Human Molecular Genetics, 1999, Vol. 8, No. 2 **185–193**

ARTICLE

PTEN is inversely correlated with the cell survival factor Akt/PKB and is inactivated via multiple mechanisms in haematological malignancies

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Received September 30, 1998; Accepted November 19, 1998

***PTEN* is a novel tumour suppressor gene that encodes a dual-specificity phosphatase with homology to adhesion molecules tensin and auxillin. It recently has been suggested that *PTEN* dephosphorylates phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3], which mediates growth factor-induced activation of intracellular signalling, in particular through the serine–threonine kinase Akt, a known cell survival-promoting factor. *PTEN* has been mapped to 10q23.3, a region disrupted in several human tumours including haematological malignancies. We have analysed *PTEN* in a series of primary acute leukaemias and non-Hodgkin's lymphomas (NHLs) as well as in cell lines. We have also examined whether a correlation could be found between PTEN and Akt levels in these samples. We show here that the majority of cell lines studied carries *PTEN* abnormalities. At the structural level, we found mutations and hemizygous deletions in 40% of these cell lines, while a smaller number of primary haematological malignancies, in particular NHLs, carries *PTEN* mutations. Moreover, one-third of the cell lines had low *PTEN* transcript levels, and 60% of these samples had low or absent PTEN protein, which could not be attributed to gene silencing by hypermethylation. In addition, we found that PTEN and phosphorylated Akt levels are inversely correlated in the large majority of the examined samples. These findings suggest that *PTEN* plays a role in the pathogenesis of haematological malignancies and that it might be inactivated through a wider range of mechanisms than initially considered. The finding that PTEN levels inversely correlate with phosphorylated Akt supports the hypothesis that PTEN regulates PtdIns(3,4,5)P_3 and suggests a role for PTEN in apoptosis.**

INTRODUCTION

The disruption of oncogenes or inactivation of tumour suppressor genes plays a central role in the pathogenesis of malignant diseases. In most cancer types, in particular leukaemias and lymphomas, the detailed characterization of such abnormalities has provided a basis for disease classification and has helped to define prognosis in individual patients. Furthermore, the identification of the targets for these disruptions has advanced our understanding of the mechanisms of tumorigenesis and has been valuable in the design of molecular approaches to diagnosis and to monitoring response to treatment. Chromosomal translocations resulting in novel fusion proteins or transcriptional activation of an oncogene by juxtaposition of a T cell receptor or immunoglobulin gene (1) are usually present at initial diagnosis of leukaemias and lymphomas. Conversely, inactivation of tumour suppressor genes seems to play a more relevant role, albeit not exclusively, in disease progression, usually with ominous prognostic implications (2,3). Moreover, the chromosomal disruptions leading to such abnormalities are not evenly distributed throughout the genome, but rather cluster in certain regions. In leukaemias and lymphomas, one such region is 10q22–24 (4,5).

A novel gene encoding a dual-specificity phosphatase, *PTEN*, also known as *MMAC1* or *TEP1*, has been identified at chromosome sub-band 10q23.3 (6–9). Abnormalities in *PTEN* have thus far been studied almost exclusively in epithelial

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malignancies. Positive findings have been reported prominently in glioblastomas and endometrial cancer (10,11). In addition, germline mutations of *PTEN* have been identified in two inherited hamartoma syndromes (12,13), one of which, Cowden syndrome, is associated with an increased risk for breast and thyroid cancers. In contrast to germline mutation, an 'early' event in these two syndromes, it has been suggested that inactivation of *PTEN* might participate as a late event in certain sporadic malignancies, such as glioblastomas, in which *PTEN* abnormalities at the DNA level have been identified frequently in more advanced tumours (10,14).

The role of phosphatases in tumour pathogenesis has not been fully explored. While several protein kinases have been implicated as oncogenes, and phosphatases have long been known frequently to antagonize their function, there has been no direct demonstration of the role of phosphatases in tumour development (15). *PTEN* characterization as a bona fide tumour suppressor gene has been confirmed by studies showing growth suppression in *PTEN*-mutated glioblastoma cell lines after transfection of wild-type *PTEN* (16).

Although a full characterization of PTEN substrates still requires further investigation, there is recent *in vitro* evidence that PTEN dephosphorylates phosphatidylinositol 3,4,5-triphosphate [PtdIns(3,4,5)P_3] and inositol phosphate via a mechanism consistent with that described for other dual-specificity phosphatases (17). PtdIns(3,4,5)P_3 mediates growth factor-induced activation of intracellular signalling, in particular through the serine–threonine kinase Akt (also known as Akt1, PKB or RAC1), a known cell survival-promoting factor (18,19). Activation of Akt by growth factors is mediated by phosphatidylinositol (PI) 3-kinase, the enzyme that catalyses the phosphorylation of PtdIns(3,4,5)P_3 from phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] (20,21). Until now, there has been no human *in vivo* evidence for an association between PTEN and Akt pathways. A recent report, however, identified high levels of phosphorylated Akt in PTEN-deficient immortalized mouse embryonic fibroblasts (22).

While most studies have focused on *PTEN* mutational analysis, little is yet known about its transcription levels and protein expression, especially in haematological malignancies. In this study, we have investigated comprehensively whether *PTEN* abnormalities are involved in the development and/or progression of these disorders. We have performed mutation analysis and examined *PTEN* expression at the RNA level in a large series of acute leukaemias and non-Hodgkin's lymphomas (NHLs), both at diagnosis and on progression, as well as in related cell lines. We also generated a rabbit polyclonal PTEN antibody and assessed PTEN protein levels in the available samples. In addition, we have examined whether a correlation would be found between the levels of PTEN and Akt in these neoplasias.

RESULTS

Mutation analysis

cDNA or genomic DNA from all primary malignancies and cell lines was analysed successfully for the presence of mutations in the whole coding region of *PTEN*. The denaturing gradient gel electrophoresis (DGGE) analysis revealed abnormalities in 11 samples (Table 1), including one acute myeloid leukaemia (AML) secondary to a myelodysplastic syndrome, four NHLs and six cell lines. Sequence analysis of the samples confirmed mutations in all but one case. An intronic sequence variant, IVS4+42C→T, was found in the AML; a nonsense mutation in codon 17 (Gln17Stp) that would lead to a premature termination of the protein was found in a diffuse large B cell lymphoma (DLBCL) transformed from a low-grade NHL. A 20 bp insertion in exon 7, c.703insCTCCCTAAGGAGGAATTTAT was detected in a T cell lymphoma. This mutation is predicted to result in a truncated PTEN protein with 260 amino acids. A neutral polymorphism, Gly40Gly, was detected in one DLBCL. A DGGE variant was found in exon 1 in a low-grade B cell lymphoma. However, no sequence variations were detected when this sample was sequenced. Finally, amongst the cell lines, a total of eight sequence variants were found in six cell lines (Table 1).

Table 1. Results of *PTEN* structural analysis in primary leukaemias, lymphomas and cell lines

Diagnosis	*PTEN* sequence	*PTEN* deletion	Abnormal *PTEN* splicing
Primary samples			
Myeloid leukaemias	25L IVS4+42C→T	0/7[a]	ND
Lymphoid leukaemias		0/6[a]	ND
NHLs	Gln17Stp		ND
	Gly40Gly		
	c.703 ins 20 bp (stop at codon 260)		
	1 DGGE variant (exon 1)[b]		
Cell lines			
Myeloid leukaemias		KU812	
		U937	
		K562	
Lymphoid leukaemias	B15; REH: R173C; N334N	B15	B15
	REH	REH	
			207
NHL	HT:IVS3–52 delT		DHL8, RAJI
	DHL8: IVS3–52delT; Leu295Leu		DHL4, DHL10
	DHL4; DHL10: IVS4+5G→T		

[a]Chronic phase–blast crisis chronic myelocytic leukaemia paired samples.
[b]No abnormality found on sequencing analysis.
ND, not done.

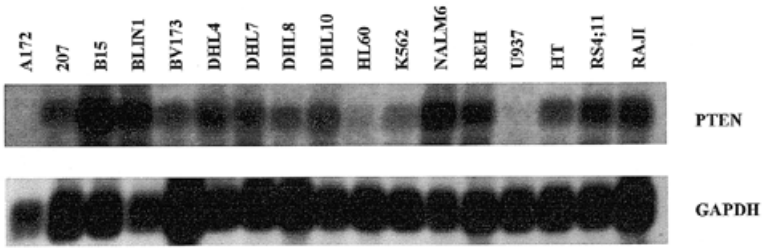

Figure 1. Northern blot analysis of *PTEN* on lymphoma and leukaemia cell lines. A *PTEN*-deleted glioblastoma cell line (A172) has been included as a negative control. A GAPDH probe was used to control for RNA loading.

Deletion analysis

PTEN dosage was assessed in paired samples by a combination of restriction analysis of a frequent single nucleotide polymorphism (SNP) present at intron 8 (IVS8+32G/T) and amplification of polymorphic markers located within or flanking the *PTEN* locus. In addition, the cell lines were also analysed for gene dosage by means of a semi-quantitative duplex PCR using *PTEN* primers alongside primers for a house-keeping gene, β-glucuronidase (*GUSB*). Fourteen paired primary samples were used for assessment of intragenic *PTEN* deletions: in 13 cases of chronic myelocytic leukaemia (CML), both chronic phase and blast crisis DNA were available for comparison; in one case of a *de novo* AML, DNA from the remission phase was also analysed (Table 1). Only one CML pair, derived from a patient with a myeloid transformation, showed a borderline loss ratio between chronic and acute phase of 1.48, with the *D10S2491* dinucleotide marker, while our operational limit was 1.5 (23,24). This might have been due to the presence of normal cells in the blast crisis sample. This sample was not informative for the SNP in intron 8.

None of the cell lines had homozygous deletion of *PTEN*. Five cell lines, including two acute lymphoid leukaemia (ALL) cell lines (B15 and REH) and three myeloid cell lines (KU812, K562 and U937), had evidence of *PTEN* hemizygous deletion based on the results of four polymorphic markers within (*D10S2491* and IVS8+32G/T) and flanking *PTEN* (*D10S1687* and *D10S1765*). Of these, only B15 and REH had mutations detected in the remaining allele (see above; Table 1).

PTEN transcription analysis

RT–PCR. All cell lines for which cDNA was available were tested for the presence of abnormally sized products indicative of alternative splicing (data not shown). In addition to a fragment of the expected size carrying a mutation in exon 6, B15 cDNA amplicon C2, spanning exons 5–7, yielded a fragment which was 141 bp shorter than the predicted size. Sequencing revealed it to lack exon 6. Skipping of exon 6 results in a truncated PTEN protein with 171 amino acids. Furthermore, two other lymphoma cell lines carrying an intronic sequence variant, DHL4 and DHL10, were found, in addition to the normal fragment, to have an alternatively spliced transcript form in which exon 4 was absent. The latter is predicted to result in a truncated 74 amino acid protein lacking the phosphatase core motif. Three other cell lines, 207, DHL8 and RAJI, were shown to carry a more complex splice variant. The first 44 bp within exon 8 were spliced out along with intron 7, and exon 7 was joined to the remaining downstream bases of exon 8, resulting in a frameshift and a truncated product of 275 amino acids. All other cell lines with sequence variation produced fragments of the expected size only.

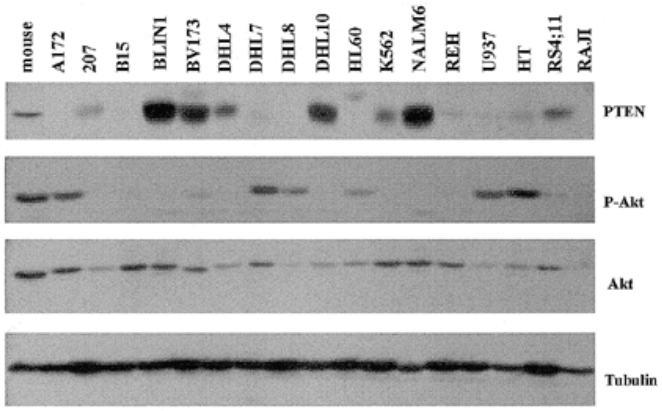

Figure 2. Western blot analysis of lymphoma and leukaemia cell lines with a rabbit polyclonal PTEN antibody recognizing the C-terminus of the protein. Analysis of the same samples with a phospho-specific Akt antibody (P-Akt) and a phosphorylation state-independent Akt antibody is shown. Sample loading was controlled by probing the membrane with a monoclonal α-tubulin antibody. A mouse fibroblast lysate was used as a positive control and the *PTEN*-deleted A172 glioblastoma cell line as a negative control.

All 23 primary lymphomas for which cDNA was available showed *PTEN* expression by RT–PCR, with no variation in the intensity of the *PTEN* band (data not shown). Because an unknown number of admixed normal cells in each sample would interfere with quantitative analysis, we did not attempt to quantify *PTEN* expression in these samples.

Northern blot. All samples analysed, except the A172 *PTEN* deleted cell line, showed expression of a major *PTEN* transcript of 5.5 kb, as well as two smaller mRNA species of ~4.5 and 2.5 kb. However, the level of *PTEN* expression varied widely amongst the samples: B15, BLIN1, DHL4, DHL10, NALM6, HT, RS4;11 and RAJI showed high transcript levels, whereas BV173, HL60 and U937 had very low transcript levels and the remaining samples (207, DHL7, DHL8 and K562) showed an intermediate level of *PTEN* transcription (Fig. 1). The GAPDH signal confirms that unequal loading of the samples did not account for the differences observed.

Table 2. Correlation between PTEN RNA and protein levels and between PTEN and Akt protein levels in the cell lines examined

Cell lines	PTEN RNA[a]	PTEN protein[a]	Concordance between PTEN RNA and protein	Inverse correspondence between PTEN and Akt protein levels
A172	–	–	Yes	Yes
BLIN1	+++	+++	Yes	Yes
DHL4	++	+	Yes	Yes
DHL10	+++	++	Yes	Yes
HL60	–/+	–/+	Yes	Yes
K562	–/+	+	Yes	Yes
NALM6	+++	+++	Yes	Yes
RS4;11	+++	+++	Yes	Yes
U937	–/+	–/+	Yes	Yes
207	+	+	Yes	No
B15	+++	–/+	No	No
BV173	–/+	++	No	No
DHL7	++	–/+	No	Yes
DHL8	++	–/+	No	Yes
RAJI	+++	+/++	No	No
REH	+++	–/+	No	No
HT	++	–/+	No	Yes

[a]Intensity of the signal.

Protein analysis. A rabbit polyclonal PTEN antibody directed against the final 15 amino acids of the PTEN protein sequence was generated and used for immunoblots. All cell lines analysed showed variable levels of PTEN protein expression: BLIN1, BV173, DHL4, DHL10, NALM6 and RS4;11 showed high or intermediate protein levels, while 207, B15, DHL7, DHL8, HL60, HT, K562, RAJI, REH and U937 had low or undetectable PTEN expression (Fig. 2). In most cases, RNA and protein levels were concordant. However, in one-third of the samples, there was some discrepancy between the RNA and protein expression: while B15, REH and RAJI had high and DHL7, DHL8, HT had moderate to high levels of *PTEN* transcript, virtually no protein was detected in these four samples on western analysis (Table 2). On the other hand, BV173 had detectable protein despite low *PTEN* transcript levels.

To determine whether PTEN levels correlated with an activated form of Akt, a phosphorylation-specific Akt antibody was used in immunoblots of the same samples described above. An antibody recognizing Ser473-phosphorylated Akt was found to correlate inversely with PTEN levels in 12 of 17 cell lines, including the PTEN-deleted cell line, A172 (Fig. 2). Analysis of the same samples with a phosphorylation state-independent Akt antibody did not reveal marked variations in Akt expression (Fig. 2). In five samples, 207, B15, BV173, REH and RAJI, PTEN and Akt did not show an inverted pattern of expression. In only two of these samples, 207 and RAJI, total Akt levels were reduced in comparison with the other cell lines.

α-Tubulin analysis revealed that the loading of the samples did not account for the differences observed in either PTEN or Akt expression.

Methylation analysis. To determine whether the low *PTEN* transcription level in some cell lines was due to methylation, we treated HL60 and U937 cells with the demethylator agent 5-aza 2′-deoxycytidine at four doses (1, 2, 5 and 10 μM) for different exposure times, from 24 to 144 h. Two cell lines with higher levels of *PTEN* transcription, 207 and DHL10, were used as controls. No increase in *PTEN* expression was noted in the cell lines examined at any of the time points analysed (24, 48, 96 and 144 h), regardless of the dose of 5-aza 2′-deoxycytidine used (data not shown). *CDKN2*/p16 mRNA and protein expression were determined in the treated samples as a control for the activity of 5-aza 2′-deoxycytidine. The increase in p16 expression was proportional to the dose and time of exposure to 5-aza 2′-deoxycytidine (data not shown).

DISCUSSION

We have analysed *PTEN* status in a panel of primary leukaemia and lymphoma samples and cell lines. We found that while only a minority of haematological malignancies had pathogenic mutations or deletions of *PTEN*, low mRNA levels were present in a subset of samples and virtually no protein was detected in 40% of the cell lines tested, most of which had high transcript levels of *PTEN*. This suggests that PTEN might be inactivated via several mechanisms, including not only the extensively reported mutations and deletions, but also transcription silencing and disruption at the protein level.

Overall, the majority of cell lines had *PTEN* abnormalities either at the structural, transcriptional or protein levels. The multiple mechanisms of *PTEN* inactivation were distributed

indistinctly amongst myeloid and lymphoid lineages. Moreover, within the same lineage, abnormalities were present regardless of the differentiation level, as seen by involvement in both multipotent cell lines (e.g. K562) and cells committed to myeloid (HL60) or monocytic differentiation (U937), as well as in pro-pre-B ALLs (REH) and B cell neoplasias (RAJI and DHLs). In addition, the majority of the samples with *PTEN* disruption previously have been shown to carry a molecular abnormality thought to be causative of the phenotype, such as *TEL–AML1* rearrangement in REH, *MLL–AF4* in RS4;11, c-*myc* fusion in RAJI and *BCR–ABL* fusion in all CML-BC cell lines (25–28). Furthermore, in several of these cell lines, disruptions of other tumour suppressor genes are present and thought to be major secondary events responsible for progression (3). This suggests that *PTEN* might be involved in late stages of neoplastic development, which is in agreement with what has been reported in glioblastomas (10), and possibly might represent an additional 'second event' in certain tumours.

CML is thought to represent an ideal model to study progression in cancer. This myeloproliferative disorder is usually identified in a 'pre-malignant' stage, where some degree of maturation, albeit discordant, is maintained (chronic phase) (2). Eventually, an overt block in differentiation arrest occurs, resembling an acute leukaemia (blast crisis). We have therefore examined CML samples in chronic phase and blast crisis for *PTEN* abnormalities. While other tumour suppressor genes, such as *p53* and *CDKN2A/p16*, have been implicated in the progression of a subset of CML in myeloid and lymphoid transformation (3,29), respectively, it is unlikely, from our findings, that *PTEN* plays a role in CML progression, as there was no clear evidence for deletion or mutation in the paired cases studied. However, due to our small sample size, a larger number of cases would need to be analysed to confirm these findings. Furthermore, *PTEN* RNA or protein levels were not analysed in these samples and might be the actual targets of *PTEN* disruption in such cases. In fact, this finding of PTEN abnormality in two CML-BC cell lines supports this notion.

Amongst the remaining primary samples, it appears from our analysis that a predominance of mutations was seen in the NHLs (10% of the cases versus 1.35% in leukaemias, $P = 0.05$ by Fisher's exact test). RNA and protein analysis of primary samples will be relevant to determine whether PTEN involvement is more extensive than a structural analysis might have suggested. A recent report on mutation analysis of *PTEN* in haematological malignancies has revealed only a small incidence of abnormalities in lymphoid samples (30). However, no RNA or protein analyses were performed in this study.

The mechanisms involved in the low *PTEN* transcription levels shown by some samples are not known. In two cell lines, U937 and K562, hemizygous deletion of *PTEN* has been detected, which might account for low RNA levels seen in this sample. In fact, a recent report on a *PTEN* knockout mouse model suggests that *PTEN* haploinsufficiency may be pathologically relevant, as malignant tumours spontaneously developed in $PTEN^{+/-}$ mice (31). However, it is unlikely that monoallelic expression of the gene accounts for the low expression levels of *PTEN* in all cases, as other hemizygously deleted cell lines (B15 and REH) had higher levels of PTEN mRNA and/or protein. In addition, a non-deleted cell line, HL60, also expressed very low levels of both mRNA and protein. This suggests that other mechanisms of transcription down-regulation might occur, such as a mutation in the promoter region of the gene, giving rise to an unstable, or even absent, transcript. Alternatively, low transcription rates could be due to methylation of *PTEN*. To investigate further the mechanisms involved in the variable PTEN mRNA and protein levels, we analysed the effect of a demethylator agent, 5-aza 2′-deoxycytidine, in cell lines with low *PTEN* transcription levels. It would be expected that mRNA and, consequently, protein levels would increase if *PTEN* were inactivated by methylation. Neither sample with low nor high baseline *PTEN* showed changes in the protein levels after exposure to the demethylator agent, whereas p16 expression, used as a control, was induced in at least one of the 5-aza 2′-deoxycytidine-treated cell lines. There have been controversial results regarding the occurrence of *PTEN* methylation in epithelial tumours. While it recently has been suggested that *PTEN* might be methylated in prostate cancer (32), an earlier study failed to find *PTEN* hypermethylation in the same type of tumour (33). It is debatable whether the low *PTEN* expression observed in the former study may in fact reflect methylation of a putative *PTEN* up-regulator rather than methylation of the *PTEN* gene itself. It is possible, however, that methylation of *PTEN*, if present, may be restricted to certain tissues only, and that factors other than methylation may account for the low mRNA levels seen in certain samples.

While, in some cases, low protein levels most likely reflect the reduced transcription levels of *PTEN*, in six samples, comprising four lymphoma cell lines (DHL7, DHL8, RAJI and HT) and two ALL cell lines (B15 and REH), the low protein levels were associated with high *PTEN* mRNA levels. This suggests that a distinct mechanism of *PTEN* inactivation might occur in these cases. Interestingly, in the two ALL cell lines, hemizygous deletion of *PTEN* with a missense mutation of the remaining allele was detected. This could indicate that an abnormally unstable protein resulted which could account for the low detection of PTEN protein in these cases. In two other samples, DHL8 and RAJI, a splicing variant resulting in a truncated *PTEN* product was identified in addition to the wild-type fragment on RT–PCR. It is possible that the low protein levels in these samples might be related to the lower levels of wild-type *PTEN*. In the remaining samples, DHL7 and HT, derived from a DLBCL and a T cell lymphoblastic lymphoma, respectively, no pathogenic mutations, deletions or aberrant splices were identified. The mechanism responsible for low PTEN levels in this case remains to be characterized.

The identification of endogenous PTEN substrates is essential for a better understanding of its intracellular role. It has been suggested recently that PtdIns(3,4,5)P_3, an important second messenger involved in cell growth signalling, is a substrate for PTEN (34). PtdIns(3,4,5)P_3 is produced from PtdIns(4,5)P_2 by PI-3 kinase on stimulation by various ligands such as insulin, platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF). One target of PtdIns(3,4,5)P_3 is the proto-oncogene *Akt*, which is the cellular homologue of the viral oncogene v-*akt* (18,35). *Akt* codes for a serine–threonine kinase with homology to protein kinase C (PKC) (36). In addition, it contains a unique N-terminal pleckstrin homology (PH) domain, which interacts with both PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 *in vitro* (35,37). Phosphorylation of Akt, in particular at Thr308 and Ser473 residues, has been proposed to be essential for its activation (20,38). Akt has been shown to promote cell survival (19,39), and its anti-apoptotic role has been characterized in differentiating neuronal cells (40). The anti-apoptotic role of Akt is mediated

through the phosphorylation of BAD, which inhibits BAD binding to Bcl-x_l, resulting in suppression of apoptosis (41). We hypothesized that if PTEN was indeed involved in the PtdIns(3,4,5)P_3 regulatory pathway, we would detect an inverse correlation between PTEN and phosphorylated Akt levels. In fact, we did note an inverse correlation between these two proteins in all but five samples. It is possible that in this minority of samples, Akt is activated through a pathway independent of PI-3 kinase, such as that initiated by heat shock or hyperosmolarity stress (42) or an as yet unidentified mechanism. In two samples, reduced PTEN levels were accompanied by low levels of both phosphorylated Akt and total Akt. The reasons for the discrepancy seen in these samples are not clear and require further investigation. On the other hand, PTEN activity may be regulated by other mechanisms, such as phosphorylation, subcellular localization or interactions with other proteins, and Akt levels might be associated with only certain specific conditions of PTEN activation. Unlike another member of the PKB subfamily, AKT2, which has been found to be overexpressed in ovarian and pancreatic cancer (43,44), abnormalities of Akt have not been observed consistently in human malignancies. This is the first human *in vivo* indication that PTEN levels are related to activated Akt levels, which reinforces the *in vitro* data by Maehama and Dixon (34) suggesting that PtdIns(3,4,5)P_3 is a substrate for PTEN. The possibility that PTEN might counteract the activity of a survival factor suggests an indirect role for PTEN in apoptosis. This has been confirmed recently by a report describing increased cell survival accompanied by high levels of phosphorylated Akt in PTEN-deficient immortalized mouse embryo fibroblasts (22). These cells were shown to resume sensitivity to apoptosis and reduce Akt levels on expression of exogenous PTEN.

The potential involvement of *PTEN* in the pathogenesis of haematological malignancies is not unexpected. Phosphatases have been implicated in several critical pathways responsible for cell growth, differentiation, cytoskeletal organization and B cell activation following antigen stimulation (45). Moreover, partial or complete absence of SHP1, a tyrosine phosphatase, is known to cause a lymphoproliferative disorder in motheaten mice (46). In addition to phosphatases, it has been suggested recently that another class of proteins, the cell adhesion molecules, might also play a role in leukaemia and lymphoma pathogenesis. There is recent evidence indicating that *PTEN* tumour suppressor function is mediated by its ability to down-regulate cell interactions with the extracellular matrix (47), which is in keeping with its structural homology to two cell adhesion molecules, tensin and auxillin (6,7). In this study, PTEN has been shown to inhibit cell migration and integrin-mediated cell spreading and formation of focal adhesions. Furthermore, two recent reports on a *PTEN* knockout mouse has revealed that chimaeric mice derived from *PTEN*$^{+/-}$ cells may develop myeloproliferative features (22,31), supporting a role for *PTEN* in haematological malignancies.

In conclusion, we have found that the majority of human haematological malignancy cell lines studied have *PTEN* abnormalities either at the structural, transcriptional or protein levels, while a smaller number of primary haematological malignancies carry *PTEN* mutations. These findings suggest that *PTEN* might be inactivated through a wider range of mechanisms than initially considered and also that *PTEN* plays a role in the pathogenesis of haematological malignancies. Further studies with primary samples will help to delineate the extent and precise subsets of tumours which have *PTEN* inactivation. Finally, the finding that PTEN levels inversely correlate with phosphorylated Akt supports the hypothesis that PTEN regulates PtdIns(3,4,5)P_3 levels *in vivo*.

MATERIALS AND METHODS

Samples

We studied a total of 136 leukaemias or lymphomas comprising 114 primary samples and 22 cell lines (48). Amongst the primary malignancies, 74 were *de novo* or secondary acute leukaemias and 40 were NHLs, derived almost exclusively from an adult population. In 14 cases, paired samples were available from the same patient, including 13 cases of CML both in chronic phase and blast crisis, and one acute myeloid leukaemia AML at diagnosis and upon remission. Table 3 describes in detail the leukaemias and lymphomas examined.

Table 3. Leukaemias, lymphomas and tumour cell lines studied

	Primary samples		Cell lines	Total
	De novo	Secondary[a]		
AML	22	21	6[b]	49
ALL	21	10	9	40
NHL	B cell	T cell		
	30	10	7	47

[a]Post-myelodysplastic syndrome or CML-blast crisis.
[b]Includes the multipotent cell K562.

DNA and RNA extraction

DNA was obtained using standard methods (2). RNA was available from 32 primary tumours and most of the cell lines and was extracted with Trizol (Gibco BRL, Gaithersburg, MD) according to the manufacturer's guidelines. cDNA was generated using random hexamers (Promega, Madison, WI) and Superscript II RNase H$^-$ reverse transcriptase (Gibco BRL) following the manufacturer's instructions.

DGGE analysis of *PTEN*

Mutation analysis was performed by DGGE. Individual *PTEN* exons were amplified and run on denaturing gels according to the conditions described previously (49).

Sequencing analysis

Abnormal samples on DGGE were reamplified with another set of primers, purified and sequenced as previously published (23). Three pairs of overlapping primers (cPTEN1F, CATCTCTCTC-CTCCTTTTTCTTCA; cPTEN1R, ATATCATTACACCAGTT-CGTCCCT; cPTEN2F, ATGGCTAAGTGAAGATGACAATCA; cPTEN2R, ACTTTGATATCACCACACACAGGT; cPTEN3F, ACGACGGGAAGACAAGTTCAT; cPTEN3R, TTTTCATGT-GTTTTATCCCTCTT) spanning the entire coding region of *PTEN* were used to analyse the integrity of the *PTEN* sequence in nine DLBCLs, for which only RNA was available.

PTEN dosage analysis

PTEN dosage was assessed in paired samples by restriction analysis of a frequent SNP present at intron 8 (IVS8+32G/T) with *Hinc*II, as previously described (23). The uninformative samples were reassessed by using a dinucleotide marker located within

PTEN, *D10S2491* (33). Cell lines were also analysed for gene dosage by means of a semi-quantitative duplex PCR. *PTEN* exon 8 was co-amplified with a non-related gene, *GUSB*. PCR was performed as described before (50) with minor modifications: 0.8 µM of *PTEN* primers and 0.1 µM of *GUSB* primers were used in a 25 cycle PCR. A standard curve of fixed amounts of a *PTEN*-deleted cell line, A172, and a *PTEN* wild-type sample was prepared to simulate *PTEN* dosage of 100, 75, 50, 25 and 0%, as described (51), and included on every PCR. A ratio of densitometric values of each band was obtained for the standard curve and every individual sample using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA). A *PTEN*:*GUSB* ratio of <1:2, corresponding to a loss >50%, was assigned hemizygous deletion status. Due to variable levels of admixed normal cells in the DNA samples, assignment of the hemizygous deletion status in the primary tumours would be less precise; therefore, primary samples were not included in this assay.

Further, to confirm the validity of the data obtained with the semi-quantitative assay, the samples were amplified with primers recognizing three microsatellite markers within and centromeric to *PTEN* *D10S2491* (see above), *D10S1687* and *D10S1765* (http://www.gdb.org), and haplotypes were determined using the GeneScan software (Applied Biosystems) as previously described (23). Due to the high heterozygosity rate of such markers, the likelihood of a given sample being homozygous for all three markers and also for the IVS8+32G/T SNP mentioned above would be <0.08. Therefore, the finding of homozygosity of all four markers and a *PTEN* dosage of <50% on the semi-quantitative assay were considered to represent hemizygous deletion of *PTEN*.

Expression analysis

RT–PCR. PCR using the overlapping cDNA primers described above was performed to determine whether splicing abnormalities would result from any intronic or splice site mutation detected in the sequencing analysis. In addition, a duplex RT–PCR was performed in 23 lymphomas and 16 cell lines, in which the *PTEN* transcript was co-amplified with the house-keeping gene *GUSB*. PCR conditions were as previously described (50).

Northern blot. Seventeen leukaemia and lymphoma cell lines, 207, B15, BLIN1, BV173, DHL4, DHL7, DHL8, DHL10, HL60, K562, NALM6, RAJI, REH, U937, HT and RS4;11, in addition to a glioblastoma multiforme cell line, A172, which has a deletion of *PTEN* (6,7), were used in this assay. Twelve micrograms of total RNA were run on an 18% formaldehyde, 1× MOPS agarose gel, transferred to a nylon membrane (Hybond; Amersham, Buckinghamshire, UK) and probed with a ^{32}P-labelled 809 bp cDNA fragment spanning *PTEN* exons 5–9. The membrane was then stripped and reprobed with a glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA probe to control for RNA loading.

Protein analysis. A peptide corresponding to the final 15 amino acids of the human PTEN sequence was synthesized at the Physiology Department at Tufts Medical School and was coupled to maleimide-activated keyhole limpet haemocyanin, using the IMJECT system (Pierce, Rockford, IL), following the manufacturer's instructions. Immune sera were generated in a New Zealand white rabbit at HRP (Denver, PA) by injecting 0.5 mg of coupled peptide in Freund's complete adjuvant (primary injection) and 0.25 mg/injection in Freund's incomplete adjuvant (subsequent injections). Immunoglobulin was isolated using protein A–Sepharose column chromatography, with elution by 100 mM glycine, pH 3.0, and used for western blots.

Immunoblot analysis was performed as follows: cell lysates from 16 cell lines, 207, B15, BLIN1, BV173, DHL4, DHL7, DHL8, DHL10, HL60, HT, K562, NALM6, RAJI, REH, RS4;11 and U937, and a *PTEN*-deleted cell line, A172, were prepared by harvesting 1×10^7 cells. After rinsing the cell pellet with phosphate-buffered saline (PBS), it was resuspended in 400 µl of extract buffer (20 mM HEPES pH 8.0, 1% NP-40, 10% glycerol) supplemented with 2.5 mM EGTA, 0.7 µg/ml pepstatin and 1× Complete Protease Inhibitor Cocktail (Boehringer Mannheim, Indianapolis, IN). Cell lysates were incubated on ice for 10 min. Samples were centrifuged at 13 000 *g* for 10 min, and supernatants were quantified for protein content by the Bradford assay (Sigma, St Louis, MO). Fifty micrograms of whole cell lysates were mixed with sample buffer (0.125 M Tris pH 6.8, 20% glycerol, 10% β-mercaptoethanol, 4% SDS), boiled for 10 min and resolved by 10% Tris–glycine SDS–PAGE. The gel was transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) with Tris–glycine transfer buffer and the membrane was blocked with Tris-buffered saline containing 0.05% Tween and 5% skim milk for 1 h. The membrane was incubated with the PTEN antibody diluted 1:1000, followed by a goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad). Detection was performed by enhanced chemiluminescence (ECL; Amersham).

To examine the levels of Akt in each sample, the membranes were also probed separately with two Akt antibodies according to the manufacturer's guidelines (New England Biolabs, Beverly, MA). One of these antibodies recognizes an activated form of Akt, phosphorylated at Ser473, while the other is a phosphorylation state-independent Akt antibody. The blots were stripped further with 2% SDS, 0.064 M Tris pH 6.8 and β-mercaptoethanol, and reprobed with a commercially available mouse monoclonal α-tubulin antibody (T-9026; Sigma) to confirm equal protein loading.

Methylation analysis. Four cell lines, HL60, U937, 207 and DHL10, were treated with the demethylator agent 5-aza 2′-deoxycytidine at 1, 2, 5 and 10 µM, for 24–144 h. After splitting, cells were harvested at 24, 48, 96 and 144 h or allowed to remain an additional 24 h in media without the drug, and harvested at the end of this period, as previously described (52). RNA and whole cell lysates were obtained as described above. RT–PCR using p16 primers was performed as previously published (53). Western blot with PTEN antibody and a commercially available p16 antibody (Pharmingen, San Diego, CA) was performed as described above.

ACKNOWLEDGEMENTS

The authors thank Margaret Shipp and Nick Cross for providing some of the cell lines or cell line DNAs used in this study; Sig Verselis for assistance with cell culture; and the Molecular Biology Core Facility at the Dana-Farber Cancer Institute for running the sequencing gels. Donna Neuberg is acknowledged for statistical advice, and Oliver Gimm for his critical review of the manuscript. This study was supported by the Susan G. Komen Breast Cancer Foundation (to P.L.M.D. and C.E.), the American Cancer Society (RPG 97-064-02VM and RPG-98-211-01-CCE),

the Concert for the Cure and a Barr Investigatorship (to C.E.). C.E. is the Lawrence and Susan Marx Investigator in Human Cancer Genetics.

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