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ORIGINAL ARTICLE

Functional characterization of *CDK5* and *CDK5R1* mutations identified in patients with non-syndromic intellectual disability

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Cyclin-dependent kinase 5 (*CDK5*) and cyclin-dependent kinase 5, regulatory subunit 1 (*CDK5R1*), encoding CDK5 activator p35, have a fundamental role in central nervous system (CNS) development and function, and are involved in the pathogenesis of several neurodegenerative disorders, thus constituting strong candidate genes for the onset of intellectual disability (ID). We carried out a mutation screening of *CDK5* and *CDK5R1* coding regions and *CDK5R1* 3′-UTR on a cohort of 360 patients with non-syndromic ID (NS-ID) using denaturing high performance liquid chromatography (DHPLC) and direct sequencing. We found one novel silent mutation in *CDK5* and one novel silent mutation in *CDK5R1* coding regions, three novel intronic variations in *CDK5*, not causing any splicing defect, and four novel heterozygous variations in *CDK5R1* 3′-UTR. None of these variations was present in 450 healthy controls and single-nucleotide polymorphism (SNP) databases. The functional study of *CDK5R1* p.A108V mutation evidenced an impaired p35 cleavage by the calcium-dependent protease calpain. Moreover, luciferase constructs containing the *CDK5R1* 3′-UTR mutations showed altered gene expression levels. Eight known polymorphisms were also identified displaying different frequencies in NS-ID patients compared with the controls. In particular, the minor allele of *CDK5R1* 3′-UTR rs735555 polymorphism was associated with increased risk for NS-ID. In conclusion, our data suggest that mutations and polymorphisms in *CDK5* and *CDK5R1* genes may contribute to the onset of the NS-ID phenotype.
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INTRODUCTION

Intellectual disability (ID), also referred as mental retardation or early-onset cognitive impairment, affects approximately 1–3% of the general population,[1] and is diagnosed upon the concomitant occurrence of an intelligence quotient below 70, significant limitations in two or more adaptive skill areas, and the presence of the condition from childhood. Although environmental influences, such as problems during pregnancy, at birth and after birth, as well as poverty and cultural deprivation can cause ID,[2] gene mutation and structural rearrangements of the genome are now considered to be the most important factors in this disorder.

Genetic ID can be subdivided into syndromic forms, which are characterized by ID accompanied by malformations or dysmorphic features or neurological abnormalities, and non-syndromic forms (NS-ID), which are characterized by ID without any additional features. The genetic basis of the latter is far to be fully understood, although about a hundred causal genes have been identified to date, taking into account both the X-linked and the autosomal forms of NS-ID.[3–6] Indeed, mutations in the currently known genes are responsible for only a small percentage of the total number of NS-ID cases, and additional genes are expected to be involved in this disorder.

Given the high genetic heterogeneity of NS-ID, *de novo* autosomal point mutations may thus explain a large fraction of cases. However, the identification of NS-ID causing mutations through linkage or association approaches is often problematic, notably in the western countries, where most of the families are small and affected individuals are typically sporadic cases. In this context, the sequencing of candidate genes with fundamental roles in brain development or function may be an effective alternative strategy for identifying the genetic basis of NS-ID. *SYNGAP1*, *STXBP1* and *SHANK3* are instances of NS-ID genes recently identified using candidate gene sequencing.[7–9] On the other hand, the advent of next-generation sequencing has opened new avenues in the elucidation of genetic defects causing NS-ID, allowing the identification of several genes. Despite the undeniable advantages of next-generation sequencing technologies compared with previous methods, the sequencing of strong candidate genes in large cohorts of patients using high-throughput techniques may still be an effective approach.

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In this paper, we considered cyclin-dependent kinase 5 (*CDK5*) and cyclin-dependent kinase 5, regulatory subunit 1 (*CDK5R1*), encoding the main neuron-specific activator of CDK5, p35, as strong candidate genes for the onset of NS-ID, owing to their key role in central nervous system (CNS) development and function,[10] and their involvement in the pathogenesis of neurodegenerative disorders.[11]

Notably, a loss-of-function in *CDK5* has been recently identified in a family with autosomal recessive lissencephaly with cerebellar hypoplasia, a condition causing abnormal gyration and organization of the cortical layers.[12] In addition, *CDK5R1* has already been proposed as a candidate gene for the onset of ID in patients affected by NF1 microdeletion syndrome.[13] Moreover, neither *CDK5* nor *CDK5R1* accumulates truncating mutations in control individuals, as the NHLBI Exome Sequencing Project (http://evs.gs.washington.edu/EVS/) reports no truncating variants in *CDK5* and only one truncating variant in *CDK5R1*. It is also worth noting that other genes encoding for proteins involved in the regulation of CDK5 activity have been implicated in the onset of CNS disorders: *CDK5R2* (cyclin-dependent kinase 5, regulatory subunit 2), the gene coding for p39, is a candidate genetic modifier in Huntington's disease[14] and mutations in *CDK5RAP2* (CDK5 regulatory subunit associated protein 2) have been found in patients affected by primary microcephaly.[15–18]

In a previous work, we screened 100 unrelated Italian patients affected by NS-ID for mutations in *CDK5R1* coding region and in two portions of its 3′-UTR in which post-transcriptional regulatory elements were predicted.[19] Four novel mutations and two novel polymorphisms in the coding region of *CDK5R1* were detected, including a missense change (c.323C>T; p.A108V), one synonymous codon variant (c.532C>T; p.(L178L)) and four variants in the 3′-UTR, one of which, the c.2254C>G change, leads to a putative structural alteration in *CDK5R1* mRNA.[19]

In this study, we report on the *CDK5* and *CDK5R1* mutation screening by denaturing high performance liquid chromatography (DHPLC) of 360 patients affected by NS-ID. The cohort is composed by 260 newly recruited NS-ID cases who have been analyzed for the coding sequences of *CDK5* and *CDK5R1* genes and for *CDK5R1* 3′-UTR, and by the 100 patients already described in the above-mentioned previous work,[19] for which we performed the mutation screening of *CDK5* coding sequence and *CDK5R1* 3′-UTR. The study led to the identification of one novel silent mutation in *CDK5* coding region and one novel silent mutation in *CDK5R1* coding region. In addition, three novel variations in the intronic regions of *CDK5* and four novel heterozygous variations in *CDK5R1* 3′-UTR were also found. The functional study of *CDK5R1* p.A108V mutation evidenced the impairment of p35 cleavage by the calcium-dependent protease calpain. Moreover, luciferase assays allowed establishing that some of the variations identified in *CDK5R1* 3′-UTR can affect *CDK5R1* expression levels. Eight known polymorphisms were also identified, which display significantly different frequencies in NS-ID patients compared with the controls. In particular, the minor allele of *CDK5R1* 3′-UTR rs735555:C>T polymorphism was associated with increased risk for NS-ID in the homozygous state. Our data indicate that these mutations and polymorphisms might constitute susceptibility variants for NS-ID.

MATERIALS AND METHODS

Subjects

The patient panel used for the mutation screening consisted of 360 Caucasian patients affected by NS-ID (100 from the previous study by Venturin *et al.*[19]). The patients were recruited by different Clinical Centers following homogeneous criteria, as previously described.[19] They were composed by 65% males and 35% females, for the most part apparently sporadic cases. With few exceptions, participants were younger than 18 years, most of them between 5 and 10. The majority of the patients were constituted by mild-to-moderate ID cases with history of speech and language delay, psychomotor delay, short attention span and behavioral abnormalities, referred to the Clinical Centers for Fragile X molecular testing. The most common genetic causes of ID (chromosome rearrangements, abnormalities in blood and urine amino acids, urine organic acids, Fragile X and Angelman and Rett syndromes) were excluded. Array-based comparative genome hybridization analysis was performed in a small proportion of patients, and was negative in all cases for the presence of copy-number variations potentially associated with ID. A few patients displayed, besides ID, additional isolated dysmorphic facial features, which however do not fit with a specific syndromic pattern.

Genomic DNA or blood samples were provided after the acquisition of a written informed consent. The genomic DNA from 450 healthy Caucasian individuals was kindly provided by Dr Rosanna Asselta.

Mutation analysis

Genomic DNA was isolated from 200 μl of whole blood using the QIAmp DNA blood mini kit (Qiagen, Hilden, Germany). Concentration and purity of DNA samples were measured using Nanodrop (Thermo Scientific, Waltham, MA, USA). *CDK5R1* (NM_003885.2) coding sequence (consisting in a single exon) was amplified in 5 overlapping PCR fragments and *CDK5R1* 3′-UTR in 12 overlapping PCR fragments. Eight PCR fragments were used to cover the 12 exons and intronic splice junctions of *CDK5* (NM_004935.3). Primers used in this study were designed using the primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and are listed in Table 1. PCR was carried out in a 30-μl reaction volume containing 25 ng of genomic DNA, 0.2 μM primers, 100 μM dNTPs, 6 μl 5× reaction buffer, 50 mM $MgCl_2$ and 2.5 U GoTaq DNA Polymerase (Promega, Madison, WI, USA) with the following cycling profile: 4 min initial denaturation at 95 °C, 35 cycles of denaturation at 95 °C for 30 s, specific annealing temperature for 30 s (Table 1) and extension at 72 °C for 30 s. The specificity of the amplified PCR products was checked by 1.5% agarose gel electrophoresis.

DHPLC analysis was performed on a WAVE Nucleic Acid Fragment Analysis System 3500HT (Transgenomic Inc., Crewe, UK). In brief, PCR products were examined for heteroduplexes by subjecting 5–10 μl of each PCR product to a denaturation step (5 min at 95 °C), followed by a cooling period at room temperature. The PCR products were then separated through a 5% linear acetonitrile gradient (flow rate of 1.5 ml per minute). Commercially available WAVE Optimized Buffers (A, B, D) and Syringe Solution (Transgenomic) were used to provide highly reproducible retention times with WAVE System instrumentation. The temperature for optimal heteroduplex analysis was determined using the Navigator software (Transgenomic). Running conditions for *CDK5* and *CDK5R1* amplicon analysis are shown in Table 1.

The identified variations were validated by resequencing an independent PCR-generated amplicon from the subject. PCR products were directly sequenced in both directions using the Big Dye Terminator kit v1.1 (Life Technologies, Monza, Italy) and resolved on a 3130xl Genetic Analyzer (Life Technologies). The data concerning the variants identified in the present study have been submitted to the public database LOVD (http://databases.lovd.nl/shared/genes/CDK5, http://databases.lovd.nl/shared/genes/CDK5R1).

Site-direct mutagenesis

The *CDK5R1* 3′-UTR was previously cloned in the *Xba*I site of the *Renilla* luciferase reporter plasmid pGL4.71P[20] and called pGL4.71P-UTR-wt. The six point mutations or small deletions were inserted in the pGL4.71P-UTR-wt by site-direct mutagenesis. In brief, complementary oligonucleotides bearing the desired mutation were designed (Table 2). A 25-μl reaction volume was prepared containing 50 ng of pGL4.71P-UTR-wt DNA, 62.5 ng oligonucleotides A and B, 100 μM dNTPs, 10× reaction buffer and 1.25 U Pfu Turbo DNA Polymerase (Stratagene, La Jolla, CA, USA) with the following cycling profile: 95 °C for 1 min, 12 cycles for single base mutations or 18 cycles for deletions as follows: 95 °C for 30 s, 55° for 30 s, extension at 68 °C for 16 min. Therefore, 5 U of *Dpn*I enzyme (Fermentas, Waltham, MA, USA) was added to the reaction for 5 h at 37°C to digest the template plasmid. The newly synthesized

Table 1 Primer sequences, PCR and DHPLC temperatures for CDK5 and CDK5R1 fragments

Amplicon name	*Size (bp)*	*Forward primer* *Reverse primer*	*Primer length (bp)*	*PCR annealing temperature (°C)*	*DHPLC analysis temperature (°C)*
CDK5-ex1	268	5′-gccagagtcttaaaaccgagg-3′	21	58	61.2–62.2–63.2
		5′-gggaaatgctaacacggggcc-3′	21		
CDK5-ex2-3	362	5′-agtgccgcatcctcaccctg-3′	20	60	61.9–62.4–62.7–63.4
		5′-cgtccagtgtcagcccccg-3′	19		
CDK5-ex4-5	276	5′-gcggacctgcctggctgag-3′	19	60	58.5–59–59.5–60
		5′-tcccccaacaccactctcct-3′	20		
CDK5-ex6	200	5′-agaggagccgacccgttgc-3′	19	58	59.1–60.1–61.1–61.6
		5′-tccattccccaccttcttccc-3′	21		
CDK5-ex7-8	439	5′-gagcacagagggaagaggac-3′	20	58	Direct sequencing
		5′-ctccctcccttccccaga-3′	19		
CDK5-ex9	201	5′-tgggggaagggagggaggc-3′	19	65.6–58.3	59.3–60.3–61.3–63
		5′-tcaaggcagaggaaagcaagg-3′	21		
CDK5-ex10-11	318	5′-cctttcccaagtgatccttga-3′	21	58	61.2–62.2
		5′-cctgacccaccctctaccc-3′	19		
CDK5-ex12	292	5′-agtgacctgtcctgaacctg-3′	20	58	60.8–61.8–62.8–64
		5′-aagtccacaaagggagtgagaa-3′	22		
CDK5R1-CDS-1	181	5′-gtgagcggttttatccctcc-3′	20	63.5–56.5	64.9–65.9–66.9
		5′-gttcttgctgttctgtacggc-3′	21		
CDK5R1-CDS-2	202	5′-gccgtacagaacagcaagaac-3′	21	64.3–57.3	61.7–62.2–62.7–63
		5′-acaggttggcgcacgacag-3′	19		
CDK5R1-CDS-3	323	5′-ctgtcgtgcgccaacctgt-3′	19	65.3–58.3	65.5–66–66.2
		5′-gtgatgaagccctggtcctg-3′	20		
CDK5R1-CDS-4	215	5′-caggaccagggcttcatcac-3′	20	64.3–57.3	61.2–62.2–63.2–63.7
		5′-aggcaacggtcccaaaaggc-3′	20		
CDK5R1-CDS-5	167	5′-ttgggaccgttgcctctctg-3′	20	65.3–58.3	61–62–62.5
		5′-caggctacagtgctcaccga-3′	20		
CDK5R1-3′UTR-1	279	5′-ggaggacaagaagcggctc-3′	19	58	56.3–58.8–60.5
		5′-caaagtgggaggtcagtgg-3′	19		
CDK5R1-3′UTR-2	274	5′-ctctgggcacttttgaactca-3′	21	61.7–54.7	60.5–61.5–62.5
		5′-cctctgggagagtgaataact-3′	21		
CDK5R1-3′UTR-3	280	5′-ccagggaggaattgggttt-3′	20	60.9–53.9	56.4–59.9–60.9–61.9
		5′-agttccatttctccactaaaga-3′	22		
CDK5R1-3′UTR-4	263	5′-tcgtttgaccacacaccgcc-3′	20	64.3–57.3	58–59–60
		5′-catttgccacagggtgactg-3′	20		
CDK5R1-3′UTR-5	281	5′-gggacagctttcctgggtg-3′	19	59.1–52.1	55.9–56.9–57.9–62.4
		5′-tctttagaagaaactcaactgtt-3′	23		
CDK5R1-3′UTR-6	268	5′-gggaaggcgatgtggtgac-3′	19	57.3–50.3	58.7–59.2–59.7–60.2
		5′-gaaagaaaatcaataaagtacac-3′	23		
CDK5R1-3′UTR-7	271	5′-ctgctggttttggtttccac-3′	20	55	56–57–61
		5′-aacttaatcctatgtaaagtgac-3′	23		
CDK5R1-3′UTR-8	389	5′-gtgctggaatggggacctgg-3′	20	58	58.5–59–62–63
		5′-tcccacctctggtcgcacg-3′	19		
CDK5R1-3′UTR-9	434	5′-acacagcctgtcttcagatc-3′	20	58–51	57.4–58.4–59.4
		5′-gtaggttttttttttattgttgatc-3′	24		
CDK5R1-3′UTR-10	289	5′-aacccatttgccatgctgct-3′	20	55	54.4–4.9
		5′-caaaactaagttctaataactgc-3′	23		
CDK5R1-3′UTR-11	280	5′-ctctttttcttgtaaacctgga-3′	22	55	52.8–54.8–55.3
		5′-caaacacactacacatacataca-3′	23		
CDK5R1-3′UTR-12	213	5′-caaactgtgggtattgttaatat-3′	23	54	52.1–55.1–56.1
		5′-cacaaaaagaggtctctgtaat-3′	22		

Abbreviations: CDK5, cyclin-dependent kinase 5; CDK5R1, cyclin-dependent kinase 5, regulatory subunit 1; DHPLC, denaturing high performance liquid chromatography.

plasmid was transformed into competent *E. coli* XL1Blue cells. The introduction of the desired mutations was verified by direct sequencing.

Cell cultures

Human neuroblastoma SK-N-BE cells were cultured in RPMI medium with 10% fetal calf serum, 100 U ml^{-1} penicillin-streptomycin, 0.01 mM L-glutamine, sodium pyruvate 11 g l^{-1} and glucose 4.5 g l^{-1}. Human embryonic kidney HEK-293 cells were maintained in DMEM with 10% fetal calf serum, 100 U ml^{-1} penicillin-streptomycin and 0.01 mM L-glutamine (all media ingredients were obtained from Sigma-Aldrich, St Louis, MO, USA). Cultures were maintained at 37 °C in a 5% CO_2 incubator.

Table 2 Oligonucleotides for site-direct mutagenesis

Name	Sequence 5′-3′
UTR-397G-A	GAGGCCCTTTTCTGGGTCCTGTGTGGAGTTA
UTR-397G-B	TAACTCCACACAGGACCCAGAAAAGGGCCTC
UTR-649-659del-A	GCCCCCCTACCCCCTCCCAGCCACGTTGGT
UTR-649-659del-B	ACCAACGTGGCTGGGAGGGGGTAGGGGGGC
UTR-1330G-A	AATGTGAAGACACTAGAGAGGATTCTGTCTT
UTR-1330G-B	AAGACAGAATCCTCTCTAGTGTCTTCACATT
UTR-1904-05del-A	GTGTCTGTTTCCTTCTTTCTCCGTATTTAT
UTR-1904-05del-B	ATAAATACGGAGAAAGAAGGAAACAGACAC
UTR-2099-101del-A	CCCATTTGCCATGCTATGAAGACTAACTTTT
UTR-2099-101del-B	AAAAGTTAGTCTTCATAGCATGGCAAATGGG
UTR-2528A-A	TTGACCAAACTGTGGATATTGTTAATATTAA
UTR-2528A-B	TTAATATTAACAATATCCACAGTTTGGTCAA

Protein overexpression and western blotting

The coding region of *CDK5R1* was PCR amplified from a control genomic DNA using the primers: fw 5′-TGACGGTTTTATCCCTCC-3′ and rev 5′-CAGGCTACAGTGCTCACC-3′ and cloned in the pTarget vector (Promega). The construct bearing the c.323C>T mutation was produced by site-direct mutagenesis as described above using the following oligonucleotides: 5′-CGGCCCAGCCTGTACCCCGGCCAGCCA-3′ and 5′-TGGCTGGCCGGG GTACAGGCGGCTGGGCCG-3′.

In all, 300 ng of each plasmid was transfected in 3×10^5 SK-N-BE or HEK-293 cells with 2 µl Lipofectamine 2000 (Invitrogen, Waltham, MA, USA). Forty-eight hours after transfection, cells were harvested, washed in PBS 1× and then centrifuged at 2000 r.p.m. for 10 min at 4 °C. The pellet was resuspended in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% Igepal, 0.5% Na deoxycholate and protease inhibitors), placed in a shaker at 4 °C for 30 min and then centrifuged at 10 000 r.p.m. for 10 min at 4 °C. The protein lysate concentration was measured using a Pierce assay (Bio-Rad Laboratories, Hercules, CA, USA). For calcium treatments, HEK-293 cells were treated for 6 h with 1 µM Ca^{2+} ionophore A23187 in the presence of 2 mM $CaCl_2$; 48 h after pTARGET-p35 transfection, and protein lysates were obtained as described above.

Western blotting was performed according to standard procedures. The following antibodies were used: anti-p35 (c-19, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-CDK5 (C-8, Santa Cruz Biotechnology), anti-APP (Millipore, Billerica, MA, USA), anti-phospho-APP (Thr668, Cell Signaling, Danvers, CA, USA) and anti-GAPDH (Novus Biologicals, Littleton, CO, USA). Horseradish peroxidase-conjugated anti-rabbit, anti-mouse or anti-goat antibodies were then added, and secondary antibodies were detected through autoradiography using enhanced chemiluminescence (ECL Plus, General Electric Healthcare, Milwaukee, WI, USA). Densitometric analyses were performed with the ImageJ software (National Institutes of Health, Bethesda, MD, USA), and the data were expressed as means ± s.d.

Splicing analysis

Genomic DNA from normal and mutated (c.484-56 A>G) *CDK5* was PCR amplified using Pfu DNA polymerase (Promega) to generate a fragment that contained the exon 7, intron 7 and exon 8 along with 150 bp of 5′ and 3′ intronic flanking sequences using the following oligonucleotides: fw 5′-CGCATATGAAGCCCCAGAACCTGCTATT-3′ and rev 5′-CGCATATG GTGAAGGACCCCTCACTCTG-3′. Both oligonucleotides carry an *Nde*I restriction enzyme site in their 5′ ends, used to clone the product into a modified version of the α-globin-fibronectin-EDB minigene.[21,22] The correct orientation of the insert as well as the presence of the mutation was verified by direct sequencing.

The splicing assay was performed by transfecting 300 ng of each minigene plasmid into 3×10^5 SK-N-BE or HEK-293 cells with 2 µl Lipofectamine 2000 (Invitrogen). Total RNA was extracted with Trizol reagent (Life Technologies) and retro-transcribed using the High Capacity cDNA archive kit (Life Technologies). PCR amplification was performed using primers complementary to sequences in the flanking fibronectin exonic sequence: fw 5′-GAGGCCCTGGAGAGGATG-3′ and rev 5′-CACCAGGAAGTTGGTT AAATCA-3′, and the length of the products was checked by 1.8% agarose gel electrophoresis.

Luciferase assay

For luciferase activity assays, 2×10^5 cells were plated in 12-well dishes 24 h before transfection. Cells were transfected with 1.5 µl of Lipofectamine 2000 (Life Technologies) and 150 ng of the different pGL4.71P-CDK5R1 3′-UTR plasmids co-transfected with 150 ng of the pGL3 plasmid containing firefly luciferase reporter gene in order to normalize for transfection efficiency.

Luciferase reporter assays were performed using the Dual-Glo Luciferase Reporter Assay System (Promega) 24 h after transfection. Relative *Renilla* luciferase light output was normalized to Firefly luciferase output. The data were expressed as means ± s.d. Statistical significance was calculated by using a Student's *t*-test.

In silico analysis

Reference Sequences of the *CDK5* (NM_004935.3) and *CDK5R1* (NM_003885.2) genes were retrieved by the NCBI Refseq Project (http://www.ncbi.nlm.nih.gov/RefSeq/). *CDK5R1* 3′-UTR secondary structure was calculated by means of Sfold, a web server for nucleic acid folding prediction (http://sfold.wadsworth.org/cgi-bin/index.pl). The splice site prediction was performed with NNSPLICE0.9 (http://www.fruitfly.org/seq_tools/splice.html). MicroRNA target sites prediction was performed using the PicTar algorithm (http://pictar.mdc-berlin.de/).

Statistical analysis

The statistical significance of the results was determined using the Student's t-test, with data considered as significant when $P<0.05$. The Hardy–Weinberg equilibrium and differences in allele frequencies between patients and controls were evaluated with the chi-squared (χ^2) test. The association between the *CDK5* or *CDK5R1* polymorphism and risk of NS-ID was estimated by odds ratios (ORs) and 95% confidence intervals (CIs).

RESULTS

Mutation analysis of *CDK5* and *CDK5R1*

We analyzed all the coding exons and intronic splice junctions of *CDK5* and the entire coding region and 3′-UTR of *CDK5R1* in 360 patients affected by NS-ID. The cohort is composed by 260 newly recruited NS-ID who have been analyzed for the coding sequences of *CDK5* and *CDK5R1* genes and *CDK5R1* 3′-UTR, and by 100 patients already described[19] for which we performed the mutation screening of *CDK5* coding sequence and *CDK5R1* 3′-UTR. The list of the variations identified by this analysis is shown in Figure 1 together with the variations previously identified[19] for an overview.

Mutation analysis of *CDK5* showed one heterozygous mutation in the coding region. This variation is a silent mutation localized in exon 12 (c.799C>T, p.(L267L)). Three novel variations in the intronic regions of *CDK5* were also found: c.126+45C>G, c.483+8A>G and c.484-56A>G.

Mutation analysis of *CDK5R1* gene allowed us to identify a novel silent mutation (c.654G>A, p.(S218S)) and a known missense mutation (c.904C>A, p.(L302I)) in the coding region. We also detected four novel heterozygous variations in *CDK5R1* 3′-UTR: among these, one was a single base substitution (c.*397C>G) and three were small deletions (c.*649_*659delCTTGTCTGCTG, c.*1904_*1905delTC and c.*2099_*2101delGCT). None of these variations was detected in 450 healthy controls, nor was present in dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) or in the NHLBI Exome Sequencing Project (http://evs.gs.washington.edu/EVS/), which incorporates data from 6500 exome analyses. In the case of *CDK5R1* 3′-UTR c.*1904_*1905delTC mutation, carried by patient MR-417,

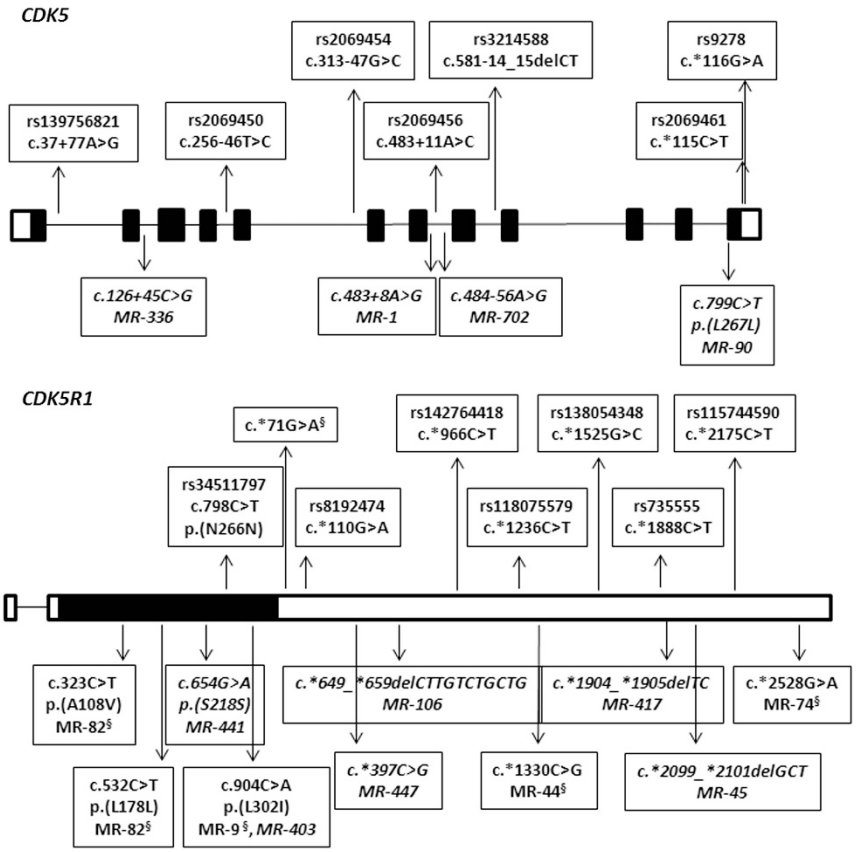

Figure 1 Schematic representation of the variations found in *CDK5* (NM_004935.3) (top panel) and *CDK5R1* (NM_003885.2) (bottom panel) genes in this study and in Venturin *et al.*[19] Squares represent exons and lines introns. Full squares are coding exons and empty squares are untranslated regions. Variations above the gene scheme are polymorphisms and variations below are single mutations. Novel variations are in italic font. §Venturin *et al.*[19]

we were also able to search for the mutation in patient's parents. The analysis showed that the mother carries the same mutation in the heterozygous state.

Several known polymorphisms were also found in both *CDK5* and *CDK5R1* genes and are reported in Figure 1 and Table 3. Seven polymorphisms were found in *CDK5* (five intronic and two in the 3′-UTR) and eight in *CDK5R1* (one in the coding sequence and seven in the 3′-UTR).

Clinical data of the mutated patients

MR-82 patient, who carries the previously described c.323C>T mutation (p.A108V),[19] is the fourth out of fifth siblings (four males and one female), born from non-consanguineous parents, all affected by NS-ID. He was referred for genetic testing (karyotype and Fragile X syndrome) at the age of 15 years, after he was adopted by his maternal uncle, as the mother could not look after him. At that age, his speech was limited to few words. This family history seems highly suggestive of an autosomal dominant form of NS-ID, whose severity shows a variable expressivity. Unfortunately, we were not able to confirm the hypothesized inheritance model by extending the mutation analysis to his NS-ID affected mother and siblings, as the family was no longer compliant to genetic investigations.

The c.*397C>G mutation is carried by patient MR-447, who is a 12-year-old male, with negative family history for neurodevelopmental conditions, affected by moderate ID and severe language delay, also manifesting behavioral problems with autistic features, such as poor social interaction, attention difficulties, repetitive use and fascination for specific objects, outburst of anger, easy and difficult to control frustration. He displayed mild fine motor coordination problems with an otherwise negative neurological examination. No epileptic manifestations were ever reported. Control EEG recordings and brain MRI were normal, as well as serial routine blood tests, extensive metabolic screening, conventional karyotype, *FMR1* gene analysis and CGH Array. Segregation analysis of the mutation and clinical evaluation in other family members has not been possible.

The c.*1904_*1905delTC mutation was found in patient MR-417, a 14-year-old male with a family history of unspecified and reportedly isolated epileptic seizures, affected by mild ID with language delay and epilepsy: focal ESES (Electrical Status Epilepticus during slow wave Sleep) on Rolandic regions. Brain MRI, routine blood workout and metabolic screening, as well as Fragile X genetic testing were negative. The proband's mother, referred as asymptomatic, was found to carry the same sequence variant. Although further clinical evaluation of this individual and segregation analysis in other family members was not possible, an autosomal dominant pattern of inheritance with reduced penetrance may be suggested.

The c.*2099_*2101delGCT mutation was identified in patient MR-45, a man affected by severe NS-ID. Fragile X syndrome as well as chromosomal unbalances were ruled out as causes of ID and his brain MRI were normal. He was referred to the genetic evaluation at the age of 45 years, because one of his relatives raised the question about her reproductive recurrence risk. In fact, MR-45 family history was apparently unremarkable, with the exception of a similarly affected sister. Both siblings, born from apparently unaffected

Table 3 Genotype and allele frequencies in patients and controls

				NS-ID population						Control population			
				Allele frequencies		Genotype frequencies				Allele frequencies			
Gene	Code	Position	Allele major (minor)	Allele 1	Allele 2	Genot. 11	Genot. 12	Genot. 22	Hardy–Weinberg equilibrium	Allele 1	Allele 2	Allele frequencies difference (χ^2 test)	OR (95% CI)
CDK5	rs139756821	intr 1 c.37+77	A (G)	0.998	0.002	0.997	0.003	0.000	Yes	0.999	0.001	No	1.573 (0.163–15.16)
CDK5	rs2069450	intr 4 c.373-46	T (C)	0.925	0.075	0.853	0.144	0.003	Yes	0.934	0.066	No	1.129 (0.805–1.583)
CDK5	rs2069454	intr 5 c.430-47	G (C)	0.937	0.063	0.877	0.120	0.003	Yes	0.956	0.044	**Yes***	**1.467 (1.009–2.133)**
CDK5	rs2069456	intr 7 c.600+11	A (C)	0.762	0.238	0.575	0.374	0.051	Yes	0.693	0.307	**Yes*****	**0.708 (0.578–0.867)**
CDK5	rs3214588	intr 8 c.581-14_15	CT (delCT)	0.998	0.002	0.997	0.003	0.000	Yes	n.d.	n.d.		
CDK5	rs2069461	3′-UTR c.*115	C (T)	0.998	0.002	0.997	0.003	0.000	Yes	0.997	0.003	No	0.535 (0.064–4.454)
CDK5	rs9278	3′UTR c.*116	G (A)	0.875	0.131	0.764	0.224	0.019	Yes	0.904	0.096	**Yes*****	**1.545 (1.187–2.011)**
CDK5R1	rs34511797	CDS c.798	C (T)	0.998	0.002	0.997	0.003	0.000	Yes	0.994	0.006	No	0.249 (0.034–1.818)
CDK5R1	Venturin et al.,[19] and this study	3′-UTR c.*71	G (A)	0.994	0.006	0.987	0.013	0.000	Yes	0.979	0.021	**Yes****	**0.194 (0.046–0.817)**
CDK5R1	rs8192474	3′-UTR c.*110	G (A)	0.710	0.290	0.503	0.415	0.082	Yes	0.792	0.208	**Yes*****	**1.569 (1.299–1.896)**
CDK5R1	rs142764418	3′-UTR c.*966	C (T)	0.998	0.002	0.997	0.003	0.000	Yes	0.999	0.001	NO	1.583 (0.164–15.25)
CDK5R1	rs118075579	3′-UTR c.*1236	C (T)	0.896	0.104	0.827	0.139	0.034	Yes	0.974	0.026	NO	1.311 (0.827–2.077)
CDK5R1	rs138054348	3′-UTR c.*1525	G (C)	0.997	0.003	0.993	0.007	0.000	Yes	0.999	0.001	**Yes***	3.876 (0.708–21.21)
CDK5R1	rs735555	3′-UTR c.*1888	C (T)	0.652	0.348	0.451	0.401	0.148	**No***	0.731	0.269	**Yes*****	**1.443 (1.196–1.740)**
CDK5R1	rs115744590	3′-UTR c.*2175	C (T)	0.998	0.002	0.997	0.003	0.000	Yes	0.990	0.010	**Yes***	0.157 (0.021–1.167)

Abbreviations: CI, confidence interval; NS-ID, non-syndromic intellectual disability; OR, odds ratio. Bold entries indicate a significant deviation from Hardy-Weinberg equilibrium and/or a significant allele frequency difference between patients and controls and/or a significant OR. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

non-consanguineous parents, had been institutionalized since their childhood. Although we cannot exclude either an autosomal recessive form of NS-ID or an X-linked dominant one, this family history may be suggestive, once again, of an autosomal dominant form of NS-ID with reduced penetrance. However, we were not able to confirm this hypothesis, as the family was no longer reachable.

Analysis of *CDK5* and *CDK5R1* polymorphism frequencies and OR risk for NS-ID

The allelic and genotypic frequencies for each polymorphism identified by mutation analysis in *CDK5* and *CDK5R1* were calculated (Table 3). The observed genotype distribution of these single-nucleotide polymorphisms (SNPs) conformed to Hardy–Weinberg equilibrium in all cases except for rs735555:C>T in *CDK5R1* 3′-UTR, for which the heterozygotic genotype was less represented and the homozygotic genotypes more represented than expected.

The allelic frequencies of the polymorphisms were compared with that of control individuals derived from both database annotations (dbSNP Caucasian population and 1000 genomes) and our 450 controls. For eight polymorphisms we found a significant allele frequency difference between patients and controls calculated with a chi-square test (Table 3).

Moreover, OR was calculated to assess the relationship between *CDK5* and *CDK5R1* polymorphisms and NS-ID risk. An increased risk for NS-ID was found for the minor allele of rs2069456:A>C, rs9278: G>A, rs8192474:G>A and rs735555:C>T and a protective role for the minor allele of rs2069454:G>C and *CDK5R1* c.*71G>A (Table 3). In particular, given the strong deviation of *CDK5R1* rs735555:C>T genotype frequencies in the NS-ID from Hardy–Weinberg equilibrium and from the control population, the OR for the different genotypes was calculated. The 'TT' genotype containing minor alleles was found to be significantly more frequent in NS-ID patients compared with controls ($P<0.001$) increasing the risk of NS-ID by almost two-folds (OR 1.958, 95% CI 1.351–2.837, $P<0.001$), while the heterozygote 'CT' showed no different risk (OR 1.102, 95% CI 0.854–4.421, $P>0.05$) and the 'CC' genotype was associated with the normal phenotype (OR 0.699, 95% CI 0.544–0.898, $P>0.01$).

The *CDK5R1* p.A108V mutation affects p35 cleavage by calpain

The *CDK5R1* c.323C>T, p.A108V missense mutation was previously identified in patient MR-82.[19] This substitution affects an amino acid conserved in mammals but not in other species and positioned 10 residues downstream the cleavage site of the calpain protease, which generates the p35 proteolytic fragment p25.

To study whether this mutation could alter p35 ability of activating the CDK5 kinase, we overexpressed either the wild-type ($p35^{wt}$) or the mutated ($p35^{A108V}$) protein in SK-N-BE and HEK-293 cells and measured the phosphorylation levels of the amyloid precursor protein (APP) Thr668, which is a known specific CDK5 target[23] (Figure 2). The overexpression of both $p35^{wt}$ and $p35^{A108V}$ led to a significant but comparable increase in CDK5 amount (Figure 2b). A slight though not significant increase in APP phosphorylation at Thr668 was observed after overexpression of both p35 forms and thus no significant difference in APP phosphorylation levels at Thr668 was detected when overexpressing $p35^{A108V}$ compared with $p35^{wt}$ (Figure 2c).

With the aim of establishing whether the p.A108V mutation could affect p35 to p25 processing by the calcium-activated calpain protease, we treated HEK-293 cells with 2 mM $CaCl_2$ and 1 μM Ca^{2+} ionophore A23187 after either $p35^{wt}$ or $p35^{A108V}$ overexpression (Figure 2d and e). The calcium treatment caused a significant increase in p25

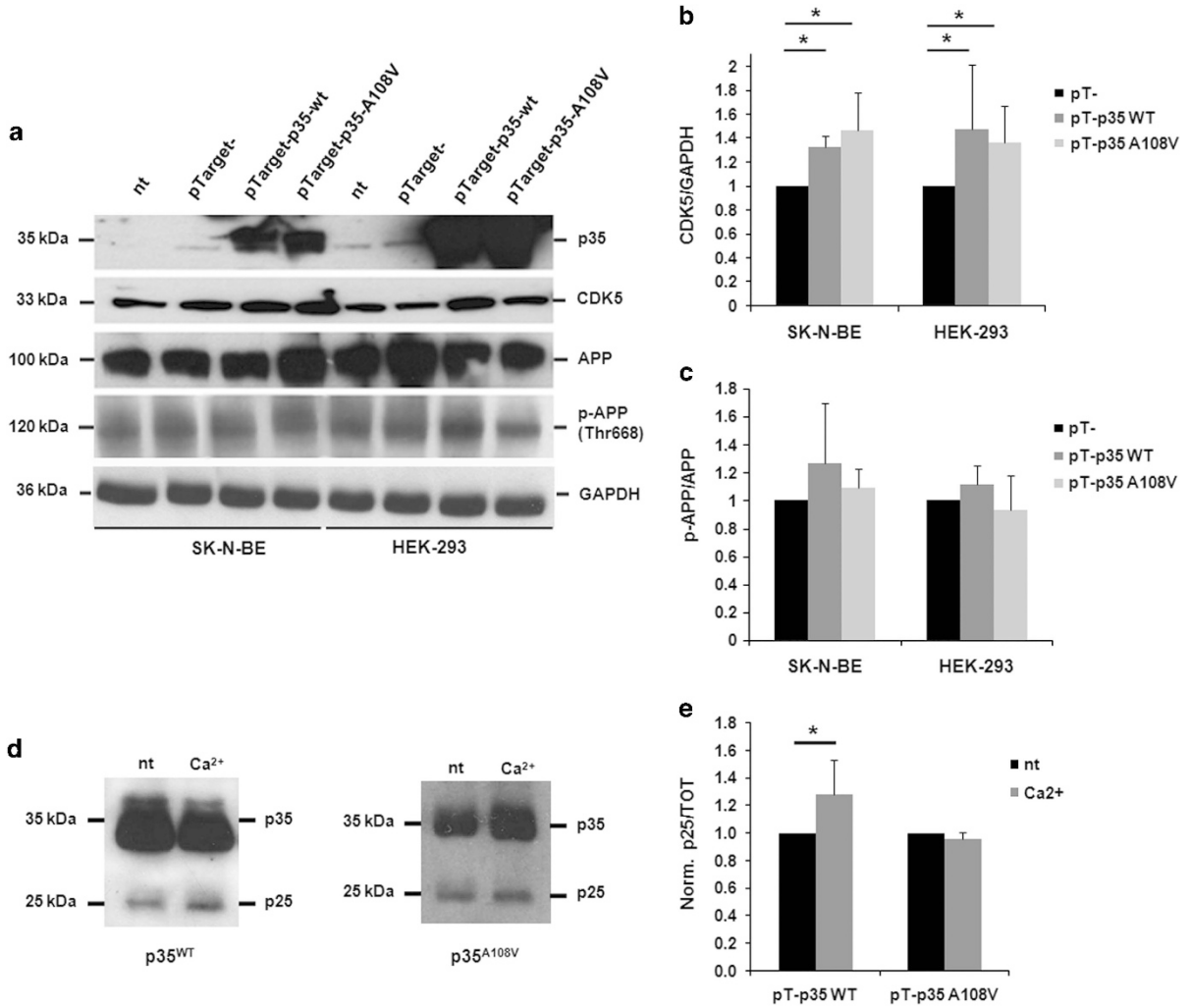

Figure 2 (**a**) Representative western blot of p35, CDK5, APP, p-APP (Thr668) and GAPDH in SK-N-BE and HEK-293 cells transfected with wild-type or mutated A108V p35. (**b**) Densitometric data of CDK5 normalized on GAPDH from (**a**). Means ± s.d. *$P<0.05$. (**c**) Densitometric data of p-APP normalized on total APP from (**a**). Means ± s.d. (**d**) Representative western blot of p35 and p25 after 6 h treatment with 1 μM Ca^{2+} ionophore A23187 in the presence of 2 mM $CaCl_2$ in HEK-293 cells transfected with wild-type or A108V mutated p35. (**e**) Densitometric data of p25 normalized on total p35 (p35 plus p25) from (**d**). Means ± s.d. *$P<0.05$.

levels, compared with untreated cells, only in the case of $p35^{wt}$ but not $p35^{A108V}$ (Figure 2e), indicating that the p.A108V change can impair p35 cleavage by calpain.

The c.484-56A>G mutation has no effect on *CDK5* pre-mRNA splicing

A bioinformatic prediction showed that none of the intronic variations affects the splicing of *CDK5* mRNA, with the exception of c.484-56A>G in intron 7, for which a possible splicing defect has been predicted. A new acceptor site with a score of 0.88 located 13 bases upstream the canonical site was predicted for the mutated mRNA using the NNSPLICE0.9 software. To verify whether this variation might lead to abnormal splicing compared to the wild-type form, minigene constructs containing CDK5 exon 7, wild-type or mutated intron 7 and exon 8, were transfected into SK-N-BE and HEK-293 cells. RT-PCR performed on total RNA from the transfected cells gave rise to four different products with no difference in height or intensity between the wild-type and mutated samples in both cell lines (data not shown). Of the four products, one corresponded to the wild-type splicing, while the product corresponding to the predicted alternative splicing possibly caused by the c.484-56A>G mutation was not detected, suggesting no effect on *CDK5* pre-mRNA splicing.

Four identified 3′-UTR variants affect *CDK5R1* expression at post-transcriptional level

To verify whether the six mutations identified in NS-ID patients in *CDK5R1* 3′-UTR (four in this study and two in the previously published analysis, Figure 1) might lead to altered *CDK5R1* expression levels, we performed a luciferase reporter assay. The pGL4.71P-UTR-wt construct, containing the whole human *CDK5R1* 3′-UTR downstream of the *Renilla* luciferase coding sequence,[20] was used to generate, by site-direct mutagenesis, six constructs each bearing one of the reported 3′-UTR variations. We then co-transfected SK-N-BE and HEK-293 cells with the pGL4.71P-UTR-wt or mutated constructs and the pGL3 plasmid, containing the firefly luciferase gene, to normalize for transfection efficiency. Dual-luciferase assays showed decreased reporter gene expression for UTR-397G and UTR-1904_5del mutants in both cell lines, and a reduced luciferase activity for UTR-2099_2101del and UTR-2528A mutants in HEK-293 cells only, compared with the wild-type UTR (Figure 3a).

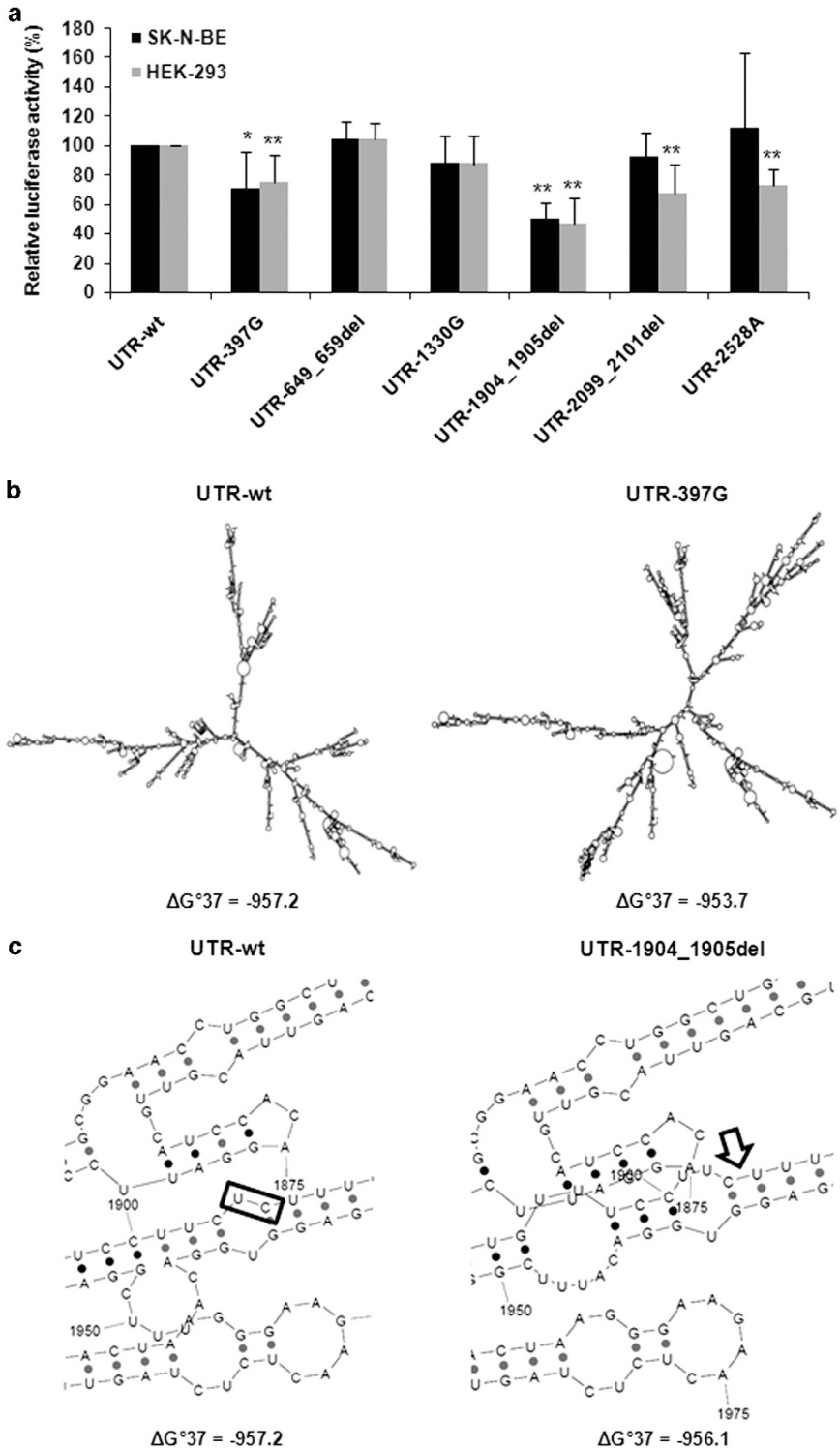

Figure 3 (**a**) Luciferase activity of pGL4.71P-UTR wild-type (UTR-wt) and mutated constructs in SK-N-BE and HEK-293 transfected cell lines. Cells were transiently co-transfected with the pGL4.71P-UTR constructs (*Renilla* luciferase) and the pGL3 (Firefly luciferase) vector and were harvested 24 h after transfection. Luciferase activity of the chimeric reporter genes, normalized for transfection efficiency against the Firefly luciferase activity, is represented as a percentage of the activity observed in cells transfected with pGL4.71P-UTR-wt (defined as 100%). Means ± s.d. luciferase values were obtained from at least four independent experiments (*$P<0.05$, **$P<0.01$ compared with the corresponding UTR-wt value for each cell line). (**b**) Secondary structure analysis by Sfold of the wild-type *CDK5R1* 3′-UTR (UTR-wt, left panel) and the mutated UTR-397G (right panel). The free energy decrease is indicated. (**c**) Secondary structure by Sfold of the wt *CDK5R1* 3′-UTR (UTR-wt) and the mutated UTR-1904_1905del for the region encompassing the deletion of the two nucleotides (highlighted by a box in the left panel and an arrow in the right panel). The free energy decrease is indicated.

To verify whether the two mutations leading to reduced luciferase activity in both cell lines (UTR-397G and UTR-1904_5del) are predicted to cause an altered *CDK5R1* 3′-UTR secondary structure, we performed a structural analysis by means of the Sfold software. The results indicated that the c.*397C>G substitution is predicted to induce a significantly different folding of the entire 3′-UTR compared with the wild type (Figure 3b) and that the c.*1904_*1905delTC can induce a different folding of the RNA in the region surrounding the deletion (Figure 3c). These results indicate that four out of six mutations identified in the 3′-UTR might affect *CDK5R1* expression at post-transcriptional level.

DISCUSSION

In this work, we carried out the mutational screening of *CDK5* and *CDK5R1* in a large cohort of NS-ID patients. The key role in CNS development and function of these two genes makes them strong candidates for the occurrence of NS-ID. CDK5 and its main activator p35,[24] encoded by the *CDK5R1* gene, are fundamental for neuronal function, as they regulate neuron maintenance and migration, cytoskeletal dynamics and neurite outgrowth.[25] In particular, they are essential for proper radial migration of neurons during the development of the mammalian cortex, and also have effects on synaptic function.[10] CDK5/p35 has a critical role in dopaminergic neuron signaling and regulates components of the synaptic vesicle cycle; recent evidence also suggests a pivotal role in synaptic plasticity, behavior and cognition.[25] Cdk5 knockout mice display perinatal lethality owing to neuronal migration deficits and impaired axonal transport of neurofilaments.[26] In p35 knockout mice, the newly generated postmitotic neurons fail to migrate past older neurons on radial glia, leading to a dramatic inversion of cortical layering.[27] The animals suffer from adult seizures and lethality, and they exhibit alterations in cell orientation as well as dendrite and axon trajectories.[27,28] It is worth to be noted that mice lacking the other CDK5 activatory subunit p39 ($p39^{-/-}$) are viable and do not display any obvious defects in CNS or other organs, indicating that the loss of p39 can be fully compensated for by p35.[24]

Importantly, it was recently observed that p25, a proteolytic product of p35 generated by calpain cleavage,[29] is generated during spatial memory formation and reduced in Alzheimer's disease.[30] Furthermore, p35 levels are reduced in post-mortem brain of patients affected by schizophrenia, a disorder that is often associated with impaired cognition.[31]

Our mutational screening of NS-ID patients led to the identification of nine unknown mutations in *CDK5* and *CDK5R1* genes. All the newly identified mutations were absent in 450 screened healthy control individuals and in the public variation databases, indicating that they are most likely private variants. Most of the probands carrying the mutations were apparent sporadic cases, whereas MR-45 and MR-82 were familial cases of NS-ID. As in most cases parents and affected relatives were unavailable for sequence analysis, we could not establish whether these variants are *de novo* in sporadic cases or inherited in familial cases.

Aside from the c.323C>T, p.A108V mutation in *CDK5R1* gene, previously observed in MR-82 patient,[19] no other significant missense variations were identified in either *CDK5* or *CDK5R1* genes in the analyzed cohort of 360 NS-ID patients. The p.A108V change is located near the p35 consensus sequence for the cleavage by calpain, the calcium-dependent protease that gives rise to the p35 truncated form, p25. p35 and p25 have distinct properties and activity: differently from p35, which is very unstable and is present throughout the entire neuron, p25 has a longer half-life and is concentrated in the cell body and in the nucleus.[32] Since it was recently demonstrated that p25 is important for memory formation,[30] we attempted to establish whether p25 generation was affected in the mutant form of p35. Our experiments showed that $p35^{A108V}$ does not respond to calcium-mediated calpain activation leading to enhanced p25 levels, suggesting that *CDK5R1* p.A108V mutation might have a role in the onset of the cognitive dysfunction displayed by the patient.

Our mutation analysis of NS-ID patients also led to the identification of mutations inside *CDK5R1* 3′-UTR. The discovery of mutations causing human disease has so far been biased toward protein-coding regions, but several works demonstrate that mutations outside these regions (for example, untranslated regions) can and should also be tackled.[33–36] It is well known that UTRs have an established role in appropriated gene control and that disorders of neuronal plasticity and learning can be due to perturbations in UTR-mediated functions.[34,37,38] As far as *CDK5R1* is concerned, we demonstrated that its 3′-UTR has a key role in the post-transcriptional regulation of CDK5R1 expression through the binding to nELAV and hnRNPA2/B1 RNA-binding proteins and to miR-103/107 microRNAs.[20,39,40] Thus, the variations found in *CDK5R1* 3′-UTR can potentially affect the control of *CDK5R1* expression at post-transcriptional level, by altering the interaction with these RNA-binding proteins and microRNAs owing to both sequence and structural changes. Indeed, four mutations led to altered reporter gene expression in our dual-luciferase assays.

In particular, two mutations (c.*397C>G and c.*1904_*1905delTC) showed decreased luciferase activity in both the analyzed cell lines and were predicted to alter the 3′-UTR secondary structure, suggesting that they might modify *CDK5R1* post-transcriptional control. It is worth to be noted that the c.*1904_*1905delTC mutation causes a considerable reduction of luciferase activity (about 50%), supporting a strong potential effect on *CDK5R1* expression levels.

The c.*2099_*2101delGCT mutation leads to a partial 'seed' sequence deletion of a predicted miR-15/107 target site.[39] Luciferase experiments showed reduced reporter gene expression in HEK-293 but not in SK-N-BE cells. As the abrogation of a miRNA target site is predicted to increase gene expression levels, we hypothesize that this reduction might be caused by the alteration of yet unknown post-transcriptional regulatory elements working in a cell line-specific manner, rather than by the loss of the miRNA target site.

Of potential interest was also the deletion c.*649_*659delCTTGTCTGCTG, which abrogates one predicted miR-15/107 microRNA group binding site.[39] This notwithstanding, the luciferase assays evidenced only a slight, though not significant, increase in reporter gene expression for the mutant construct compared with the wild-type, and do not thereby support a deleterious role for this mutation.

The other mutations detected in *CDK5R1* 3′-UTR fall outside the post-transcriptional regulatory elements validated or predicted in our previous works.[20,39,40]

A homozygous loss-of-function *CDK5* mutation was recently identified in a large family with autosomal recessive lissencephaly with cerebellar hypoplasia.[12] This mutation caused a very severe phenotype, which considerably affected both the CNS and the other organs, and was lethal. Our cohort of NS-ID patients generally displays a milder phenotype, and ID is often the sole or the most relevant clinical manifestation, although a few of them can present isolated dysmorphic facial features, which however are not clearly ascribable to any specific syndromic pattern. Microcephaly and seizures are present in a minority of our ID patients and are the only clinical signs shared

with the lissencephaly with cerebellar hypoplasia patients. Nevertheless, the severity of the phenotype caused by the complete absence of *CDK5* expression strengthens the hypothesis that heterozygous mutations in the *CDK5* or *CDK5R1* gene that cause even a partial haploinsufficiency can lead to milder clinical conditions, such as NS-ID.

Fifteen known polymorphisms were also detected in the two screened genes in our cohort of NS-ID patients, and eight of them displayed significantly different frequencies compared with the healthy control population (see Table 3). Among these, five are mapped to *CDK5R1* 3′-UTR. In particular, the rs735555:C>T polymorphism showed a significant difference in allele and genotype frequencies between the NS-ID group and the control population. Moreover, a deviation from the Hardy–Weinberg equilibrium of genotype frequencies was observed in the NS-ID population, which is characterized by a higher incidence of minor allele 'TT' homozygotes compared with the controls. We thus hypothesize that an increased risk of NS-ID is associated with the 'TT' genotype, while 'CC' might act as a protective genotype. The distribution of the allele and genotype frequencies of the rs735555:C>T polymorphism has been previously analyzed in two large cohorts of patients affected by Alzheimer's disease[41] and Parkinson's disease,[42] respectively. In both cases there was no evidence for deviation from Hardy–Weinberg equilibrium in the affected population and the distribution of the allele and genotype frequencies did not differ significantly between patients and control groups. However, in both cases, an association was found when considering the *CDK5R1* rs735555:C>T polymorphism and a second polymorphism in the *GSK-3β* gene. Our findings suggest that the rs735555:C>T polymorphism alone could represent a susceptibility variant for NS-ID.

Taken together, our data suggest that mutations and polymorphisms in *CDK5* and *CDK5R1* genes, either alone or in combination with other genetic alterations, could have a role in the occurrence of NS-ID in some patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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