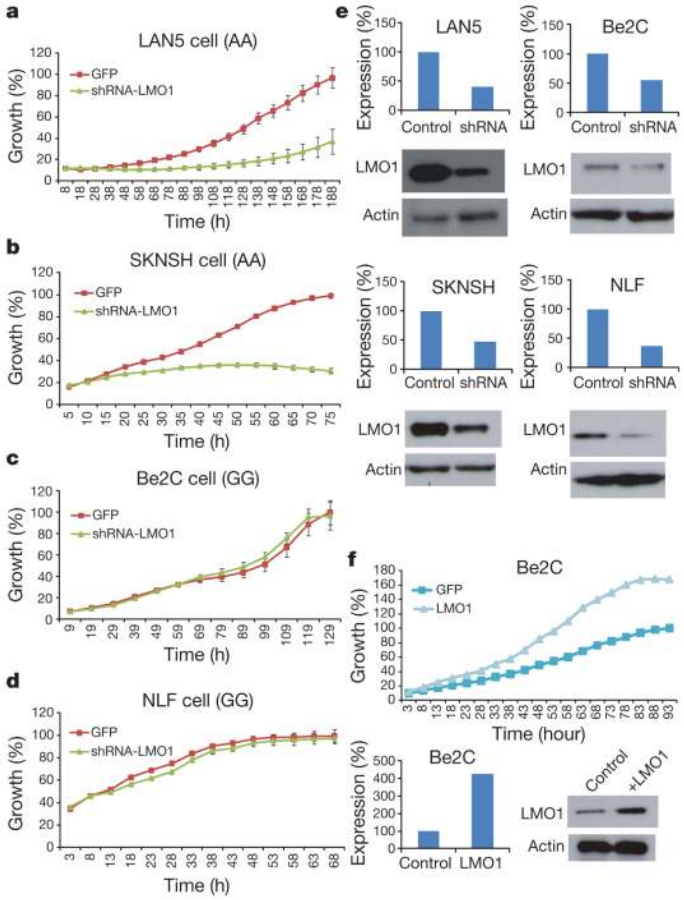

Figure 3. Genetic manipulation of *LMO1* expression in neuroblastoma cell line models influences proliferative phenotype in an expression-specific manner

a–**d**, In cells with neuroblastoma risk alleles and higher *LMO1* expression levels, *LMO1* knockdown leads to inhibition of cellular proliferation. **e**, *LMO1* knockdown as measured by quantitative reverse-transcription PCR and western blot for experiments **a–d. f**, In SK-N-BE2C cells with non-risk alleles and low *LMO1* expression levels, forced overexpression of *LMO1* leads to enhanced cellular proliferation. Approximate fourfold overexpression of *LMO1* RNA and protein are shown. Error bars, s.e.m.

Table 1

Significantly associated SNPs at the *LMO1* locus on 11p15.4

SNP[†]	Risk/non-risk allele	Discovery (HumanHap550)[*]			US replication (Human610)[*]			UK replication (TaqMan)[*]		
		Frequency of cases (n = 1,627)	Frequency of controls (n = 3,254)	P[‡]	Frequency of cases (n = 190)	Frequency of controls (n = 1,507)	P[‡]	Frequency of cases (n = 253)	Frequency of controls (n = 845)	P[‡]
rs4758051	G/A	0.51	0.45	2.8×10^{-8}	0.55	0.45	2.1×10^{-4}	0.51	0.45	0.039
rs10840002	A/G	0.42	0.37	6.0×10^{-6}	0.44	0.38	0.019	0.37	0.36	0.61
rs110419	A/G	0.55	0.49	5.1×10^{-10}	0.61	0.49	1.0×10^{-5}	0.53	0.48	0.057
rs204938	C/T	0.49	0.44	1.2×10^{-5}	0.50	0.45	0.058	0.50	0.44	0.032

SNP[†]	Risk/non-risk allele	Italian replication (TaqMan)[*]			Combined	
		Frequency of cases (n = 181)	Frequency of controls (n = 491)	P[‡]	CMH[§] P	CMH OR (95% confidence interval)[‖]
rs4758051	G/A	0.45	0.42	0.45	1.4×10^{-11}	1.28 (1.19–1.37)
rs10840002	A/G	—	—	—	8.5×10^{-7}	1.21 (1.12–1.30)
rs110419	A/G	0.49	0.41	0.004	5.2×10^{-16}	1.34 (1.25–1.44)
rs204938	C/T	—	—	—	1.7×10^{-7}	1.22 (1.13–1.31)

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

[†] SNP: r^2 in controls between rs110419 and each of rs4758051, rs10840002 and rs204938 was 0.30, 0.17 and 0.29, respectively.

[‡] P values were calculated by allelic test.

[§] CMH, Cochran–Mantel–Haenszel test.

[‖] OR, odds ratio of risk allele.

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