PTEN expression controls cellular response to cetuximab by mediating PI3K/AKT and RAS/RAF/MAPK downstream signaling in *KRAS* wild-type, hormone refractory prostate cancer cells

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Abstract. Overexpression of epidermal growth factor receptor (EGFR) and mutation of pten tumor suppressor gene in human cancer cells leads to activated EGFR downstream signaling including PI3-kinase/AKT (PI3K/AKT) and/or mitogen-activated protein kinases (RAS/RAF/MAPK) and have been linked to resistance to anti-EGFR targeted therapies. Cetuximab is a chimeric IgG1 monoclonal antibody that binds the EGFR with high specificity and have been developed as promising therapeutic anticancer treatments in several solid tumors, including colorectal and head and neck squamous cell carcinomas. Cetuximab activity is related to PI3K/AKT and RAS/RAF/MAPK signaling pathways functionality and its activity has been shown to be higher in wild-type *KRAS* tumors. To study the influence of PTEN expression on cell response to cetuximab, we used wild-type *KRAS*, PTEN-null, EGFR overexpressing PC3 prostate cancer cells. Reintroduction of PTEN significantly reduced the constitutive overexpression of phosphorylated-AKT (p-AKT) and downstream kinases (p-GSK3ß and p-P70S6 kinase) as well as phosphorylated-ERK1/2 (p-ERK1/2) and consequently significantly restored cetuximab-induced cell growth inhibition and apoptosis induction. Taken together, the results achieved in the present study show that PTEN controls the cellular response to cetuximab in *KRAS* wild-type prostate carcinoma PC3 cells through the regulation of AKT phosphorylation and restoration of the functionality of EGFR downstream signaling. Extrapolation of these findings to clinical situation, suggests that the assessment of EGFR downstream signaling functionality could be proposed as a diagnostic response predictive marker for anti-EGFR targeted therapies.

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Introduction

EGFR is overexpressed in many tumor types and its inhibition represents an innovative therapeutic approach of targeted therapy. EGFR inhibition can be achieved by monoclonal antibody prevention of ligand binding to the extracellular part of the receptor and tyrosine kinase inhibition at the intracellular level. Cetuximab is a chimeric monoclonal antibody binding EGFR with high affinity and abrogating the ligand-induced dimerization and phosphorylation of the receptor (1). EGFR inhibition by cetuximab decreases cell proliferation, increases apoptosis, and decreases angiogenesis (2). However, number of EGFR overexpressing tumors fail to respond to EGFR-targeted therapy. Recently, in colorectal cancer, resistance to cetuximab was associated with *KRAS* mutations (3,4). However, in *KRAS* wild-type tumors, some additional functional alterations of EGFR downstream signaling have been related to shorter progression-free survival (5). *KRAS* mutations have been reported in prostate cancer (6). Additionally, alteration of pten gene has been shown to promote prostate cancer progression (7). PTEN antagonizes the action of PI3-kinase (PI3K) and negatively controls downstream signaling by inhibiting the phosphorylation of AKT (8). PI3K/AKT pathway has also been reported to cross talk with MAP kinase (MAPK) signaling pathway by autocrine signaling through RAS (9). Consequently, the loss of PTEN expression could putatively affect the response to cetuximab by impairing PI3K/AKT and RAS/RAF/MAPK signaling downstream to EGFR.

A recent study investigating protein expression demonstrated that PTEN plays a fundamental role in predicting the response to cetuximab against EGFR in metastatic colorectal

cancer (10). In the present study we used the PC3 human prostate carcinoma cell line harboring *PTEN* deletion. Our investigation was designed to evaluate how restoring PTEN expression would affect cetuximab-mediated growth inhibition and apoptosis induction, regarding the implication of PI3K/AKT and MAPK signaling pathways.

Materials and methods

Cell lines. PC3 cell line was obtained from the American Type Culture Collection. The cells were maintained at 37˚C/ 5% CO_2 in RPMI-1640 culture medium, supplemented with 10% heat inactivated fetal calf serum (Dutscher, Brumath, France) and 1% glutamine. Culture media and additives were from Life Technologies (Eragny, France).

KRAS genotyping. PC3 cells were checked for *KRAS* mutations (codon 12 and 13) using two-step PCR-RFLP according to Schimanski *et al* (11). Briefly, DNA was extracted from 10^9 cell suspension. The amount of DNA isolated was determined spectrometrically and 100 ng DNA was used as template for the first PCR (Master Cycler Gradient, Eppendorf, Germany) with the oligonucleotide primers Ras A (sense; 5'-ACTGAATATAAACTTGTGGTC CATGGAGCT-3') and Ras B (antisense; 5'-TTATCTGTA TCAAAGAATGGTCCTGCACCA-3'). PCR products were then submitted to enzymatic digestion with either *Bst*XI or *Xcm*I, restricting the amplicon if the first two bases of codon 12 (*Bst*XI) and codon 13 (*Xcm*I) were wild-type. The first digest (2 μl) was used as template for the second PCR in which primer Ras C (antisense; 5'-GGATGGTCCTCCACC AGTAATATGGATATTA-3') was used instead of Ras B. Second PCR (7 μl) was digested with either *Bst*XI or *Xcm*I. The digest (10 μl) was submitted to PAGE stained with ethidium bromide and analyzed under UV light (GelDoc EQ, Bio-Rad). DNA extracts from cell lines bearing codon 12 or/and 13 mutations were added as positive controls.

Gene transfer. Linear polyethylenimine (PEI) was kindly provided by Polyplus-Transfection (Illkirch, France). pCMV-Luc (Dr P. Erbacher, Polyplus-Transfection), and pPTEN (Dr K. Yamada, NIH, Bethesda, MD) plasmids, encoding luciferase and *PTEN* genes respectively, were purified from *Escherichia coli* using Marligen Maxiprep columns (Life Technologies).

Gene transfer was performed as already published (12) with slight modifications. Briefly, the cells were seeded at the density of $5x10^4$/ml in 6-well plates 48 h before transfection, to reach 60-70% confluence at the time of transfection. pCMV-Luc or pPTEN plasmids (5 μg/ml) and corresponding amount of PEI were separately prepared and the resulting mixture was added to the cells. Twenty-four hours after transfection cells were submitted to 100 μg/ml cetuximab (Merck Lipha Sante, Lyon, France) and analyzed for cell growth inhibition and apotosis induction.

Western blot analysis of EGFR and PTEN expression. EGFR and PTEN protein analysis was performed using Western blotting with monoclonal antibodies directed against EGFR and PTEN (dilution 1/500, Dako, Trappes, France). Tubulin was used as internal loading control (dilution 1/500, Santa Cruz).

Bio-Plex phosphoprotein array. Phosphoproteins were analyzed using multiplex sandwich bead immunoassays (Bio-Plex® phosphoprotein array, Bio-Rad, Hercules CA) in cells exposed to EGF (100 ng/ml, for 5 min). Phosphoprotein determination kits (Bio-Rad) were used according to the manufacturer's recommendations. Briefly, protein extracts were transferred into 96-well dishes then fluorescent capturing beads coupled to antibodies directed against the RAS/RAF/MAPK and PI3K/AKT signaling phosphoproteins (phospho-AKT, phospho-GSK3ß, phospho-p70S6K, phospho-ERK1/2) were mixed, and added into each well and incubated overnight. Following incubation, the plates were washed and incubated with biotinylated antibodies fixing each target protein. Streptavidin-phycoerythrin solution was then added. The analysis consisted in a double laser fluorescence detection allowing simultaneous identification of the target protein through the red fluorescence emission signal of the bead and quantification of the target protein through the fluorescence intensity of phycoerythrin. Results were recorded as mean fluorescence intensities and compared to negative controls. Positive control consisting in standard protein extract from cell lines were added to each series. This technique was previously cross-validated with Western blot analysis (13).

Cell growth inhibition assay. Cell growth inhibition assays were performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Briefly, the cells were seeded in 24-well dishes and allowed to grow for 24 h before being transfected then exposed to cetuximab for 24 h. Following incubation 250 μl of MTT was added into each well. The reaction was allowed to proceed for 3 h at 37˚C, then the formazan crystals were dissolved by adding 250 μl of sodium dodecylsulfate and the absorbance was measured at 540 nm (Multiskan Ascent LabSystem).

Apoptosis induction. Apoptosis induction was first determined by the analysis of DNA fragmentation (subG1 peak) by flow cytometry (FACS calibur, Becton Dickinson) using propidium iodide labeling. Briefly, cetuximab treated cells were trypsinized, washed with PBS, fixed in 70% ethanol for 30 min at 4˚C, and suspended in PBS containing propidium iodide (1 mg/ml) and RNase A (20 mg/ml). Fixed cells were subjected to flow cytometry and subG1 cell population, identified as hypoDNA-diploid cell population was calculated.

Active caspase-3 expression was measured as molecular marker for apoptosis induction using flow cytometry in cells fixed in Cytofix/Cytoperm™ (Becton Dickinson, Le Pont de Claix, France), then were incubated with phycoerythrin-conjugated anti-active caspase-3 antibodies (PharMingen-Becton Dickinson) for 30 min. Cells were washed and 500 μl of 1X Perm/Wash™ was added before analysis by flow cytometry. Active caspase-3 expressing cell population identified as phycoerythrin-labeled cell population was quantified.

Statistical analysis. Differences between mean values were evaluated using either one-way ANOVA with Dunnett's test

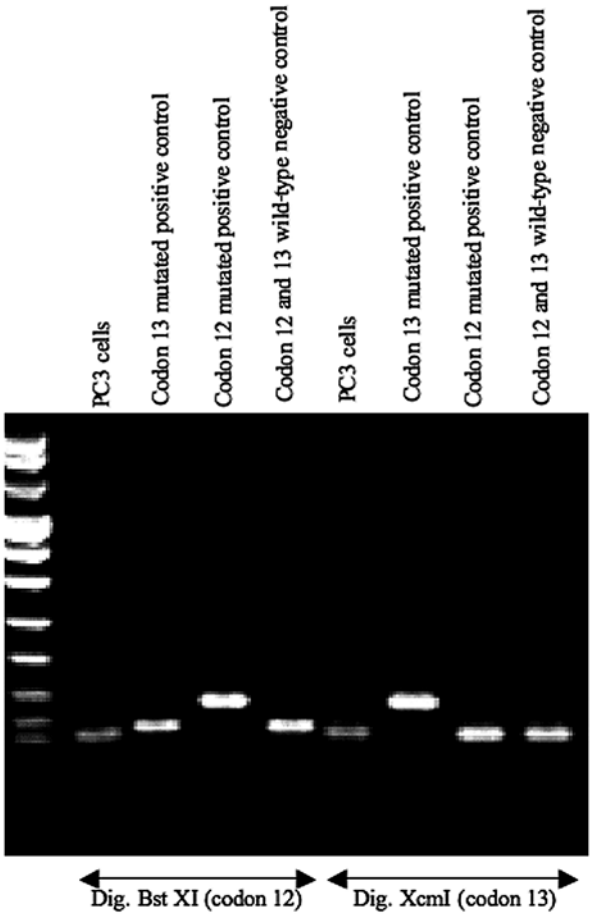

Figure 1. *KRAS* mutation analysis using PCR-RFLP. DNA extract from PC3 cells were submitted to double PCR amplification after *Bst*XI and *Xcm*I enzymatic digestion allowing discrimination of codon 12 and 13 mutations. Codon 12 and 13-mutated cell lines were used as positive controls.

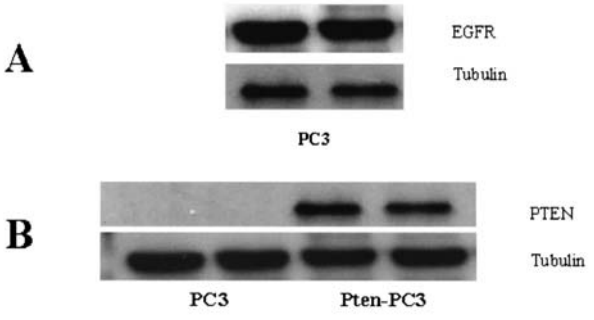

Figure 2. EGFR (A) and PTEN (B) protein expression analysis using Western blotting. ß-tubulin was used as loading control.

or Kruskal Wallis with Mann-Whitney test according to data distribution. P≤0.05 was considered as statistically significant.

Results

KRAS genotyping. As revealed by PCR-RFLP (Fig. 1), no mutation in either codon 12 or 13 was detected in the PC3 cell DNA extracts.

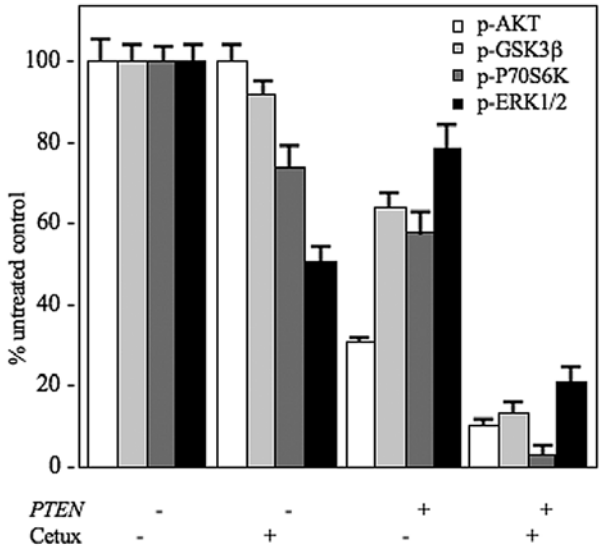

Figure 3. Analysis of AKT and MAPK signaling pathway functionality using Bio-Plex phosphoprotein array. Effect of *PTEN* restoration and exposure to cetuximab on AKT and MAPK signaling pathways. In each gene transfer condition, p-AKT, p-GSK3ß, p-P70S6K, p-ERK1/2 expression was analyzed using Bio-Plex phosphoprotein array in total protein extracts from cell lysates. Results are expressed as mean values of three independent experiments, relative to untransfected controls.

EGFR, PTEN expression. In untransfected PC3 cells, EGFR overexpression was observed in PC3 cells (Fig. 2A). No PTEN expression was observed (Fig. 2B). Following gene transfer, pten gene was found to be restored (Fig. 2B).

RAS/RAF/MAPK and PI3K/AKT signaling. Constitutive overexpression of phosphorylated-AKT (p-AKT) and consequent overexpression of AKT downstream signaling phosphoproteins, phosphorylated-GSK3ß (p-GSK3ß and phosphorylated-P70S6K (p-P70S6K) was observed in untransfected control cells as expected in relation with pten-null phenotype (Fig. 3). Constitutive overexpression of phosphorylated-ERK1/2 (p-ERK1/2) was also observed. Exposure of control cells (untransfected control cells and cells transfected with pCMV-Luc irrelevant plasmid) to cetuximab did not induce any significant (P>0.05) variation of p-AKT and p-GSK3ß expression. Cetuximab induced significant decrease in p-P70S6K and p-ERK1/2 expression, respectively down to 67% (P<0.05) and to 46% (P<0.01) of the initial values achieved in untreated controls.

Pten gene transfer was found to significantly restore inhibition of AKT signaling. p-AKT expression was significantly (P<0.01) reduced down to 37% of the value achieved in untranstected controls. Decreased p-GSK3ß, and p-P70S6K expression was also observed down to 62 and 55% of the value measured in untransfected controls. Additionally, p-ERK1/2 expression was significantly (P<0.01) reduced down to 73% of the value measured in untransfected controls. The expression of all phosphoproteins was found to be further significantly (P<0.05) reduced, down to 5-20% of the values observed in untransfected controls, when the pten-transfected cells were exposed to cetuximab.

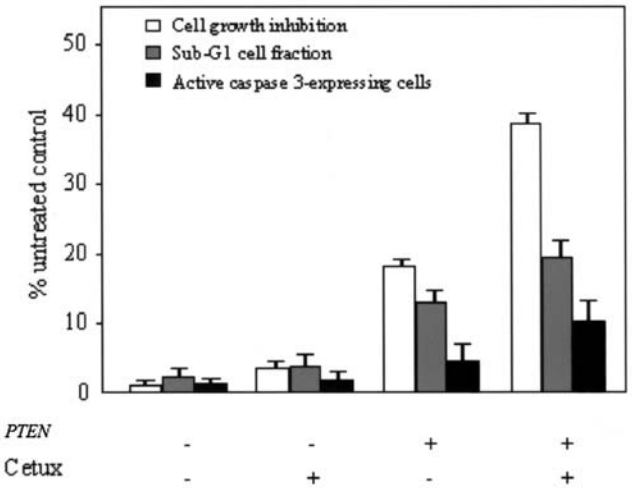

Figure 4. Analysis of cell growth inhibition and apoptosis induction in *PTEN*-transfected PC3 cells exposed to cetuximab for 24 h. Cell growth inhibition was assessed using MTT assay. Apoptosis was analyzed by quantifying sub G1 cell population determination after propidium iodide staining of cells and by detection of active caspase-3 expression using flow cytometry. Results are expressed as mean values of three independent experiments, relative to untransfected controls.

Growth inhibition and apoptosis induction by cetuximab. Pten-transfected PC3 cells were exposed to cetuximab (100 μg/ml for 24 h) and assayed for cell growth inhibition and apoptosis induction. Negative controls consisted in untransfected cells. No significant difference was found between untransfected cells and cells transfected with irrelevant plasmid (pCMV-Luc) (data not shown).

No significant growth inhibition and apoptosis induction was observed in untransfected control cells exposed to cetuximab (Fig. 4).

PTEN-transfected cells were found to be significantly ($P<0.01$) sensitized to cetuximab with significant increase in cell growth inhibition, DNA fragmentation (sub G1) and caspase-3 expressing cell fraction.

Discussion

The present study was performed in PC3 human prostate cancer cell line which is referenced as *PTEN* null also because of a deletion of the 3'-end of *PTEN* gene and promoting the absence of the full length *PTEN* m-RNA (14). No mutation of PI3K has been described in these cells illustrating that PI3K/AKT signaling pathway functionality should be extremely dependent on *PTEN* expression. Consistently, pten mutated status was found to be associated with constitutive activation of AKT in PC3 cells and the constitutive high level of expression of phosphorylated AKT was associated with a reduced sensitivity of PC3 cells to cetuximab. The data achieved in the present study are fully consistent with results showing that PC3 are resistant to apoptosis induction (15).

As expected, reinstating *PTEN* in PC3 cells promoted a decrease in phosphorylation of AKT and its downstream signaling, and consequently induced the inhibition of cell growth and the induction of apoptosis, confirming the key role of *PTEN* expression in the control of the cell survival pathway. These results are consistent with the now well-established model in which PTEN was shown to be essential for con-trolling cell growth in prostate human cancer cell lines (16) by regulating PI3K/AKT signaling pathways by decreasing the pool of phosphorylated proteins. Many studies have demonstrated that p-AKT overexpression is associated with resistance to EGFR inhibitors. Further, it has been showed (17) that resistance to cetuximab was associated with p-AKT overexpression and repression of AKT expression restored the cell sensitivity to cetuximab. Accordingly, we showed here that *PTEN* reintroduction restored the cell response to cetuximab by restoring inhibition of p-AKT and to a lesser extent by promoting p-ERK1/2 inhibition. Therefore, these data further confirmed that AKT signaling pathway functionality is controlled by PTEN and contribute to cetuximab activity.

These results strongly suggest that, in *KRAS* wild-type cells, *PTEN*-mutated status, could be predictive of cellular resistance to cetuximab and warrant clinical investigation to evaluate whether patients with *PTEN*-mutated prostate tumors, are unlikely to respond to this treatment as already shown with *KRAS* mutation in colorectal cancer treated by cetuximab (3) or with *PTEN* in breast cancer treated by trastuzumab (18). In such a situation, common diagnostic criteria could be the determination of signaling phosphoprotein expression in tumors (19). Pre-liminary results achieved in breast (20), head and neck (21) and colon carcinoma (5) using Bio-Plex phosphoprotein array demonstrated that large variation in phosphoprotein expression can be found in clinical specimens at diagnosis. Such variations were further demonstrated to contribute to variation in progression-free survival of patients with metastatic colorectal cancer treated by cetuximab (5). In the case of signaling alteration, addition of targeted therapy directed against PI3K downstream signaling kinases could be proposed to obtain greater antitumor signaling inhibition. As already proposed in preclinical *in vivo* models (15,22), gene therapy could be envisaged in tumors with gene alteration.

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