

Distinct pattern of E2F1 expression in human lung tumours: E2F1 is upregulated in small cell lung carcinoma

Béatrice Eymin, Sylvie Gazzeri, Christian Brambilla, Elisabeth Brambilla, Pr Elisabeth Brambilla

▶ **To cite this version:**

Béatrice Eymin, Sylvie Gazzeri, Christian Brambilla, Elisabeth Brambilla, Pr Elisabeth Brambilla. Distinct pattern of E2F1 expression in human lung tumours: E2F1 is upregulated in small cell lung carcinoma: E2F1 expression in human lung tumours. Oncogene, 2001, 20 (14), pp.1678-87. 10.1038/sj.onc.1204242 . inserm-02345421

HAL Id: inserm-02345421
https://inserm.hal.science/inserm-02345421

Submitted on 4 Nov 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distinct pattern of E2F1 expression in human lung tumours: E2F1 is upregulated in small cell lung carcinoma

Béatrice Eymin, Sylvie Gazzeri, Christian Brambilla and Elisabeth Brambilla [1].

Groupe de Recherche sur le Cancer du Poumon, EA 2021, Equipe INSERM 9924,

Institut Albert Bonniot, 38706 La Tronche Cedex, France

Running title: E2F1 expression in human lung tumours

Keywords: carcinogenesis, cell cycle, E2F1, expression, lung tumour

[1] To whom requests should be addressed.

Pr. Elisabeth Brambilla

Tel: (33) 4 76 76 54 86

Fax: (33) 4 76 76 59 49

E-mail: Elisabeth.Brambilla@ujf-grenoble.fr

Abstract

The transcription factor E2F1 is a key component of cell cycle that acts to transactivate genes required for S phase entry. Thus, it plays an important role in cellular proliferation, oncogenesis and differentiation. In order to investigate its potential implication in human lung carcinogenesis, we studied E2F1 protein expression by western blotting and immunohistochemistry in a series of 58 human lung tumours of all histological types. We showed that E2F1 product was overexpressed in 92% (24/26) of small cell lung carcinoma (SCLC) and in 50% (5/10) of large cell neuroendocrine carcinoma (LCNEC) whereas it was undetectable in 90% (10/11) of adenocarcinoma and 82% (9/11) of squamous carcinoma when compared to corresponding normal lung. No amplification was found but an increase in *E2F1* mRNA expression was detected in 75% (18/24) of SCLC overexpressing E2F1 product. In these tumours and in contrast with NSCLC, upregulation of E2F1 product was associated with its nuclear accumulation and with overexpression of several of its target-genes. Moreover, E2F1 overexpression in NE lung tumours was significantly associated with a high KI67 index ($p<0.0001$) as well as a Bcl-2:Bax ratio > 1 ($p<0.001$). Overall, these results demonstrate a distinct pattern of E2F1 expression in human lung tumours and suggest that its deregulation could be involved in the carcinogenesis of SCLC.

Introduction

E2F1, a critical regulator of cell cycle progression, was the first identified member of a family of transcription factors generically referred to as E2F (Nevins, 1992). This protein plays a pivotal role in G_1 to S phase transition by transactivating specific target-genes (Dyson, 1998; Helin, 1998 for review). The majority of E2F1-regulated genes encode proteins involved in DNA replication and/or in S phase progression such as DNA polymerase α, thymidine kinase (TK), dihydrofolate reductase (DHFR), Mini Chromosome Maintenance 2 to 7 (MCM), cdc6, cyclin A and cyclin E, cdc2, c-myc and E2F1 itself (DeGregori *et al*., 1995; Helin, 1998). E2F1 function is tightly regulated during cell cycle by the product of the retinoblastoma tumour suppressor gene (pRb). In G_1, unphosphorylated pRb represses the transactivating activity of E2F1/DP1 heterodimeric complexes (Flemington *et al*., 1993; Weintraub *et al*., 1995; Zhang *et al*., 1999). In late G_1, phosphorylation of pRb by cyclin-dependent kinases (cdk) induces its dissociation from E2F1/DP1 heterodimers and progression into S phase (Mittnacht,

1998). In late S, cyclin A/cdk2 complex binds to E2F1 and phosphorylates DP1, thereby inactivating E2F1/DP1 activity.

Ectopic expression of E2F1 induces DNA synthesis in quiescent immortal rodent cells (Johnson *et al*., 1993; Qin *et al*., 1994; Shan *et al*., 1994; Shan *et al*., 1996), confers neoplastic properties to immortalized cells (DeGregori *et al*. 1997), and increases tumorigenesis in transgenic mice that lack p53 function (Pierce *et al*., 1998). Moreover, old transgenic mice overexpressing E2F1 developp spontaneous tumours in a variety of tissues (Pierce *et al*., 1999). Altogether, these results confer oncogenic properties to E2F1. Paradoxically, mice lacking E2F1 also developp lymphoma or pulmonary adenocarcinoma suggesting that E2F1 can function as a tumour suppressor (Field *et al*., 1996; Yamasaki *et al*., 1996), probably via its ability to induce p53-dependent or independent apoptosis (Wu and Levine, 1994; DeGregori *et al*., 1997; Phillips *et al*., 1999). Thus, the ability of E2F1 to promote cell cycle progression and tumour growth or apoptosis and tumour suppression might be tissue-specific.

Human lung cancers are divided into two different sub-classes based on the 1999 WHO classification of lung cancer (Travis *et al*., 1999): (1) non small cell lung carcinoma (NSCLC) that include squamous carcinoma, adenocarcinoma and large cell carcinoma; (2) neuroendocrine (NE) lung tumours that include carcinoid (typical and atypical), large cell neuroendocrine carcinoma (LCNEC) and small cell lung carcinoma (SCLC), the last one being the most aggressive lung tumours. Pathways involving p53 and pRb tumour suppressor genes are frequently deregulated during lung tumorigenesis. p53 is inactivated in 70% of human lung tumours either by missense (Chiba *et al*., 1990; Brambilla *et al*.,

1993) or null mutations (Gazzeri *et al*., 1994). pRb pathway is differentially disrupted in NSCLC and high grade NE tumours. Whereas loss of pRb protein expression (through mutations and/or loss of transcription) occurs in 85% of SCLC and LCNEC (Gouyer *et al*., 1998), pRb function is abolished in 85% of NSCLC either through loss of $p16^{INK4a}$ protein expression (Xu *et al*., 1996; Gazzeri *et al*., 1998), and/or cyclin D1 overexpression (Brambilla *et al*., 1997). As E2F1 is one key component of these two pathways (O'Connor *et al*., 1995, Bates *et al*., 1998; Kowalik *et al*., 1998), any alterations affecting them, especially pRb inactivation, are thought to deregulate its activity. However, since E2F1 activation could contribute to either proliferation or apoptosis depending on the tissular context, the consequences of pRb inactivation on E2F1 expression and activity might differ during tumorigenesis according to each category of tumour.

E2F1 was poorly analyzed in lung tumours (DeMuth *et al*., 1998; Volm *et al*., 1998). To get further into its putative role in lung carcinogenesis, we studied E2F1 protein expression and mRNA in a series of 58 lung tumours of all histological types, in comparison with normal lung tissue. To assess E2F1 transactivating activity in each tumour category, we also studied the expression level of several of its known target-genes. Our results identify a distinct pattern of E2F1 expression between NSCLC and SCLC and suggest that E2F1 upregulation and hyperactivity could contribute to the carcinogenesis of SCLC.

Results

Distinct pattern of E2F1 expression in human lung tumours

E2F1 protein expression was studied on a panel of 58 lung tumour tissue samples by western-blotting using two monoclonal antibodies (Table 1, Figure 1). E2F1 product was undetectable in all normal lung tissues studied (Figure 1A) and poorly detectable in 2/11 (18%) squamous carcinoma and 1/11 (9%) adenocarcinoma (Figure 1B & 1C). In contrast, when compared to normal lung, a significative overexpression of E2F1 was observed in 24/26 (92%) small cell lung carcinoma and 5/10 (50%) large cell NE carcinoma (Table 1, Figure 1D). Same results were obtained with the two anti-E2F1 antibodies used. Overall, E2F1 product was overexpressed in 29 of 36 (81%) NE lung carcinoma whereas it was undetectable in our conditions in 19 of 22 (87%) NSCLC.

Immunohistochemical (IHC) analysis was performed using KH95 monoclonal antibody on 47 cases of this series where a frozen block was available. When compared to normal lung cells, overexpression of E2F1 was observed in 26/30 (86%) high grade NE tumors and in only 1/17 (6%) NSCLC, demonstrating that our unability to detect E2F1 protein by western blotting in NSCLC was not linked to a high proportion of contaminating stromal cells. IHC results were consistent with those of western blotting in 91% (43/47) of cases (Table 1, Figure 2). Four cases displayed discordance: in 3 of them where necrosis was intense, E2F1 staining was positive using IHC analysis whereas Western analysis was negative. In the last case, a faint band was observed by Western blot but tumour cells were negative in IHC analysis. Taken together, our results demonstrated a distinct pattern of E2F1 protein expression in human lung tumours.

Subcellular distribution of E2F1 in human lung tumours

As subcellular localization of E2F members could influence their activity, their interaction with target-genes as well as their degradation by the ubiquitin-proteasome pathway (Hateboer *et al*., 1996; Hofmann *et al*., 1996; Lindeman *et al*., 1997; Vigo *et al*., 1999), we next investigated the subcellular distribution of E2F1 in lung tumours on whole imprint preparations using indirect immunofluorescence in addition to classical immunohistochemistry revealed with peroxydase on tissue sections. Twenty of the 58 samples included in this study comprising 1 normal lung tissue, 5 SCLC and 5 LCNEC that overexpressed E2F1 product, 5 squamous carcinoma and 5 adenocarcinoma that expressed undetectable level of E2F1 by western blotting were analyzed. In all NSCLC tested, E2F1 product was never detected in the nucleus of tumour cells (Figure 3B). Absence of nuclear staining was confirmed by counterstaining with Hoechst. However, we observed a faint E2F1 cytoplasmic staining in many NSCLC cells (Figure 3B) when compared to negative control. In contrast, all SCLC cells exhibited a strong nuclear staining thereby confirming overexpression of E2F1 in these set of tumours (Figure 3C). Neither normal lung cells (Figure 3A) nor stromal cells exhibited nuclear staining, although the last cells might display some cytoplasmic staining. Same results were obtained using IHC analysis (see Figure 2).

Overexpression of E2F1 is associated with increased *E2F1* mRNA transcripts

We next investigated if E2F1 protein upregulation in SCLC could be the consequence of *E2F1* gene amplification and/or increase in *E2F1* gene transcription. Using Southern blotting, we did not detect any amplification of *E2F1* gene in all SCLC tested when compared to normal lung tissues (data not shown). Analysis of *E2F1* mRNA was performed by Northern blotting in the 24 SCLC overexpressing E2F1 protein. High levels of E2F1 transcripts were detected in 18 of those tumours (75%) when compared to normal lung tissues where E2F1 transcripts were undetectable using this technique (Figure 4). In the 6/24 remaining cases, E2F1 transcriptional level was undetectable as in normal lung despite high level of E2F1 protein expression.

Overexpression of E2F1 correlates with upregulation of some of its target-genes

To further analyze E2F1 activity in lung tumours, we studied by western blot analysis the expression of several well-known E2F1-target genes. When compared to normal lung tissues (Figure 5A), high levels of cyclins A and E, cdc2 and DHFR products were detected in 22/24 (90%) SCLC and 5/5 (100%) LCNEC that overexpressed E2F1 (Figure 5B). In one case (4%) SCLC, cyclin A was undetectable despite overexpression of other target-genes. In contrast, expression of these four proteins was weak in 17/19 (95%) NSCLC that exhibited undetectable E2F1 product (Figure 5C & 5D). Again, in 3/11 (27%) squamous lung carcinoma, cyclin A was slightly detectable despite low level of E2F1 expression (Figure 5D; lanes 9-11) suggesting that additional factors might regulate its expression in this subset of tumour. Taken together, these results indicated

that upregulation of E2F1 protein expression in SCLC was associated with overexpression of some E2F1-targets genes.

Relationships between E2F1, p53, pRb and hp14^{ARF} status in lung carcinoma

Status of p53, pRb and hp14^{ARF} had been previously analyzed in this series of tumours (Brambilla *et al*., 1993; Gazzeri *et al*., 1994; Gazzeri *et al*., 1998a; Gazzeri *et al*., 1998b; Gouyer *et al*., 1998). In these tumours, pRb function was inactivated, either by loss-of-function mutations or p16^{INK4a} loss and/or cyclin D1 overexpression in 29/36 (80%) NE tumours and 18/22 (82%) NSCLC respectively. However, we did not find any correlation between the status of E2F1 and pRb inactivation in all tumours studied (data not shown). There was no inverse nor direct correlation linking E2F1 and hp14^{ARF} or p53 expression in any histological tumour class. However, we noticed that overexpression of E2F1 was frequently associated with inactivation of hp14^{ARF} [as detected by negative immunostaining (Gazzeri *et al*., 1998b)] (20/36, 69%) and/or of p53 [as indicated by p53-positive immunoreactivity (Gazzeri *et al*., 1994)] (18/36, 50%) in NE lung tumours (Table 2).

Correlation between E2F1 , KI67 and Bcl-2:Bax levels in human lung carcinoma

In human lung tumours, E2F1 might mediate either apoptosis or cellular proliferation depending on the context. Thus, we finally investigated in each tumoral subtype, the relationships between E2F1 protein expression, the well-known proliferative index KI67 (Gerdes *et al*., 1984) and the equilibrium of two main factors of cell

susceptibility to apoptosis, Bcl-2 and Bax (Brambilla *et al*., 1996). KI67 proliferative index was significantly higher in NE lung tumours when compared to NSCLC (44 ± 19 versus 21 ± 13; $p<0.0001$; Table 3). Furthermore, a high KI67 index correlated with overexpression of E2F1 product in all histological subtypes ($p<0.0001$).

Susceptibility to apoptosis was investigated through IHC analysis of both Bcl-2 and Bax products. Indeed, we had previously demonstrated that a Bcl-2:Bax ratio > 1 correlated with a lower apoptotic index in NE lung tumours (Brambilla *et al*., 1996). We showed that Bcl-2 mean score was significantly higher in NE lung tumours when compared to NSCLC (202 ± 109 versus 41 ± 54; $p<0.0001$; Table 3). Furthermore, a Bcl-2:Bax ratio > 1 was significantly associated with NE phenotype ($p<0.001$) and E2F1 overexpression ($p<0.001$) whereas NSCLC exhibited a Bcl-2:Bax ratio < 1 and low level of E2F1 product (Table 3). Overall, these results strongly suggest that E2F1 overexpression in high grade neuroendocrine lung tumours might mediate cellular proliferation rather than apoptosis.

Discussion

We have investigated E2F1 protein expression in a series of 58 human lung tumours and demonstrated that NSCLC and NE carcinoma displayed distinct pattern of E2F1 expression. Whereas E2F1 product was undetectable by western blot analysis and IHC in nearly all NSCLC analyzed, it was upregulated in high grade NE lung tumours. As

we previously showed that pRb function was invalidated (either by mutations or loss of $p16^{INK4a}$ and/or cyclin D1 overexpression) in 80% of all lung tumours tested in this study (Gazzeri *et al*., 1998a; Gouyer *et al*., 1998), our present data suggest that inactivation of pRb pathway does not predict deregulated levels of E2F1 expression, at least during lung tumorigenesis.

To get clues onto the mechanism of E2F1 upregulation in SCLC, we analyzed DNA amplification and mRNA expression. Although amplification was not found, we detected an increase in *E2F1* mRNA expression in the majority of those tumours overexpressing E2F1 protein. An increase in E2F1 transcription normally occurs in late G_1 in response to growth stimuli (Sherr, 1996) but can also be detected in response to oncogenic stimuli such as myc (Leone *et al*., 1997). Moreover, E2F1 has been reported to stimulate its own transcription (Johnson *et al*., 1994; Neuman *et al*., 1994). These data and the present study suggest that, in SCLC, deregulated oncogenic signals could induce a primary increase of E2F1 gene transcription further amplified by protein accumulation. Interestingly, some tumours exhibited a high E2F1 protein level despite absence of mRNA overexpression. E2F1 protein level is tightly controlled by ubiquitin-proteasome mediated proteolysis (Campanero *et al*., 1997; Vandel *et al*., 1999) and association with pRb has been shown to protect E2F1 from degradation (Hateboer *et al*., 1996; Hofmann *et al*., 1996). Since pRb was lost in 80% of the NE tumours studied, E2F1 accumulation in these tumours is probably not the result of protection by pRb. Additionally, the protein $p45^{SKP2}$ was recently shown to directly interact with E2F1 and to negatively regulate its stability (Marti *et al*., 1999; Wade-Harper and Elledge, 1999). We have studied $p45^{SKP2}$

protein expression by IHC in our series of tumours but we could not find any correlation between $p45^{SKP2}$ and E2F1 expression (data not shown). Thus, it remains to elucidate if accumulation of E2F1 in SCLC could be associated with a defect in its proteolysis.

We observed E2F1 accumulation in the nuclei of SCLC tumoral cells that was consistent with the expression of an active E2F1 product (de la Luna *et al*., 1996; Allen *et al*., 1997) and found a strong correlation between the expression of E2F1 and several of its target-genes including cyclins A and E. Interestingly, in SCLC overexpressing E2F1, we also detected heterodimeric complexes between E2F1 and DP1 or E2F1 and mdm2 (data not shown). Since E2F1 transactivating activity is highly enhanced upon dimerization with DP1 (Wu *et al*., 1995) and binding of mdm2 (Martin *et al*., 1995), our results highly suggest that E2F1 is not only upregulated but also transcriptionally active in SCLC. In contrast and interestingly, absence of E2F1 nuclear staining correlated with weak level of E2F1 target-genes expression in NSCLC. Thus, despite loss of pRb repression, free E2F1 product might be transcriptionally inactive in this subset of tumors.

Therefore, what might be the biological consequences of E2F1 expression in lung carcinogenesis? During tumorigenesis, E2F1 has been implicated both as an oncogene (Johnson *et al*., 1993; Qin *et al*., 1994; Pierce *et al*., 1998; Pierce *et al*., 1999) or a tumour suppressor gene (Field *et al*., 1996; Yamasaki *et al*., 1996; Pierce *et al*., 1999) depending on the context and on its ability to induce cell proliferation and/or p53-dependent or -independent apoptosis. In lung tissues, we noticed that E2F1 protein expression displayed opposite patterns between NSCLC and SCLC making attractive the possibility that this protein could play a distinct role during tumorigenesis of these two tumour subsets. Since

E2F1 knock-out mice developp pulmonary adenocarcinoma (Field *et al*., 1996), its inactivation in NSCLC could contribute to a defective apoptosis of tumoral cells. Conversely, unbalance between apoptosis and cell proliferation could be implicated in the tumorigenesis of SCLC. In this subset of tumour, we observed a correlation between E2F1 expression and KI67 proliferative index suggesting that E2F1 might be involved in their high proliferative pattern. In contrast, the ability of E2F1 to induce apoptosis might be counteracted. Consistent with this, we previously showed that p53 is inactivated and/or $hp14^{ARF}$ lost in most SCLC (Gazzeri *et al*., 1998b) and although statistically not significant, a correlation between E2F1 overexpression and p53 inactivation and/or $hp14^{ARF}$ loss was detected in SCLC. As E2F1 can induce p53-dependent apoptosis via its ability to transactivate $hp14^{ARF}$ (Bates *et al*., 1998), inactivation of p53 or $hp14^{ARF}$ could give an advantage to the proliferative effects of E2F1 versus its pro-apoptotic functions. Moreover, as we observed that high grade neuroendocrine lung tumours including SCLC expressed a Bcl-2:Bax ratio > 1 (Brambilla *et al*., 1996 and this study), Bcl-2 could further inhibit the apoptosis of SCLC tumor cells overexpressing E2F1 and contribute to their proliferation. According to this, sensitivity of oesophageal cancer cells to E2F1-mediated apoptosis was recently related to differential expression of Bcl-2 family member proteins (Yang *et al*., 2000).

Overall, our data identify a distinct pattern of E2F1 protein expression in human lung tumours and demonstrate its upregulation in SCLC, the most proliferative and aggressive lung tumours. In these tumours, E2F1 could therefore act as an oncogene being an essential actor of uncontrolled cellular proliferation, for example by

counteracting the negative effects of cyclin-cdk inhibitors. This could explain why these tumours are so actively proliferative despite their high content in $p16^{INK4a}$ (Kelley *et al*., 1997; data not shown) and $p27^{Kip1}$ products (Yatabe *et al*., 1998; data not shown). These findings have obvious conceptual implications in the promising strategic therapy targeting E2F1 function. They should aim at neutralizing E2F1 production in SCLC in order to inhibit their proliferation and inducing its expression in NSCLC in order to restitute apoptosis.

Material and methods

Tissue samples

Tissue samples were taken at surgical resection of lung tumours or node metastases at mediastinoscopy. They were immediately frozen and kept at -80°C until use. They consisted of 11 squamous carcinoma; 11 adenocarcinoma; 10 LCNEC and 26 SCLC according to the 1999 WHO international histological classification of lung tumours (Travis *et al*., 1999).

Antibodies

Monoclonal antibodies (mAbs) included anti-cdc2p34 (sc-54, Santa-Cruz Biotechnology, Santa Cruz, CA), anti-cyclin E (clone HE12, Euromedex, Souffelweyersheim), anti-DHFR (Dihydrofolate Reductase; clone 49, Transduction Laboratories, Lexington, KY) and two anti-human E2F1 (clone Ab-4, Neomarkers and clone KH95, Pharmingen). Rabbit polyclonal antibodies included anti-actin (20-33, Sigma-Aldrich, L'Isle d'Abeau) and anti-cyclin A (C-22, sc-160, Santa-Cruz).

Immunohistochemistry (IHC)

E2F1, KI67, Bcl-2 and Bax immunostaining were performed on frozen sections. For E2F1 immunostaining, two monoclonal E2F1 antibodies KH95 (Santa-Cruz; 1/100) and Ab-4 (Neomarkers; 1/50; data not shown) were used. Three step immunohistochemical method was applied on 5 μm thick frozen sections taken in the immediate vicinity of samples used for western-blot and immunoprecipitation studies. After E2F1 primary antibodies, the secondary antibody was anti-mouse (1/1250) and third step was ABC complex (Streptavidin-Biotin peroxidase; Dako). Cases were recovered as positive when at least 20% of tumour cells showed obvious nuclear staining. Only nuclear staining was considered to assess immunoreactivity. Cytoplasmic staining was never considered in the assessment of E2F1 expression. It was observed in addition to nuclear staining in a subset of squamous carcinoma and adenocarcinoma. Normal lung cells, lymphocytes and vessels were all negative. KI67 immunostaining was performed using a mouse monoclonal antibody from Immunotech (1/100). KI67 percentage of positive cells was established on

300 cells count. Bcl-2 and Bax immunostaining were performed as previously described (Brambilla *et al*., 1996) using a Bcl-2 monoclonal antibody from Dako (1/100) and a Bax polyclonal antibody N-19 from Santa-Cruz (1/200). Scores of immunostaining were calculated by multiplying the percentage of labeled cells with the intensity (1+, 2+, 3+) of staining for a total score gain from 0 to 300. The Bcl-2/Bax ratio was established by dividing the 2 total scores.

Indirect immunofluorescence

Whole imprint preparations were performed by gentle pressing tumoural frozen tissue to silane-coated glass slides, air dried and stored at -80°C until use. Slides were treated for 10 min at room temperature in ice-cold nuclei isolation buffer [5 mM HEPES (pH 8), 50 mM KCl, 10 mM $MgSO_4$, and 3 mM DTT], incubated at -20°C during 10 min in nuclei isolation buffer supplemented with 0.25% Triton 100X, washed twice with PBS and fixed for 5 min in acetone at - 20°C. After fixation, slides were washed, saturated and incubated overnight with primary antibody (anti-human E2F1, Ab-4, 1/200) at 4°C. After three PBS washes, slides were exposed to Alexa^{TM} 488 goat anti-mouse-IgG (H+L) conjugate (2 mg/ml; 1/500, Interchim, Montluçon) for 30 min at room temperature and washed twice in PBS. Normal mouse IgG at the same concentration as the primary antibody served as a negative control. The slides were counterstained with Hoechst and preparations were observed using an Olympus microscope (20x magnification), captured by a Coolview CCD camera (Photonic Science) and digitally saved using Visilog. The images were further processed by using Photoshop (Adobe).

Western-blot analysis

Tissues samples were lysed in ice-cold lysis buffer [5 mM EDTA, 150 mM NaCl, 100mM Tris (pH 8), 0.5% Na deoxycholate, 0.5% NP40, 0.5% SDS, 0.1% aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin, and 1 mM PMSF] for 30 min and centrifugated (30 min, 15 000g). In order to ensure appropriate detection, a molecular weight marker was loaded onto each gel. Proteins (40 μg) were denatured in Laemmli buffer [60 mM Tris HCl (pH 6.8), 20% glycerol, 10% ß-mercaptoethanol, 4.6% SDS, and 0.003% bromophenol blue], separated by 10-12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and electroblotted on PVDF membrane (Hybond P, Amersham, Les Ulis, France). The membrane was then incubated for 2-3 h at room temperature with primary Ab in 2% non-fat milk TPBS, washed three times, incubated with horseradish peroxydase-conjugated goat anti-mouse or anti-rabbit Abs (Jackson ImmunResearch Laboratories , West Grove, PE) for 30 min and revealed using enhanced chemoluminescence detection kit (ECL; Amersham).

Northern Blot Analysis

Total RNA was extracted according to the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). 10 μg of total RNA were fractionated by electrophoresis in 1% agarose-formaldehyde gels and transferred onto a nylon membrane (Hybond N, Amersham). Equivalent RNA loading was verified by ethidium-bromide staining of gels before transfer onto membranes. A Bam H1-1.35-kb full lenght human

E2F1 cDNA fragment (kindly provided by Dr. K. Helin, Milan) was used as a probe and was radiolabeled using the random primed DNA labeling kit (Roche Diagnostics). The membranes were prehybridized and hybridized in 50% formamide, 5X Denhardt's solution, 50 mM phosphate buffer (pH 6.5), 5X SSC, 0.1% SDS, and 2.5 μg/ml salmon sperm DNA for 42 h at 42°C. The most stringent final wash was for 20 min at 56°C in 0.2X SSC, and 0.1% SDS. Membranes were exposed for autoradiography at -80°C for at least 3 days.

Aknowledgements

This work was supported by grants from the Association pour la Recherche sur le Cancer, from the Comité Départemental de la Ligue contre le Cancer de l'Isère and from the Groupement des Entreprises Francaises dans la Lutte contre le Cancer. B.E. was supported by INSERM (Poste Accueil).

References

Allen KE, de la Luna S, Kerkhoven RM, Bernards R and La Thangue NB. (1997). *J. Cell Sci.*, **110,** 2819-2831.

Bates S, Phillips AC, Clark PA, Stott F, Peters G, Ludwig RL and Vousden KH. (1998). $p14^{ARF}$ links the tumour suppressors RB and p53. *Nature*, **395,** 124-125.

Brambilla E, Gazzeri S, Moro D, Caron de Fromentel C, Gouyer V, Jacrot M and Brambilla C. (1993). *Am. J. Pathol.,* **143,** 199-210.

Brambilla E, Negoescu A, Gazzeri S, Lantuejoul S, Moro D, Brambilla C and Coll, J.L. (1996). *Am. J. Pathol.*, **149,** 1941-1952.

Brambilla E. Moro D, Gazzeri S and Brambilla C. (1997). *J. Path.*, **188,** 351-360.

Campanero MR and Flemington EK. (1997). *Proc. Natl. Acad. Sci. USA.*, **94,** 2221-2226.

Chiba I, Takahashi T and Nau M. (1990). *Oncogene*, **5,** 1603-1610.

Chomczynski PT and Sacchi N. (1987). *Ann. Biochem.*, **162,** 156-159.

De La Luna S, Burden MJ, Lee CW and La Thangue NB. (1996). *J. Cell Sci.*, **109,** 2443-2452.

DeGregori J, Kowalik T and Nevins JR. (1995). *Mol. Cell. Biol.,* **15,** 4215-4224.

DeGregori, J, Leone, G, Miron, A, Jakoi, L and Nevins, JR. (1997). *Proc.Natl.Acad.Sci.USA.*, **94,** 7245-7250.

DeMuth JP, Jackson CM, Weaver DA, Crawford EL, Durzinsky DS, Durham SJ, Zaher A, Phillips ER, Khuder SA and Willey JC. (1998). *Am. J. Respir. Cell. Mol. Biol.*, **19,** 18-24.

Dyson N. (1998). *Genes Dev.,* **12**, 2245-2262.

Field SJ, Tsai FY, Kuo F, Zubagia AM, Kaelin WG, Livingston DM, Orkin SH and Greenberg ME. (1996). *Cell,* **85,** 549-561.

Flemington EK, Speck SH and Kaelin WG. (1993). *Proc.Natl.Acad.Sci.USA.*, **90,** 6914-6918.

Gazzeri S, Brambilla E, Caron de Fromentel C, Gouyer V, Moro D, Perron P, Berger F and Brambilla C. (1994). *Int.J. Cancer*, **58,** 24-32.

Gazzeri S, Gouyer V, Vourc'h C, Brambilla C and Brambilla E. (1998a). *Oncogene,* **16,** 497-505.

Gazzeri S, Della Valle V, Chaussade L, Brambilla C, Larsen CJ and Brambilla E. (1998b). *Cancer Res.*, **58,** 3926-3931.

Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U and Stein H. (1984). *J. Immunol.*, **133**, 1710-1715.

Gouyer V, Gazzeri S, Bolon I, Drevet C, Brambilla C and Brambilla E. (1998). *Am. J. Respir. Cell Mol. Biol.*, **17,** 1-9.

Hateboer G, Kerkhoven RM, Shvarts A, Bernards R and Beijersbergen RL. (1996). *Genes Dev.*, **10,** 2960-2970.

Helin K. (1998). *Curr.Opin.Genet.Dev.*, **8**, 28-35.

Hofmann F, Martelli F, Livingston DM and Wang ZY. (1996). *Genes Dev.*, **10,** 2949-2959.

Johnson DG, Schwarz JK, Cress WD and Nevins JR. (1993). *Nature*, **365,** 349-352.

Johnson DG, Ohtani K and Nevins JR. (1994). *Genes Dev.,* **8,** 1514-1525.

Kelley MJ, Nakagawa K, Steinberg SM, Mulshine JL, Kamb A and Johnson BE. (1997). *J. Nat. Cancer.Inst.*, **87**, 756-761.

Kowalik TF, DeGregori J, Leone G, Jakoi L and Nevins JR. (1998). *Cell Growth Differ.*, **9,** 113-118.

Leone G, DeGregori J, Sears R, Jakoi L and Nevins JR. (1997). *Nature*, **387,** 422-426.

Lindeman GJ, Gaubatz S, Livingston DM and Ginsberg D. (1997). *Proc. Natl. Acad. Sci. USA.*, **94,** 5095-5100.

Marti A, Wirbelauer C, Scheffner M and Krek W. (1999). *Nature Cell Biol.,* **1,** 14-19.

Martin K, Trouche D, Hagemeier C, Sorensen TS, La Thangue NB and Kouzarides T. (1995). *Nature*, **375,** 691-694.

Mittnacht S. (1998). *Curr.Opin.Genet.Dev.*, **8,** 21-27.

Neuman E, Flemington EK, Sellers WR and Kaeli,n WG. (1994). *Mol. Cell. Biol.*, **14,** 6607-6615.

Nevins JR. (1992). *Science*, **258,** 424-429.

O'Connor DJ, Lam EWF, Griffin S, Zhong S, Leighton LC, Burbidge SA and Lu X. (1995). *EMBO J*., **14,** 6184-6192.

Phillips AC, Ernst MK, Bates S, Rice NR and Vousden KH. (1999). *Mol.Cell.,* **4,** 771-781.

Pierce AM., Gimenez-Conti, IB, Schneider-Broussard R, Martinez LA, Conti CJ and Johnson DG. (1998). *Proc.Natl.Acad.Sci.USA*., **95,** 8858-8863.

Pierce AM, Schneider-Broussard R, Gimenez-Conti, IB, Russell JL, Conti CJ and Johnson DG. (1999). *Mol.Cell.Biol*., **19,** 6408-6414.

Qin XQ, Livingston DM, Kaelin WG and Adams P. (1994). *Proc.Natl.Acad.Sci.USA.,* **91,** 10918-10922.

Shan B and Lee WH. (1994). *Mol.Cell.Biol*., **14,** 8166-8173.

Shan B, Durfee T, and Lee WH. (1996). *Proc.Natl.Acad.Sci.USA*., **93,** 679-684.

Sherr CJ. (1996). *Science* (Washington DC), **274,** 1672-1677.

Travis WD, Colby TV, Corrin B, Shimosato Y and Brambilla E. (1999). *WHO International Histological Classification of Tumours: Histological Typing of Lung and Pleural Tumours*. Third Edition. Ed Springer.

Vandel L and Kouzarides T. (1999). *EMBO J*., **18,** 4280-4291.

Vigo E, Muller H, Prosperini E, Hateboer G, Cartwright P, Moroni MC and Helin K. (1999). *Mol. Cell. Biol*., **19,** 6379-6395.

Volm M, Koomagi R and Rittgen W. (1998). *Int.J. Cancer*, **79,** 294-299.

Wade Harper J and Elledge SJ. (1999). *Nature Cell Biol*., **1,** E5-E7.

Weintraub SJ, Chow K, Luo RX, Zhang SH, He S and Dean DC. (1995). *Nature*, ***375*,** 812-815.

Wu CL, Zukerberg LR, Ngwu C, Harlow E and Lees JA. (1995). *Mol. Cell. Biol*., **15,** 2536-2546.

Wu X and Levine AJ. (1994). *Proc.Natl.Acad.Sci.USA*., **91,** 3601-3606.

Xu HJ, Cagle PT, Hu SX, Li J and Benedict WF. (1996). *Clin. Cancer. Res.*, **2,** 1169-1176.

Yamasaki L, Jacks T, Bronson R, Goillot E, Harlow E and Dyson NJ. (1996). *Cell*, **85,** 537-548.

Yang HL, Dong YB, Elliott MJ, Liu TJ and McMasters KM. (2000). *Clin. Cancer Res.,* **6,** 1579-1589.

Yatabe Y, Masuda A, Koshikawa T, Nakamura S, Kuroishi T, Osada H, Takahashi T, Mitsudowi T and Takahashi, T. (1998). *Cancer Res.*, **58**, 1042-1047.

Zhang HS, Postigo AA and Dean DC. (1999). *Cell*, **97,** 53-61.

Legends to figures

Figure 1. E2F1 expression in normal lung tissues, small cell lung carcinoma (SCLC) and non small cell lung carcinoma (NSCLC). Western-blot analysis was performed as described in material and methods and actin was used as a loading control. Results obtained with clone Ab-4 are shown but same results were obtained using clone KH-95. To allow comparison between each histological type, western-blots were revealed in the

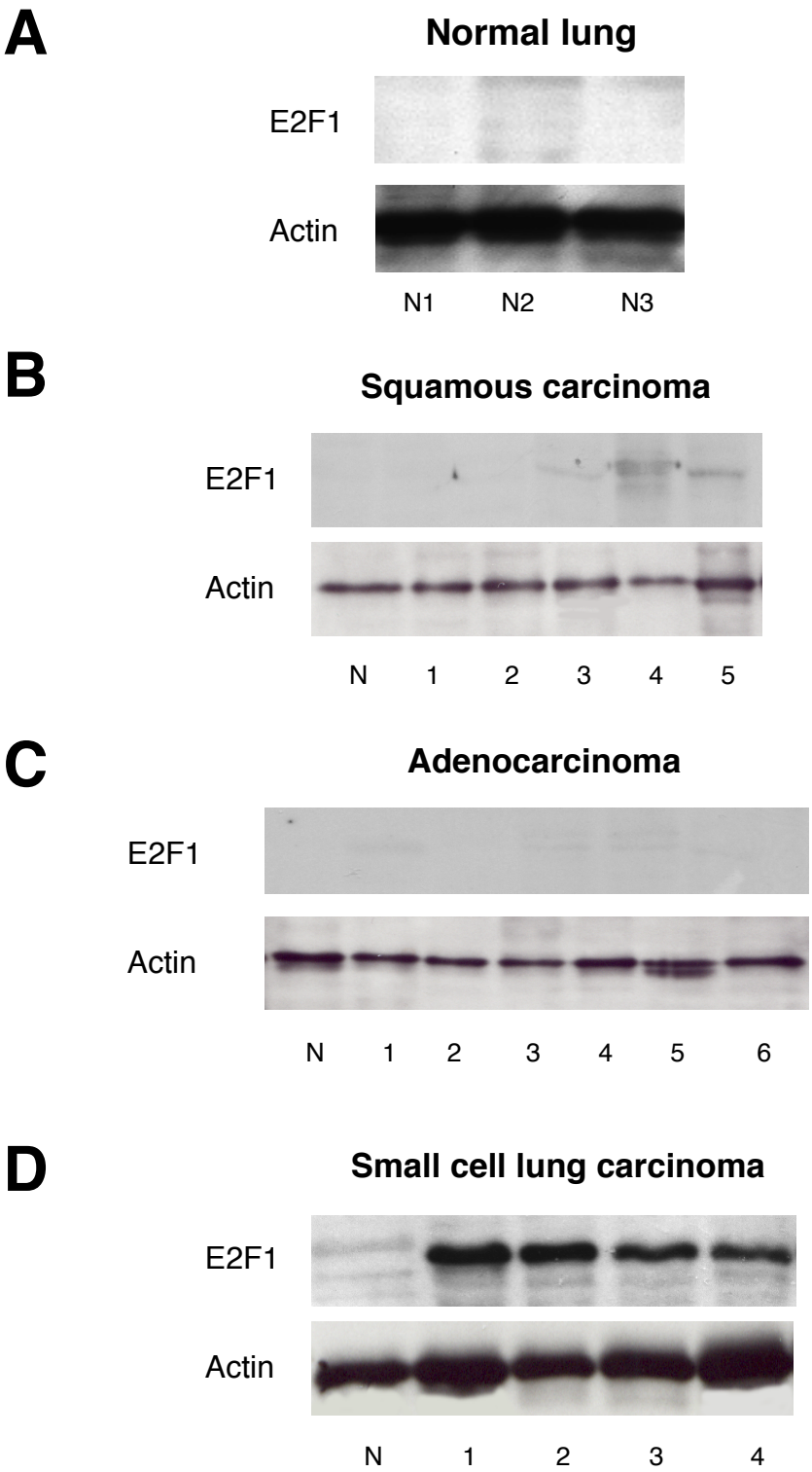
same conditions and E2F1 expression for each case was confirmed in at least two independent experiments. **(A)** Representative experiment of E2F1 protein expression in 3 normal lung tissues. **(B, C)** Representative experiment of E2F1 protein expression in 5 squamous carcinoma **(B)** and 6 adenocarcinoma **(C)**. N: normal tissue. **(D)** Example of E2F1 protein upregulation in 4 SCLC. N: normal tissue.

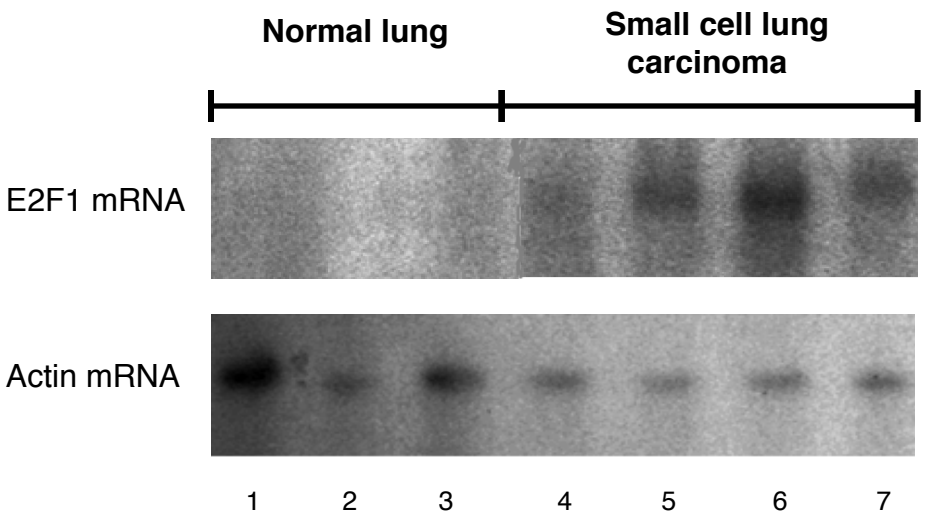
Figure 2. E2F1 immunostaining of lung cancer tissue on frozen sections using anti-E2F1 monoclonal antibody (KH-95). Same results were obtained using clone Ab-4 (Neomarkers). **(A)** Negative E2F1 immunostaining in normal lung alveoli (x 200). **(B)** Negative E2F1 immunostaining in normal bronchioli (x 200). **(C)** Positive E2F1 nuclear immunostaining in small cell lung carcinoma (x 400). **(D)** Negative E2F1 immunostaining in a squamous cell carcinoma (x 200).

Figure 3. Indirect immunofluorescence study of E2F1 subcellular localization. E2F1 staining (in red) was performed as described in material and methods. A representative staining is shown in normal lung tissues **(A)**, adenocarcinoma **(B)** and SCLC **(C)**.

Figure 4. Expression of E2F1 transcripts by northern blot analysis. Some representative cases are shown. SCLC tumour samples (4 to 7) expressed high level of E2F1 mRNA when compared to normal tissues (1 to 3). Notice the existence of variations in E2F1 mRNA levels between each tumoral sample.

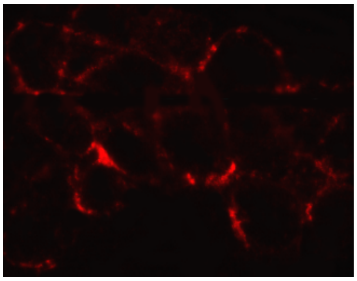
Figure 5. E2F1, cyclin A, cyclin E, DHRF and cdc2 protein expressions in normal lung tissues **(A)**, SCLC **(B)**, adenocarcinoma **(C)** and squamous carcinoma **(D)**. Western-blot analysis was performed as described in material and methods and actin was used as a loading control. To allow comparison between each histological type, western-blots were revealed in the same conditions and results for each case were confirmed in at least two independent experiments. **(B)** Overexpression of E2F1 was associated with high level of cyclin E, A, cdc2 and DHFR (tumours 1, 3 to 7). Lane 2: tumour representative of 2/26 SCLC that do not overexpress E2F1 and subsequent cyclin E, cyclin A, cdc2 and DHFR expression. Lane 8: tumour representative of 2/24 SCLC tested in which overexpression of E2F1 does not correlate with overexpression of cyclin A despite overexpression of cyclin E, cdc2 and DHFR. **(C, D)** In NSCLC, as in SCLC, correlation was observed between levels of E2F1 and several of its targets.


A
Normal lung
E2F1
Actin
N1
N2
N3
B
Squamous carcinoma
E2F1
Actin
N
1
2
3
4
5
C
Adenocarcinoma
E2F1
Actin
N
1
2
3
4
5
6
D
Small cell lung carcinoma
E2F1
Actin
N
1
2
3
4


Normal lung
Small cell lung carcinoma
E2F1 mRNA
Actin mRNA
1
2
3
4
5
6
7

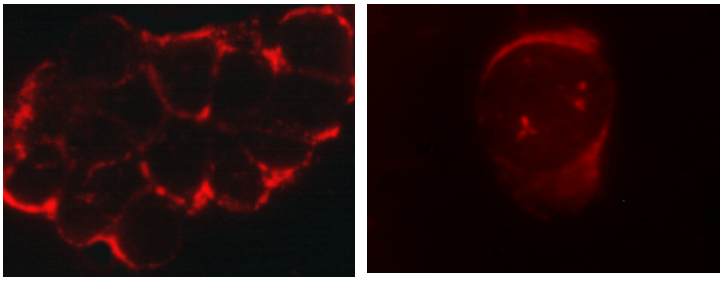
A

Normal lung



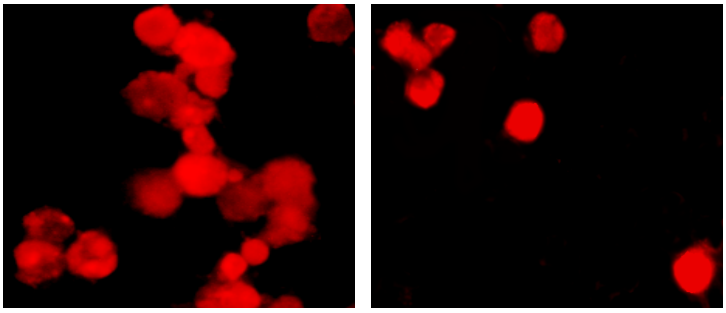
B

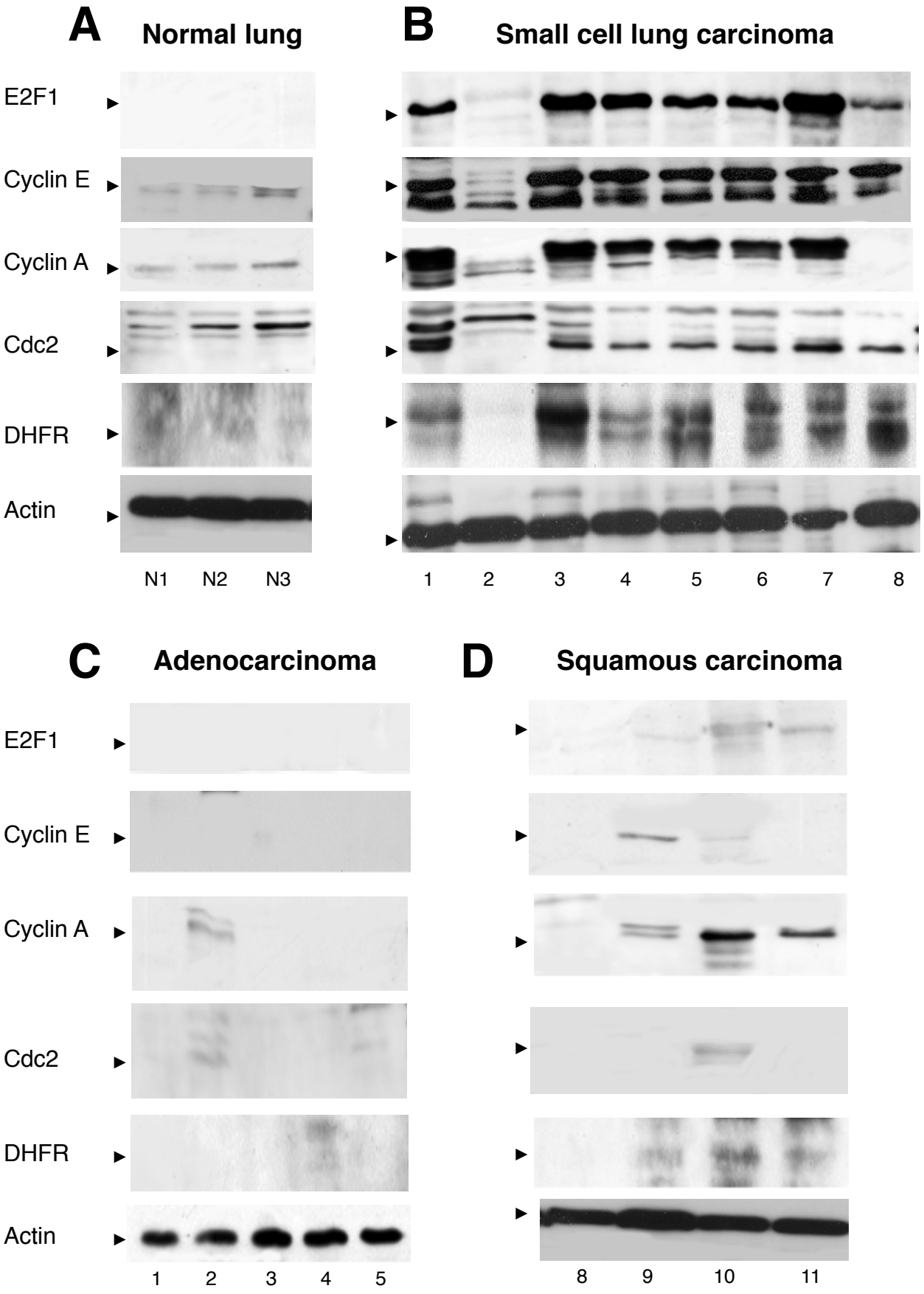
Non small cell lung carcinoma



C

Small cell lung carcinoma




A
Normal lung
B
Small cell lung carcinoma
E2F1
Cyclin E
Cyclin A
Cdc2
DHFR
Actin
N1
N2
N3
1
2
3
4
5
6
7
8
C
Adenocarcinoma
D
Squamous carcinoma
E2F1
Cyclin E
Cyclin A
Cdc2
DHFR
Actin
1
2
3
4
5
8
9
10
11