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# Telomerase activation in a model of lung adenocarcinoma

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**Abstract (198 words)**

Ovine pulmonary adenocarcinoma (OPA) is a lung cancer strikingly similar to the pneumonic-type mixed invasive adenocarcinoma with a predominant bronchioloalveolar component in humans. We investigated telomerase activity in OPA and the potential involvement of the kinase Akt in telomerase activation and regulation of cell proliferation.

Lung tissues were collected from sheep with a histopathological diagnosis of OPA or controls. Epithelial cell cultures were derived *in vitro* from lung tissues. Telomerase activity was evaluated by the Telomeric Repeat Amplification Protocol method. Phosphorylation of Akt was detected by western-blotting.

Telomerase activity was significantly higher in OPA lung tissues as compared with control lung tissues. A high telomerase activity was detected in 8/12 (67%) primary cell cultures derived from tumours. A high level of expression of phosphorylated Akt was found in 10/27 (37%) tumours, with abolition of Akt activation in response to EGF stimulation demonstrated in primary cell cultures derived from tumours.

Telomerase activation takes place in OPA tumour cells and may be partly attributable to Akt activation. Telomerase may inhibit cellular senescence and contribute to the accumulation of tumour cells in mixed adenocarcinoma with bronchioloalveolar component. Further work is necessary to identify alternative signalling pathways of telomerase activation in tumours.

Keywords: bronchioloalveolar carcinoma, lung cancer, Akt, telomerase, type-II pneumocyte, JSRV

Lung cancer is the first cause of cancer mortality in developed countries. Non-small cell lung cancer represents approximately 80% of lung cancers, adenocarcinoma being the most frequent cell type, accounting for ~40% of all cases of lung cancer. Lung adenocarcinoma may present as the pneumonic-type adenocarcinoma (pADC), that associates typical radiological features with diffuse or disseminated alveolar condensation with or without air bronchogram evolving towards pulmonary right-to-left shunting, together with evidence of adenocarcinoma tumour cells in the lung (1). The histological pattern in pADC may include bronchioloalveolar carcinoma (BAC) (defined as an adenocarcinoma with a pure bronchioloalveolar growth pattern with no evidence of stromal, vascular, or pleural invasion) (2), or more commonly a mixed-type adenocarcinoma with a predominant bronchioloalveolar component and papillary or acinar invasive component (1, 3).

Ovine pulmonary adenocarcinoma (OPA) is a naturally occurring lung cancer that occurs spontaneously in sheep infected by the Jaagsiekte Sheep RetroVirus (JSRV), and that may be reproduced by the experimental inoculation of lambs with the virus (4). It grows with a disseminated pattern at the periphery of the lung. OPA is a mixed type adenocarcinoma containing a significant proportion of BAC component, together with papillary and acinar growth patterns (2, 3). OPA also shares striking clinical and radiological homology with human pADC, including progressive intrapulmonary spread and lack of distant extrathoracic metastasis (3), and thus represents a unique natural and reproducible animal model of peripheral lung cancer, especially pADC.

In contrast with previous studies that focused on defining oncogenic properties of JSRV structural proteins in rodents (5), we aimed at identifying pathogenic processes taking place in spontaneous tumours *in vivo*. Mechanisms potentially involved in tumour formation include extensive cell division as a result of oncogenic mutations, and inactivation of cellular senescence, tumour suppressor pathways, or apoptosis mechanisms that may otherwise arrest the

proliferation or induce the death of potential cancer cells (6). Cell senescence is a process mostly described *in vitro*, whereby primary normal cells grown in culture do not proliferate indefinitely, but withdraw from the cell cycle (after a period of rapid proliferation) in response to diverse regulatory mechanism including dysfunctional telomeres (7). More recently, cellular senescence has been also demonstrated *in vivo* in premalignant but not malignant cells, suggesting that it may be an important anticancer defence (8).

Cellular senescence is mainly regulated by telomerase, a ribonucleoprotein enzyme able to stabilize telomere length by *de novo* synthesis of telomeres and elongation of existing telomeres. Telomerase activation is considered mandatory for tumour cells to escape cell senescence and gain increased proliferative capacities (9). The telomerase reverse transcriptase (TERT) catalytic subunit is the major determinant of telomerase activity *in vitro* and *in vivo*. Activation of TERT has been well established in human cancer cell lines and tumours including lung cancer (10), while telomerase activity is repressed in most normal somatic cells. Complex regulation of telomerase activity may include the phosphatidylinositol 3-kinase (PI3-K) pathway through phosphorylation of TERT by Akt (11). Involved in regulation of cell survival and cell cycle progression, Akt is constitutively activated in a variety of human tumours including lung cancer (12).

In this study, we demonstrate increased telomerase activity in tumours and in primary cultures of tumour cells derived from OPA, suggesting that inhibition of cell senescence may be involved in tumorigenesis and accumulation of tumoral cells within the lung. We next show that the regulatory kinase Akt is constitutively activated in OPA tumours and disregulated in primary cultures derived from OPA, suggesting that Akt may be involved in telomerase activation in a proportion of tumours.

## Materials and methods

### Lung tissues

Lung tissues were collected immediately *post-mortem* from sheep presenting loss of weight, dypsnea, profuse lung secretions suggestive of OPA or without signs of OPA. Tissue sections were sampled, stored at -70°C until use or fixed in formol for histopathological examination. The tissues were classified as tumoral and non-tumoral lung (henceforth referred to as “controls”) following the current 2004 World Health Organization (2).

### Culture of alveolar epithelial type II cells

Tumour cells and normal type-II pneumocytes were isolated and characterized from ovine lungs as described elsewhere (Archer *et al*, manuscript in preparation). Briefly tissue samples were digested overnight at 4°C in Eagle’s minimum essential medium (MEM, Eurobio, France) with 0.025% collagenase I, 10 µg/ml deoxyribonuclease, 1 mg/ml protease XIV (Sigma), 50 µg/ml streptomycin, 50 U/ml penicillin, 50 µg/ml gentamycine, 2.5 µg/ml amphotericin B and 100 U/ml nystatin. The cells were then homogenized and filtered through a 40 µm filter to eliminate cell debris. After centrifugation at 430g for 10 min at 4°C, pulmonary cells were plated onto collagen, laminin and fibronectin coated-plates in selective medium for epithelial cells (Quantum 286, PAA, Austria) with 5 ng/ml Hepatocyte growth factor and 10 ng/ml Keratinocyte Growth Factor (Abcys). Cells derived from OPA tumours (n=12) and non tumoral lungs (n=4) are referred to as tumoral and control cells respectively.

### Detection of JSRV proviral DNA

Total genomic DNA from lung tumours and control lungs was prepared with the Fastprep device following recommendations of the supplier (BIO 101, France). A semi-nested PCR protocol was used to detect proviral DNA. Briefly, reaction mixtures contained 500 ng of total genomic DNA, 50 µl of 1X buffer (1.5 mM $MgCl_2$, 67 mM Tris-HCl pH 8.8, 16 mM $(NH4)_2SO4$, 0.01% Tween 20), 0.2

mM of each desoxynucleotide triphosphate, 0.2 mM of each primer and 1.25 units of Taq polymerase (Eurobio, France). The first round of PCR was performed with the primers JSRV42 (sense) 5'-CTTTGTATTTCCCTGTGTCG-3' corresponding to nucleotides 7041-60 in the *env* gene of JSRV genomic sequence (Genbank accession number AF105220), and JSRV53 (antisense) 5'-GGATTCTTACACAATCACC-3' corresponding to nucleotides 7381-62 in the LTR U3 region of JSRV genomic sequence (Genbank accession number AF105220). The second round of PCR was performed with 1-5 µl of PCR product using JSRV42 and JSRV52 (antisense) 5'-CACCGGATTCTTATATAATC-3' corresponding to nucleotides 7366-46 in the LTR U3 region of JSRV genomic sequence (Genbank accession number AF105220). PCR reactions were performed as followed: 5 min at 95°C, 35 cycles of 30 s at 95°C, 30 s at 50°C and 1 min at 72°C with a final elongation of 10 min at 95°C.

Cell proliferation assay

Cell numbers were determined by an MTT (3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyl tetrazolium bromide) assay. Briefly, 5000 cells per well were plated in 96-well plates, incubated for 96h at 37°C with 5% $CO_2$. MTT was incorporated as recommended with the "MTT Cell Proliferation Assay kit" (Chemicon, France). Ten microliters of MTT were added to each well for 4h at 37°C. After solubilization with 100 µl of isopropanol 1N - HCl 0.04N solution, absorbance was read at 595 nm with a Wallac Victor II device (Perkin-Elmer, France). Each measurement was performed in triplicates.

Flow cytometry

Quantitative measurement of cell cycle was performed by flow cytometry analysis of nuclear DNA contents following propidium iodide staining. Briefly, $10^6$ cells were harvested following dissociation with trypsin, washed twice in Phosphate Buffered Saline (PBS), and then fixed with 70% ethanol at -20°C. After one wash, cells were treated with RNAse A (1mg/ml) (Sigma-Aldrich, France) for 30 min at 4°C, incubated in 20 µg of propidium iodide (Sigma-Aldrich,

France) and analysed by flow cytometry. DNA content was analysed on >10,000 events by FACScan flow cytometry (Becton-Dickinson, France) with a 488-nm argon ion laser.

Measurement of telomerase activity

Frozen tissues or cells were lysed in 250 µl ice-cold 1X Chaps lysis buffer (Intergen Company, USA) and pulverized with the Fastprep device (BIO 101, France). The lysates were incubated for 30 min on ice, centrifuged at 18,000 g for 30 min at 4°C. The supernatants were collected and proteins concentrations were measured by a modified Lowry protein assay (Pierce, USA). Telomerase activity was assayed by the TRAP (Telomeric Repeat Amplification Protocol) method using the TRAP-eze ELISA Telomerase Detection Kit (Intergen Company, USA) following recommendations of the manufacturer. Briefly, 200 ng of total proteins were used. Lysis buffer without protein was used as negative control. Control cell extracts containing telomerase activity and a synthetic oligonucleotide with 8 telomeric repeats (supplied with the kit) were used as positive controls. For each cell sample a heat-inactivated (10 min at 85°C) negative control sample was also prepared. After amplification by PCR, the TRAP products were resolved on 12% polyacrylamide gels and visualized with 1:10,000 SYBR Gold (Molecular Probes, USA). Amplification efficiency in each reaction was determined using the provided internal control oligonucleotides forming a 36-bp band. Telomerase activity was evaluated using a semiquantitative ELISA method and expressed as the ratio of telomerase activity in sample over telomerase activity in telomerase-positive control cells. All experiments were performed at least in duplicate. Measurements were reported as mean ± standard error of the mean (SEM).

Immunodetection of P-Akt, total Akt and capsid protein

Frozen tissues or cells were lysed in 250 µl lysis buffer (0.5M Tris pH 8.0, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1mM $Na_3VO_4$, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptine and 10 µg/ml aprotinine),

homogenized with the Fastprep system device (BIO 101, France) and incubated for 30 min on ice. The lysates were centrifuged at 18,000 g for 30 min at 4°C and 50 µg of protein were separated on a 12% SDS-polyacrylamide gel and transferred onto a 0.2 µM nitrocellulose membrane (Biorad Laboratories, France). The membranes were pre-incubated with TSBT (25 mM Tris pH 7.6, 0.15 M NaCl, and 0.05% Tween 20) containing 5% nonfat dry milk for 1h at room temperature. After three washes in TSBT, the membranes were incubated overnight at 4°C with 1:1,000 rabbit polyclonal antibodies against P-Akt (serine 473) or total Akt (Cell Signalling, USA) diluted in TSBT containing 5% bovine serum albumine (BSA). For detection of capsid antigenic protein, membranes were incubated for 1 h at room temperature with 1:10,000 rabbit polyclonal antibody to JSRV capsid protein (generously provided by Dr J. De Martini, Fort Collins, Co, USA). Membranes were washed 3 times for 5 minutes at room temperature with TBST and incubated with horseradish peroxidase-labeled anti-rabbit IgG antibody (Sigma, France) diluted in TBST containing 5% nonfat dry milk (1:10,000) for 1 h at room temperature. The immunoreactive bands were detected with an enhanced chemiluminescence detection kit (Pierce, France), quantified with the Un-Scan-It software (Silk Scientific Corporation, USA) and expressed as percentage of sample in comparison to A549 control cells. Measurements of expression of P-Akt were reported as mean ± SEM. Protein quantity loaded onto gels was controlled by immunodetection of actin (Sigma, France) with a mouse monoclonal antibody against ß-actin (Sigma, France).

Statistical analysis

Statistical analyses were performed with the Prism4 software (GraphPad Software Inc, USA). The values for telomerase activity and expression level of P-Akt in tumoral and non tumoral samples were compared using the Mann-Whitney U-test. Tests were two-tailed except mentioned otherwise. Only p-values $< 0.05$ were considered as significant.

## Results

### Characterisation of tumours

Lung tissue was obtained from OPA tumours and non-tumoral control lungs. The diagnosis of OPA (invasive mixed adenocarcinoma with a bronchioloalveolar component, associated with acinar and (or) papillary growth patterns) was confirmed by histopathological examination in all tumours, whereas absence of tumour was confirmed in all control lungs. In addition, lung tissues were assessed for the presence of the JSRV provirus and of the capsid antigenic protein as described in Methods. JSRV proviral DNA was detected by PCR analysis in 11/12 (92%) tumour lungs, and none of the 4 control lungs. Similarly, the JSRV capsid protein was detected by western-blot analysis in 12/12 OPA lungs, but none of the 4 control lungs.

### Telomerase activity in tumours

In a first approach to study the involvement of telomerase activation in OPA, we measured telomerase activity in lung tissues from OPA tumours (n=12) and non-tumoral control lungs (n=4), using the TRAP assay as described. As illustrated in figure 1A, PCR products visualised as a DNA ladder on polyacrylamide gels indicated the presence of telomerase activity in all OPA samples (n=12), but none of the samples from control lungs. Semi-quantification using ELISA demonstrated that the telomerase activity was significantly higher in OPA tissues as compared to control lungs ($p=0.03$, one-tailed Mann-Whitney test) (Figure 1B). These results thus demonstrated that a high telomerase activity was present in tumours (invasive mixed adenocarcinoma with a bronchioloalveolar component) in which JSRV infection was confirmed.

### Characterisation of tumoral cell cultures

We next aimed at demonstrating that the telomerase activity observed in whole tumours was attributable to tumour cells *per se* (and not to accompanying non-tumoral cells present in the tumoral lung). Primary cultures were derived from tumours (n=12) and from control lungs (n=4) as described in Methods. These cells were characterized by immunocytochemistry using antibodies against specific markers of type-II pneumocytes (surfactant proteins A and C), and transmission electron microscopy (Archer et *al*, manuscript in preparation). As expected, primary cells derived from tumours and from normal lungs had a cuboidal morphology typical of epithelial cells. The purity of the cultures was confirmed by expression of the surfactant proteins A and C in >95% of the cells, and was maintained over all passages. The JSRV genome was detected by PCR analysis in cell cultures derived from all OPA lungs, but in none of the control type-II pneumocytes derived from control lungs. Cultured cells derived from tumours could be maintained for 7 to 10 passages, as compared to only 2 to 3 passages for control cells.

To assess the biological relevance of cell cultures derived from tumours and control lungs, we next analysed cell proliferation and distribution throughout the cell cycle of tumour and control cells. Using a MTT assay, we showed that cells derived from lung tumours had a statistically significant proliferative advantage as compared to control type-II pneumocytes derived from control lungs (Figure 2). Similarly, cell cycle analysis using flow cytometry indicated that the proportion of cells in S-phase was higher in cultures of tumoral cells (17% ± 3.2) than in control type-II pneumocytes (8.5% ± 1.9).

Telomerase activity in tumoral cell cultures

To demonstrate that the telomerase activity observed in whole tumours was attributable to tumour cells *per se*, telomerase activity was assessed in primary cell cultures derived from tumours and controls. Semi-quantitative measurement of telomerase activity by the TRAP assay followed by ELISA demonstrated a particularly high level of telomerase activity in 8 / 12 (67%)

primary cultures derived from tumours (Figure 1C), as compared with control type-II pneumocytes, in which a low level of enzymatic activity could be detected. Surprisingly, no telomerase activity was found in 4 / 12 (33%) cell cultures derived from tumours (in which telomerase activity had been detected in whole tumours). Therefore, these results showed that a high level of telomerase activity was present in a majority of cell cultures derived from lung tumours, and indicated that the telomerase activity observed in whole tumours was indeed attributable to tumour cells *per se* in a majority of tumours.

Disregulation of Akt in tumoral cell cultures

Regulation of telomerase activity is complex and may involve activation of TERT through its phosphorylation by the kinase Akt. In order to determine whether telomerase activation may be related to the Akt pathway, we next studied the phosphorylation state of Akt on serine-473 in OPA-derived cell cultures. Primary cells derived from tumours and control type-II pneumocytes were cultured in the absence of growth factors during 24 h, and then exposed or not for 30 min to epidermal growth factor (EGF) (100 ng/ml), a known activator of Akt in mammalian cells. Moderate expression of phosphorylated Akt was detected by western blotting at similar levels in unstimulated cells derived from lung tumours and control type-II pneumocytes (Figure 3), suggesting that culture conditions (including deprivation of growth factors) might lead to basal phosphorylation and activation of Akt in unstimulated cells. However, EGF stimulation *in vitro* lead to a dramatic increase in Akt activation in control cells, while cells derived from lung tumours were not responsive to stimulation by EGF (Figure 3). These results showed that the EGF - Akt pathway was disregulated in OPA cell cultures, with lack of Akt activation in response to growth factor stimulation in tumoral cell cultures.

Activation of Akt in tumours

To further characterise the disregulation of Akt in OPA, Akt activation was next studied in whole tumours (n=27) and normal lung tissues (n=14) using

western blotting analysis, and using human A549 epithelial cells as a reference. As shown in figure 4, significant expression of phosphorylated Akt was demonstrated in 10 / 27 (37%) OPA tumours, and none of 14 control lungs (table 1), while expression of total Akt and actin was similar between both groups. Thus, Akt is activated and may thus participate in telomerase activation in a significant proportion of OPA tumours.

## Discussion

In this study, we showed that telomerase is activated in OPA, a mixed-type adenocarcinoma of the lung with prominent bronchioloalveolar component. Telomerase activation was demonstrated in tumours as well as epithelial cell cultures derived from the tumours. We next investigated the activation of the regulatory kinase Akt as a potential activator of telomerase, and demonstrated that Akt is indeed activated in a proportion of tumours. Taken together, these results suggest that inhibition of cell senescence may be involved in tumorigenesis and accumulation of tumoral cells within the lung in OPA, and that Akt activation may participate in telomerase activation in a proportion of tumours.

pADC differs from other types of non-small cell lung cancer in several ways, including higher incidence in women, a lesser role of tobacco smoking, lack of distant metastatic spread, and higher rate of sensitivity to EGF-tyrosine kinase inhibitors. The clinical syndrome of pADC remains a rare presentation of non-small cell lung cancer, hampering research.

Ovine pulmonary adenocarcinoma (OPA) was chosen as a model of pADC since it shares with the human disease a variety of key features, including similar clinical and radiological presentation with progressive dyspnea, and abundant bronchorrhea ; multifocal pulmonary disease with alveolar consolidation and nodules ; and almost identical pathology (3). As in BAC, tumour cells in OPA derive from type-II pneumocytes and to a lesser extent Clara

cells. The BAC component of both the human and the ovine tumours is characterised by lepidic spread, where tumoral cells grow following the alveolar septa; as opposed to the rare form of pure BAC, mixed adenocarcinoma with bronchioloalveolar component includes evidence of stromal, vascular and (or) pleural invasion, together with associated acinar and mostly papillary growth patterns (1, 3). We have evidence that OPA is immunohistochemically similar to human mixed BAC (13), with expression of cytokeratin-7, nuclear expression of thyroid transcription factor-1, lack of expression of cytokeratin-20, and by electronic microscopy studies (data not shown). As opposed to previous works on OPA where studies were mostly conducted in immortalised cell lines or rodent fibroblasts tranfected with JSRV gene expression vectors (14-16), in the work reported herein we were able to derive primary cell cultures from naturally occurring tumours and control lungs. Genomic proviral DNA of the causative agent JSRV was detected only in cells derived from tumours. In addition, cells derived from OPA tumours expressed surfactant pulmonary-associated proteins A and C (markers of type-II pneumocytes), and thyroid transcription factor-1, indicating that their specific phenotype was maintained *in vitro*. Importantly, concordant results of telomerase activation were obtained in parallel in tumours and in epithelial cell cultures derived from the tumours, doubtlessly increasing the biological relevance of this observation.

We demonstrated a high level of telomerase activity in OPA lung tumours as compared to control non-tumoral lungs. Telomerase activity was detected in all OPA tumours, and was likely due to telomerase activation within tumour cells. As cells located within the tumour other than cancer cells *per se* (17-19) such as lymphocytes of the bronchial mucosa (20) may also express telomerase activity, we derived primary cell cultures from the same lung tumours; telomerase activity was found in two thirds of epithelial cell cultures derived from tumours, thus indicating that the telomerase activity observed in whole tumours was at least partly attributable to tumour cells *per se*. No telomerase activity was found in a third of cell cultures derived from telomerase-positive tumours; we hypothesize that activated lymphocytes or other inflammatory cells infiltrating the tumour may

have been responsible for telomerase activity in such cases, as previously reported (17, 18). It remains to be determined whether alternative pathways to telomerase activation may also contribute to escaping cell senescence in telomerase-negative tumour-cell cultures (21). Conversely, a low telomerase activity was found in control lung tissues and cell cultures, potentially resulting from a subpopulation of type-II pneumocytes with self-renewal capacities that are assumed to repair lung alveolar epithelium after injury (22 113). Alternatively, telomerase activity in control lungs may be due to activated lymphocytes within non-tumoral lungs as a result of a variety of infraclinical infections that are common in naturally bred animals, although this was not suggested by the pathological analysis of control lungs in our study.

Telomerase activation in OPA suggests that inhibition of cell senescence may be involved in tumorigenesis and in the process of accumulation of tumour cells along the alveolar septa. Telomeres terminate eukaryotic chromosomes and are involved in chromosome integrity. Continued telomere shortening in normal somatic cells eventually results in an arrest of cell proliferation, a physiological process referred to as cell senescence that controls cell lifespan and limits the number of cell divisions (9). Activation of telomerase activity, mainly dependent on its catalytic subunit TERT, contributes to telomere length maintenance and inhibition of cell senescence, and thus to cell immortalization and cancer (9). Hence, we found maintained telomere length in OPA (Leroux et al, manuscript in preparation). In addition to its enzymatic activity, the TERT subunit may enhance genomic stability by direct interaction with telomeres (23), and may participate in the regulation of p53-induced apoptosis (24). Telomerase activation is an early event in carcinogenesis, concomitant with P53 overexpression, Rb inactivation, and decrease in Bcl-2/Bax ratio in high grade preinvasive bronchial lesions (beginning at the level of moderate dysplasia), suggesting a coupling between telomerase activation, proliferation, and resistance to apoptosis (20). Although telomerase activation has been described in a variety of human cancers including lung cancer (10), it has not been extensively studied in pADC and mixed adenocarcinoma with BAC features. Some telomerase activity as assessed by a

non-quantitative method was detected in 4/10 cases of human BAC presenting as solitary nodules (25), and in 97% of peripheral and small-sized nonmucinous BAC (26), but such tumours were unlikely representative of the clinically-defined pADC (1). Interestingly, telomerase activation alone is not sufficient to transform human cells *in vitro* (27). Mechanisms other than cell senescence inhibition (such as inhibition of apoptosis, or deregulation of cell proliferation) may also take place in pADC and OPA, as suggested by the increased proliferation of tumour-derived cell cultures which was observed as compared to control cells.

The complex regulation of telomerase activity involves several pathways including the phosphorylation and activation of TERT by Akt (11), a kinase involved in the regulation of processes characteristic of cancer such as cell proliferation and survival, cell size, response to nutrient availability, angiogenesis and tissue invasion (28). Overexpression of Akt can transform NIH3T3 cells, indicating that Akt is a potential oncogene (29). We have studied the potential role of Akt in telomerase activation, and observed disregulation of the EGF - Akt pathway in OPA tumors. Hence, Akt activation was present in a significant proportion of OPA tumours but none of control lungs. Lack of Akt activation in response to EGF stimulation was further demonstrated in cell cultures derived from tumours, as compared to control cells (in which EGF stimulation dramatically induced Akt phosphorylation), demonstrating that the EGF - Akt is disregulated in cells derived from tumours. The basal level of Akt activation was comparable in cell cultures derived from tumours and non tumoral lung, a finding likely related to artefacts of cell culture (deprivation in growth factors or replacement of medium may have contributed to moderate activation of Akt in both tumoral and non tumoral cell cultures (28)). Taken together, our results suggest that Akt activation may participate in telomerase activation and regulation of cell senescence in OPA, as recently shown in human mixed adenocarcinoma with BAC features (30). Alternative pathways such as the Ras-MEK-MAPK pathway (15) are also likely to participate to telomerase regulation in this tumour, as Akt activation was not found in all telomerase-positive tumours.

Several studies have identified the gene encoding the envelope of JSRV, (the causative agent of OPA) as a potential oncogene, and have shown that its overexpression was sufficient to transform rodent fibroblasts (31) and epithelial cell lines *in vitro* (14). Transfection of JSRV env in mammalian cells induces constitutive activation of Akt; studies using chemical inhibitors of the PI3K-Akt-mTOR pathway have further demonstrated the central role of Akt and of the Ras-MEK-MAPK pathway in JSRV-induced cell transformation (15, 32), although the precise mechanism of Akt activation in naturally occurring OPA remains speculative.

Recently, activating mutations of the EGF receptor (EGFR) mutually exclusive of K-ras mutations have been reported in lung adenocarcinoma especially with a prominent nonmucinous BAC component, and may be associated with clinical response to the EGFR tyrosine kinase inhibitors gefitinib and erlotinib (33-35). Activation of EGFR is associated with activation of downstream signalling pathways including the PI3K-Akt and the Ras-MEK-ERK pathways in this tumour (30, 36). In addition, increased Akt phosphorylation (not inhibited by EGFR tyrosine kinase inhibitors) has been demonstrated *in vitro* in specific subpopulations of adenocarcinoma cell lines that have become naturally resistant to gefitinib, despite the loss of the EGFR gene mutation when compared with parental cell lines (37). These observations thus indicate that constitutive activation of the PI3K-Akt-pathway may occur independently of EGFR mutations, and may be an attractive therapeutic target in lung adenocarcinoma. Studies are currently undertaken to determine whether disregulation of the EGF – Akt pathway in cells derived from OPA may be similarly related to mutations of the EGFR gene.

In conclusion, we have shown that telomerase activation takes place in OPA tumour cells and may be partly attributable to Akt activation. Telomerase activation may contribute to the accumulation of tumour cells within the lung through inhibition of cellular senescence. Future strategies for the treatment of human pADC with BAC features may be envisioned, through telomerase specific

inhibition and (or) modulation of the Akt pathway. OPA provides an attractive model for the preclinical assessment of the efficacy of innovative approaches to treat this incurable disease.

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Table 1. Activation of Akt in OPA tumours. Phosphorylated Akt was detected by western-blotting, quantified by densitometry, and expressed in comparison to phosphorylated Akt in human A549 epithelial cells.

| Level of phosphorylated Akt | Non tumoral lung | Tumours |
|---|---|---|
| - | 10/14 (71.4%) | 16/27 (59.3%) |
| + | 4/14 (28.6%) | 1/27 (3.7%) |
| ++ | 0/14 (0%) | 10/27 (37%) |

(-) 0-9% of the level of phosphorylated Akt in A549 cells; (+) 10-49% of the level of phosphorylated Akt in A549 cells; (++) >50% of the level of phosphorylated Akt in A549 cells;

## Figure Legends

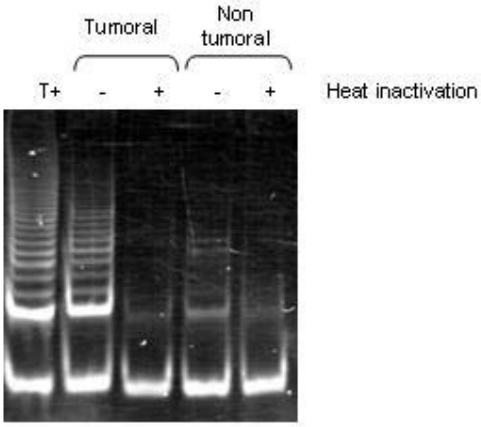
Figure 1: Activation of telomerase in lung tissues and primary cell cultures derived from lung tumours and control lungs. Telomerase activity was measured by a TRAP assay and expressed as the ratio of telomerase activity in sample / positive control cells (mean ± SEM) in duplicate experiments. (A) Polyacrylamide gel analysis of PCR products of the TRAP assay in lung tumours and control lung tissues. Heat-inactivated samples were used as negative controls. T+: telomerase-positive control cell line. (B) Telomerase activity in OPA and control lung tissues. (C) Telomerase activity in cell cultures derived from tumours and control lungs.

Figure 2: Cell proliferation of cultures derived from OPA tumours and normal lungs (MTT assay).

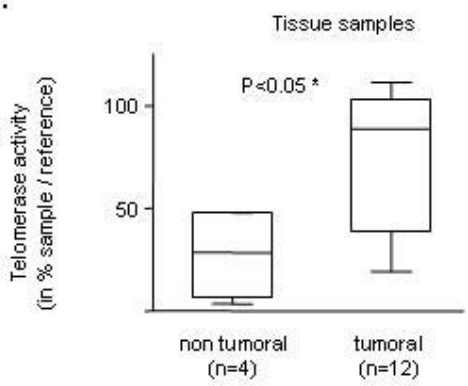
Figure 3: Disregulation of Akt in cells derived from OPA tumours. (A) Western blot analysis of phosphorylated-Akt protein (Ser-473) in cell lysates from cultures derived from OPA lung tumours and normal lungs. Cells were deprived of serum and growth factors for 24h and exposed or not to stimulation by EGF (100 ng/ml) for 30 minutes. The amount of total Akt was determined by reprobing the membranes with an anti-Akt antibody (Cell Signalling). (B) Quantification by densitometry of phosphorylated and total Akt (mean ± SEM).

Figure 4: Activation of Akt in OPA tumours. (A) Detection by western-blot of phosphorylated Akt (Ser-473), total Akt and β-actin in lysates of tumoral and control lung tissues. (B) Quantification by densitometry, expressed as percentage of phosphorylated Akt in sample compared to EGF-stimulated A549 cells as reference. Each dot represents the mean of 2 to 3 independent experiments ; horizontal bars indicate the median value.

A:


Tumoral
Non tumoral
T+
-
+
-
+
Heat inactivation

B:


Tissue samples
P<0.05 *
Telomerase activity (in % sample / reference)
100
50
non tumoral (n=4)
tumoral (n=12)

C :

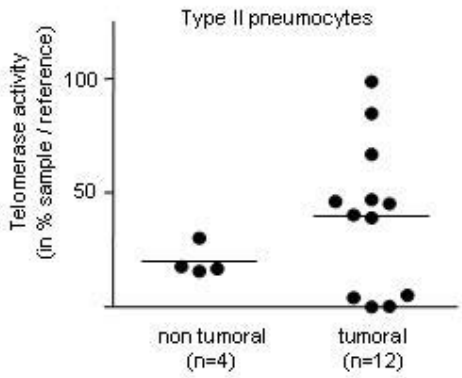

Type II pneumocytes
Telomerase activity (in % sample / reference)
100
50
non tumoral (n=4)
tumoral (n=12)

Fig1

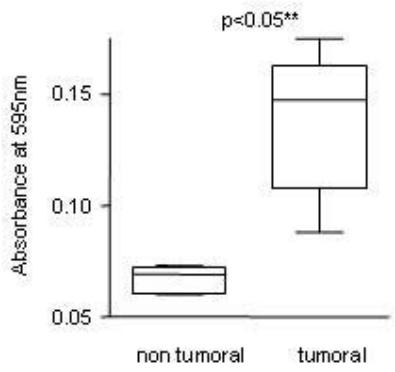
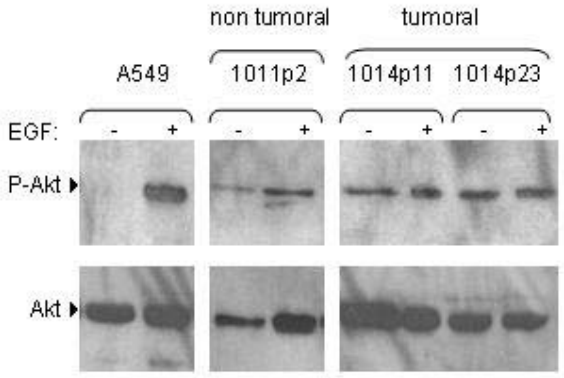



Fig2

**A:**


non tumoral
tumoral
A549
1011p2
1014p11
1014p23
EGF:
- + - + - + - +
P-Akt
Akt

**B:**

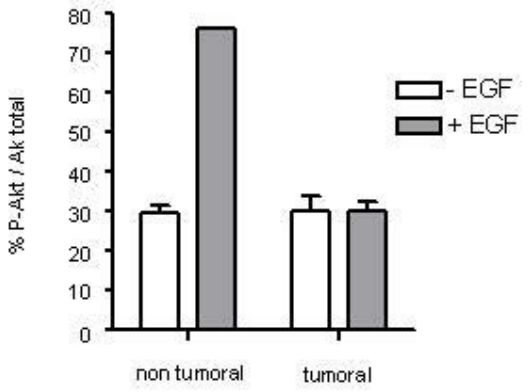
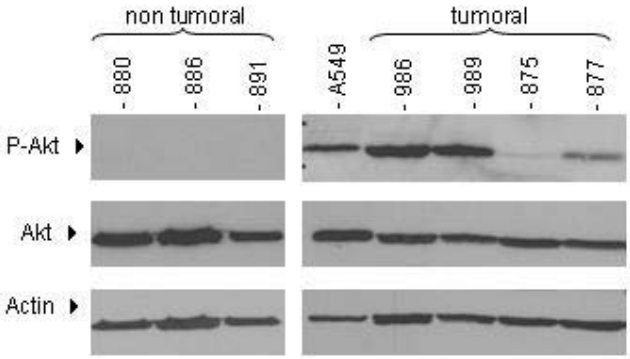

% P-Akt / Ak total
80
70
60
50
40
30
20
10
0
- EGF
+ EGF
non tumoral
tumoral

Fig3

**A:**


non tumoral
tumoral
- 880
- 886
- 891
- A549
- 986
- 989
- 875
- 877
P-Akt
Akt
Actin

**B:**

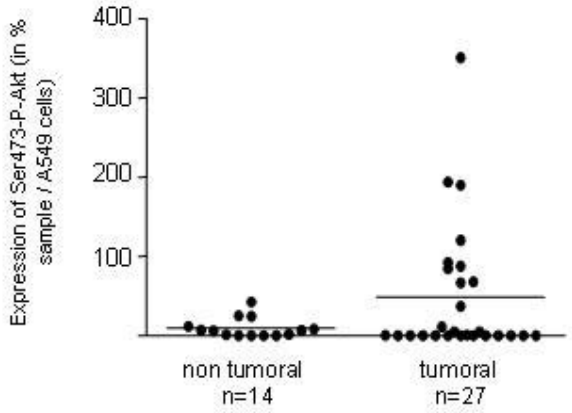

Expression of Ser473-P-Akt (in % sample / A549 cells)
400
300
200
100
non tumoral
n=14
tumoral
n=27

Fig4