

ORIGINAL ARTICLE

LRRTM1 on chromosome 2p12 is a maternally suppressed gene that is associated paternally with handedness and schizophrenia

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Left–right asymmetrical brain function underlies much of human cognition, behavior and emotion. Abnormalities of cerebral asymmetry are associated with schizophrenia and other neuropsychiatric disorders. The molecular, developmental and evolutionary origins of human brain asymmetry are unknown. We found significant association of a haplotype upstream of the gene LRRTM1 (Leucine-rich repeat transmembrane neuronal 1) with a quantitative measure of human handedness in a set of dyslexic siblings, when the haplotype was inherited paternally ($P=0.00002$). While we were unable to find this effect in an epidemiological set of twin-based sibships, we did find that the same haplotype is overtransmitted paternally to individuals with schizophrenia/schizoaffective disorder in a study of 1002 affected families ($P=0.0014$). We then found direct confirmatory evidence that LRRTM1 is an imprinted gene in humans that shows a variable pattern of maternal downregulation. We also showed that LRRTM1 is expressed during the development of specific forebrain structures, and thus could influence neuronal differentiation and connectivity. This is the first potential genetic influence on human handedness to be identified, and the first putative genetic effect on variability in human brain asymmetry. LRRTM1 is a candidate gene for involvement in several common neurodevelopmental disorders, and may have played a role in human cognitive and behavioral evolution.

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Introduction

Left–right asymmetrical function is a conserved feature of vertebrate central nervous systems.¹ Asymmetrical brain function is pronounced in humans, and underlies much of our behavior, cognition and

emotion.² Approximately 90% of humans are right-handed.² This is the strongest population-level bias in handedness for any primate.^{1,2} Left-handedness in humans is associated with reductions or reversals of normal brain asymmetries,³ particularly of cerebral cortical areas related to language perception and production. This association is likely to be partly genetic in etiology.⁴ Handedness and complex cognition in humans may, therefore, be related developmentally and evolutionarily, although the nature and extent of these relationships remain uncertain.

We performed previously the first genome-wide linkage scan for a quantitative measure of human handedness⁵ (relative hand skill; assessed with timed peg moving⁵), and found linkage on 2p12–q11.⁵ This analysis was performed in 222 reading-disabled siblings,⁶ although there was no relationship detectable between reading disability (RD) and relative hand skill in this sample.^{5,7} We then replicated the 2p12–q11 linkage,⁸ with the same measure of relative hand skill, in 105 pairs of left-handed brothers ($P=0.0009$).⁸ We found that the linkage to relative hand skill, in the RD sibs, was derived entirely from paternal inheritance of 2p12–q11 ($P=0.000037$),⁹ whereas the maternally inherited locus was not linked.⁹ This suggested that the underlying gene was imprinted, and inactivated or downregulated on the maternally inherited chromosome.⁹ We did not have parental genotypes to assign parent-of-origin for analysis of the left-handed brother sample.⁹ However, under standard linkage analysis, the peak location of linkage to relative hand skill in the left-handed brothers was the same as the peak paternal-specific location in the dyslexic siblings,⁹ and was within the gene CTNNA2 (α -N-catenin) on 2p12.

Schizophrenia is a heterogeneous neuropsychiatric disorder that affects roughly 1% of the adult human population. Schizophrenia is associated with reductions or reversals of normal cerebral asymmetries, including the medial temporal lobe, superior temporal gyrus, planum temporale and the overall brain anterior–posterior torque.^{10,11} Schizophrenia is also associated with a slightly elevated rate of mixed or left-handedness.^{12,13} Crow drew attention to the association of abnormal brain asymmetry with schizophrenia-like psychoses,¹⁴ and was drawn to a human-specific rearrangement of the sex chromosomes and a candidate gene there,¹⁵ Protocadherin X-Y. However, this gene has not so far been shown to be linked to asymmetry, language or psychosis.

In a recent meta-analysis of 20 linkage screens for schizophrenia, 2p12–q22 was the only location to reach significance when adjusted for genome-wide testing.¹⁶ This linkage was derived primarily, but not exclusively, from a sample of affected sibling pairs of European descent that were recruited in New York and Oxford.¹⁷ We showed previously that the 2p12–q11 linkage in this sample was derived predominantly from paternal sharing (log of the odds, LOD=4.72), not maternal sharing (LOD=0.6).⁹ As parent-of-origin effects are unusual in the genome, we

proposed in 2003 that a single paternally expressed gene was responsible for the linkages of 2p12–q11 to handedness and schizophrenia.⁹

In the present study we have used genetic association mapping and gene-functional analysis to identify a novel imprinted gene on 2p12, LRRTM1, that we propose is responsible for causing the linkages of this chromosomal region to human handedness and schizophrenia.

Materials and methods

All work involving human samples and materials was approved by the appropriate institutional review boards, and appropriate informed consent was obtained from all human subjects.

Details of the Materials and methods are given in Supplementary information online.

Results

We genotyped 87 single-nucleotide polymorphisms (SNPs) in 222 RD siblings and their parents, within a region of paternal-specific linkage to relative hand skill that we had detected previously in this sample (Figure 1). The SNPs were targeted within four positional and functional candidate genes on 2p12–p11 (LRRTM4, CTNNA2, LRRTM1 and DNAH6) (Figure 1 and Table S1). Following our parent-of-origin linkage data (Figure 1), we tested for quantitative association of paternally inherited SNP alleles with relative hand skill (using QTDT¹⁸). Four SNPs in distinct locations (rs1517771, rs290015, rs2063436 and rs723524; Table S1), which were not in significant linkage disequilibrium (LD) with one another, showed nominally significant paternal-specific association with relative hand skill ($0.05 > P > 0.01$). This was no more than the expected false-positive rate.

We then aimed to interrogate the underlying haplotype structure by testing haplotypes for association, that were constructed in 'sliding windows' representing all sets of three consecutive SNPs (disregarding any instances where SNPs were not in significant LD, that is, $P > 0.05$ based on χ^2 -test of pairwise LD). We used the total, paternal and multi-allelic options of QTDT together for the association tests. Significance levels for paternal-specific association were all $P > 0.05$, except for haplotypes derived from the SNPs rs1446109-rs1007371-rs723524 ($P=0.00002$), and the overlapping window rs1007371-rs723524-rs1025947 ($P=0.0007$). These SNPs are on 2p12 within a region of strong inter-marker LD that spans at least the first exon and 137 kb upstream of the gene LRRTM1, which is located within an intron of CTNNA2 (Figure 1). There was no significant maternal-specific haplotype association at any location (all $P > 0.05$).

The rs1446109-rs1007371-rs723524 haplotype 2-2-2 (where 2 is the minor allele of each SNP) was responsible primarily for the paternal-specific association observed with haplotypes derived from these

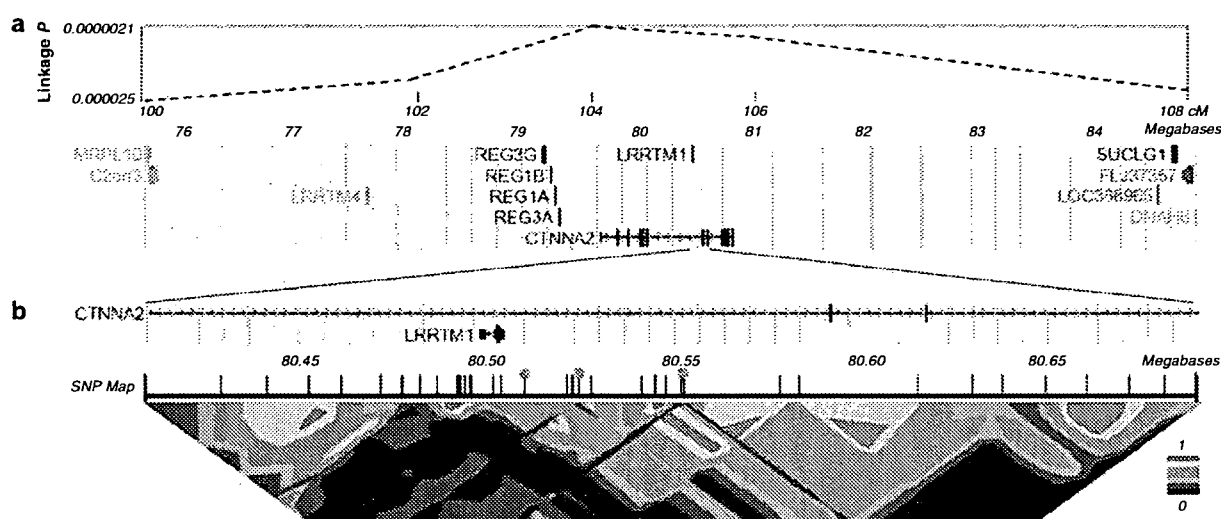


Figure 1 Mapping of an imprinted quantitative-trait-locus (QTL) for human handedness. (a) Top: pointwise significance of linkage derived from paternal sharing across 8 cM of chromosome 2p12-p11.¹⁸ This corresponds roughly to a 1-LOD unit support interval for the QTL. Significance of linkage is shown on a logarithmic scale. Bottom: known genes within the region (adapted from UCSC Genome Browser). Note LRRTM1 (transcribed proximal to distal (right to left)) sited within an intron of CTNNA2 (transcribed distal to proximal). (b) Close up of 286 kb around LRRTM1. Two exons of CTNNA2 are also visible. The final SNP map that we used in this region is shown (see also Table S1), and the three SNPs that showed the initial paternal-specific haplotype associations are highlighted by pink circles (rs1446109-rs1007371-rs723524). Pairwise intermarker LD (Cramer's V) is shown at the bottom.

three SNPs. The 2-2-2 haplotype had 9% frequency, and was associated with a mean shift of 1.1 s.d. toward left-handedness in the relative hand skill distribution^{5,7} when inherited paternally, compared to all other haplotypes. Genotyping of 32 more SNPs, including 12 within 137 kb upstream of LRRTM1, confirmed that rs1446109-rs1007371-rs723524 haplotype 2-2-2 represents a distinct haplotype clade for at least 76 kb upstream of, and including, the predicted promoter of LRRTM1 (Figure S1 and Table S1).

We screened the first two exons and predicted promoter of LRRTM1 for polymorphisms in 26 left-handers from the RD sample by denaturing high-performance liquid chromatography and sequencing, but we did not detect any polymorphisms that tagged rs1446109-rs1007371-rs723524 haplotype 2-2-2, or that had overt disruptive effects on the predicted LRRTM1 protein (entirely coded within exon 2). The only novel SNP that we identified was upstream of the first LRRTM1 exon (Table S1).

We analyzed the SNPs rs1007371 and rs723524, which together can be used to construct a close proxy to the 'risk' haplotype described above (see Figure S1), in a sample of normal twin-based sibships from Brisbane, Australia, that was derived from 215 independent families. We found no significant evidence for paternal association of the risk haplotype with our quantitative measure of human handedness ($P > 0.1$).

We genotyped the SNPs rs1446109 and either rs723524 or rs718466 (the latter are equivalent tagging SNPs according to international HapMap data) in four

family samples of white European descent that included individuals with schizophrenia or poor-outcome schizoaffective disorder. These comprised a subset of the New York/Oxford sample¹⁷ (226 families), an Irish 'High Density' sample¹⁹ (236 families), a sample collected in Montreal²⁰ (124 families) and an Afrikaner sample²¹ (416 families) (Table 1). The 2-2 haplotype defined by the rare alleles of these SNPs is equivalent to the risk haplotype for left-handedness defined above (Figure S1). The 2-2 haplotype varied in frequency between 7.6 and 12.1% in the four sample sets. We found that haplotype 2-2 was overtransmitted paternally to affected individuals in a combined analysis of the four samples, $P = 0.0014$ (one-tailed test, 38 transmissions to 16 nontransmissions; Table 1). This was a specific hypothesis test that requires no statistical adjustment. There was no significant paternal overtransmission of any other haplotype, nor was there maternal overtransmission of any haplotype. The paternal 2-2 result was derived roughly equally from three of the four samples (Table 1).

We genotyped rs1446109 (almost tagging for the risk haplotype; Figure S1), in two case-control collections of European descent (461 cases and 459 controls from Munich, Germany, and 429 cases and 428 controls from Scotland (all cases had *Diagnostic and Statistical Manual of Mental Disorders*, fourth edition diagnoses of schizophrenia, and both sample sets were recruited according to the same protocol)).²² As the parental origin of the alleles could not be established and the paternal and maternal alleles

Table 1 Transmission disequilibrium statistics for two-marker haplotypes (see main text), for families affected with schizophrenia/schizoaffective disorder

	N	%	Paternal			Maternal			Both parents		
			TR	NT	χ^2	TR	NT	χ^2	TR	NT	χ^2
<i>New York/Oxford</i>											
Haplo.1	504	81.8	18	27	1.80	21	19	0.10	40	47	0.56
Haplo.2	50	8.1	15	6	3.86	8	9	0.06	23	15	1.68
Haplo.3	61	9.9	13	13	0.00	15	14	0.03	29	28	0.02
Haplo.4	1	0.2	0	0	0.00	0	2	2.00	0	2	2.0
<i>Irish high density</i>											
Haplo.1	336	79.6	6	14	3.20	15	17	0.12	23	33	1.79
Haplo.2	51	12.1	10	1	7.36	11	6	1.47	22	8	6.53
Haplo.3	30	7.1	5	5	0.00	5	8	0.69	11	14	0.36
Haplo.4	5	1.2	0	1	1.00	1	1	0.00	1	2	0.33
<i>Montreal</i>											
Haplo.1	327	86.5	4	4	0.00	11	9	0.20	15	13	0.14
Haplo.2	31	8.2	3	3	0.00	5	12	2.88	8	15	2.13
Haplo.3	19	5.0	2	2	0.00	7	2	2.78	9	4	1.92
Haplo.4	1	0.3	0	0	0.00	0	0	0.00	0	0	0.00
<i>Afrikaner</i>											
Haplo.1	1013	81.0	16	19	0.26	21	18	0.23	39	39	0.00
Haplo.2	95	7.6	10	6	1.00	4	14	5.56	15	21	1.00
Haplo.3	118	9.4	11	9	0.20	15	8	2.13	27	18	1.80
Haplo.4	24	1.9	2	5	1.29	3	3	0.00	5	8	0.69
<i>Total sample</i>											
Haplo.1	2180	81.8	44	64	3.70	68	63	0.19	117	132	0.90
Haplo.2	227	8.5	38	16	8.96	28	41	2.45	68	59	0.64
Haplo.3	228	8.6	31	29	0.07	42	32	1.35	76	64	1.03
Haplo.4	31	1.2	2	6	2.00	4	6	0.40	6	12	2.00

χ^2 , χ^2 statistic; haplo.1, haplotype 1-1; haplo.2, haplotype 2-2; haplo.3, haplotype 1-2; haplo.4, haplotype 2-1; N, number of instances of the haplotype in the sample; NT, number of non-transmitted haplotypes from heterozygous parents; TR, number of transmitted haplotypes from heterozygous parents; %, haplotype frequency. The left-handedness risk haplotype 2-2 is highlighted in bold.

were confounded, we expected this analysis to be low-powered to detect an imprinted effect. Nonetheless, this SNP showed a trend toward association ($P=0.09$) when tested under a genotypic 2-df logistic regression model using covariates for gender and the collection site (Munich/Aberdeen). The additive component of this model was significant at $P=0.036$, and the direction of allelic effect was the same as in the family samples. When we repeated the analysis using only those cases (151) who reported a positive family history of schizophrenia or bipolar disorder, with the aim of removing sporadic environmental cases, rs1446109 showed significant association with $P=0.013$, and the additive component of the model showed $P=0.0038$, again in the expected direction. We also tested for association in a sample of 270 Han

Chinese families²³ but we found no significant bias in paternal or maternal transmission of any haplotype to schizophrenic people (there were 65 paternal transmissions to 78 paternal nontransmissions of the 2-2 haplotype).

No imprinted genes were known previously on chromosome 2p. We found that human LRRTM1 is imprinted (paternal-only expression) in hybrid A9 cells²⁴ (mouse cell lines containing single human chromosomes of known parental origin) (Figure 2). Despite certain limitations with hybrid cells in the

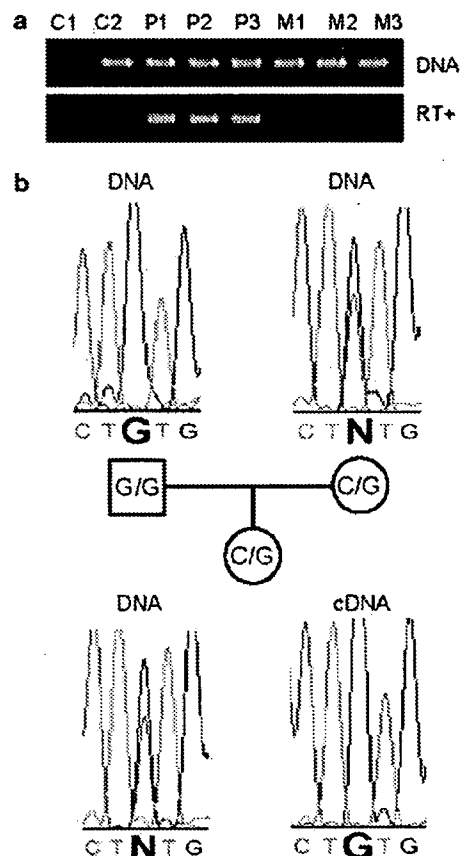


Figure 2 LRRTM1 expression is downregulated maternally in humans. (a) Data are shown from A9 cells that each contains a single human chromosome 2 of known parental origin. Products are shown from PCR using primers specific for human LRRTM1. Upper panel: the human gene is present in genomic DNA (labeled 'DNA') from all cell lines tested, apart from C1 (mouse A9 cell line containing no human chromosome). Lower panel: the human gene is only expressed (detected by RT-PCR) in three cell lines containing paternally derived human chromosome 2s (P1, P2, P3), and not in three cell lines containing maternally derived human chromosome 2s (M1, M2, M3). (C2 is a human fibroblast cell line). (b) Monoallelic paternal expression of LRRTM1 in a human EBV-transformed lymphoblastoid cell line. Sequence traces derived from genomic DNA (labeled 'DNA') surrounding a C/G SNP are shown for a father, mother and child. The child is heterozygous, but the cDNA prepared from mRNA showed expression of only the paternally inherited G allele of LRRTM1.

context of imprinting studies,²⁵ mouse A9 cells have been used for the reliable identification and verification of human imprinted genes.²⁴ To validate the chromosome 2 hybrid cells, we analyzed the expression of four additional biallelically expressed genes, GGCX (2p11.2), BCL2L11 (2q13), TCF7L1 (2p11.2) and RALB (2q14.2). We detected the expression of all four genes in chromosome 2 hybrid cells, regardless of the parental origin of the human chromosome 2 (Figure S2). No detection of these genes was obtained in RT- controls. We also used transcribed polymorphisms to show mono-allelic LRRTM1 expression in tissue samples from a minority of unrelated post-mortem human brains (3 out of 18) by RT-PCR and sequencing or restriction digestion (not shown). However, biallelic expression was found in 15 out of 18 brains, and LRRTM1 was expressed at similar levels from both alleles in the adult human cerebral cortex of five individuals showing biallelic expression (analyzed by quantitative PCR). All brain tissue was obtained from normal controls that were unaffected by major neurological or psychiatric disease. These results suggest that imprinting of LRRTM1 is either variable between individuals (as for the imprinted genes IMPT1 or IGF2²⁶), and/or variable between different brain regions or cell/tissue types (as for UBE3A²⁷). We also found mono-allelic paternal expression of LRRTM1 in four out of four unrelated EBV-transformed human lymphoblastoid cell lines that were heterozygous for a transcribed SNP (Figure 2).

By use of *in situ* hybridization in the mouse, we found that *Lrrtm1* is expressed predominantly in the nervous system by postmitotic neurons, but also in some nonneuronal tissues (Figure 3; Figure S3). Expression is upregulated in the mouse brain during embryonic development and early postnatally. In adult brain, *Lrrtm1* expression is most prominent in the forebrain, particularly in the thalamus (in most or all nuclei), and in cortical areas including hippocampus, piriform and posterior cingulate (Figure 3; Figure S3).

In northern blot analysis of adult human brain, LRRTM1 also showed predominant expression in forebrain regions including thalamus and cerebral cortex (Figure S4). By *in situ* hybridization in coronal sections of the post-mortem developing human brain (14–16 weeks gestation; Figure 4), strong expression was observed in anterior sections throughout the cortical plate and in septum, caudate and putamen. The absence of signal in the subventricular zone argues against a direct involvement in neurogenesis (Figure 4). Transcript distribution was similar in more caudal sections with the addition of signal in dorsolateral thalamus (Figure 4). More caudal still, thalamic signal shifted ventrally to a structure consistent with the lateral geniculate body (Figure 4). The striking signal in caudate and putamen in human (Figure 4) was not present in mouse, at least not at E15 and in adult. In addition, expression within the human thalamus was more restricted as

compared to the mouse, with staining relatively limited to dorsomedial regions.

No consistent asymmetric expression was observed in any of three human developing brains examined by *in situ* hybridization (14–16 weeks' gestation), regardless of whether cerebral hemispheres were analyzed in aggregate or cortical sub-regions were examined in isolation (dorsolateral, temporal, ventrolateral or cingulate). Similarly to embryonic brain, LRRTM1 was expressed at similar levels (that is, symmetrically) in all analyzed regions of left and right adult human cerebral cortex (several different cortical regions from five individuals were analyzed by quantitative PCR). We also quantified left- and right-brain *Lrrtm1* mRNA expression levels in rats and embryonic mice (see Materials and methods), but did not detect evidence for asymmetrical expression in rodents. In addition, we tested cerebral cortex, cerebellum, brain stem, olfactory bulb, thymus, heart, lung, liver, intestine, pancreas, spleen, kidney, muscle and testis of two reciprocal crossed F1 mice between C57BL/6J and JF1 strains for allele-specific expression. Each of the F1 mice was 30 weeks old. Expression of *Lrrtm1* was detected in cerebral cortex, cerebellum and brain stem, but it was biallelic (Figure S5).

In rodent primary sensory (DRG) and cortical neurons (Figure 3), and in cerebellar granular neurons (data not shown), overexpressed LRRTM1 localized to the cell soma, neurites and lamellipodia of growth cones, suggesting a function in axon guidance and/or synaptogenesis. Unexpectedly, in transfected MRC5, Cos-7 and Neuro-2a cells, LRRTM1 colocalized with endoplasmic reticulum (ER) markers (Figure 5 and Figure S6). Live-cell staining for overexpressed LRRTM1 in DRG neurons revealed that the protein is not accessible on the plasma membrane under conditions that allowed surface detection of a related member of the LRR protein superfamily, Lingo1 (Figure 5). These results suggest that endogenous LRRTM1 may have a role in intracellular trafficking within axons. However, it remains possible that LRRTM1 is localized to plasma membrane in cells expressing an unidentified LRRTM1 chaperone or coreceptor protein that promotes its processing and/or transport.

We analyzed methylation within 2 CpG islands that correspond to the predicted promoter and coding exon of LRRTM1, and a third island roughly 18 kb upstream of LRRTM1, in 17 lymphoblastoid cell lines and 17 human post-mortem brain samples, but we did not find evidence that these CpG islands are differentially methylated regions (DMRs) (data not shown).

We tested CTNNA2 for mono-allelic expression in 10 post-mortem brain samples from normal, unrelated individuals, by use of a transcribed SNP in exon 12, but expression was always biallelic, in contrast to LRRTM1 (data not shown). We did not detect CTNNA2 expression in the A9 hybrids, or in human lymphoblastoid cell lines, by RT-PCR. The first CTNNA2 exon is over 750 kb from LRRTM1 (Figure 1), and several LD blocks away, further

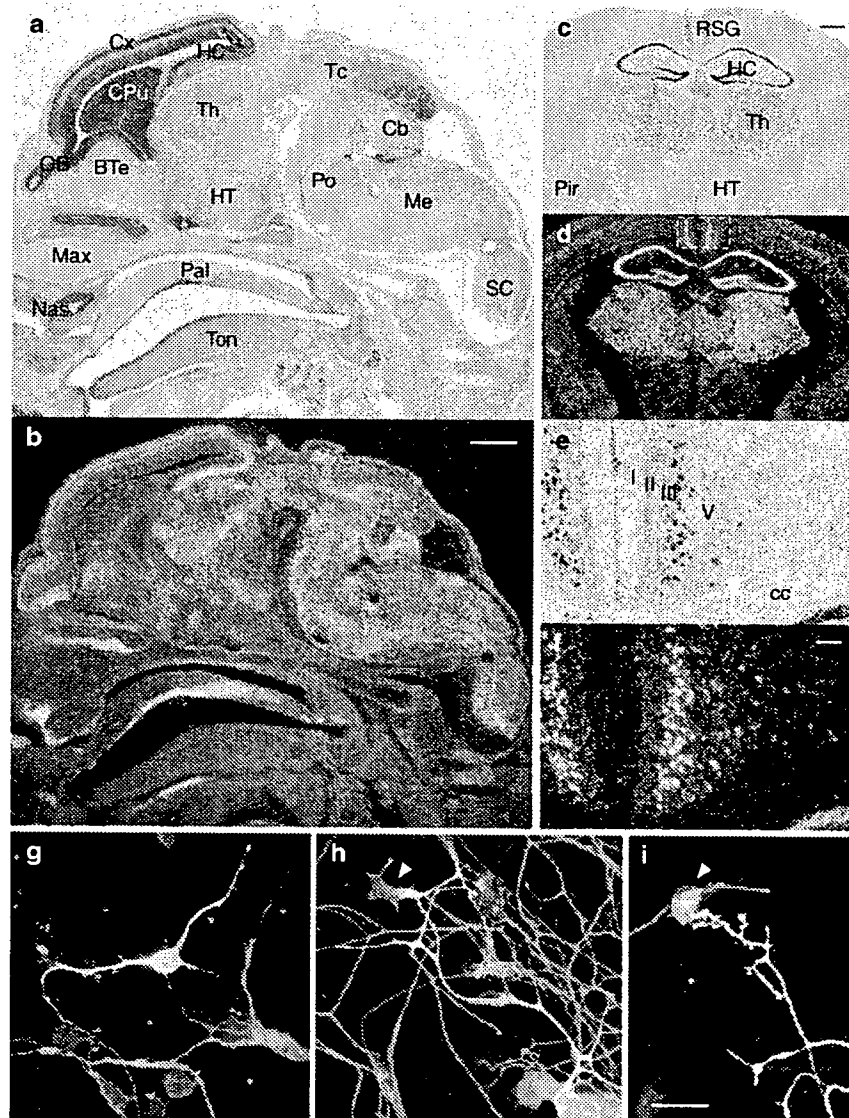


Figure 3 Developmental increase of *Lrrtm1* mRNA expression in mouse thalamus, hippocampus and retrosplenial cortex, and localization of LRRTM1 in neurons. *In situ* hybridization analysis in sagittal sections of E15 mouse embryos (a and b) and coronal sections of adult mouse brain (c–f). Bright-field images counterstained with hematoxylin (a, c and e) and corresponding dark-field autoradiographs are shown (b, d and f). The white signal in the dark-field images indicates *Lrrtm1* expression. (e and f) Higher magnification of the retrosplenial granular cortex (boxed area in d) shows a high level of *Lrrtm1* expression in layer III–IV neurons. (g–i) Myc-LRRTM1 was transfected into E17 mouse cortical neurons (g) and nucleofected into rat P18 dorsal root ganglion neurons (h and i) and detected 40 h later with anti-myc antibody. Neurons were visualized with anti- β -tubulin antibody. LRRTM1 is localized to the cell soma and in the neurites; in neurites it is also localized to lamellipodia of the growth cones (arrowheads in h and i). BTe, basal telencephalon; Cb, cerebellum; cc, corpus callosum; CPu, caudate-putamen; Cx, cerebral cortex; HC, hippocampus; HT, hypothalamus; Max, maxilla; Me, medulla; Nas, nasal cavity; OB, olfactory bulb; Pal, palatine; Pir, piriform cortex; Po, pons; RSG, retrosplenial granular cortex; Sc, Spinal cord; Tc, tectum; Th, thalamus; Ton, tongue. Scale bar is 500 μ m in (a–d), 100 μ m in (e–f) and 20 μ m in (g–i).

suggesting that the regulation of these genes is distinct. There are no other genes within 1.13 Mb of LRRTM1, and the next closest gene expressed in the forebrain (expression data available via the UCSC genome browser) is over 2.75 Mb distally (LRRTM4; Figure 1).

In order to analyze the evolution of LRRTM1, we compared it between humans and chimpanzees. There is no fixed amino acid difference between human LRRTM1 and chimpanzee LRRTM1, and it is not among the genes that are differently expressed between adult humans and adult chimpanzees in

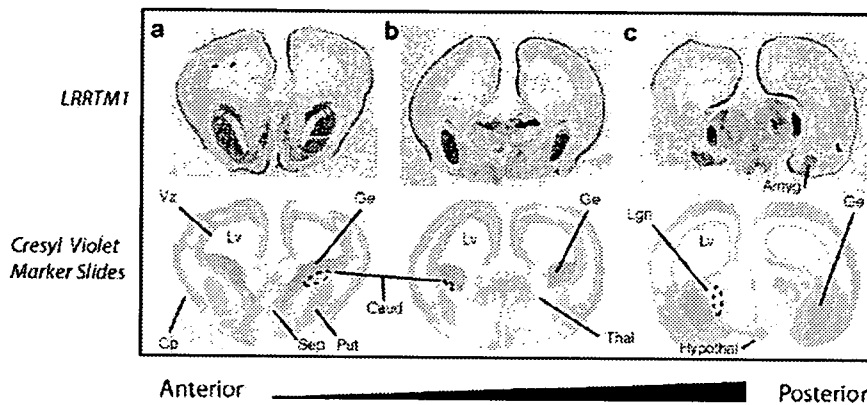


Figure 4 *In situ* hybridization analysis of *LRRTM1* expression in the developing human brain at 15 weeks' gestation. In coronal sections of anterior brain (a), expression is strong throughout the cortical plate and otherwise restricted to septum, caudate, and putamen. Transcript distribution is similar in more caudal sections (b) with the addition of signal in dorsolateral thalamus. More caudal still (c), thalamic signal shifts ventrally to a structure consistent with the lateral geniculate body. Abbreviations are as follows: Amyg (Amygdala), Caud (Caudate), Cp (Cortical), Ge (Ganglionic Eminence), Hypothal (Hypothalamus), Lgn (Lateral Geniculate Nucleus), Lv (Lateral Ventricle), Thal (Thalamus), Vz (Ventricular Zone).

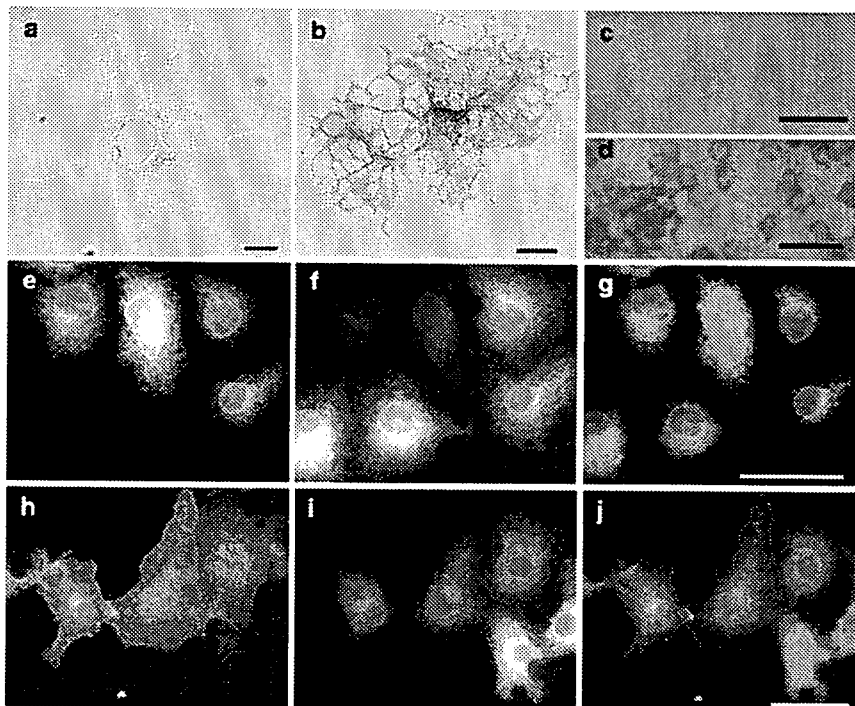


Figure 5 *LRRTM1* is not localized on the plasma membrane. No cell-surface expression of *LRRTM1* is seen in primary sensory neurons electroporated with myc-*LRRTM1* construct and detected by live-cell staining with alkaline phosphatase-conjugated anti-myc antibody (a); strong cell-surface staining of myc-*Lingo1* expressing neuron is seen (b). Live-cell staining resulted in 20–30 strongly stained DRG neurons per well for myc-*Lingo1* but none for myc-*LRRTM1*. Similarly, in neuro-2a neuroblastoma cells myc-*LRRTM1* (c) is not, but myc-*lingo1* is (d) localized to plasma membrane as detected by live-cell staining. In permeabilized Cos-7 cells *LRRTM1* (anti-myc antibody; e) is colocalized (merge picture; g) with ER-marked dsRED2-ER (f), whereas *lingo1* (anti-myc antibody; h) is only partially colocalized with dsRED2-ER, and it is partially localized on the plasma membrane (i; merge picture: j). Scale bars are 50 μm .

brain, liver, kidney, heart or testis.²⁸ Furthermore, biallelic expression of *LRRTM1* was detected in two out of two chimpanzee brain samples (see Materials and methods).

Discussion

Crow proposed that handedness, brain asymmetry and schizophrenia share an underlying genetic

relationship.¹⁴ However, no genes have previously been shown to associate across these traits. We carried out SNP-based association screening for human relative hand skill, under a parent-of-origin model, within four candidate genes close to or within 6 mb of a peak of paternal-specific linkage on 2p12, in a sample of sib pairs recruited for the presence of RD. We found strong paternal-specific association with relative hand skill implicating the gene LRRTM1 (leucine-rich repeat transmembrane neuronal 1) and a region of 80 kb upstream of this gene (which is located within an intron of CTNNA2). We did not find this association in a sample of normal twin-based sibships from Australia. However, consistent with our parent-of-origin association data, we went on to show that LRRTM1 is downregulated on the maternally inherited chromosome, and is therefore a newly identified imprinted locus in the human genome. We also found that the same haplotype that drove the paternal association with handedness was overtransmitted paternally, but not maternally, to schizophrenic/schizoaffective individuals in 1002 affected families of European descent ($P=0.0014$). This was a specific hypothesis test that requires no statistical adjustment, and indicates that LRRTM1 is a novel susceptibility factor for schizophrenia. We also found consistent evidence for association in a large case-control sample of European descent that was collected in Scotland and Germany.

We did not find that the 'risk' 2-2 haplotype that we identified in European family-based samples was associated with schizophrenia in a Han Chinese family-based sample. The frequency of the 2-2 risk haplotype was somewhat higher in the Han Chinese, at 18%. The failure to replicate may nonetheless be related to modest power in the Chinese sample of 270 families. Alternatively, the nonreplication in the Chinese may indicate that the 2-2 haplotype does not carry risk variation in this population (it may also be relevant that native Chinese speakers show some morphological differences to English speakers in language-related areas of the brain²⁹). A further study in a larger Chinese sample will be required to distinguish these possibilities, together with identification of the functional variants in European populations.

Of a total of approximately 35 000 human genes, only roughly 40 were previously known to be imprinted, and the total number is unlikely to exceed this greatly.³⁰⁻³² The finding that LRRTM1 is imprinted, and maternally suppressed, was a confirmation of the parent-of-origin genetic data that led to this locus, because the *a priori* probability that LRRTM1 would be imprinted was very low (roughly 1 in 1000). Our association data suggest that allelic variation upstream of LRRTM1, that may affect the gene's expression, is relevant functionally for brain asymmetry when inherited on the active paternal chromosome, but not on the relatively inactive maternal chromosome, at certain locations and time points during human brain development. However, the 'risk'

haplotype that we identified at LRRTM1 cannot explain the majority of the paternal-specific linkage across the genomic region of 2p12 (Figure 1), and therefore additional genetic or epigenetic variability at LRRTM1, or neighboring genes, may also be responsible partly for the linkages of handedness and schizophrenia to 2p12.⁹ Although we found evidence that LRRTM1 is variably imprinted in the post-mortem adult human brain (as is the imprinted gene 5HT2A),³³ we cannot conclude directly, on the basis of our data, that variability in imprinting of LRRTM1 is involved in individual differences in human handedness and schizophrenia liability. Our data indicate a role for the 2-2 haplotype in handedness variability and susceptibility to schizophrenia, in European populations, while the data on imprinting are consistent with the paternal-specific nature of this effect.

Our data suggest that a subtype of schizophrenia, linked to misregulation of human LRRTM1, may have its origins in fetal neurodevelopment. Since LRRTM1 appears to underlie the strongest linkage to schizophrenia in the genome, as identified by a meta-analysis of 20 genomewide linkage scans,¹⁶ it is possible that LRRTM1 dysfunction causes a major, common subtype of schizophrenia. Assessing the frequency of this subtype will require studies in further clinical and epidemiological samples, together with a better definition of the functional genetic and epigenetic variation at the LRRTM1 locus. This information may have a substantial impact on pharmacogenetic studies and the development of new treatments for schizophrenia, by allowing patient heterogeneity to be accounted for in clinical trial studies. The receptor-like structure of LRRTM1 also suggests that it may be a drug-tractable target in its own right.

LRRTM1 is one of a four-member family of type I transmembrane proteins containing leucine-rich repeat (LRR) domains, which are commonly involved in protein-protein interactions (LRRs are present in the Slits and Nogo-receptor,^{34,35} involved in axonal pathfinding). Each LRRTM member has a specific, dynamically regulated regional brain-expression distribution.³⁵ Using several gene-functional approaches, we found that LRRTM1 is likely to play a role during the development of specific forebrain structures by influencing neuronal differentiation and connectivity, with a possible role in intracellular trafficking in axons. Thus, LRRTM1 is an ideal candidate gene for having an involvement in subtle developmental abnormalities of the central nervous system.

When comparing expression between human and mouse, it seems that there is a striking absence of signal in caudate and putamen in mouse, at least at e15 and in adult. Also, expression within the fetal human thalamus is more restricted as compared to the mouse, with staining relatively limited to dorso-medial regions that have been implicated in schizophrenia.^{36,37} We found no evidence for overtly asymmetrical expression of *Lrrtm1* in the developing

or adult brain of rodents or humans, using *in situ* hybridization and/or quantitative PCR. However, we cannot rule out a subtle asymmetry of function or expression at some restricted time point during human brain development, as for the transcription factor LMO4.³⁸ It remains possible that LRRTM1 is important in the establishment, consolidation or elaboration of the left–right axis during human brain growth and development, particularly before 14 weeks' gestation, which was the earliest stage that we were able to analyze in human. (Population-level morphological asymmetries of the cerebral cortex are already noticeable shortly after this time, and are visible by ultrasound in normal fetuses at 20–22 weeks' gestation.³⁹) Therefore, detailed studies of the roles of LRRTM1 and the pathways in which it functions in mammalian brain development are warranted, particularly at developmental time points earlier than 14 weeks in humans, that may reveal critical new insights into the establishment and/or maintenance of normal and abnormal human brain function and asymmetry.

Humans have the strongest population-level bias in handedness of any primate,^{1,2} and LRRTM1 is a candidate for having had a role in the evolution of this trait. As the human and mouse predicted LRRTM1 proteins are 96% identical, and the human and chimpanzee proteins are 100% identical, any potential human-specific properties of LRRTM1 may involve the spatiotemporal control of its imprinted expression. (It is interesting that recent analysis of conserved noncoding elements demonstrated that a sequence element 130 kb downstream of LRRTM1 displays accelerated evolution in the human lineage, which may be important for the gene's regulation).⁴⁰ We detected only biallelic, nonimprinted expression of *Lrrtm1* in cerebral cortex, cerebellum, and brain stem from two 30-week-old reciprocal crossed F1 mice between C57BL/6J and JF1 strains (data not shown), and only biallelic expression in the post-mortem brains of two adult chimpanzees. Larger sample sizes will be needed to test whether imprinting of LRRTM1 is found only in humans. (There is a high level of discordance in imprinting between humans and mice;³² the gene *DLX5* is an example.⁴¹) It will also be interesting to compare the regulation of LRRTM1 longitudinally during the development of humans and other species, as the imprinted regulation may be restricted to certain developmental periods.³⁸ In addition, human genetic variation at this locus in nonclinical populations could be analyzed with dense marker genotyping, to test for evidence of selection, particularly with regard to the risk haplotype.

Genomic imprinting can arise when the optimal level of maternal resource investment in offspring differs between the two parental sexes, in polygamous mating systems.^{31,42} Paternally inherited alleles of imprinted genes often sequester more maternal resources than do maternally inherited alleles, especially when influencing growth *in utero*.^{31,42}

The imprinting of LRRTM1 in humans, therefore, suggests an intriguing evolutionary scenario, in which the parental sexes have conflicting interests in relation to the outcome of lateralized brain development in their offspring, which underlies much of human cognition and behavior. Further study of the neural systems and behaviors that LRRTM1 influences will be required to understand the selective forces that drove LRRTM1 to be imprinted.

We did not detect evidence for differential methylation of the paternal and maternal alleles at the CpG islands associated directly with LRRTM1, and therefore the mechanism underlying imprinted expression of LRRTM1 remains unclear. However, many other imprinted genes are not regulated by differential methylation within their own promoters, but are regulated instead by imprinted control centers which may be located many kilobases distant.³¹ Also, allele-specific histone modifications are emerging currently as equally important determinants of imprinted gene expression.⁴³ The lack of methylation does not, therefore, refute the imprinted expression of LRRTM1, which we observed in three different tissues and experimental settings. We recommend that the mechanism of imprinted regulation at this locus should be investigated as a priority, as epigenetic misregulation of LRRTM1 may have clinical relevance and could underlie much of the paternal linkage of 2p12 to schizophrenia.

We analyzed schizophrenia and schizoaffective disorder jointly in the New York/Oxford sample to maximize the numbers of informative paternal transmissions for the risk haplotype (Table 1). However, this sample was not sufficiently large to test for heterogeneity of the association effect between these two patient groups. Our previous linkage analysis⁹ of 2p12 had indicated that the linkage was stronger for the subset of sibling pairs with schizophrenia only (paternal LOD 4.72), but this remains to be investigated more thoroughly by targeted association analysis in additional samples.

LRRTM1 is the first genetic influence on human handedness to be identified. Handedness, brain asymmetry and schizophrenia are likely to be etiologically complex traits with several, or many, genetic and environmental influences. As we detected an effect of LRRTM1 on handedness in dyslexic siblings, but not in normal twin-based sibships, it is possible that LRRTM1 influences behavioral lateralization more strongly in clinically selected populations for psychiatric/neurodevelopmental dysfunctions, than in the general population. This would suggest an interactive, nonadditive effect of LRRTM1 with other genetic and/or environmental effects that predispose to neuropsychiatric dysfunction, such that the effect of LRRTM1 depends on other risk factors in order to manifest fully. However, it is also possible that the failure to replicate the association with handedness was due to low power and a relatively small replication sample in our study. It is a well-described

statistical feature of genetic association analysis that follow-up samples should usually be much larger than initial samples to provide adequate power, as the genetic effect in the initial sample is typically inflated.⁴⁴ Unfortunately, we are unaware of other family-based collections with accurate quantitative assessments of handedness, that are suitable for genetic studies.

In addition to schizophrenia, other neurodevelopmental disorders including bipolar disorder, autism and language impairment have shown evidence for associations with left-handedness and/or abnormal asymmetrical brain structure/function.^{2,10,11,45–49} LRRTM1 is, therefore, also a candidate for involvement in these traits. The LRRTMs are a four-member gene family first described in 2003, and it is interesting to note that LRRTM3 has recently been proposed as a susceptibility factor for late-onset Alzheimer's disease.⁵⁰ We recommend, therefore, that the whole LRRTM gene family is investigated in relation to psychiatric and neurological disorders.

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References

- 1 Rogers LJ, Andrew R (eds). *Comparative Vertebrate Lateralization*. Cambridge University Press: Cambridge, UK, 2002.
- 2 Hugdahl K, Davidson RJ (eds). *The Asymmetrical Brain*. MIT Press: Cambridge, MA, 2003.
- 3 Mevorach C, Humphreys GW, Shalev L. Attending to local form while ignoring global aspects depends on handedness: evidence from TMS. *Nat Neurosci* 2005; **8**: 276–277.
- 4 Geschwind DH, Miller BL, DeCarli C, Carmelli D. Heritability of lobar brain volumes in twins supports genetic models of cerebral laterality and handedness. *Proc Natl Acad Sci USA* 2002; **99**: 3176–3181.
- 5 Francks C, Fisher SE, MacPhie IL, Richardson AJ, Marlow AJ, Stein JF *et al*. A genome-wide linkage screen for relative hand skill in sibling pairs. *Am J Hum Genet* 2002; **70**: 800–805.
- 6 Fisher SE, Francks C, Marlow AJ, MacPhie IL, Newbury DF, Cardon LR *et al*. Independent genome-wide scans identify a chromosome 18 quantitative-trait locus influencing dyslexia. *Nat Genet* 2002; **30**: 86–91.
- 7 Francks C, Fisher SE, Marlow AJ, MacPhie IL, Taylor KE, Richardson AJ *et al*. Familial and genetic effects on motor coordination, laterality, and reading-related cognition. *Am J Psychiatry* 2003; **160**: 1970–1977.
- 8 Francks C, DeLisi LE, Fisher SE, Laval SH, Rue JE, Stein JF *et al*. Confirmatory evidence for linkage of relative hand skill to 2p12-q11. *Am J Hum Genet* 2003; **72**: 499–502.
- 9 Francks C, DeLisi LE, Shaw SH, Fisher SE, Richardson AJ, Stein JF *et al*. Parent-of-origin effects on handedness and schizophrenia susceptibility on chromosome 2p12-q11. *Hum Mol Genet* 2003; **12**: 3225–3230.
- 10 DeLisi LE, Sakuma M, Kushner M, Finer DL, Hoff AL, Crow TJ. Anomalous cerebral asymmetry and language processing in schizophrenia. *Schizophr Bull* 1997; **23**: 255–271.
- 11 Shenton ME, Dickey CC, Frumin M, McCarley RW. A review of MRI findings in schizophrenia. *Schizophr Res* 2001; **49**: 1–52.
- 12 DeLisi LE, Svetina C, Razi K, Shields G, Wellman N, Crow TJ. Hand preference and hand skill in families with schizophrenia. *Laterality* 2002; **7**: 321–332.
- 13 Orr KG, Cannon M, Gilvarry CM, Jones PB, Murray RM. Schizophrenic patients and their first-degree relatives show an excess of mixed-handedness. *Schizophr Res* 1999; **39**: 167–176.
- 14 Berlim MT, Mattevi BS, Belmonte-de-Abreu P, Crow TJ. The etiology of schizophrenia and the origin of language: overview of a theory. *Compr Psychiatry* 2003; **44**: 7–14.
- 15 Williams NA, Close JP, Giouzeli M, Crow TJ. Accelerated evolution of Protocadherin11X/Y: a candidate gene-pair for cerebral asymmetry and language. *Am J Med Genet B Neuropsychiatr Genet* 2006; **141**: 623–633.
- 16 Lewis CM, Levinson DF, Wise LH, DeLisi LE, Straub RE, Hovatta I *et al*. Genome scan meta-analysis of schizophrenia and bipolar disorder, part II: schizophrenia. *Am J Hum Genet* 2003; **73**: 34–48.

- 17 DeLisi LE, Shaw SH, Crow TJ, Shields G, Smith AB, Larach WV *et al*. A genome-wide scan for linkage to chromosomal regions in 382 sibling pairs with schizophrenia or schizoaffective disorder. *Am J Psychiatry* 2002; **159**: 803–812.
- 18 Abecasis GR, Cookson WO, Cardon LR. Pedigree tests of transmission disequilibrium. *Eur J Hum Genet* 2000; **8**: 545–551.
- 19 Thiselton DL, Webb BT, Neale BM, Ribble RC, O'Neill FA, Walsh D *et al*. No evidence for linkage or association of neuregulin-1 (NRG1) with disease in the Irish study of high-density schizophrenia families (ISHDSF). *Mol Psychiatry* 2004; **9**: 777–783.
- 20 Xiong L, Rouleau GA, DeLisi LE, St Onge J, Najafee R, Riviere JB *et al*. CAA insertion polymorphism in the 3'UTR of Nogo gene on 2p14 is not associated with schizophrenia. *Mol Brain Res* 2005; **133**: 153–156.
- 21 Abecasis GR, Burt RA, Hall D, Bochum S, Doheny KF, Lundy SL *et al*. Genomewide scan in families with schizophrenia from the founder population of Afrikaners reveals evidence for linkage and uniparental disomy on chromosome 1. *Am J Hum Genet* 2004; **74**: 403–417.
- 22 Van den Oord EJCG, Rujescu D, Robles JR, Giegling I, Birrell C, Bukszar J *et al*. Factor structure and external validity of the PANSS revisited. *Schizophr Res* 2006; **82**: 213–223.
- 23 Takahashi S, Cui YH, Kojima T, Han YH, Zhou RL, Kamioka M *et al*. Family-based association study of markers on chromosome 22 in schizophrenia using African-American, European-American, and Chinese families. *Am J Med Genet B Neuropsychiatr Genet* 2003; **120**: 11–17.
- 24 Kugoh H, Mitsuya K, Meguro M, Shigenami K, Schulz TC, Oshimura M. Mouse A9 cells containing single human chromosomes for analysis of genomic imprinting. *DNA Res* 1999; **6**: 165–172.
- 25 Mergenthaler S, Hitchins MP, Blagitko-Dorfs N, Monk D, Wollmann HA, Ranke MB *et al*. Conflicting reports of imprinting status of human GRB10 in developing brain: how reliable are somatic cell hybrids for predicting allelic origin of expression? *Am J Hum Genet* 2001; **68**: 543–545.
- 26 Sakatani T, Wei M, Katoh M, Okita C, Wada D, Mitsuya K *et al*. Epigenetic heterogeneity at imprinted loci in normal populations. *Biochem Biophys Res Commun* 2001; **283**: 1124–1130.
- 27 Yamasaki K, Joh K, Ohta T, Masuzaki H, Ishimaru T, Mukai T *et al*. Neurons but not glial cells show reciprocal imprinting of sense and antisense transcripts of Ube3a. *Hum Mol Genet* 2003; **12**: 837–847.
- 28 Khaitovich P, Hellmann I, Enard W, Nowick K, Leinweber M, Franz H *et al*. Parallel patterns of evolution in the genomes and transcriptomes of humans and chimpanzees. *Science* 2005; **309**: 1850–1854.
- 29 Kochunov P, Fox P, Lancaster J, Tan LH, Amunts K, Zilles K *et al*. Localized morphological brain differences between English-speaking Caucasians and Chinese-speaking Asians: new evidence of anatomical plasticity. *Neuroreport* 2003; **14**: 961–964.
- 30 Imprinted Gene Catalogue. <http://igc.otago.ac.nz/home.html>. Web 2006.
- 31 Reik W, Walter J. Genomic imprinting: parental influence on the genome. *Nat Rev Genet* 2001; **2**: 21–32.
- 32 Morison IM, Ramsay JP, Spencer HG. A census of mammalian imprinting. *Trends Genet* 2005; **21**: 457–465.
- 33 Bunzel R, Blumcke I, Cichon S, Normann S, Schramm J, Propping P *et al*. Polymorphic imprinting of the serotonin-2A (5-HT_{2A}) receptor gene in human adult brain. *Mol Brain Res* 1998; **59**: 90–92.
- 34 Fournier AE, GrandPre T, Gould G, Wang X, Strittmatter SM. Nogo and the Nogo-66 receptor. *Prog Brain Res* 2002; **137**: 361–369.
- 35 Lauren J, Airaksinen MS, Saarna M, Timmusk T. A novel gene family encoding leucine-rich repeat transmembrane proteins differentially expressed in the nervous system. *Genomics* 2003; **81**: 411–421.
- 36 Andrews J, Wang L, Csernansky JG, Gado MH, Barch DM. Abnormalities of thalamic activation and cognition in schizophrenia. *Am J Psychiatry* 2006; **163**: 463–469.
- 37 Harrison PJ, Weinberger DR. Schizophrenia genes, gene expression, and neuropathology: on the matter of their convergence. *Mol Psychiatry* 2004; **10**: 40–68.
- 38 Sun T, Patoine C, Abu-Khalil A, Visvader J, Sum E, Cherry TJ *et al*. Early asymmetry of gene transcription in embryonic human left and right cerebral cortex. *Science* 2005; **308**: 1794–1798.
- 39 Hering-Hanit R, Achiron R, Lipitz S, Achiron A. Asymmetry of fetal cerebral hemispheres: in utero ultrasound study. *Arch Dis Child Fetal Neonat Ed* 2001; **85**: F194–F196.
- 40 Prabhakar S, Noonan JP, Paabo S, Rubin EM. Accelerated evolution of conserved noncoding sequences in humans. *Science* 2006; **314**: 786.
- 41 Kimura MI, Kazuki Y, Kashiwagi A, Kai Y, Abe S, Barbieri O *et al*. Dlx5, the mouse homologue of the human-imprinted DLX5 gene, is biallelically expressed in the mouse brain. *J Hum Genet* 2004; **49**: 273–277.
- 42 Wilkins JF, Haig D. What good is genomic imprinting: the function of parent-specific gene expression. *Nat Rev Genet* 2003; **4**: 359–368.
- 43 Peters AH, Schubeler D. Methylation of histones: playing memory with DNA. *Curr Opin Cell Biol* 2005; **17**: 230–238.
- 44 Ioannidis JPA, Ntzani EE, Trikalinos TA, Contopoulos-Ioannidis DG. Replication validity of genetic association studies. *Nat Genet* 2001; **29**: 306–309.
- 45 Csernansky JG, Schindler MK, Splinter NR, Wang L, Gado M, Seimon LD *et al*. Abnormalities of thalamic volume and shape in schizophrenia. *Am J Psychiatry* 2004; **161**: 896–902.
- 46 De Fosse L, Hodge SM, Makris N, Kennedy DN, Caviness Jr VS, McGrath L *et al*. Language-association cortex asymmetry in autism and specific language impairment. *Ann Neurol* 2004; **56**: 757–766.
- 47 Herbert MR, Harris GJ, Adrien KT, Ziegler DA, Makris N, Kennedy DN *et al*. Abnormal asymmetry in language association cortex in autism. *Ann Neurol* 2002; **52**: 588–596.
- 48 Paulesu E, Demonet JF, Fazio F, McCrory E, Chanoine V, Brunswick N *et al*. Dyslexia: cultural diversity and biological unity. *Science* 2001; **291**: 2165–2167.
- 49 Sommer IE, Ramsey NF, Mandl RC, Kahn RS. Language lateralization in monozygotic twin pairs concordant and discordant for handedness. *Brain* 2002; **125**: 2710–2718.
- 50 Majercak J, Ray WJ, Espeseth A, Simon A, Shi XP, Wolffe C *et al*. LRRTM3 promotes processing of amyloid-precursor protein by BACE1 and is a positional candidate gene for late-onset Alzheimer's disease. *Proc Natl Acad Sci USA* 2006; **103**: 17967–17972.
- 51 Abecasis GR, Cherny SS, Cookson WO, Cardon LR. Merlin—rapid analysis of dense genetic maps using sparse gene flow trees. *Nat Genet* 2002; **30**: 97–101.
- 52 Wright M, De Geus E, Ando J, Luciano M, Posthuma D, Ono Y *et al*. Genetics of cognition: outline of a collaborative twin study. *Twin Res* 2001; **4**: 48–56.
- 53 Maegawa S, Yoshioka H, Itaba N, Kubota N, Nishihara S, Shirayoshi Y *et al*. Epigenetic silencing of PEG3 gene expression in human glioma cell lines. *Mol Carcinog* 2001; **31**: 1–9.
- 54 Stanssens P, Zabeau M, Meersseman G, Remes G, Gansemans Y, Storm N *et al*. High-throughput MALDI-TOF discovery of genomic sequence polymorphisms. *Genome Res* 2004; **14**: 126–133.
- 55 Ehrich M, Nelson MR, Stanssens P, Zabeau M, Liloglou T, Xinarianos G *et al*. Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry. *Proc Natl Acad Sci USA* 2005; **102**: 15785–15790.
- 56 Klumperman J, Schweizer A, Clausen H, Tang BL, Hong W, Oorschot V *et al*. The recycling pathway of protein ERGIC-53 and dynamics of the ER-Golgi intermediate compartment. *J Cell Sci* 1998; **111**: 3411–3425.

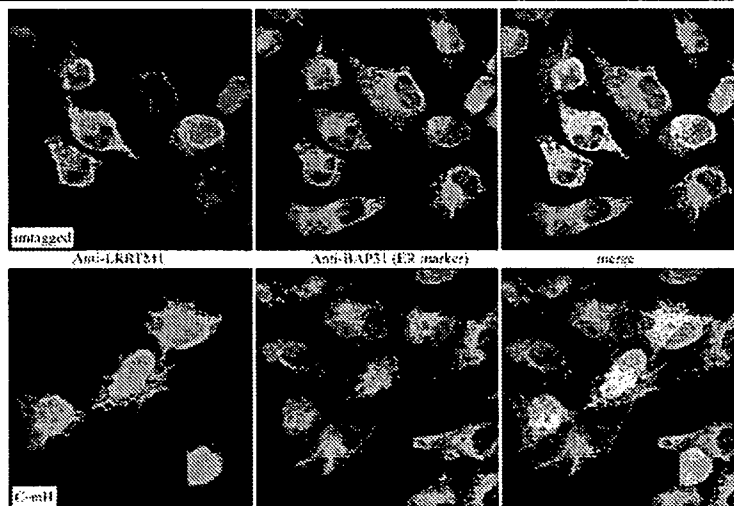
Supplementary Information accompanies the paper on the Molecular Psychiatry website (<http://www.nature.com/mp>)

IMAGE

LRRTM1 protein is located in the endoplasmic reticulum (ER) in mammalian cells

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LRRTM1 is an imprinted gene that shows parent-of-origin association with human handedness and schizophrenia. The left-hand panels (green) show localization of LRRTM1 within MRC-5 SV2 cells, following transfection with untagged (top) or myc + His-tagged (bottom) LRRTM1, and detection with an LRRTM1 antipeptide. The red panels (middle) show the localization of BAP31, an ER marker. In the right-hand panels, the images for LRRTM1 and BAP31 are overlaid, and the yellow areas indicate the colocalization of the two proteins. LRRTM1 was also detected in the axons and growth cones of neurons but not at the cell surface membrane. Together, these data suggest that LRRTM1 may be involved in intracellular trafficking within axons. For more information on this topic, see the paper by Francks *et al.* on pages 1129–1139.

Original article

Antisense suppression of *TSC1* gene product, hamartin, enhances neurite outgrowth in NGF-treated PC12h cells

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Abstract

Tuberous sclerosis complex (TSC) is an autosomal dominant inherited disorder characterized by benign tumors (hamartomas) in various organs. The brain is one of the most severely affected organs with neuropsychiatric disorders including epilepsy, mental retardation and autism. The identification of TSC genes (*TSC1* and *TSC2*) and their gene products (hamartin and tuberlin, respectively), revealed that they function together as a complex. However, mutations in *TSC2* are often accompanied by more severe neurologic deficits. Here, we show that hamartin and tuberlin play different roles in NGF-treated cultured neuronal cells PC12h. The level of hamartin in PC12h cells was slightly and gradually increased, while those of tuberlin rapidly increased upon NGF-induced neuronal differentiation in PC12h cells. Antisense for *TSC1* (*TSC1*-AS) or *TSC2*-AS reduced expression of hamartin or tuberlin, respectively, and enhanced S-phase of cell cycle in PC12h cells. Suppression of hamartin significantly enhanced neurite outgrowth after NGF-treatment in PC12h cells, while suppression of tuberlin inhibited neurite outgrowth. Expression of activated V14RhoA reverted *TSC1*-AS induced abnormal neurite development. These results suggest that loss of hamartin results in abnormal neurite elongation through Rho inactivation in NGF-treated PC12h cells, which may be associated with the neurologic manifestations of TSC.

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Keywords: Hamartin; Tuberlin; Cell cycle; Neurite outgrowth; RhoA

1. Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder affecting 1 in 6000–10,000 births [1,2]. Two causative genes were identified as tumor suppressor genes, *TSC1* (chromosome 9q34) and *TSC2* (chromosome 16p13.3), which encode hamartin and tuberlin, respectively [3,4]. Both familial and sporadic TSC cases occur. Among sporadic TSC cases, mutations in *TSC2* are more frequent and often accompanied by more

severe neurologic deficits [5,6]. TSC is characterized pathologically by the occurrence of multiple hamartomas in various organs, such as renal angiomyolipomas and cardiac rhabdomyomas [1,7]. The neurologic manifestations are often quite disabling and include epilepsy, developmental delay, mental retardation and autism [1,8]. The cerebrum is most frequently affected in TSC. Three types of nodular lesions are identified as cortical tubers, subcortical heterotopic nodules and subependymal giant cell astrocytomas (SEGAs). Histologically, these hamartomas contain abnormal giant cells that show evidence of abnormal differentiation of immature neuronal cells [9,10]. Tumorigenesis in TSC is explained on the basis of loss of heterozygosity (LOH) involving either the

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TSC1 or *TSC2* gene [6]. However, the molecular pathogenesis of cortical tubers and other TSC associated hamartomas remains unclear. In the present study, we examined the role of hamartin on neurite outgrowth in NGF-treated cultured neuronal cell line, PC12h.

2. Materials and methods

2.1. PC12h cell culture

Rat pheochromocytoma cell line PC12 cells, have been used as a model cells for neuronal differentiation and neurite outgrowth [11]. Since PC12 cells express detectable levels of hamartin and tuberlin, we used this cell line for this study [12]. PC12h, a subclone of PC12, was maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) supplemented with 5% fetal bovine serum (FBS; HyClone, Logan, UT) and 5% heat-inactivated horse serum (Invitrogen, Carlsbad, CA) as previously described [24]. For induction of neuronal differentiation, PC12h cells were trypsinized, plated onto collagen IV (Sigma, St. Louis, MO) coated 35 mm plastic dishes (BD, Franklin Lakes, NJ) at 50% confluence and allowed to proceed overnight. Then medium was replaced by serum-free DMEM supplemented with 50 ng/ml nerve growth factor (NGF; Wako, Tokyo, Japan) and incubated for an additional 24 h.

2.2. Transfection

V14RhoA cDNA was inserted into mammalian expression vector pME18sf. Transfection of mammalian V14RhoA expression vector (pV14RhoA) was performed with Lipofectamine Plus (Invitrogen) following manufacturer's protocol. Empty pME18sf was used for control transfection. Antisense (AS) oligonucleotides against rat *TSC1* (*TSC1*-AS; 5'-ATGTTGGCTAGCTGGGCCA T-3') and *TSC2* (*TSC2*-AS; 5'-TTGCTAGTTG GTTTGGCCAT-3') were purchased from Greiner (Frickenhäusen, Germany). We used phosphorothioate oligonucleotides with high performance liquid chromatography (HPLC) purification. For transfection, oligonucleotides were mixed with Lipofectamine Plus and added into the cultured medium at the concentration of 10 μ M. We also used sense (*TSC1*-S and *TSC2*-S) or random oligonucleotides as control, which induced no changes in tuberlin and hamartin expression. Mock transfections were also used as a control.

2.3. Immunofluorescence staining

For immunofluorescence microscopy, PC12h cells were plated onto collagen IV coated 22 mm coverslips in 35 mm plastic dishes. Cells were fixed in 4% neutral phosphate-buffered paraformaldehyde for 20 min at room

temperature (RT), rinsed in phosphate-buffered saline (PBS), permeabilized with 0.1% Triton X-100 in PBS for 15 min at RT and incubated with primary antibodies for 1 h, followed by 1 h incubation with secondary antibodies at RT. Primary anti-hamartin (H-300, rabbit polyclonal) and anti-tuberlin (C-20, rabbit polyclonal) were obtained from Santa Cruz biotech. Inc. (Santa Cruz, CA). Specificity of these antibodies has been confirmed by immunostaining and immunoblotting of cultured normal skin fibroblast and angiofibroma stroma cells from TSC patients, or alternatively by transfection experiments with *TSC1* and *TSC2* cDNA into PC12h cells (data not shown). Secondary antibody was Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR). Dilutions were 1:100 and 1:2000 for primary and secondary antibodies, respectively. Actin filaments were stained with Alexa Fluor 594-conjugated phalloidin (Molecular Probes) in PBS for 1 h at RT. Cells on coverslips were mounted in Gel Mounting medium (Biomedica, Foster City, CA) and images were obtained using confocal microscopy (Leica TCS SP-2, Wetzlar, Germany).

2.4. Western blotting

For Western blot analysis, cells were harvested and lysed by sonication in a buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and protease inhibitor cocktail (Roche, Basel, Switzerland). Proteins were quantified using Protein assay kit (Bio-Rad, Hercules, CA), run on 10% SDS-PAGE gel and transferred on PVDF membranes (Millipore, Billerica, MA) by a semi-dry blotter (Bio-Rad). Membranes were incubated with antibodies and signals were detected using ECL reagent (Amersham-Pharmacia Biotech) on X-ray films (Fuji). For quantification, images were analyzed by NIH-image software.

2.5. BrdU labeling

In vitro labeling with bromodeoxyuridine (BrdU) was performed as described previously [13]. Briefly, cells plated on coverslips were labeled with BrdU (Roche). Detection of BrdU-positive cells was performed by immunofluorescence staining as described above using anti-BrdU (clone BRD.2, NeoMarkers, Fremont CA). Cells from 10 randomly selected fields were counted in a phase setting and then BrdU-positive cells were counted in a fluorescent setting in the same fields (Leica DMIRE2). The percentage of BrdU-positive cells from a total of 300 cells was then computed. Each experiment was performed independently in triplicate.

2.6. Morphometric analysis of neurite outgrowth

PC12h cells were plated onto collagen IV coated 35 mm plastic dishes at low densities of 5×10^3 cells/cm² so that

individual neurites could be traced. Phase microscopy (CK2, Olympus, Tokyo, Japan) was used to collect images of the culture. Ten fields containing a total of at least 100 cells were chosen randomly and cells with processes longer than one cell body were considered as neurite-bearing cells. Every experiment was performed three times. Statistical difference was determined by paired *t* test.

3. Results

3.1. Expression of hamartin and tuberin proteins during neuronal differentiation

NGF-induced a rapid increase in the amount of tuberin in PC12h cells (Fig. 1). The level decreased gradually but remained significantly high for 72 h after stimula-

tion. On the other hand, NGF-induced a gradual but modest increase in the amount of hamartin. We also examined the subcellular distribution of hamartin, tuberin and F-actin after stimulation with NGF. Immunofluorescence staining revealed that in unstimulated PC12h cells both hamartin and tuberin were present mainly in the cytoplasm (Fig. 1d). At 5 min after the addition of NGF, PC12h cells produced a few protrusions from the cell membrane, and both hamartin and tuberin were partially co-localized with F-actin at these protrusions.

3.2. Antisense suppression for hamartin and tuberin

Treatment of PC12h cells with either TSC1-AS or TSC2-AS but not with mock or sense-oligonucleotides resulted in significant reduction of hamartin and tuberin, respectively (Fig. 2a and b). These suppressions

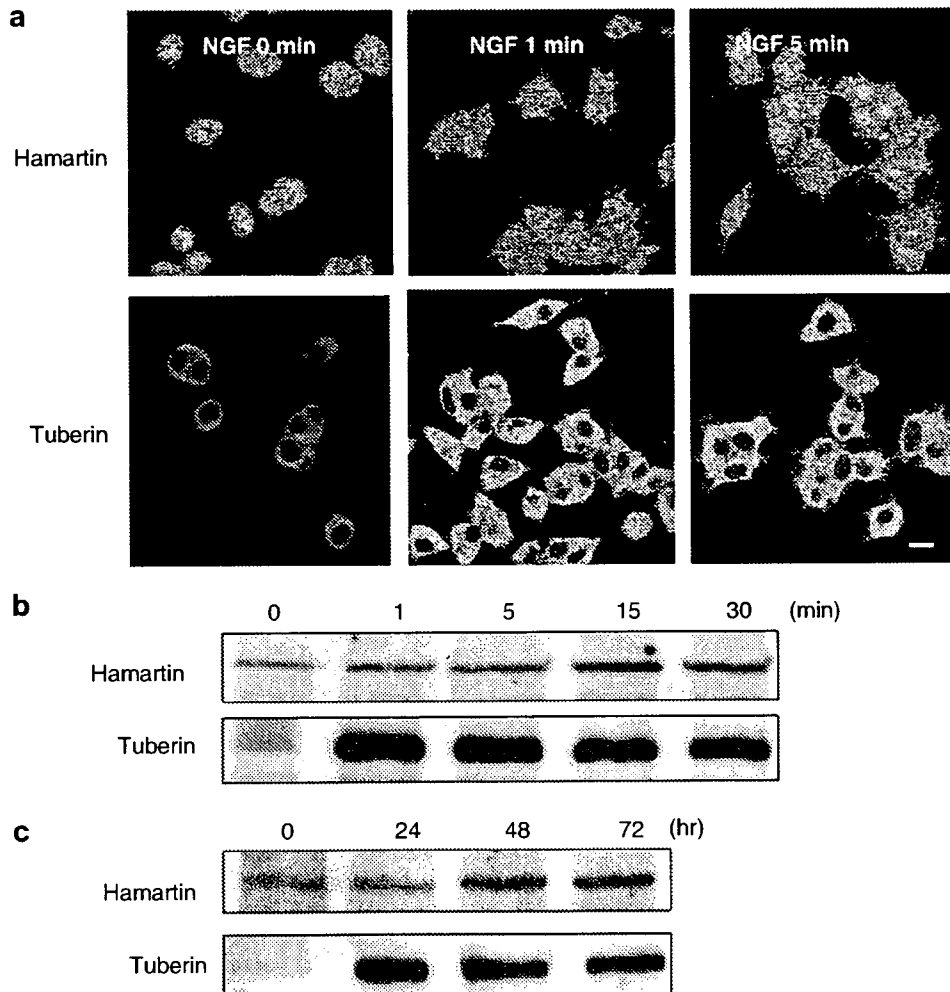


Fig. 1. Expression and localization of hamartin and tuberin during NGF-induced neuronal differentiation of PC12h cells. PC12h cells were plated on collagen type IV-coated dishes and treated with NGF for the indicated periods and examined. (a) Cells were fixed and immunostained with anti-hamartin and anti-tuberin. Representative images of hamartin and tuberin labelings are shown in upper and lower panels, respectively. Cells were collected and cell lysates were subjected to immunoblotting with anti-hamartin (b) and anti-tuberin (c), respectively. (d) Cells were fixed and stained with anti-hamartin, anti-tuberin and alexa 596-conjugated phalloidin. Arrowheads indicate protrusions. Bar, 10 μ m.

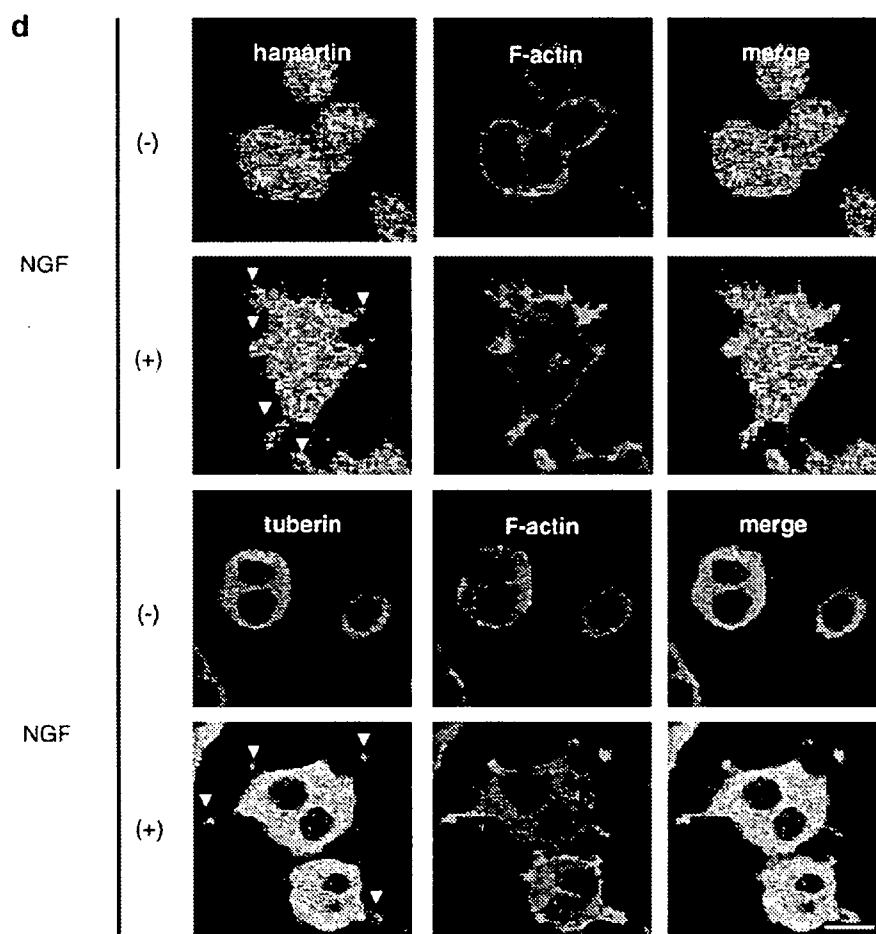


Fig. 1 (continued)

were also confirmed by immunostaining until 48 h of NGF-treatment (data not shown). The level of tuberin expression was modestly but significantly reduced in TSC1-AS treated cells when compared to control and TSC1-S transfected cells. We next sought to examine incorporation of BrdU in AS-treated PC12h cells. Percentage of BrdU-positive cells was significantly reduced when cells were incubated with 0.5% low serum. However, down-regulation of hamartin or tuberin led to a higher percentage of BrdU incorporation under low serum conditions (Fig. 3). The rate of BrdU-positive cells with TSC1-AS did not differ significantly from that of TSC2-AS or normal serum control.

3.3. NGF-induced neurite outgrowth in antisense-treated PC12h cells

Suppression of hamartin expression by TSC1-AS enhanced neurite outgrowth when compared to mock or TSC1-S transfected cells (Fig. 4a). In contrast, suppression of tuberin expression by TSC2-AS treatment in PC12h cells significantly inhibited neuronal differentiation after NGF stimulation (Fig. 4b). We next exam-

ined actin structures in AS-treated cells. TSC1-AS treated cells extended neurite processes heavily invested with actin cytoskeltons, while TSC2-AS treated cells induced a marked decrease in cortical actin filaments at the cell periphery after NGF stimulation, when compared to that of mock or sense transfected cells with NGF stimulation (Fig. 4c). Then we assessed whether the abnormal enhancement of neurite outgrowth in TSC1-AS treated PC12h cells is caused by inactivation of RhoA. For that purpose we used forced expression of constitutively activated form of RhoA (V14RhoA) and sought to observe whether this treatment could restore the extension of neurites. Combined treatment of PC12h cells with TSC1-AS, NGF and V14RhoA expression significantly reduced the number of neurite-bearing cells, which was similar to mock transfected NGF stimulated cells (Fig. 4d).

4. Discussion

Recent evidence indicates that hamartin and tuberin directly interact and are thought to stabilize each other

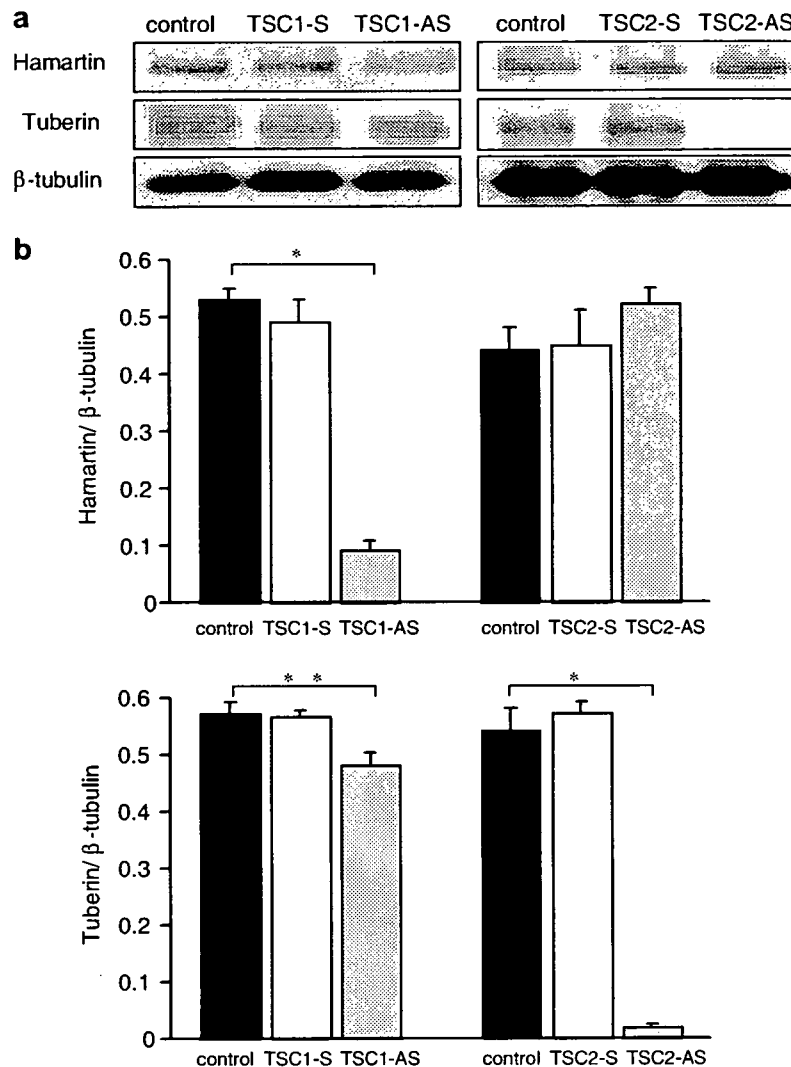


Fig. 2. Antisense oligonucleotide-mediated suppression of hamartin and tuberin in PC12h cells. PC12h cells were transfected with anti-rat TSC1 or anti-rat TSC2 antisense oligonucleotide (AS). Mock (control) or sense oligonucleotide (S) transfections were performed as control. (a) Cells were collected and lysates were subjected to immunoblotting with anti-hamartin, anti-tuberin and anti- β -tubulin, respectively, at 48 h after transfection. (b) Relative intensities of hamartin/ β -tubulin and tuberin/ β -tubulin were shown. Values are means \pm SEM, $n = 3$, * $p < 0.01$; ** $p < 0.05$.

[4,7,14]. Phosphorylation of tuberin by Akt modulates its binding activity with hamartin. Tuberin/hamartin complex has also strong and highly specific GAP activity toward Rheb (Ras homolog enriched in brains), which activate mammalian target of rapamycin (mTOR) [7,15,16]. Several lines of evidence have revealed that hyperactivation of Akt signaling leads to hyperactive mTOR signaling through inhibition of *TSC1* or *TSC2*, thus potentially explaining the nearly identical clinical courses caused by *TSC1* and *TSC2* mutations [5,6]. However, these are two distinct molecules and the possibility of independent functions of each molecule should not be excluded.

In this study, we found that the level of hamartin and tuberin expression was differently regulated in dif-

ferentiating PC12h cells (Fig. 1). During neuronal differentiation, cells withdraw permanently from the cell cycle. Tuberin contains a conserved carboxy terminal region that exhibits sequence homology to the catalytic domain of a GAP for Rap1 and Rheb [3,7]. Like other Rap-related protein, Rap1 is known to function in regulation of DNA synthesis and cell cycle transition [17], and the proliferation status may be regulated by the up-regulation of tuberin. Another line of evidence is that ectopic overexpression of tuberin in human neuroblastoma SK-N-SH cells accelerated the process of neuronal differentiation in these cells [18]. Here, we reported also that loss of tuberin expression with antisense oligonucleotides led to re-entering of the cell cycle and inhibited neuronal differentiation

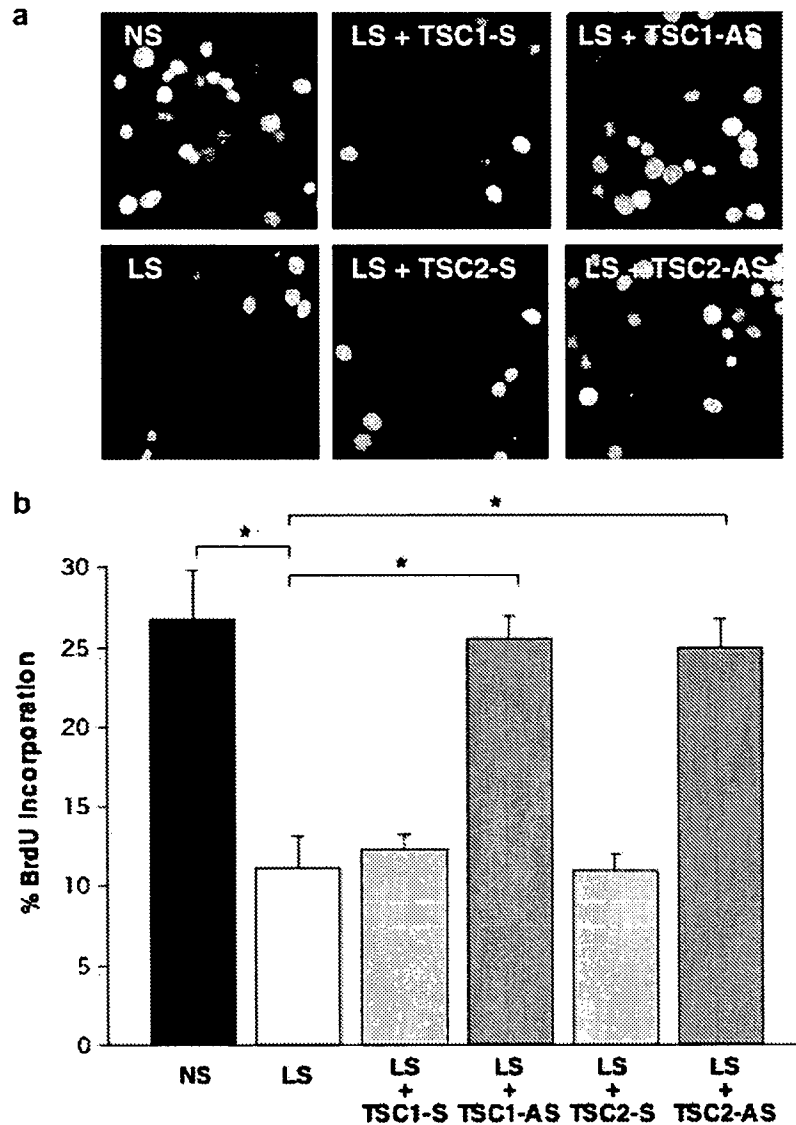


Fig. 3. BrdU labeling of antisense oligonucleotide-treated PC12h cells. PC12h cells were cultured with 10% normal serum (NS). Forty-eight hours after transfection with mock, sense (S)- or antisense (AS)-oligonucleotide, cells were cultured with 0.5% serum (low serum; LS) for 12 h and labeled with BrdU for another 12 h. Incorporated BrdU was detected by immunostaining with anti-BrdU Ab. (a) Representative images of BrdU in PC12h cells. (b) Percentage of BrdU-positive cells was calculated by counting 300 cells from each sample. Values are means \pm SEM, $n = 3$, $*p < 0.01$.

in PC12h cells treated with NGF (Figs. 3 and 4b). In HeLa cells, TSC2 down-regulation has been shown to cause an increase in cell size [19]. We did not find this to be the case in PC12h cells. TSC2-AS treated cells seem to have a size similar to that of untreated cells (Fig. 4c). Although, we did not investigate further this finding, we cannot rule out at this point tuberin may play a different role in PC12h cells when compared with HeLa with respect to the cell size control regulation. These data show that loss of tuberin expression is sufficient to block neuronal differentiation and makes it very likely that tubers in TSC patients may reflect disruption in neuronal maturation.

TSC1 gene product hamartin binds to the ezrin-radixin-moesin (ERM) family of actin-binding proteins and regulates the small GTP-binding protein Rho [20]. As shown in Fig. 1e, hamartin was partially co-localized with actin in the periphery of PC12h cells after NGF stimulation, which suggests that hamartin may function in neuronal differentiation through regulation of the actin cytoskeleton. Interestingly, tuberin distributed predominantly into the cytoplasm and also partially co-localized with actin at the edge of neurite processes. Loss of hamartin expression after TSC1-AS treatment enhanced neurite outgrowth in PC12h cells after NGF stimulation, the process being independent of the cell

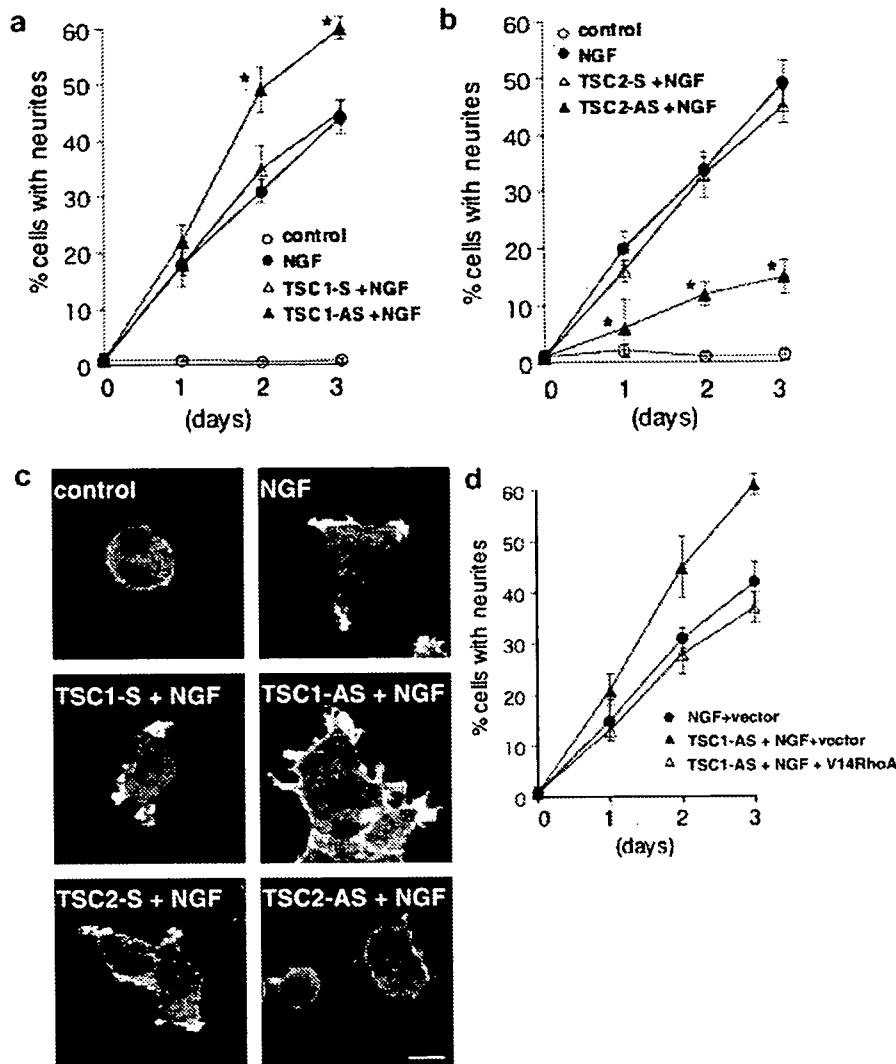


Fig. 4. Effect of antisense suppression of hamartin and tuberin on NGF-induced neurite outgrowth in PC12h cells. PC12h cells were plated on collagen type IV-coated dishes and transfected with sense (S) or antisense (AS) oligonucleotides. After 48 h of transfection, cells were treated with or without NGF for another intervals. (a and b) Phase images of the cultures were obtained and percentage of neurite-bearing cells were determined for each of the 300 cells. (c) Cells were fixed and stained with alexa 596-conjugated phalloidin. (d) PC12h cells were transfected with AS-oligonucleotide with control empty vector (vector) or V14RhoA expression vector and treated with NGF for another indicated periods. Percentage of neurite-bearing cells was calculated as above. Values are means \pm SEM, $n = 3$, $*p < 0.01$. Bar, 10 μ M.

cycle status (Fig. 4a). In addition, loss of hamartin resulted in a heavy accumulation of actin filaments at the neurites' periphery, suggesting inactivation of RhoA (Fig. 4c). Further, abnormal enhancement of neurite outgrowth was reverted by forced expression of activated V14RhoA (Fig. 4d). Our findings regarding to neurite outgrowth in AS-treated PC12h cells was obtained only after NGF treatment (data not shown). At this stage of our work, we have no direct evidence of hamartin and tuberin involving on the regulation of RhoA. RhoA has been undoubtedly proved to be involved in neuritis outgrowth [21]. Recent emerging data shows that tuberin also modulates RhoA activity through TSC1-binding domain [22]. Although, addi-

tional work is needed to prove a direct involvement of these hamartin and tuberin in RhoA regulation in neurons, our initial data suggest at least in part that hamartin links RhoA activity in NGF-induced neuronal differentiated PC12h cells.

Hamartin and tuberin function as a heterodimer to inhibit cell growth and proliferation [7]. Although, it remains unclear whether hamartin contributes to the GAP activity of tuberin on Rheb, Inoki et al. has shown that tuberin alone is sufficient to promote GTP hydrolysis of Rheb [15]. It has also been shown that hamartin can bind to tuberin to prevent tuberin ubiquitination and degradation. [23,24]. Our finding is also consistent of these reports that TSC1-AS resulted in decreased

level of tuberin in PC12h cells (Fig. 4a and b). Therefore, tuberin might be unstable or somehow degraded in TSC1-deficient cells, and we observed an increased rate of progression through G1-S phase in both TSC1-AS and TSC2-AS treated cells. Hamartin may also activate RhoA in TSC2-AS treated cell, which results in neurite retraction, although these ideas are experimentally untested at present. Although, there is an overlap in the spectrum of many features of patients with *TSC1* and *TSC2* mutations, *TSC1* disease is often milder than *TSC2* disease [5,25] and that might correlate with our finding regarding the abnormal neurite development in TSC1-AS treated PC12h cells.

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References

- [1] Gomez MR, Sampson JR, Wittmore VH. Tuberous sclerosis complex. 3rd ed. New York: Oxford University Press; 1999.
- [2] Ohno K, Yakura N, Zhang H, Ninomiya H, Nanba E. Molecular epidemiology of tuberous sclerosis. In: Niimura M, Otsuka F, Hino O, editors. Phacomatosis in Japan-epidemiology, clinical picture, and molecular biology. Tokyo: Japan Scientific Societies Press; 1999. p. 53–9.
- [3] The European Chromosome 16 Tuberous Sclerosis Consortium. Tuberous sclerosis gene on chromosome 16. *Cell* 1993;75:1305–15.
- [4] van Slechtenhorst M, de Hoogt R, Hermans C, Nellist M, Jansen B. Identification of the tuberous sclerosis gene *TSC1* on chromosome 9q34. *Science* 1997;277:805–8.
- [5] Dabora SL, Jozwiak S, Franz DN, Roberts PS, Nieto A, Chung J, et al. Mutational analysis in a cohort 224 tuberous sclerosis patients indicates increased severity of *TSC2*, compared with *TSC1*, disease in multiple organs. *Am J Hum Genet* 2001;68:64–80.
- [6] Jones AC, Shyamsundar MM, Thomas MW, Maynard J, Idziaszczyk JS, Tomkins S, et al. Comprehensive mutational analysis of *TSC1* and *TSC2*-and phenotypic correlations in 150 families with tuberous sclerosis. *Am J Hum Genet* 1999;64:1305–15.
- [7] Kwiatkowski DJ. Tuberous sclerosis: from tubers to mTOR. *Ann Hum Genet* 2003;67:87–96.
- [8] Ess KC. The neurobiology of tuberous sclerosis complex. *Semin Pediatr Neurol* 2006;13:37–42.
- [9] Crino PB. Molecular pathogenesis of tuber formation in tuberous sclerosis complex. *J Child Neurol* 2004;19:716–25.
- [10] Mizuguchi M, Takashima S. Neuropathology of tuberous sclerosis. *Brain Dev* 2001;23:508–15.
- [11] Yamada M, Ikeuchi T, Aimoto S, Hatanaka H. PC12h-R cells, a subclone of PC12 cells, shows EGF-neuronal differentiation and sustained signaling. *J Neurosci Res* 1996;43:355–64.
- [12] Catania MG, Johnson MW, Liao LM, Kremen TJ, de Vellis JS, Vinters HV. Hamartin expression and interaction with tuberin in tumor cell lines and primary cultures. *J Neurosci Res* 2001;63:276–83.
- [13] Wataya-Kaneda M, Kaneda Y, Hino O, Adachi H, Hirayama Y, Seyama K, et al. Cells derived from tuberous sclerosis show a prolonged S phase of the cell cycle and increased apoptosis. *Arch Dermatol Res* 2001;293:460–9.
- [14] Plank TL, Yeung RS, Henske EP. Hamartin, the product of the tuberous sclerosis 1 (*TSC1*) gene, interacts with tuberin and appears to be localized to cytoplasmic vesicles. *Cancer Res* 1998;58:4766–70.
- [15] Inoki K, Li Y, Xu T, Guan KL. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. *Gene Dev* 2003;17:1829–34.
- [16] Zhang Y, Gao X, Saucedo LJ, Ru B, Edgar BA, Pan D. Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. *Nat Cell Biol* 2003;5:578–81.
- [17] Apicelli AJ, Uhlmann EJ, Baldwin RL, Ding H, Nagy A, Guha A, et al. Role of the Rap1 GTPase in astrocyte growth regulation. *Glia* 2003;42:225–34.
- [18] Soucek T, Holz G, Bernaschek G, Hengstschläge M. A role of the tuberous sclerosis gene-2 product during neuronal differentiation. *Oncogene* 1998;16:2197–204.
- [19] Rosner M, Hofer K, Kubista M, Hengstschläge M. Cell size regulation by the human TSC tumor suppressor proteins depends on PI3K and FKBP38. *Oncogene* 2003;22:4786–98.
- [20] Lamb RF, Roy C, Diefenbach TJ, Vinters HV, Johnson MW, Jay DG, et al. The *TSC1* tumor suppressor hamartin regulates cell adhesion through ERM proteins and the GTPase Rho. *Nat Cell Biol* 2000;2:281–7.
- [21] Etienne-Manneville S, Hall A. Rho GTPases in cell biology. *Nature* 2002;420:629–35.
- [22] Goncharova E, Goncharov D, Noonan D, Krymskaya VP. *TSC2* modulates actin cytoskeleton and focal adhesion through TSC1-binding domain and the Rac1 GTPase. *J Cell Biol* 2004;167:1171–82.
- [23] Benvenuto G, Li S, Brown SJ, Braverman R, Vass WC, Cheadle JP, et al. The tuberous sclerosis-1 (*TSC1*) gene product hamartin suppresses cell growth and augments the expression of the *TSC2* product tuberin by inhibiting its ubiquitination. *Oncogene* 2000;19:6306–16.
- [24] Inoki K, Li Y, Zhu T, Wu J, Guan KL. *TSC2* is phosphorylated and inhibited by Akt and suppresses mTOR signaling. *Nat Cell Biol* 2002;4:648–57.
- [25] Jones AC, Daniells CE, Snell RG, Tachataki M, Idziaszczyk SA, Krawczak M, et al. Molecular genetic and phenotypic analysis reveals differences between *TSC1* and *TSC2* associated familial and sporadic tuberous sclerosis. *Hum Mol Genet* 1997;6:2155–61.