Telomerase activation in a model of lung adenocarcinoma

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

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 using the telomeric repeat amplification protocol method. Phosphorylation of Akt was detected by Western blotting.

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

Telomerase activation takes place in ovine pulmonary adenocarcinoma 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 a bronchioloalveolar component. Further work is necessary to identify alternative signalling pathways of telomerase activation in tumours.

KEYWORDS: Akt, bronchioloalveolar carcinoma, jaagsiekte sheep retrovirus, lung cancer, telomerase, type-II pneumocyte

Lung cancer is the leading cause of cancer mortality in developed countries. Non-small cell lung cancer represents ~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 pneumonic-type adenocarcinoma (P-ADC), which associates typical radiological features with diffuse or disseminated alveolar condensation with or without an air broncho-gram showing progression towards pulmonary right-to-left shunting, together with evidence of adenocarcinoma tumour cells in the lung [1]. The histological pattern in P-ADC 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 a 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 which 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 a BAC component, together with papillary and acinar growth patterns [2, 3]. OPA also shares striking clinical and radiological homology with human P-ADC, including progressive intrapulmonary spread and a lack of distant extrathoracic metastasis [3], and thus represents a unique natural and reproducible animal model of per-ipheral lung cancer, especially P-ADC.

In contrast with previous studies, which focused on defining the oncogenic properties of JSRV structural proteins in rodents [5], the aim of the present study was to identify 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 apoptotic 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 mechanisms including dysfunctional telomeres [7]. More recently, cellular senescence has been also demonstrated *in vivo* in pre-malignant but not malignant cells, suggesting that it may be an important anti-cancer defence [8].

Cellular senescence is mainly regulated by telomerase, a ribonucleoprotein enzyme able to stabilise telomere length by *de novo* synthesis of telomeres and elongation of existing telomeres. Telomerase activation is considered mandatory in order 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], whereas telomerase activity is repressed in most normal somatic cells. Complex regulation of telomerase activity may include the phosphatidylinositol-3′-kinase (PI3K) pathway through phosphorylation (P) 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 the present study, increased telomerase activity was demonstrated in tumours and 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. It was next shown that the regulatory kinase Akt is constitutively activated in OPA tumours and dysregulated 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 with loss of weight, dypsnoea and profuse lung secretions suggestive of OPA or without signs of OPA. Tissue sections were sampled and stored at -70°C until use or fixed in formalin for histopathological examination. The tissues were classified as tumoral and nontumoral lung (or controls) following the current 2004 World Health Organization classification [2].

Culture of alveolar epithelial type II cells
Tumour cells and normal type-II pneumocytes were isolated from ovine lungs and characterised. Briefly, tissue samples were digested overnight at 4°C in Eagle's minimum essential medium (Eurobio, Courtaboeuf, France) containing 0.025% collagenase I, 10 $\mu g \cdot mL^{-1}$ deoxyribonuclease, 1 $mg \cdot mL^{-1}$ protease XIV (Sigma-Aldrich, Saint-Quentin-Fallavier, France), 50 $\mu g \cdot mL^{-1}$ streptomycin, 50 $U \cdot mL^{-1}$ penicillin, 50 $\mu g \cdot mL^{-1}$ gentamicin, 2.5 $\mu g \cdot mL^{-1}$ amphotericin B and 100 $U \cdot mL^{-1}$ nystatin. The cells were then homogenised and filtered through a 40-μm filter in order to eliminate cell debris. After centrifugation for 10 min at $430 \times g$ at 4°C, pulmonary cells were plated onto collagen, laminin and fibronectin-coated plates in selective medium for epithelial cells (Quantum 286; PAA Laboratories, Pasching, Austria) containing 5 $ng \cdot mL^{-1}$ hepatocyte growth factor and 10 $ng \cdot mL^{-1}$ keratinocyte growth factor (Abcys, Paris, France). Cells derived from OPA tumours (n=12) and nontumoral lungs (n=4) are referred to as tumoral and control cells, respectively.

Detection of jaagsiekte sheep retrovirus proviral DNA
Total genomic DNA from lung tumours and control lungs was prepared using the Fastprep device following the recommendations of the supplier (Qbiogene, Carlsbad, CA, USA). A semi-nested PCR protocol was used to detect proviral DNA. Briefly, reaction mixtures contained 500 ng total genomic DNA, 50 μL 1× buffer (1.5 mM $MgCl_2$, 67 mM Tris-HCl (pH 8.8), 16 mM $(NH_4)_2SO_4$, 0.01% Tween 20), 0.2 mM each deoxynucleotide triphosphate, 0.2 mM each primer and 1.25 U Taq polymerase (Eurobio). The first round of PCR was performed with the primers JSRV42 (sense), 5′-CTTTGTATTTCCCTGTGTCG-3′, corresponding to nucleotides 7041-7060 in the *env* gene of the JSRV genomic sequence (GenBank accession number AF105220), and JSRV53 (antisense), 5′-GGATTCTTACACAATCACC-3′, corresponding to nucleotides 7381-7362 in the long terminal repeat (LTR) U3 region of the JSRV genomic sequence. The second round of PCR was performed with 1–5 μL PCR product using JSRV42 and JSRV52 (antisense), 5′-CACCGGATTCTTATATAATC-3′, corresponding to nucleotides 7366-7346 in the LTR U3 region of the JSRV genomic sequence. PCR reactions were performed as follows: 5 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 50°C and 1 min at 72°C, and with a final elongation of 10 min at 95°C.

Cell proliferation assay
Cell numbers were determined using a 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyl tetrazolium bromide (MTT) assay. Briefly, 5,000 $cells \cdot well^{-1}$ were plated in 96-well plates and incubated in a 5% carbon dioxide atmosphere for 96 h at 37°C. MTT was incorporated as recommended for the MTT Cell Proliferation Assay kit (Chemicon, Temecula, CA, USA). MTT (10 μL) was added to each well for 4 h at 37°C. After solubilisation in 100 μL 1N isopropanol/0.04N HCl, absorbance was read at 595 nm using a Wallac Victor II device (Perkin-Elmer, Wellesley, MA, USA). Each measurement was performed in triplicate.

Flow cytometry
Quantitative measurement of the cell cycle was performed using flow cytometric analysis of nuclear DNA contents following propidium iodide staining. Briefly, 1×10^6 cells were harvested following dissociation with trypsin, washed twice in PBS and then fixed with 70% ethanol at -20°C. After one wash, cells were treated with 1 $mg \cdot mL^{-1}$ ribonuclease A (Sigma-Aldrich) for 30 min at 4°C, incubated in 20 μg propidium iodide (Sigma-Aldrich) and analysed by flow cytometry. The DNA content was analysed for >10,000 events using FACScan flow cytometry (Becton-Dickinson, Franklin Lakes, NJ, USA) with a 488-nm argon ion laser.

Measurement of telomerase activity
Frozen tissues or cells were lysed in 250 μl ice-cold 1×3-((3-cholamidopropyl)diethylammonio)-1-propane sulphonate

(CHAPS) lysis buffer (Intergen Company, Purchase, NY, USA) and pulverised using the Fastprep device. The lysates were incubated on ice for 30 min and then centrifuged at $18,000 \times g$ for 30 min at 4°C. The supernatants were collected and protein concentrations were measured with a modified Lowry protein assay (Perbio Science, Brébières, France). Telomerase activity was assayed using the telomeric repeat amplification protocol (TRAP) method using the TRAP-eze ELISA Telomerase Detection Kit (Intergen Company) following the manufacturer's recommendations. Briefly, 200 ng total protein was used. Lysis buffer without protein was used as negative control. Control cell extracts containing telomerase activity and a synthetic oligonucleotide with eight 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 visualised with 1:10,000 SYBR Gold (Molecular Probes (Invitrogen), Eugene, OR, 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 semi-quantitative ELISA method and expressed as the ratio of telomerase activity in the sample to telomerase activity in telomerase-positive control cells. All experiments were performed at least in duplicate. Measurements were reported as mean±SEM.

Immunodetection of phosphorylated 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, 1 mM Na_3VO_4, 1 mM phenylmethylsulphonyl fluoride, 10 μg·mL^{-1} leupeptin,10 μg·mL^{-1} aprotinin), homogenised with the Fastprep system device (Qbiogene) and incubated for 30 min on ice. The lysates were centrifuged at $18,000 \times g$ for 30 min at 4°C and 50 μg protein were separated on a 12% sodium dodecylsulphate-polyacrylamide gel and transferred onto a 0.2-μM nitrocellulose membrane (Biorad Laboratories, Marnes-la-Coquette, France). The membranes were pre-incubated with 25 mM Tris (pH 7.6)/0.15 M NaCl/ 0.05% Tween 20 (TSBT) containing 5% non-fat dry milk for 1 h at room temperature (20°C). After three washes in TSBT, the membranes were incubated overnight at 4°C with a 1:1,000 dilution of rabbit polyclonal antibodies directed against P-Akt (serine 473) or total Akt (Cell Signalling, Danvers, MA, USA) diluted in TSBT containing 5% bovine serum albumin. For detection of capsid antigenic protein, membranes were incubated for 1 h at room temperature with 1:10,000 diluted rabbit polyclonal antibody directed against JSRV capsid protein (generously provided by J.C. De Martini, Dept of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO, USA). Membranes were washed three times for 5 min at room temperature with TBST and incubated with horseradish peroxidase-labelled anti-rabbit immunoglobulin G (Sigma-Aldrich) diluted in TBST containing 5% non-fat dry milk (1:10,000) for 1 h at room temperature. The immunoreactive bands were detected using an enhanced chemiluminescence detection kit (Perbio Science), quantified with the Un-Scan-It software (Silk Scientific Corporation, Orem, UT, USA) and expressed as a percentage compared to A549 control cells. Measurements of expression of P-Akt were

reported as mean±SEM. The protein quantity loaded on to gels was controlled by immunodetection of actin (Sigma-Aldrich) with a mouse monoclonal antibody directed against β-actin (Sigma-Adlrich).

Statistical analysis

Telomerase activity and P-Akt expression in tumoral and nontumoral samples were compared using the Mann–Whitney U-test. Tests were two-tailed unless otherwise detailed. Only p-values of <0.05 were considered significant.

RESULTS

Characterisation of tumours

Lung tissue was obtained from OPA tumours and nontumoral 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 for 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 the capsid antigenic protein. JSRV proviral DNA was detected by PCR analysis in 11 out of 12 (92%) tumour lungs and none of the four control lungs. Similarly, the JSRV capsid protein was detected by Western blot analysis in 12 out of 12 OPA lungs but none of the four control lungs.

Telomerase activity in tumours

As a first approach to studying the involvement of telomerase activation in OPA, telomerase activity was measured in lung tissues from OPA tumours (n=12) and nontumoral control lungs (n=4) using the TRAP assay. 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 compared to control lungs (p=0.03 (one-tailed Mann–Whitney test); fig. 1b). These results thus demonstrate that high telomerase activity was present in the tumours (invasive mixed adenocarcinoma with a bronchioloalveolar component) in which JSRV infection was confirmed.

Characterisation of tumoral cell cultures

The next aim was to demonstrate that the telomerase activity observed in whole tumours was attributable to tumour cells *per se* (and not accompanying nontumoral cells present in the tumoral lung). Primary cultures were derived from tumours (n=12) and control lungs (n=4). These cells were characterised immunocytochemically using antibodies directed against specific markers of type-II pneumocytes (surfactant proteins A and C) and transmission electron microscopy. As expected, primary cells derived from tumours and normal lungs showed the cuboidal morphology typical of epithelial cells. The purity of the cultures was confirmed by expression of 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 seven to ten passages, as compared to only two or three passages for control cells.
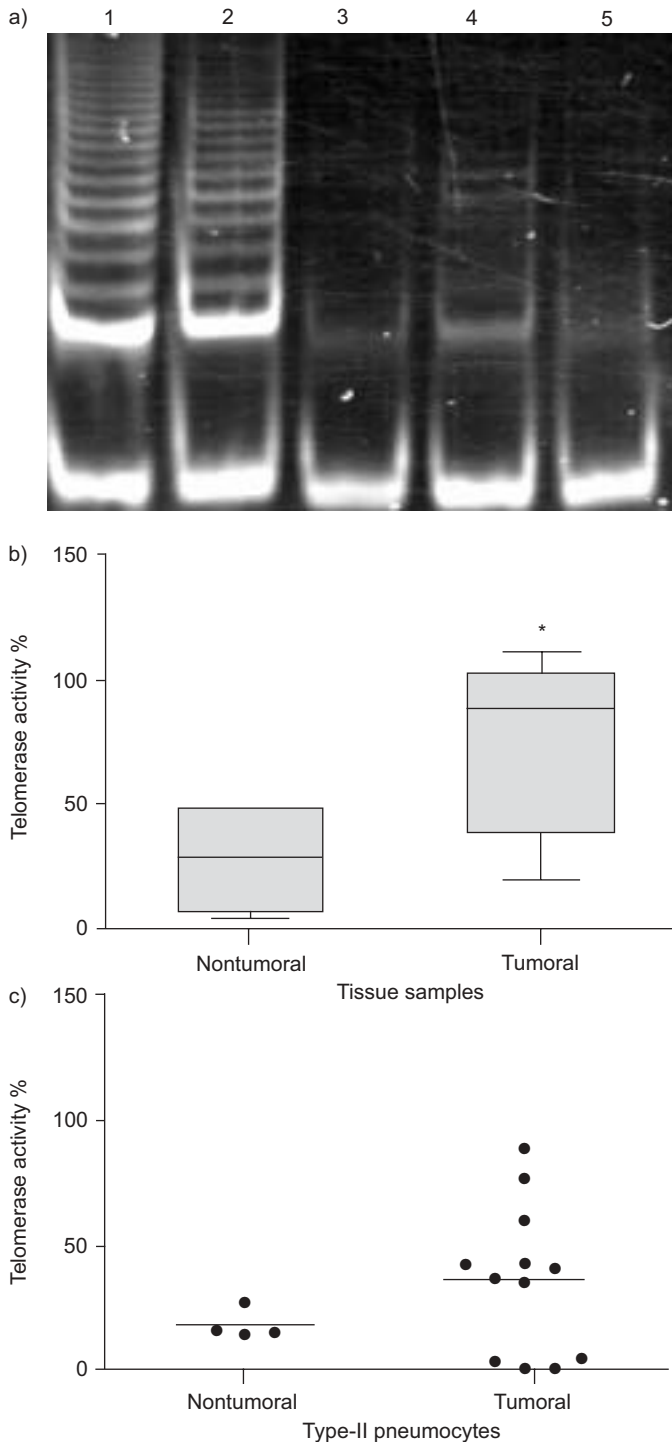
In order to assess the biological relevance of cell cultures derived from tumours and control lungs, cell proliferation and distribution were analysed throughout the cell cycle of tumour and control cells. Using an MTT assay, it was shown that cells derived from lung tumours exhibited a significant proliferative advantage compared to control type-II pneumocytes derived from control lungs (fig. 2). Similarly, cell cycle analysis using flow cytometry indicated that the proportion of cells in the S phase was higher in cultures of tumoral cells ($17 \pm 3.2\%$) than in control type-II pneumocytes ($8.5 \pm 1.9\%$).

Telomerase activity in tumoral cell cultures

In order 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 using the TRAP assay followed by an ELISA demonstrated a particularly high level of telomerase activity compared to control type-II pneumocytes, in which a low level of enzymatic activity could be detected, in eight out of 12 (67%) primary cultures derived from tumours (fig. 1c). Surprisingly, no telomerase activity was found in four out of 12 (33%) cell cultures derived from tumours (in which telomerase activity had been detected in the whole tumour). Therefore, these results show that a high level of telomerase activity was present in the 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 the majority of tumours.

Dysregulation 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 might be related to the Akt pathway, the phosphorylation state of serine 473 of Akt was studied in OPA-derived cell cultures. Type-II pneumocytes derived from tumours and normal lungs were cultured in the absence of growth factors for 24 h, and
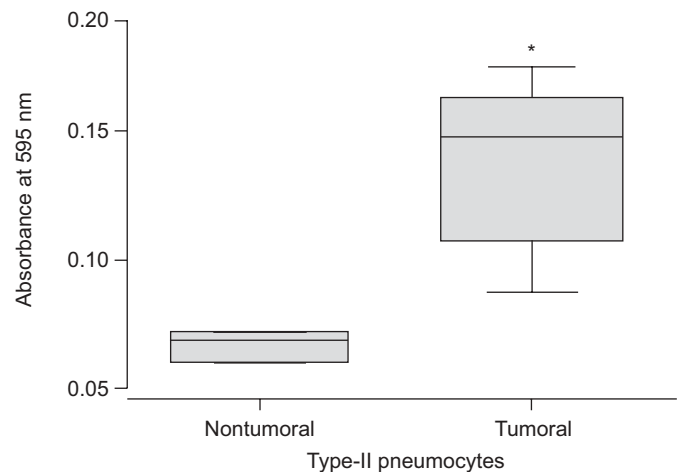

FIGURE 1. a) Polyacrylamide gel analysis of PCR products of the telomeric repeat amplification protocol assay in lung tumours (lanes 2 and 3) and control lung tissues (lanes 4 and 5). Heat-inactivated samples (lanes 3 and 5) were used as negative controls, and a telomerase-positive control cell line as a positive control (lane 1). Telomerase activity in: b) ovine pulmonary adenocarcinoma (n=12) and control lung tissues (n=4); and c) cell cultures (type-II pneumocytes) derived from tumours (n=12) and control lungs (n=4). Boxes represent median and vertical bars represent interquartile range. Horizontal bars represent medians *: p<0.05.

FIGURE 2. Cell proliferation of cultures derived from ovine pulmonary adenocarcinoma tumours and normal lungs (3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyl tetrazolium bromide assay). Boxes represent median and interquartile range, with vertical bars representing range. *: p<0.05.

then exposed or not to 100 ng·mL^{-1} epidermal growth factor (EGF), a known activator of Akt in mammalian cells, for 30 min. Similar moderate expression of P-Akt was detected by Western blotting in unstimulated cells derived from lung tumours and control type-II pneumocytes (fig. 3), suggesting that the culture conditions (including deprivation of growth factors) might lead to basal phosphorylation and activation of Akt in unstimulated cells. However, EGF stimulation *in vitro* led to a dramatic increase in Akt activation in control cells, whereas cells derived from lung tumours were not responsive to stimulation by EGF (fig. 3). These results showed that the EGF–Akt pathway was dysregulated in OPA cell cultures, with lack of Akt activation in response to growth factor stimulation in tumoral cell cultures.

Activation of Akt in tumours

In order to further characterise the dysregulation of Akt in OPA, Akt activation was next studied in whole tumours (n=27) and normal lung tissues (n=14) using Western blotting analysis, with human A549 epithelial cells as a reference. As shown in figure 4, significant expression of P-Akt was
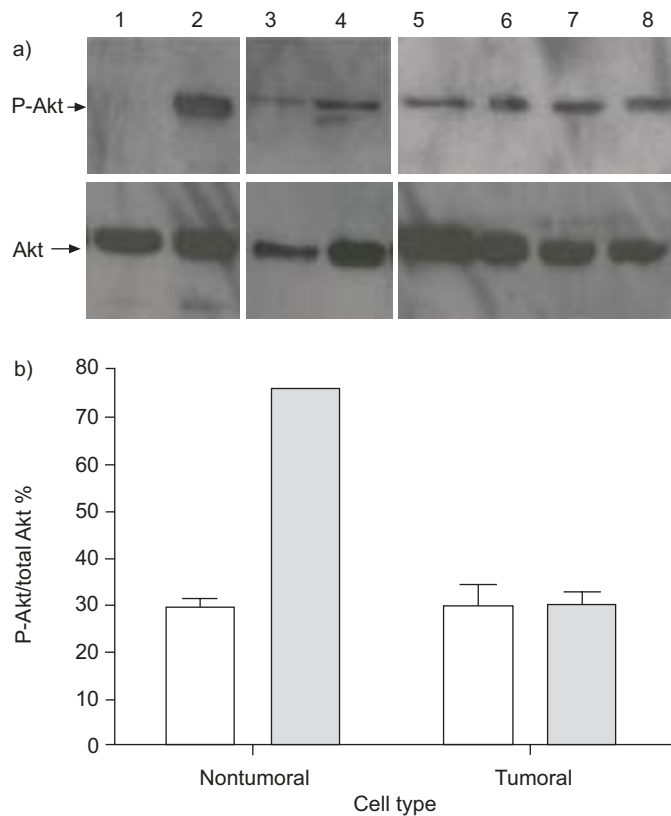
demonstrated in 10 of 27 (37%) OPA tumours and none of 14 control lungs (table 1), whereas expression of total Akt and actin were similar between the two groups. Thus Akt is activated and may thus participate in telomerase activation in a significant proportion of OPA tumours.

DISCUSSION

In the present study, it was shown that telomerase is activated in OPA, a mixed-type adenocarcinoma of the lung with a prominent bronchioloalveolar component. Telomerase activation was demonstrated in tumours as well as in epithelial cell cultures derived from those tumours. Next the activation of the regulatory kinase Akt, as a potential activator of telomerase, was investigated, and it was 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.

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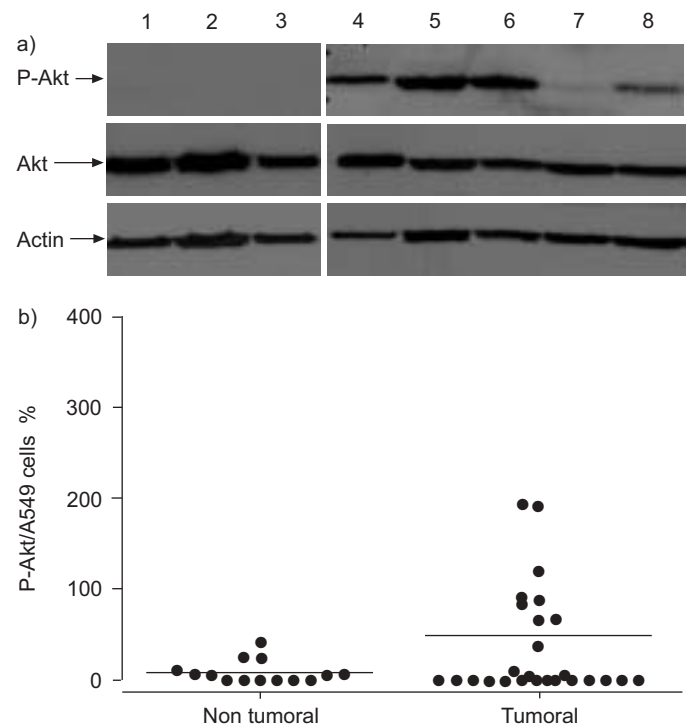

FIGURE 3. Dysregulation of Akt in cells derived from ovine pulmonary adenocarcinoma (OPA) tumours. Cells were deprived of serum and growth factors for 24 h and then exposed (lanes 2, 4, 6 and 8; ▒) or not (lanes 1, 3, 5 and 7; □) to stimulation with 100 ng·mL^{-1} epidermal growth factor (EGF) for 30 min. a) Western blot analysis of phosphorylated (P)-Akt (serine 473) in cell lysates from cultures derived from OPA lung tumours (lanes 5–8), A549 control cells (lanes 1 and 2) and normal lungs (lanes 3 and 4). The amount of total Akt was determined by re-probing the membranes with an antibody directed against Akt. b) Densitometric quantification of P-Akt and total Akt detected by Western blot analysis. Data are presented as mean±SEM.

FIGURE 4. Activation of Akt in ovine pulmonary adenocarcinoma tumours. a) Western blot analysis of phosphorylated (P)-Akt (serine 473), total Akt and β-actin in lysates of tumoral (lanes 5–8) and control lung tissues (lanes 1–3) and A549 control cells (lane 4). b) Densitometric quantification of P-Akt in sample (tumoral (n=27) and nontumoral (n=14)) relative to reference epidermal growth factor-stimulated A549 cells. Points represent means of two or three independent experiments; horizontal bars indicate medians.

TABLE 1 Activation (phosphorylation (P)) of Akt# in ovine pulmonary adenocarcinoma tumours

P-Akt	Nontumoral lung	Tumours
-	10 (71.4)	16 (59.3)
+	4 (28.6)	1 (3.7)
++	0 (0)	10 (37)

Data are presented as n (%). -: 0–9%; +: 10–49%; ++: >50% of P-Akt level in A549 cells. #: P-Akt was detected by Western blotting (whole tumours (n=27) and normal lung tissues (n=14)), quantified densitometrically and expressed relative to P-Akt levels in human A549 epithelial cells.

OPA was chosen as a model of P-ADC since it shares with the human disease a variety of key features, including similar clinical and radiological presentation with progressive dyspnoea, and abundant bronchorrhoea; 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, whereby 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]. There is evidence that OPA is immunohistochemically similar to human mixed BAC [13], with expression of cytokeratin 7, nuclear expression of thyroid transcription factor-1 and lack of expression of cytokeratin 20, and by electronic microscopic studies (data not shown). As opposed to previous work on OPA, in which studies were mostly conducted in immortalised cell lines or rodent fibroblasts transfected with JSRV gene expression vectors [14–16], in the present study, it was possible 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 epithelial cell cultures derived from the tumours, doubtlessly increasing the biological relevance of the present observation.

A high level of telomerase activity was demonstrated in OPA lung tumours compared to control nontumoral lungs. Telomerase activity was detected in all OPA tumours, and was probably 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, primary cell cultures were derived 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; the present authors hypothesise 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, 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]. Alternatively, telomerase activity in control lungs may be due to activated lymphocytes within nontumoral 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 the present 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 chromosomal integrity. Continued telomere shortening in normal somatic cells eventually results in an arrest of cell proliferation, a physiological process referred to as cell senescence, which 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 immortalisation and cancer [9]. Hence, maintained telomere length was found in OPA (data not shown). 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, retinoblastoma protein (Rb) inactivation and a decrease in B-cell leukaemia/lymphoma 2 gene product (Bcl-2)/Bcl-2-associated X protein (Bax) ratio in high-grade pre-invasive 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 P-ADC and mixed adenocarcinoma with BAC features. Some telomerase activity, as assessed by a nonquantitative method, was detected in four of 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 probably not representative of the clinically defined P-ADC [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 P-ADC and OPA, as suggested by the increased proliferation of tumour-derived cell cultures that was observed 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]. The potential role of Akt in telomerase activation was studied, and dysregulation of the EGF–Akt pathway observed in OPA tumours. Hence, Akt activation was present in a significant proportion of OPA tumours but none of the 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 pathway is dysregulated in cells derived from tumours. The basal level of Akt activation was comparable in cell cultures derived from tumours and nontumoral lung, a finding probably related to artefacts of cell culture (deprivation of growth factors or replacement of medium may have contributed to moderate activation of Akt in both tumoral and nontumoral cell cultures [28]). Taken together, the present 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–mitogen-activated protein kinase kinase (MEK)–mitogen-activated protein kinase (MAPK) pathway [15] are also likely to participate in 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 (env), the causative agent of OPA, as a potential oncogene, and have shown that its overexpression is 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–mammalian-target-of-rapamycin 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–extracellular-signal-regulated-kinase 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 compared to 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 being undertaken in order to determine whether dysregulation of the EGF–Akt pathway in cells derived from OPA may be similarly related to mutations of the EGFR gene.

In conclusion, it has been shown that telomerase activation takes place in ovine pulmonary adenocarcinoma 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 pneumonic-type adenocarcinoma with bronchioloalveolar carcinoma features may be envisioned, through telomerase specific inhibition and/or modulation of the Akt pathway. Ovine pulmonary adenocarcinoma provides an attractive model for the preclinical assessment of the efficacy of innovative approaches to treat this incurable disease.

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