

# Transient ectopic expression of *PTEN* in thyroid cancer cell lines induces cell cycle arrest and cell type-dependent cell death

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The tumour suppressor gene *PTEN/MMAC1/TEP1* has been implicated in a variety of human cancers and several inherited hamartoma tumour syndromes, including Cowden syndrome, which has a high risk of breast and thyroid cancer. We have previously reported that overexpression of *PTEN* in MCF-7 breast cancer cells induces cell cycle arrest and apoptosis. In this study, we analysed *PTEN* status at both the structural and expression levels and explored *PTEN*'s growth-suppressive effects on thyroid. We found that 1 of 10 thyroid cancer lines [follicular thyroid carcinoma FTC-133] had hemizygous deletion and a splice variant IVS4–19G→A in the remaining allele. Four lines, including FTC-133, express *PTEN* mRNA at low levels. In general, *PTEN* protein levels correlated with mRNA levels, except for NPA87, which has low levels of transcript and relatively high levels of *PTEN* protein. Transient expression of *PTEN* in seven thyroid cancer cell lines resulted in G<sub>1</sub> arrest in two well differentiated papillary thyroid cancer lines (PTCs) and both G<sub>1</sub> arrest and cell death in the remaining five lines, including three FTCs, one poorly differentiated PTC and one undifferentiated thyroid cancer. The level of phosphorylated Akt was inversely correlated with the endogenous level of *PTEN* protein and overexpression of *PTEN*-blocked Akt phosphorylation in all cells analysed. Our results suggest that downregulation of *PTEN* expression at the mRNA level plays a role in *PTEN* inactivation in thyroid cancer and *PTEN* exerts its tumour-suppressive effect on thyroid cancer through the inhibition of cell cycle progression alone or both cell cycle progression and cell death.

## INTRODUCTION

*PTEN*, a tumour suppressor gene also known as *MMAC1* and *TEP1*, was identified based on homozygous deletions in several cancers (1,2) and through its homology to tyrosine phosphatases (3). Germline mutations in *PTEN* are found in patients with Cowden syndrome (CS; MIM 158350), characterized by multiple hamartomas and a high risk of breast, thyroid and endometrial cancers, those with Bannayan–Riley–Ruvalcaba syndrome (BRR; MIM 153480), a congenital hamartoma syndrome characterized by macrocephaly, lipomatosis, haemangiomas and pigmented macules on the glans penis, and possibly those with Proteus-like syndromes as well (4–7). Mutation analysis in sporadic tumours revealed that *PTEN* might play a role in the pathogenesis of various tumours (8–12). Interestingly, some sporadic tumours, which are currently not believed to be part of CS, have some frequency of somatic mutations: 17–44% in glioblastoma multiforme (11,13), 10–25% in lung carcinoma (14,15), 20% in Burkitt lymphoma (16) and 6–13% in kidney cancer (10,17). In contrast, somatic intragenic mutations have rarely been found in primary non-cultured breast cancer (1–10%) (10,18–20) and thyroid cancer (2–8%) (21,22), which are the hallmark component cancers of CS.

Thyroid cancer is a rare human malignancy, accounting for ~1% of all malignant neoplasias. Generally, three different types deriving from the follicular thyroid cell are distinguished: papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC) and undifferentiated thyroid carcinoma (UTC). PTC has the best prognosis whereas patients with UTC usually die within 1 year after diagnosis (23,24). Somatic mutations in *PTEN* are found only in a minority of thyroid carcinomas (21,22), whereas loss of heterozygosity (LOH) at 10q23 has been reported in 20–60%, depending on the histological type analysed, with the more aggressive tumours harbouring a higher percentage of LOH (21,25,26). Immunohistochemistry revealed strong nuclear and weaker cytoplasmic *PTEN* expression in normal thyroid tissue (25). In

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general, the more aggressive the tumour, the less intense was *PTEN* expression, particularly in the nucleus. However, the functional role of *PTEN* in thyroid carcinoma remains unclear.

Although the complete functional role of *PTEN* is as yet unknown, studies have revealed that *PTEN* is a dual-specificity phosphatase and a major lipid phosphatase, which acts on the PI3-kinase and Akt apoptotic pathways (27–30). *PTEN* has subsequently been shown to participate in G<sub>1</sub> arrest and/or apoptosis. Transient ectopic expression in glioma lines resulted in G<sub>1</sub> cell cycle arrest (31), whereas transient expression in breast cancer lines resulted in apoptosis (32). Stable transfection of *PTEN* into the MCF-7 breast cancer line was shown to result in early G<sub>1</sub> arrest followed by both cell cycle arrest and apoptosis (30). In the present study, we examined *PTEN* status at both the structural and expression levels in a panel of 10 non-medullary thyroid cancer cell lines originating from PTC, FTC and UTC and investigated the effect of ectopic *PTEN* expression on Akt phosphorylation, cell growth, cell cycle and cell death in seven selected thyroid cancer cell lines.

## RESULTS

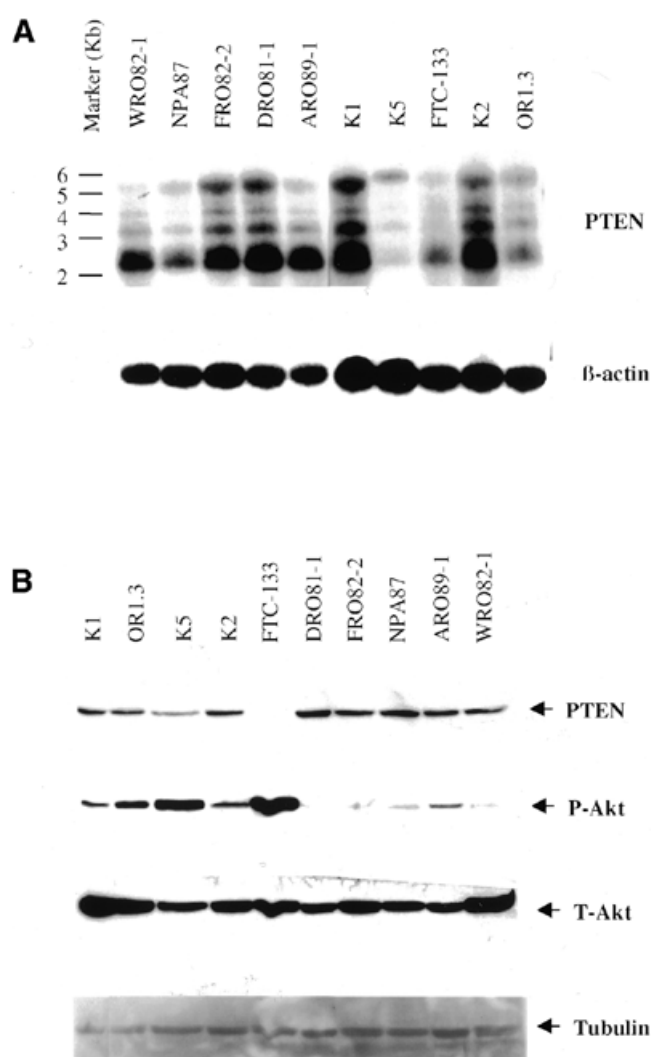
### Mutation analysis

At the gene level, all but one cell line were wild-type. FTC-133 had one *PTEN* allele deleted and the remaining allele harboured a splice variant IVS4–19G→A.

### *PTEN* expression

To analyse *PTEN* expression in thyroid cancer, we examined the expression of *PTEN* mRNA by northern blot and *PTEN* protein by western blot in a panel of 10 thyroid cancer cell lines. As Figure 1A shows, *PTEN* transcripts comprised multiple isoforms, with the smallest transcript of 2.5 kb being the major one. The longest isoform, represented by the 5.5 kb transcript, is the second most abundant and, in between, there appeared another two transcripts of low abundance. In general, the relative intensity of the four transcripts was consistent among all cell lines except K5, in which the largest transcript (5.5 kb) was dominant. The abundance of *PTEN* mRNA varied among cell lines, with FTC-133 and K5 having the lowest levels, NPA87 and OR1.3 having relatively low levels and the others showing relatively high levels of *PTEN* transcripts. In these cell lines, it is likely that none of the northern blot-evident messages represents the highly conserved *PTEN* pseudogene. Although it is believed that the pseudogene can be transcribed (but not translated) in certain cell types (33) but not in others (34), the pseudogene transcript is not thought to undergo post-transcriptional modification [alternative splicing, different poly(A) tails etc.] and should therefore be ~1.2–1.3 kb in size. There are no distinct northern signals below the 2.5 kb dominant band and hence it is believed that the pseudogene is not transcribed at any significant levels in these 10 thyroid cancer lines.

Western blot analysis using *PTEN*-specific 6H2.1 monoclonal antibody revealed that FTC-133 had no detectable *PTEN* protein, OR1.3 and K5 had relatively low levels and the remaining cell lines had relatively high levels (Fig. 1B, top). In general, the protein levels of *PTEN* correlated with the relative abundance of mRNA in most of the cell lines analysed except

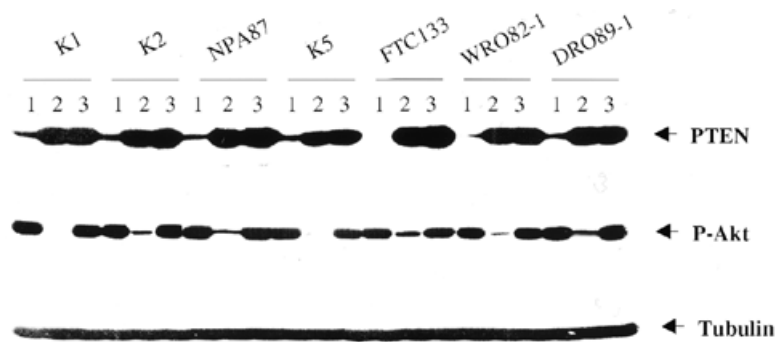


**Figure 1.** Expression of *PTEN* in 10 thyroid cancer cell lines. A panel of 10 thyroid cancer cell lines were grown to subconfluency, replated on p 100 mm plates and grown for 48 h in DMEM containing 10% FBS. Cells were then harvested for cell lysates and for isolation of RNA. (A) Expression of *PTEN* mRNA in thyroid cancer cell lines. A total of 10 µg of RNA was electrophoresed on a 1.5% agarose gel, blotted and hybridized with *PTEN* probe (top) and the same blot was reprobed with β-actin (bottom). (B) Endogenous *PTEN* protein, phosphorylated Akt and total *Akt* expression levels in thyroid cancer lines. A total of 25 µg of protein was separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with an anti-*PTEN* antibody (top), anti-phospho-Akt antibody (upper middle), anti-Akt antibody (lower middle) and anti-tubulin antibody (bottom), sequentially. Note that levels of *PTEN* are inversely correlated with phosphorylated Akt (P-Akt) levels in the context of similar levels of total Akt and tubulin across all lines.

NPA87, which expressed low levels of mRNA but high levels of protein.

### Correlation of *PTEN* and *Akt* expression

We have previously shown that Akt phosphorylation levels are inversely correlated with the endogenous levels of *PTEN* in breast cancer (30) and haematological malignancies (35). To see whether this is also the case in thyroid cancer, we analysed *PTEN* protein levels against the level of phosphorylated Akt in



**Figure 2.** The effect of wild-type (wt) and phosphatase-dead mutant (cs) *PTEN* on Akt phosphorylation. Cells were infected with Ad/GFP (lanes 1), Ad/*PTEN*.wt (lanes 2) and Ad/*PTEN*.cs (lanes 3) at a concentration of  $1 \times 10^5$  m.o.i. After transfection (24 h), cells were lysed for analysis of *PTEN* (top), phosphorylated Akt (middle) and tubulin (bottom) expression by western blot. In the endogenous state (empty shuttle, lanes 1) as well as after infection with wild-type *PTEN* (lanes 2), *PTEN* levels are inversely correlated with phosphorylated Akt levels. However, overexpression of phosphatase-dead mutant *PTEN* (lanes 3) has no effect on Akt phosphorylation (compare lanes 3 with lanes 1).

a panel of thyroid cancer cell lines by western blot. In general, the levels of phosphorylated Akt detected by an anti-phospho-Akt specific antibody were inversely correlated with those of *PTEN*. OR1.3 and K5 with low levels of *PTEN* showed high levels of phosphorylated Akt (Fig. 1B). FTC-133 had the highest levels of phosphorylated Akt in accordance with its *PTEN*-null status. The remaining cell lines with high levels of *PTEN* showed low levels of phosphorylated Akt (Fig. 1B). The difference in the levels of phosphorylated Akt was not secondary to the absolute abundance of Akt protein, as all cell lines examined expressed relatively equal amounts of Akt protein as revealed by western blot using anti-Akt antibody that recognizes both phosphorylated and non-phosphorylated forms (Fig. 1B, lower middle).

To further investigate whether *PTEN* inhibits Akt phosphorylation in thyroid cancer, we overexpressed both wild-type and phosphatase-dead mutant *PTEN* in a panel of thyroid cancer cell lines and examined the status of Akt phosphorylation. Overexpression of *PTEN* resulted in dramatic decreases in Akt phosphorylation in a phosphatase-dependent manner in all cell lines analysed (Fig. 2).

### ***PTEN* inhibits cell growth in thyroid cancer**

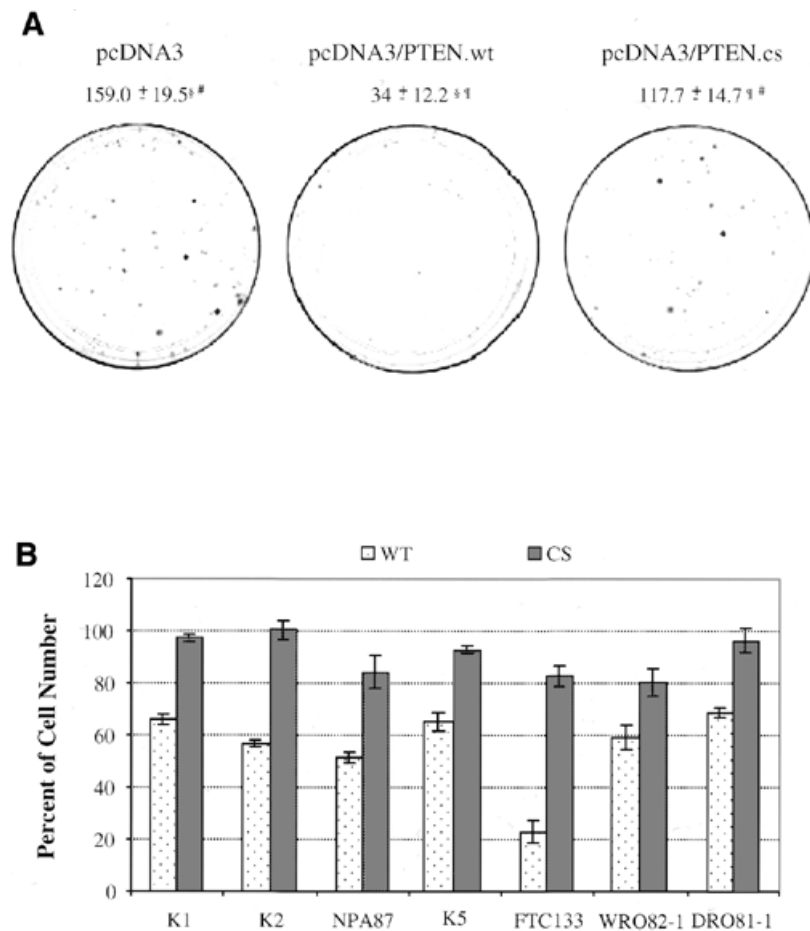
We and others have shown that ectopic expression of *PTEN* inhibits cell growth in a variety of cancer cell lines. To investigate whether the loss of *PTEN* has any growth advantage in thyroid cancer, we re-introduced *PTEN* into *PTEN* protein-null FTC-133 cells and examined its effect on cell growth. The wild-type and the C124S mutant *PTEN* were cloned into a mammalian expression vector, pcDNA3, which carries a neomycin selection marker. The FTC-133 cells were transfected with the wild-type and the mutant *PTEN* constructs as well as the empty vectors and selected in neomycin-containing media for 2 weeks. As shown in Figure 3A, FTC-133 cells with empty vectors produced many large colonies after 2 weeks of selection. However, wild-type transfected cells resulted in a few small colonies. The number of colonies from mutant *PTEN*-transfected cells was four times greater than that from wild-type *PTEN*-transfected cells but less than that from empty vector-transfected cells, although the difference was small but significant. These results suggested that the inhibition of cell growth by *PTEN* in

FTC-133 cells was both phosphatase activity dependent and independent.

To investigate whether *PTEN* could have growth-suppressive effects on thyroid cancer in general, we introduced both wild-type and phosphatase-dead mutant *PTEN* into seven selected thyroid cancer cell lines by an adenoviral gene delivery system and examined the effect of *PTEN* on cell growth. Adenovirus carrying wild-type and C124S mutant *PTEN* as well as empty virus were used to infect seven thyroid cancer cell lines, which were derived from different origins, namely FTC-133, K5 and WRO82-1 from FTC, and NPA87, K1 and K2 from PTC and DRO81-1 from UTC. Except for endogenous *PTEN*-null FTC-133, the ratio of ectopically generated *PTEN* protein levels to endogenous levels in the remaining six lines was 10–25:1 (Fig. 2, top, lanes 2 versus lanes 1). As shown in Figure 3B, infection with wild-type *PTEN* virus for 48 h resulted in ~30–50% growth suppression in most cells and ~80% in FTC-133 cells. The variation of *PTEN*-mediated growth inhibition among thyroid cell lines was not due to the expression levels, as *PTEN* virus produced similar amounts of *PTEN* in all cell lines tested (Fig. 2). Interestingly, a phosphatase activity-independent growth suppression was also observed in NPA87, K5, FTC-133 and WRO82-1, as infection with C124S mutant *PTEN* virus in these cells still resulted in significant decreases in cell numbers.

### ***PTEN* induces G<sub>1</sub> arrest and cell type-dependent apoptosis**

Having shown that the ectopic expression of *PTEN* suppresses cell growth in thyroid cancer, we next investigated whether inhibition of cell growth was due to either cell death or cell cycle arrest. Infection of cells with wild-type *PTEN* virus resulted in an increase in the G<sub>1</sub> cell population in most of the cell lines except FTC-133 (Fig. 4), although there were increases in the G<sub>1</sub> population when FTC-133 was infected with low concentrations of virus or for a brief time (data not shown). The effect of *PTEN* on the cell cycle was phosphatase activity dependent in all lines, since the cell cycle phase distribution in cells infected with the C124S mutant virus revealed no difference from that infected with empty virus. However, the effect of *PTEN* on cell death varied among cell lines (Fig. 5). Infection with wild-type *PTEN* virus resulted in



**Figure 3.** PTEN inhibits cell growth in FTC-133. **(A)** The effect of PTEN on cell growth was analysed by colony formation assay. FTC-133 cells were transfected with pcDNA3, pcDNA3/PTEN.wt and pcDNA3/PTEN.cs and selected by neomycin for 2 weeks. The formed colonies were photographed. The numbers represent averaged colony numbers ( $\pm$  95% confidence interval) from triplicate plates. Average colony numbers were compared between plates and a  $P$  value statistic was assigned ( $^{\S}P = 0.00102$ ;  $^{\P}P = 0.00131$ ,  $^{\#}P = 0.180447$ ). The number of colonies formed after infection with wild-type PTEN was significantly more than after infection with empty vector ( $P = 0.00102$ ) or infection with phosphatase-dead mutant PTEN ( $P = 0.00131$ ). The number of colonies formed after infection with mutant PTEN were statistically similar to that after infection with empty vector ( $P = 0.181$ ) although inspection of colonies clearly showed fewer colonies in the former compared with the latter. **(B)** The effect of overexpression of wild-type PTEN versus phosphatase-dead mutant C124S in seven thyroid cancer lines. Growth suppression is expressed as percentage of cell number after infection with either wild-type PTEN or C124S-PTEN to cell number at baseline. Note that wild-type PTEN overexpression results in growth suppression but to a variable extent among cell lines. Interestingly, overexpression of phosphatase-dead mutant is without effect, as expected, in K1, K2 and DRO81-1, but results in mild growth suppression in the remaining four.

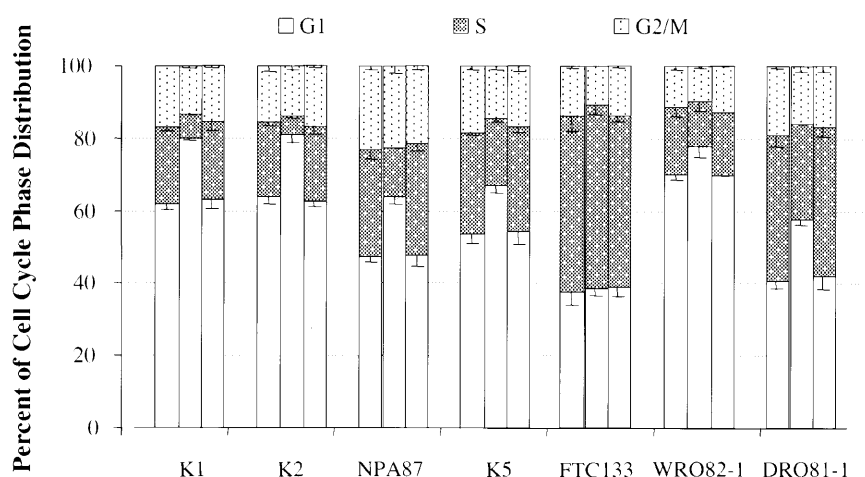
>50% and >30% of dead cells, respectively, in FTC-133 and NPA87; ~10–15% dead cells in WRO82-1, DRO81-1 and K5 and no detectable cell death in K1 and K2 cells (Fig. 5). This variation was not due to exogenous PTEN protein levels, as western blot demonstrated relatively equal amounts of PTEN in all cell lines infected with wild-type and mutant PTEN virus (Fig. 2, top). Infection of cells with C124S mutant virus also caused cell death to a much reduced extent compared with that of wild-type PTEN infection, but significant compared with empty virus. These results suggest that PTEN plays a general role in the suppression of cell growth in thyroid cancer; the inhibition of cell cycle progression by PTEN is universal but induction of cell death depends on cell type.

## DISCUSSION

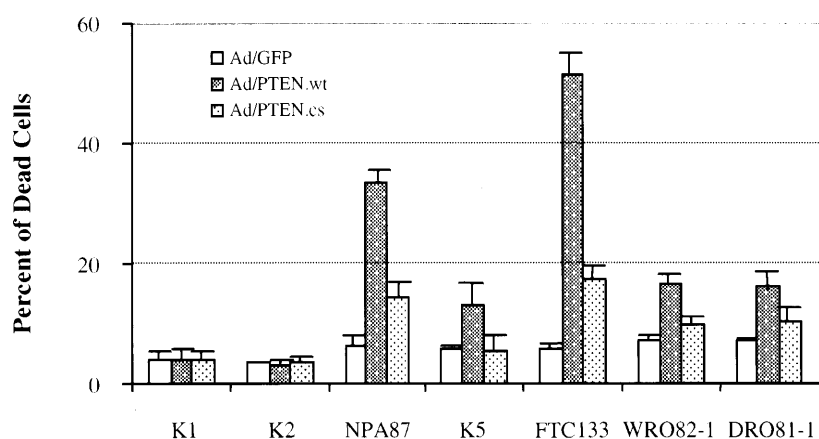
Similar to the low frequency of somatic PTEN mutation in sporadic thyroid cancer, only one cell line (FTC-133) among

10 thyroid cancer cell lines was found to have hemizygous deletion and mutation in the other allele. As expected, FTC-133 expresses low levels of mutant PTEN mRNA and extremely low levels of or no PTEN protein. Interestingly, three other lines (K5, NPA87 and OR1.3) also express low levels of PTEN transcript, although they carry two normal alleles of PTEN, indicating that downregulation of PTEN at the transcription level plays some role in PTEN inactivation in the thyroid cancer model.

Our results clearly demonstrate that PTEN has a general negative effect on cell growth in thyroid cancer. Earlier studies of PTEN function in glioma lines suggest that the growth-inhibitory function of PTEN depends on endogenous PTEN status (31). Introduction of PTEN into thyroid cancer cells led to growth inhibition in all seven cell lines regardless of endogenous PTEN status, similar to the data for ectopic expression of PTEN in breast cancer cell lines, although PTEN has the greatest growth-suppressive effect in endogenous PTEN-null



**Figure 4.** Overexpression of *PTEN* induces phosphatase-dependent  $G_1$  arrest. Cells were infected with Ad/GFP (left bar over each line), Ad/PTEN.wt (middle bar) and Ad/PTEN.cs (right bar) at concentrations of  $1 \times 10^5$  m.o.i. Twenty-four hours after transfection, cells were harvested for cell cycle distribution analysis by FACS. The indicated values are means of triplicate of dishes. Overexpression of wild-type *PTEN* resulted in  $G_1$  arrest among all thyroid cancer lines (middle bars versus left bars). Note that high levels of wild-type *PTEN* did not produce  $G_1$  arrest in FTC-133 although low level infection with *PTEN* adenovirus or short exposure to virus resulted in  $G_1$  arrest (latter, data not shown). Phosphatase-dead mutant *PTEN*.cs could not induce  $G_1$  arrest (right bars).



**Figure 5.** The effect of *PTEN* overexpression on apoptosis in thyroid cancer lines. Cells were infected with Ad/GFP (left white bar over each line), Ad/PTEN.wt (middle bar) and Ad/PTEN.cs (right bar) at concentrations of  $1 \times 10^5$  m.o.i. Twenty-four hours after transfection, both floating and adherent cells were collected and stained with Trypan blue. The number of dead cells and total cells were counted and presented as a percentage of dead cells/total cells. The indicated values are means of triplicates of dishes. Overexpression of wild-type *PTEN* results in cell death in all thyroid cancer lines except in PTC lines K1 and K2. Note that *PTEN*-mediated apoptosis can be phosphatase dependent and independent.

FTC-133 cells. These data suggest that the overall phosphatase activity of *PTEN* seems more important in conferring sensitivity to *PTEN* than the endogenous *PTEN* status.

Cell growth requires both proliferation signals and survival signals. Tumour cells gain a growth advantage due to abnormal proliferation and defects in the regulation of cell death. Tumour suppressors p53 (36) and Rb (37) exert direct effects on both cell cycle progression and cell viability. Cell death and cell cycle are tightly linked. Apoptosis is often accompanied by growth arrest and checkpoint proteins have been shown to be involved in the apoptotic response. For example, activation of p53 by DNA damage results in cell cycle arrest or cell death, depending on the type and circumstances of the cell (38). Activation of the cell cycle arrests the function of p53 in a cell

that is simultaneously being driven through this checkpoint by expression of E1A, myc or E2F and results in cell death. In general, mutations that abolish the cell cycle arrest function of p53 also inactivate its cell death function, although several exceptions exist. On the other hand, a tumour-derived mutation of p53, which retains wild-type ability to activate p21 and induce cell cycle arrest, is unable to activate apoptosis (39). p53 can induce apoptosis but not  $G_1$  arrest in p21-deficient mice (40,41). Therefore the induction of  $G_1$  arrest is not necessarily a prerequisite for p53-induced apoptosis (42). These pieces of evidence suggest that the cell cycle arrest and apoptosis mediated by p53 are independent and separable.

Early studies have also suggested a tissue-specific effect of *PTEN*. Overexpression of *PTEN* in different tumour cell lines

results in either cell cycle arrest in glioma cell lines (43–45) or cell death in breast cancer lines (32), although G<sub>1</sub> arrest and apoptosis within a single cell are not mutually exclusive (30). In thyroid cancer lines, the cell cycle arrest induced by *PTEN* is not necessarily specific to histologic subtype. Overexpression of *PTEN* resulted in the inhibition of cell cycle progression in seven epithelial thyroid cancer cell lines, all of which are of different histologic subtype, suggesting that *PTEN* plays a universal role in the regulation of the cell cycle.

Corroborating our previous report that overexpression of *PTEN* in MCF-7 breast cancer cells can induce both G<sub>1</sub> arrest and apoptosis (30), overexpression of *PTEN* in thyroid cancer lines induces both cell death and G<sub>1</sub> arrest in three FTC lines (FTC-133, WRO82-1 and K5). Interestingly, *PTEN* induces G<sub>1</sub> arrest without cell death in two PTC lines (K1 and K2), but not in the poorly differentiated PTC line (NPA87), which does undergo some apoptosis. The proto-oncogene *RET* has been shown to be rearranged in up to 60% of PTCs (46). Whether the absence of cell death response to *PTEN* expression in the two well differentiated PTCs is determined by the presence of a RET/PTC translocation and, hence, constitutive activation of cell survival signals, remains to be investigated further.

PI3K has been implicated in the transduction of both proliferation and survival signals. It is required for G<sub>1</sub> to S phase progression stimulated by a variety of growth factors (47). Activation of PI3K or Akt protects various cell types from apoptosis induced by withdrawing survival factors (48). An inverse correlation between endogenous *PTEN* levels and phosphorylated Akt was evident in our panel of 10 thyroid cancer cell lines. Furthermore, infection of these thyroid cancer cells with wild-type *PTEN* resulted in a dramatic reduction in the phosphorylation of Akt. These observations suggest that the blockage of the PI3K/Akt signalling pathway may be important for *PTEN*'s growth-suppressive effects. *PTEN* also seems to be able to induce cell death independent of its phosphatase activity, since overexpression of the phosphatase-dead mutant *PTEN*, C124S, also caused cell death, although much less than that induced by wild-type *PTEN*. PI3K/Akt-dependent and -independent *PTEN*-mediated growth arrest is also evident in a breast cancer model (49). The cell death caused by the C124S mutant is not simply due to the general toxicity of the viral infection, as it produces equivalent amounts of *PTEN* protein as wild-type *PTEN* virus and has no effect on the cell cycle. In addition to its well documented lipid phosphatase activity, *PTEN* possesses protein phosphatase activity as well. The importance of its protein phosphatase is poorly understood. It is possible that *PTEN* may interact with multiple signalling pathways, which are involved in the regulation of cell survival and cell proliferation in a separate and coordinate way.

## MATERIALS AND METHODS

### Cell culture

The thyroid cancer cell lines, K1 and K2, are derived from PTC (50); NPA87 is derived from a poorly differentiated PTC; FTC-133 (50), K5 and WRO82-1 are derived from FTC and ARO89-1 and DRO81-1 are derived from UTC. WRO82-1, ARO89-1, DRO81-1, FRO82-2 and NPA87 cell lines are gifts from Dr Danilo Canlanan. All the cells were grown in Dulbecco's modified Eagle's medium (DMEM)/10% fetal

bovine serum (FBS) (Gibco BRL, Life Technologies) with 100 U/ml penicillin G (Sigma) and 100 µg/ml streptomycin sulphate (Sigma).

### Northern blot analysis

RNA extraction was performed using TRIzol reagent (Gibco BRL, Life Technologies) according to the manufacturer's instructions. Northern blot analysis was performed according to the glyoxal/(CH<sub>3</sub>)<sub>2</sub>SO denaturation method (51). A total of 10 µg of RNA was electrophoresed on 1.5% agarose gel, blotted and hybridized according to standard procedures (51). The *PTEN* probe was a 1.3 kb cDNA fragment containing the *PTEN* coding region and labelled with <sup>32</sup>P by the hexamer-random primer method (52). Hybridization was done in 50% v/v formamide solution containing 1 × 10<sup>6</sup> c.p.m./ml probe overnight at 42°C. The hybridized membrane was washed twice in 2× SSC/0.2% SDS at 65°C for 1 h and once in 0.2× SSC/0.2% SDS at 65°C for 30 min and then exposed to X-ray film (Kodak XAR-5) for 24 h at -70°C.

### Mutation analysis

DNA extraction was performed using the Qiamp DNA Mini kit (Qiagen) according to the manufacturer's recommendation. Mutation analysis was performed using denaturing gradient gel electrophoresis (DGGE) as described by Mutter *et al.* (12) and Marsh *et al.* (53). DNA samples showing DGGE variation were subjected to standard PCR-based semi-automated sequence analysis using the ABI-377 or Perkin-Elmer 3700.

### Immunoblotting

Western blots were performed as previously described (30). In brief, cell lysates were mixed with equal volumes of 2× Laemmli sample buffer, boiled for 10 min, resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane. The membranes were blocked with 5% non-fat dry milk in TBST (10 mM Tris-HCl pH 8.0, 100 mM NaCl and 0.05% Tween 20) for 1 h at room temperature followed by incubation with appropriate primary antibody for 2 h at room temperature or overnight at 4°C. Subsequently, incubation with horseradish peroxidase-conjugated secondary antibody (Promega) was performed at 1:500 dilution for 1 h at room temperature. Protein signals were detected by enhanced chemiluminescence (Amersham). The anti-*PTEN* monoclonal antibody 6H2.1 (54) was used to detect *PTEN*. The polyclonal anti-phospho-Akt, anti-Akt (New England Biolabs) and monoclonal anti-α-tubulin (Sigma) were used at 1:1000, 1:1000 and at 1:5000 dilutions, respectively.

### Plasmid construction, transfection and colony formation assay

Wild-type (wt) and C124S mutant (cs) *PTEN* cDNA was obtained by PCR and PCR-based site-directed mutagenesis as previously described (30). The mammalian cell expression vector pcDNA3 (Invitrogen) was used to generate two sets of *PTEN* expression constructs, pcDNA3/*PTEN*.wt and pcDNA3/*PTEN*.cs. FTC-133 cells were transfected with pcDNA3, pcDNA3/*PTEN*.wt and pcDNA3/*PTEN*.cs. Transfections were performed using lipofectamine (Gibco BRL). Exponentially growing cells (5 × 10<sup>4</sup>) were transfected with 1 µg

of plasmid DNA on a 35 mm dish. The cells were transferred to p 100 mm dishes 24 h after transfection and selected by neomycin (Sigma) at 500 µg/ml. After 2 weeks of selection, the medium was removed and cells were washed with phosphate-buffered NaCl solution and fixed with 12.5% glutaraldehyde (Fisher) for 20 min at room temperature. Cells were rinsed with distilled water and incubated with 0.05% methylene blue (Sigma) for 30 min. Colonies measuring >50 µm in diameter were counted.

### Adenovirus preparation and infection

Generation of recombinant adenoviruses was performed following the protocol described by He *et al.* (55). Wild-type and C124S mutant *PTEN* cDNA were subcloned into the shuttle vector, pAdTrack-CMV, to generate pAdTrack/*PTEN*.wt and pAdTrack/*PTEN*.cs, respectively. Recombinant *PTEN* adenoviruses, Ad/*PTEN*.wt and Ad/*PTEN*.cs, were generated by homologous recombination of *PTEN* shuttle vectors with the adenovirus backbone vector, pAdEasy-2. The virus expressing the enhanced fluorescent protein, Ad/GFP, derived from the same vector as Ad-*PTEN*, was used as a control. All of the viruses were grown in 293 cells and purified by caesium gradient ultra-centrifugation.

Infection of the cells with recombinant adenovirus was performed as previously described (55). Briefly, cells at 30% confluence were washed with phosphate-buffered NaCl solution and then incubated with medium containing adenovirus and 20 mM of HEPES for 75 min. Medium containing 10% FBS was then added back. Subsequent cell death and fluorescence-activated cell sorting (FACS) analysis were performed after 48 h of incubation at 37°C.

### Cell death assay

Dead cells were determined by trypan blue staining as previously used (30). Both floating cells and trypsinized attached cells were collected and incubated with 0.2% Trypan blue for 5 min at room temperature. Blue cells and total cells were counted. Cell death was presented as percentage of blue cells versus total cells. Apoptotic cell death was confirmed by TUNEL assay. TUNEL analysis of DNA fragmentation was performed using an *in situ* apoptosis detection kit (ApopTag), following the procedures recommended by the manufacturer (Intergen).

### Flow-cytometric analysis

Assays were performed in p 100 mm dishes. At the end of incubation, cells were trypsinized and washed into ice-cold phosphate-buffered NaCl solution. Cells were then fixed by adding them drop-wise into ice-cold 80% ethanol while vortexing, followed by incubation on ice for 60 min. The fixed cells were washed with cold phosphate-buffered NaCl solution and incubated at 37°C for 30 min in 0.5 ml phosphate-buffered NaCl solution containing 10 µg/ml propidium iodide (Sigma) and 5 µg/ml Rnase A (New England Biolabs). DNA content was determined by FACS analysis (Becton Dickinson).

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