ORIGINAL ARTICLE

Functionally distinct groups of inherited PTEN mutations in autism and tumour syndromes

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ABSTRACT

Background Germline mutations in the phosphatase PTEN are associated with diverse human pathologies, including tumour susceptibility, developmental abnormalities and autism, but any genotype-phenotype relationships are poorly understood.

Methods We have studied the functional consequences of seven PTEN mutations identified in patients diagnosed with autism and macrocephaly and five mutations from severe tumour bearing sufferers of PTEN hamartoma tumour syndrome (PHTS).

Results All seven autism-associated PTEN mutations investigated retained the ability to suppress cellular AKT signalling, although five were highly unstable. Observed effects on AKT also correlated with the ability to suppress soma size and the length and density of dendritic spines in primary neurons. Conversely, all five PHTN mutations from severe cases of PHTS appeared to directly and strongly disrupt the ability to inhibit AKT signalling.

Conclusions Our work implies that alleles causing incomplete loss of PTEN function are more commonly linked to autism than to severe PHTS cases.

INTRODUCTION

Phosphatase and tensin homologue deleted on chromosome ten (PTEN) is a phosphatase that suppresses the activity of the class I phosphoinositide 3-kinase/AKT signalling pathway.1 It has been heavily studied due to its status as a tumour suppressor gene in which loss of function mutations are identified in many sporadic tumours and in the germline of patients with diverse phenotypes. Inherited dominant PTEN mutations have been identified in patients with Cowden syndrome and Bannayan-Riley-Ruvalcaba syndrome, conditions that are often grouped together as PTEN hamartoma tumour syndrome (PHTS). They have also been identified in a fraction of patients with autism spectrum disorder (ASD) who also display macrocephaly.2–8 Supporting the causality of PTEN mutations in these phenotypes, a similar tumour spectrum and autism-like phenotypes have been identified in mice either carrying a single null Pten allele or with tissue-specific deletion of Pten.9–11

The major diagnostic criteria for PHTS include malignancies of the breast, thyroid and endometrium in addition to benign hamartomas, skin lesions and macrocephaly.12 However, the symptoms associated with PTEN mutations are diverse and in some cases, germline mutations have been identified in adult patients only upon presentation with malignancy,13–14 and in patients with macrocephaly, autism and/or learning disability without further symptoms.15 A series of clinical and laboratory based studies have presented evidence that mutational functional diversity and genetic background may each contribute to the phenotypic diversity observed in patients carrying PTEN mutations. In some families, individuals carrying the same PTEN mutation have been noted to display very different phenotypes, to the extent of separate diagnoses of Cowden syndrome and Bannayan-Riley-Ruvalcaba syndrome.16,17 Additionally, the spectrum of tumours arising in heterozygous mice carrying a null allele of Pten also appears to be strongly dependent on genetic background.18 Studies of the mutation types (missense vs truncation) and positions (phosphatase vs C2 domain) within PTEN have argued for19 and against20 genotype-phenotype relationships. On the other hand, evidence that not all PTEN mutations cause one phenotypic profile has been provided by studies of heterozygous mice expressing a stable Pten mutant protein either lacking all phosphatase activity, Pten C124S or that selectively lacks lipid phosphatase activity, Pten G129E. These mice display a more severe tumour phenotype than mice carrying a Pten deletion allele21,22 indicating that inactive Pten can aggravate phenotypes, particularly tumour severity, through dominant negative mechanisms. However, whether this represents a consistent genotype-phenotype relationship within PTEN mutation carriers, in particular relating to the occurrence of ASD, is unclear.

RESULTS

PTEN missense mutations identified in patients with autism are catalytically competent

Recombinant PTEN protein purified from bacteria has been used in many previous studies to demonstrate that phosphatase activity is critical for its tumour suppressor function. In preliminary experiments studying three PTEN mutants identified in patients with autism, we could detect little or no catalytic activity in vitro from protein purified from bacteria, yet transient expression of these mutants in mammalian cells led to a robust suppression of the phosphorylation of the PTEN regulated kinase AKT (see online supplementary figure S1A, B). This apparent difference between assay formats encouraged us to analyse a larger group of seven mutant proteins identified in patients with autism (figure 1A) lacking other manifestations of PHTS.3,15 Therefore, we used
lentiviruses to express either PTEN wild-type (WT) or individual missense PTEN mutants (C124S, G129R, H118P, H123Q, E157G, F241S, D252G, N276S and D326N) in PTEN-null U87MG glioblastoma cells. These include two recognised active site PTEN mutants that lack catalytic activity: PTEN C124S and PTEN G129R. We expressed PTEN WT and each mutant using five increasing doses of each viral vector (figure 1C). Investigating effects on cellular AKT phosphorylation, when expressed at similar levels, all seven of the autism-related mutants showed similar (or in some cases possibly greater) effects to PTEN WT or mutants. (D) Relative PTEN expression was detected using real-time qPCR. Data are shown as mean PTEN ΔCt values relative to GAPDH±SEM. from three experiments each performed in duplicate. (E) Parallel PTEN protein immunoblot. GFP, green fluorescent protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Reduced protein stability of autism-associated PTEN mutants

To investigate the relationship between the cellular abundance of PTEN mRNA and protein for the mutants, we expressed PTEN WT and mutants in U87MG cells and measured PTEN mRNA levels by real-time qPCR and protein by western blotting. In all cases, the mRNA abundance corresponded to the titre of lentivirus applied in the experiment. The expression of PTEN WT and the widely used stable mutants, C124S and G129R was robust and similar, as was the expression of two of the autism-associated mutants, H123Q and E157G. However, we observed that for five of the autism-associated mutants (H118P, F241S, D252G, N276S and D326N) the amount of protein expressed was very low (figure 1D, E).

To assess more directly the stability of these mutant proteins relative to PTEN WT, we used the protein synthesis inhibitor cycloheximide (200 μg/mL). Transduced cells were incubated with the inhibitor for 0 h, 2 h, 4 h, 6 h and 8 h, followed by immunodetection using anti-PTEN antibody (figure 2A, B). Whereas the protein level of PTEN WT and some mutants (C124S, G129R, H123Q and E157G) was reduced by ∼20% after 8 h of inhibition of protein synthesis, the protein level for the PTEN mutants H118P, F241S, D252G, N276S and D326N was reduced by ∼20%.
was decreased by ~60% after 8 h (figure 2A, B), and was significantly lower than PTEN WT, supporting the hypothesis that these five mutants are unstable.

**Molecular characterisation in primary neurons of the PTEN mutations identified in patients with autism**

Macrocephaly is one of the several anatomical and cellular abnormalities that have been suggested to be important factors in the pathogenesis of ASD. Work carried out in mice showed that PTEN can control neuronal morphology and growth, with hypertrophy and dendritic overgrowth and development of macrocephaly and additional behavioural phenotypes similar to human autism observed in mice lacking PTEN in neurons. We have therefore used neurons from which endogenous Pten is deleted to investigate whether the physiological level re-expression of PTEN WT or autism-associated PTEN mutants is able to support a wild type phenotype of normal dendritic growth and neuronal size. Hippocampal neurons prepared from E15.5 flox/flox Pten mice were transduced simultaneously with lentiviruses expressing Cre recombinase (RFP-Cre) to delete endogenous Pten and with viruses encoding PTEN WT or PTEN mutants. The mutants PTEN H123Q, F241S and D326N were selected to represent a range of protein stabilities (see figure 2D, E). ***p<0.001, **p<0.01, *p<0.05 compared with PTEN WT (Student’s t test using GraphPad Prism software).

PTEN mutations identified in severe cases of PHTS lack activity

Considering the view that patients diagnosed only with ASD and macrocephaly may reflect a group at the milder end of the spectrum of phenotypes associated with PTEN mutations, we chose to select a comparator group of mutations associated with a severe tumour bearing PHTS phenotype. To do this, we conducted an extensive search of data published in journals and online resources associating specific PTEN mutations with phenotype (see online supplementary tables S1–S3). Although inconsistencies in the categorisation of patients’ severity are likely to be introduced by diversity in the diagnosis and recording of separate phenotypical characteristics between publications, our analysis identified 112 missense mutations that had sufficient associated phenotypical data and which had been identified in a total of 204 patients (see online supplementary tables S2, S3). This analysis shows that missense mutations (rather than truncating mutations) were overrepresented among all PTEN mutation carriers described with ASD compared with all carriers of PTEN mutations, consistent with the hypothesis that more of these mutations may retain some activity (figure S3).
Five PTEN missense mutations associated with the most severe phenotype (score >6) were selected for characterisation (PTEN A39P, N48K, L108P, L112P and R130L (figure 4A)). These were expressed in U87MG cells together with the already described stable and inactive PTEN mutants C124S, G129R and G129E. The latter two have been identified in Cowden syndrome sufferers and in our classification were associated with a mild/moderate phenotype. When transduced into U87MG cells using lentiviruses, two of these mutant cDNAs (A39P and N48K) were expressed with similar efficiency to WT PTEN but did not suppress cellular AKT phosphorylation (figure 4C). The other three mutants (L108P, L112P and R130L) expressed less efficiency compared with PTEN WT, but when expression was forced to this level, they also failed to downregulate AKT phosphorylation (figure 4C and see online supplementary figure S3B). Analysis of the half-life of PTEN mutant proteins showed that two of the mutations (L108P and L112P) destabilise the protein with a decrease in protein levels by ∼60–80% after 8 h of protein synthesis inhibition, whereas the protein level for the mutants A39P, N48K and R130L as well as for PTEN WT, C124S, G129E and G129R was reduced by ∼20% (figure 4D, E). This finding that when expressed at the same level as wild type PTEN, all the seven PTEN mutations associated with ASD, but none of the five associated with severe Cowden syndrome analysed was able to regulate AKT is significant (two-tailed Fisher’s exact test p=0.0013).

DISCUSSION

Our data show that all seven PTEN mutant proteins that we investigated due to their identification in patients with ASD retained the capacity to suppress cellular phosphoinositide 3-kinase-AKT signalling when they were expressed to the same level as wild type PTEN. However, most of these mutant PTEN proteins are unstable and it would be expected that in vivo this instability would lead to a very considerable reduction in protein abundance relative to PTEN WT and a resultant reduction in biological activity. Functional evaluation of PTEN mutants in a yeast based system has previously found significantly lower activity in a set of PHTS/Cowden-associated PTEN mutations than in autism-associated mutations, although it was unclear whether this effect related to direct inactivating mutations or poor protein stability. It has already been reported for another mutant associated with ASD, PTEN H93R, that this mutation caused a significant reduction in activity in vitro and in cells, to approximately 15% of wild type activity in vitro. In agreement with our own data, it seems possible that strong

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but incomplete loss of activity may be related to the observation of ASD symptoms in the absence of some of the more severe phenotypes seen in some patients with Cowden syndrome. In this regard, we have observed mutations that would lead to loss of function through instability and through direct incomplete loss of activity.

In contrast, when a set of PTEN mutations associated with the most severe cases of Cowden syndrome were tested for their cellular activity, all of these five mutant proteins were found to display no detectable effect on AKT phosphorylation even when overexpressed. This implies that complete loss of activity may associate with a more severe PHTS/Cowden phenotype. Of these five proteins, three showed stability similar to wild type PTEN, whereas two were unstable. In the cases of the stable mutants, this supports a hypothesis that the expression of stable inactive protein may exacerbate the phenotype caused by PTEN loss of function, through interference with the functions of the remaining WT PTEN protein and possibly other proteins.

Strong evidence supporting the potential importance of this effect comes from the observed phenotypes of Pten knockin mice, in which the most severe tumour phenotype was observed in mice expressing stable PTEN mutants lacking lipid phosphatase activity, G129E or C124S, relative to either mice carrying a Pten deletion or with an unstable PTEN mutation. However, other mechanisms may be involved as this would not seem to explain the occurrence of unstable catalytically inactive PTEN mutants (L108P and L112P) in severe PHTS. Therefore, although the mechanisms mediating these effects are unclear, our work characterises a novel correlation between specific human patient groups and the features of the PTEN mutations they carry.

Our work implies that mutation-specific factors do contribute to the severity of PTEN mutation carriers’ symptoms. These effects may relate to the retention of some biological PTEN activity in the case of patients displaying macrocephaly and ASD in the absence of more severe developmental phenotypes, and to the expression of dominant negative inactive PTEN protein in some cases combining severe developmental and eventually tumour phenotypes. Biochemically, potential dominant negative effects of mutant PTEN proteins have not been extensively studied, although data supporting the functional importance of PTEN dimerisation has very recently been published. The first trials are underway of potential targeted therapies, such as inhibitors of the mammalian target of rapamycin (mTOR) kinase activated downstream of PTEN loss, to treat patients with inherited PTEN mutations and severe phenotypes. Through a deeper understanding of the signalling mechanisms driving specific phenotypes, it may eventually be possible to tailor treatments to individual patient groups based upon their early symptoms and on an understanding of the cellular effects of defined classes of mutation.

**MATERIALS AND METHODS**

**Cell culture**

U87MG glioblastoma cells culture, lentivirus preparation and titration were as previously described. 30 U87MG cells were transduced with lentiviruses encoding PTEN WT or mutants. Specific lentiviral doses were used for each mutant to standardise PTEN expression level (5 units are equal to 50 μL of viral supernatant). Cell expressing GFP and cells treated with the phosphoinositide 3-kinase (PI3K) inhibitor PI103 (1 μM, 30 min) were controls. PTEN expression and AKT and PRAS40 phosphorylation were investigated by immunoblotting of total cell lysates. (D and E) U87MG cells were transduced with PTEN WT or mutants, treated with 200 μg/mL cycloheximide, lysed at the indicated times and then immunoblotted for PTEN and GAPDH. (D) PTEN protein levels normalised to GAPDH from three independent experiments (mean±SEM). Six independent experiments for PTEN WT, C124S, H118P and G129R (including the data shown in figure 2). ***p<0.001, **p<0.01, *p<0.05 compared with PTEN WT (Student’s t test using GraphPad Prism software). (E) The blots shown are representative of three independent experiments.

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transduced with lentivirus particles in a medium supplemented with polybrene (16 μg/mL; hexadimethrine bromide, Sigma). Media were changed 24 h after transduction, and after a further 24 h cells were processed. To delete the Pten gene from cultured Pten flox-flox murine neurons, a modified lentiviral vector was used in which the expression of the RFP-Cre transgene is driven by a human synapsin-1 promoter. RFP-Cre lentiviruses were produced by triple cotransfection of 6.5 million HEK293 T cells in 75 cm² flasks with 10 μg lentiviral vector, 7.5 μg pHCMV-8.9 deltaR packaging vector and 5 μg pCMV VSV-G envelope vector using X-tremeGENE 9 DNA transfection reagent (Roche). The 10 mL of viral supernatant was harvested by using Lipofectamine 2000 as transfection reagent (0.6 B-27 (serum-free supplement) and cultured on poly-L-ornithine coated coverslips at 37°C in 5% CO₂ for immunocytochemistry and biochemical analysis.

RFP-Cre lentivirus particles were added to the neuronal culture 7 days after seeding, together with lentivirus particles encoding WT or mutant forms of PTEN and left for 6 days. Every 2–3 days half of the medium was replaced by fresh medium. Neuronal cells used for immunocytochemistry were transfected with yellow fluorescent protein 24 h before fixation by using Lipofectamine 2000 as transfection reagent (0.6 μg DNA/0.8 μL Lipofectamine in OptiMEM). Of the medium 250 μL was removed, collected in a tube, mixed with fresh media in a 1:1 ratio and incubated at 37°C. Transfection mix was added to the cells for 20 min and then removed and replaced by the incubated media. Cells used for biochemical analysis were washed in phosphate buffered saline (PBS) and lysed using RIPA buffer (RIPA lysis and extraction buffer, Thermo Scientific, 25 mM Tris-HCL pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecylphosphate (SDS), protease inhibitors and phosphatase inhibitors).

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