Overexpression of E2F1 associated with LOH at RB locus and hyperphosphorylation of RB in non-small cell lung carcinoma

Overexpression of E2F1 Associated with LOH at RB Locus and Hyperphosphorylation of RB in Non-small Cell Lung Carcinoma

Miwa Akasofu Imai, Yoshio Oda, Makoto Oda, Isao Nakanishi, and Ei Kawahara

---

This work was supported by grants from the Japan Ministry of Education, Culture, Sports, Science and Technology.

M. A. Imai

Department of Health Science, Ishikawa Prefectural Nursing University, 7-1 Nakanuma Tsu, Takamatsu-machi, Kahoku-gun, Ishikawa 929-1212, Japan

Y. Oda

Department of Pathology, School of Medicine, Kanazawa University Faculty of Medicine, 13-1 Takara-machi, Kanazawa 920-8640, Japan

M. Oda

Department of Surgery, School of Medicine, Kanazawa University Faculty of Medicine, 13-1 Takara-machi, Kanazawa 920-8640, Japan

I. Nakanishi

Kanazawa Laboratories, ALP Co., Ltd, 309 Chikaoka-machi, Kanazawa 920-8217, Japan

E. Kawahara (Corresponding author)

Department of Laboratory Sciences, School of Health Sciences, Kanazawa University Faculty of Medicine, 5-11-80 Kodatsuno, Kanazawa 920-0942, Japan

E-mail kawahara@kenroku.kanazawa-u.ac.jp

Tel.: +81-76-265-2598 Fax.: +81-76-234-4369

Abstract

Purpose: E2F1 plays a critical role in cell proliferation, and its function is controlled by the retinoblastoma (RB) protein. We examined the expression of E2F1 and aberration of RB gene and protein to elucidate what factors contribute to the overexpression of E2F1 in non-small cell lung carcinomas. *Methods*: The expression level of E2F1 in tissues of non-small cell lung carcinomas was measured by means of quantitative reverse transcription-polymerase chain reaction and immunohistochemistry. For RB, we examined loss of heterozygosity (LOH) by PCR-restriction fragment length polymorphism and variable number of tandem repeats, and protein expression by immunohistochemistry. *Results*: Fifteen cases of carcinoma (46%) showed high transcription levels of E2F1 gene. Immunohistochemically, almost all (14 of 15) cases overexpressing E2F1 mRNA were positive for E2F1 protein. LOH at the RB locus was found in 13 of 30 informative cases. In 13 cases with LOH, 10 showed overexpression of E2F1 mRNA and protein. Immunohistochemical positivity for phosphorylated RB protein was also closely correlated with overexpression of E2F1. *Conclusions*: Our results suggest that overexpression of E2F1, induced both by LOH at the RB locus and anomalous phosphorylation of the RB protein, is involved in the development of non-small cell lung carcinoma.

Key words E2F – Non-small cell lung carcinoma – RB – Loss of heterozygosity-Hyperphosphorylation

Introduction

E2F1 protein functions as a transcription factor that enhances cell proliferation by binding to the promoter region of several genes that are important for cell growth control. Dephosphorylated RB protein binds to the transcriptionally active region of E2F1 protein, and inactivates E2F1. In turn, phosphorylated RB protein releases E2F1 protein and the resultant free E2F1 acts as a transcription factor (Dyson 1995; Sellers et al. 1995; Weinberg 1995; Lee et al. 2002). In vitro studies have revealed that overexpression of E2F1 mediates cell transformation (Johnson et al. 1994; Xu et al. 1995). Gene amplification and overexpression of E2F1 have been reported in an erythroleukemia cell line (Saito et al. 1995). Overexpression of E2F1 has also been reported in tumor tissues including gastrointestinal carcinoma (Suzuki et al. 1999) and malignant lymphoma (Lai et al. 1998). Thus, E2F1 is believed to act as an oncogene, and to play an important role in tumor progression in various organs. Recently, upregulation of E2F1 has also been demonstrated in small cell lung carcinoma (Eymin et al. 2001) and in non-small cell lung carcinoma (Gorgoulis et al. 2002).

The aberrant expression of E2F1 could be caused by abnormality of E2F1 itself or by aberrant regulation by RB protein. The rate of gene amplification of E2F1 is low in various organs, including lung carcinoma (Suzuki et al. 1999, Rabbani et al. 1999, Gorgoulis et al. 2002). Mutation of E2F1 occurs rarely (Suzuki et al. 1999, Gorgoulis et al. 2002), whereas mutation of E2F4 is frequent (Ikeda et al. 1998). These observations suggest that the main mechanism of E2F-1 protein overexpression is deregulation at the transcriptional level due to factor(s) upstream of E2F1. The loss of function of RB protein, for example, due to a defect of the RB gene at 13q14, is expected to induce overexpression of E2F1. Indeed, small cell lung carcinoma frequently shows allelic loss of the RB gene (Gouyer et al. 1994). Loss of heterozygosity (LOH) at the RB locus has also been detected and is not a rare phenomenon even in non-small cell carcinoma (Xu et al. 1991; Reissmann et al. 1993; Tamura et al. 1997).

In addition, inactivating mutation of the other allele, leading to total loss of function of RB protein, is frequent in small cell lung carcinoma (Gouyer et al. 1994; Higashiyama et al. 1994). However, mutation is infrequent in non-small cell lung carcinoma (Sachse et al. 1994, Tamura et al. 1997). Thus, the mechanism of genesis of non-small cell carcinoma could be different from that of small cell carcinoma. The incidence of aberration of the RB gene and protein in non-small cell carcinoma is lower than that in small cell carcinoma, and it has been believed that loss of RB function is due to the loss of function of RB protein, which is regulated by phosphorylation status (Kaye 2002). Cyclin-dependent kinase and cyclin complex phosphorylate RB, and p16 is known to inhibit the complex. Therefore, these factors are likely to affect expression of E2F1. Inactivation of the RB pathway may occur through inactivation of p16 and upregulation of cyclin D1 (Brambilla 1999). Hyperphosphorylation of RB protein could be a marker for such aberrant control by cyclin-dependent kinases, cyclins and p16. However, the phosphorylation status of RB in vivo in non-small cell lung carcinoma is poorly understood.

In the present study, we examined abnormalities of E2F1 and RB in non-small cell lung carcinomas. We observed overexpression of E2F1, and found a strong correlation of the overexpression with LOH at the RB locus and hyperphosphorylation of RB protein.

Materials and methods

Tissue samples

Tissue samples were obtained from 33 patients with non-small cell lung carcinoma (17 cases of squamous cell carcinoma and 16 cases of adenocarcinoma), who were operated at Kanazawa University Hospital between 1996 and 1998. Tumor tissues and non-tumor tissues were dissected immediately after operation. The tissues adjacent to the area from which the fresh samples were dissected were fixed with 10% neutral-buffered formalin and

embedded in paraffin. Informed consent was obtained from all patients enrolled in the study.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from carcinoma tissue and non-tumor tissue with a total RNA Separator Kit (Stratagene, CA, USA), and mRNA was further purified using a poly (A) Quick™ mRNA purification kit (Stratagene). The amount of mRNA of E2F1 was quantified by using a competitive RT-PCR method described by Riedy et al. (1995). An RNA competitive reference standard (RNA-CRS) template with internal nucleotide deletions was created from the mRNA extracted from cultured OSC-19 cells (Kawahara et al. 1993). The mRNA was reverse-transcribed to cDNA with the Reverse Transcription System™ (Promega, WI, USA), and the resultant cDNA mixture was added to a standard PCR mixture. The primer sets were designed to amplify the E2F1 cDNA sequence (Helin et al. 1992), and to produce amplified cDNA which contains sequences of T7 RNA polymerase and an 80-bp deletion as compared with the native mRNA strand (Table I). The sequence coding for the DNA binding region of the T7 RNA polymerase (underlined) was added to the upstream primer 5'-AATTTAATACGACTCACTATAGGGAGAAGTCCAAGAACCACATCCAGT-3'. The downstream primer 5'-CTCAGGGCACAGGAAAACATCGATCACCATAACCATCTGCTGCTGC-3' was designed to create the 80-base deletion. The specificity of the primers was confirmed by sequencing the PCR product of cDNA of OSC-19 cells. The resultant mutant cDNA was transcribed to RNA with a T7-MEGA Shortscript™ kit (Ambion, Texas, USA). Decreasing amounts of RNA-CRS mixed with 5 ng of sample mRNA were added to tubes along with upstream primer 5'-GAAGTCCAAGAACCACATCCAGT-3' for the RT followed by the addition of downstream primer 5'-CTCAGGGCACAGGAAAACATCG-3' for the PCR. A

negative control contained all the reagents for the RT except for the RNA templates, to detect any contamination of RNA or DNA. The PCR product was run in 4% agarose gel. The gel was stained with ethidium bromide and visualized with an UV transilluminator. The densities of the bands of RNA-CRS cDNA and the wild-type cDNA were measured using a densitometer. The concentration of RNA-CRS was plotted against the ratio of the RNA-CRS: native RNA band densities. Linear regression was performed and the equivalence point was determined. The point where the ratio of RNA-CRS: E2F1 is equal to 1 gives the concentration of E2F1 mRNA. The regression plot was linear over a broad range and we could quantify E2F1 mRNA accurately down to the femtogram level. To verify the quality and quantity of mRNA, extracts were also processed in parallel for amplification of a 115-base pair segment of the human β2-microglobulin gene (Noonan et al. 1990).

Restriction fragment length polymorphism (RFLP) and variable number of tandem repeats (VNTR)

Genomic DNA was extracted from carcinoma tissue and non-tumor tissue with a DNA extraction kit (Stratagene). Three previously published polymorphisms (introns 1, 17, 25) in the RB gene (Bookstein et al. 1990; McGee et al. 1990; Wadayama et al. 1994) were analyzed by PCR-RFLP using endonucleases, BamHI, XbaI and DraI. The PCR products were digested and run in an agarose gel containing ethidium bromide. VNTR (CTTT)n in intron 20 was also analyzed (Wadayama et al. 1994). The PCR product was run in 2% Metaphor^{TM} agarose gel (FMC, ME, USA), and the gel was stained with ethidium bromide. The results of RFLP were classified into two groups: informative case when one allele was digested by the endonucleases and the other allele was not digested; not informative case when two alleles were restricted or were not restricted. In informative cases, the cases were recorded as allelic loss when the tumor signal was reduced by more than 30% from the normal signal, in accordance with Matsumura et al. (1992), because normal cells were

included within the tumor. For VNTR, the lengths of the two alleles were different in informative cases, but the same in non-informative cases.

Direct Sequencing for Identification of RB Mutation

RB cDNA was submitted to direct sequencing of exons 12-23 using three primer sets (Liu et al. 1993). The PCR products were electrophoresed in 2 % agarose gels and the appropriate bands were cut out. The DNA was extracted with SUPREC^{TM}-01 (Takara, Ootsu, Japan), precipitated with ethanol, and dissolved in Tris-EDTA buffer. Direct sequencing was performed using a Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin Elmer, Foster City, CA, USA). Samples were electrophoresed on an autosequencer (ABI PRISM^{TM} 377 DNA Sequencer, Perkin Elmer).

Immunohistochemistry

E2F1, RB and phosphorylated RB proteins in formalin-fixed and paraffin-embedded tissue sections were detected by the streptavidin-biotin-peroxidase complex method or tyramide signal amplification method. The sections were incubated with a monoclonal mouse antibody against E2F1 protein (clone KH95, diluted at 1 : 40, Santa Cruz Biotechnology, CA, USA) and RB protein (clone 3H9, diluted at 1 : 40, MBL, Nagoya, Japan) at 4˚C overnight and sequentially with a biotinylated anti-mouse antibody (diluted at 1 : 200, Vector Laboratories, CA, USA) at room temperature for 30 min. They were next incubated with streptavidin-biotin peroxidase complex (Jackson ImmunoResearch Laboratories, PA, USA). The tyramide signal amplification method (CSAII System, DakoCytomation, Kyoto, Japan) using polyclonal anti-phospho-Rb (Ser$^{807/811}$) antibodies (diluted at 1: 100, Cell Signaling Technology, Beverly, MA, USA) was applied for detection of phosphorylated RB protein. Immunohistochemical nuclear stainability, regardless of staining intensity, was regarded as positive. Normal mouse IgG instead of the primary antibody served as the negative control.

Statistical analysis

Statistical analyses were made using Mann-Whitney's U test or the χ^2 test.

Results

High levels of E2F-1 mRNA in Carcinoma Tissue

The amount of E2F1 mRNA in non-tumor tissues was always less than 2 fg/ng total mRNA. Nine cases (53 %) of squamous cell carcinoma and six cases (38 %) of adenocarcinoma showed higher transcription levels of mRNA than the maximum level of non-tumor tissues, i.e., 2.0 - 41.7 fg / ng total mRNA, and this was designated as overexpression (Fig. 1). The level of E2F1 mRNA in tumor tissues was significantly higher ($p<0.005$) than in non-tumor tissues, and the differences were significant in both squamous cell carcinoma ($p<0.05$) and adenocarcinoma ($p<0.05$). The level tended to be higher in squamous cell carcinomas than in adenocarcinomas, though not significantly so.

Correlation of E2F-1 mRNA Expression with E2F-1 Protein Expression

The E2F1 protein was not immunohistochemically detectable in normal tissue. We preliminarily examined 2 cases of small cell carcinomas, which have been reported to be stained positively at high frequency (Eymin et al. 2001). Since both of them showed intense nuclear staining, we used the small cell carcinoma as a positive control. In the present cases of non-small cell carcinoma, eight of nine squamous cell carcinomas with E2F1 mRNA overexpression and all six adenocarcinomas with E2F1 mRNA overexpression were positive with anti-E2F1 antibody (Fig. 2A, B). The immunohistochemically positive cases and mRNA-overexpressing cases were thus coincident in all cases except one (Table 1).

Correlation of E2F1 Overexpression with LOH at the RB Locus

Four sites were examined for LOH at the RB locus, and 30 cases were constitutionally heterozygous in at least one of the four sites. LOH at the RB locus was

found in 13 cases (42%) (Fig. 3). Among them, seven cases (47%) of squamous cell carcinoma in 15 informative cases and six cases (40%) of adenocarcinoma in 15 informative cases showed LOH. In 13 cases with LOH, 10 (77%) showed overexpression of E2F1 mRNA and protein; 5 squamous cell carcinomas (70%) and 5 adenocarcinomas (83%). Conversely, in 14 RB-informative cases with E2F1 overexpression, 10 (71%) were LOH-positive, consisting of 5 squamous cell carcinomas (56%) , and 5 adenocarcinoma (83%), and 4 cases were negative. In 18 cases of normal E2F1 expression, only 3 (12%) were LOH-positive. There was significant difference ($p<0.05$) between LOH-positive cases and LOH-negative cases with regard to E2F1 overexpression.

Correlation of Phosphorylated RB Protein with E2F1 Overexpression

RB protein and phosphorylated RB protein were also examined by an immunohistochemical method (Table 1). All cases of squamous cell carcinoma and 11 cases of adenocarcinoma were positive. Five cases of adenocarcinoma were judged negative, because RB protein-positive cells were not seen among carcinoma cells, although they were scattered among basal cells, endothelial cells, lymphocytes and fibroblasts. There was no correlation of E2F-1 expression with immunohistochemical RB expression.

We used the colon tissue as a positive control for phosphorylated RB. The crypt epithelia of the normal colon mucosa showed scattered immunostaining, as did germinal center cells in lymphoid follicles in the mucosa. In the normal lung tissues no epithelial cell was immunostained, and positive germinal center cells occasionally appeared in inflamed lung tissue. In carcinoma cells, phosphorylated RB protein was detected in seven cases out of 15 with overexpression of E2F1 (Fig. 2C, D). Cases negative for RB protein did not show positive for phosphorylated RB protein. Only 2 cases out of 18 cases without overexpression of E2F1 were positive. The χ^2 test showed a significant correlation between E2F1 overexpression and positive immunostaining for phosphorylated RB.

Among 21 cases (Cases 1-7, 9, 11, 14, 17-19, 23, 24, 26, 28, 29, 31-33) for which RB cDNA was sequenced over exons 12-23, no mutation was found.

Discussion

In the present quantitative study we observed at high rate of E2F1 overexpression in both squamous cell carcinoma and adenocarcinoma, in agreement with the findings of Gourgoulis et al. (2002). E2F1 may be implicated in neoplastic transformation in a broad spectrum of tumors, since overexpression of E2F1 occurs in a variety of carcinomas (Eymin et al. 2001; Suzuki et al. 1999; Lai et al. 1998; Zhang et al. 2000).

LOH at the RB locus, which correlated with overexpression of E2F1, was seen in 43% of non-small cell lung carcinoma in the present study. Similar frequencies of LOH in non-small cell lung carcinoma have been reported by others: 35% (Gouyer et al. 1994), 17% (Xu et al. 1991), 58% (Tamura et al. 1997), and 30% (Sachse et al. 1994). LOH on chromosome 13q is extremely frequent in small cell carcinoma at 92% (Gouyer et al. 1994) or 88% (Higashiyama et al. 1994). Though the frequency of LOH was lower than in small cell carcinoma, it is noteworthy that 63% of squamous cell carcinomas and 83 % of adenocarcinomas which overexpressed E2F1 mRNA showed LOH at the RB locus. The results suggest that decrease of RB protein due to LOH plays an important role in upregulation of E2F1, though other factors probably also contribute.

Mutation of RB along with LOH may result in further loss of RB function in E2F-1 regulation in small cell lung carcinoma. We sequenced a key region called the “RB pocket” (exons 12 – 22), which is the binding region to E2F-1 (Nevins 1992; Xu et al. 1990), but found no mutation. It is reported that mutations are infrequent in non-small cell lung carcinoma (Sachse et al. 1994; Tamura et al. 1997). However, we did not examine the regions forward from exon 12 or back from exon 23. Secondly, since we examined cDNA,

the sequence of tumor RB would not have been amplified and RB in non-tumor cells alone would have been amplified by RT-PCR, if the exon sequence had been altered constitutively due to frame shift or altered splicing, which are frequently detected in small cell carcinoma (Mori et al. 1990).

Although the possibility of mutation could not be excluded, it might be unlikely in non-small cell lung carcinomas. However, we could detect aberrant expression of phosphorylated RB protein in many cases with E2F1 overexpression. Inactivation of the RB pathway may result from hypermethylation of RB protein, and may occur through inactivation of the p16 protein and upregulation of cyclin D1. Therefore, these factors are likely to affect expression of E2F1 in addition to LOH of RB presented here. Actually, methylation in the promoter region of the p16 gene and allelic loss of the p16 gene leading to loss of p16 protein have been frequently detected in non-small cell lung carcinoma (Kurakawa et al. 2001; Chen et al. 2002). In contrast, abnormal expression of cyclin D and p16 are infrequent in small cell lung carcinomas (Zöchbauer-Müller et al. 2002). Since both overexpression of cyclin D and loss of p16 upregulate phosphorylation of RB, hyperphosphorylation of RB could be a characteristic abnormality of non-small cell lung carcinomas.

E2F1 activity as a transcription factor is controlled by phosphorylation and dephosphorylation of RB protein, and free E2F1 acts as an ultimate effector of G1/S progression (Weinberg et al. 1995). We did not examine the level of free E2F1, but rather the overall expression level of E2F1. However, E2F1 overexpression has been thought to be sufficient to override the negative control of cell growth by RB protein. It is suggested that LOH at the RB locus and hyperphosphorylation of RB induced the increased E2F1 level. An E2F1 binding domain exists in the promoter of E2F1, providing positive feedback, which normally induces a rapid increase of E2F1 in the G1/S transition (Johnson et al. 1994), and

the increase of free E2F1 stimulates E2F synthesis, enabling the overall level of E2F to exceed that of RB protein.

References

Bookstein R, Lai C-C, To H, Lee WH (1990) PCR-based detection of polymorphic BamHI site in intron 1 of the human retinoblastoma (RB) gene. Nucl Acids Res 18: 1660.

Brambilla E, Moro D, Gazzeri S, Brambilla C (1999) Alterations of expression of Rb p16 (INK4A) and cyclin D1 in non-small cell lung carcinoma and their clinical significance. J Pathol 188: 351-360.

Chen JT, Chen YC, Wang YC, Tseng RC, Chen CY, Wang YC (2002) Alterations of the p16(ink4a) gene in resected nonsmall cell lung tumors and exfoliated cells within sputum. Int J Cancer 98:724-31.

Dyson N (1995) The regulation of E2F by pRB-family proteins. Genes Dev 12: 2245-2262.

Eymin B, Gazzeri S, Brambilla C, Brambilla E (2001) Distinct pattern of E2F1 expression in human lung tumors: E2F1 is upregulated in small lung carcinoma. Oncogene 20: 1678-1687.

Gorgoulis VG, Zacharatos P, Mariatos G, Kotsinas A, Bouda M, Kletsas D, Asimacopoulos PJ, Agnantis N, Kittas C, Papavassiliou AG (2002) Transcriptional factor E2F-1 acts as a growth-promoting factor and is associated with adverse prognosis in non-small cell lung carcinomas. J Pathol 198: 142-156.

Gouyer V, Gazzeri S, Brambilla E, Bolon I, Moro D, Perron P, Benabid AL, Brambilla C (1994) Loss of heterozygosity at the RB locus correlates with loss of RB protein in primary malignant neuroendocrine lung carcinomas. Int J Cancer 58: 818-824.

Helin K, Lees JA, Vidal M, Dyson N, Harlow E, Fattaey A (1992) A cDNA encoding a pRB-binding protein with protein with properties of the transcription factor E2F. Cell 70: 337-350.

Higashiyama M, Doi O, Kodama K, Yokouchi H, Tateishi R (1994) Retinoblastoma protein expression in lung cancer: an immunohistochemical analysis. Oncology 51: 544-551.

Ikeda M, Orimo H, Moriyama H, Nakajima E, Matsubara N, Mibu R, Tanaka N, Shimada T, Kimura A, Shimizu K (1998) Close correlation between mutations of E2F4 and hMSH3 genes in colorectal cancers with microsatellite instability. Cancer Res 58: 594-598.

Johnson DG, Ohtani K, Nevins JR (1994) Autoregulatory control of E2F1 expression in response to positive and negative regulators of cell cycle progression. Genes Dev 8: 1514-1525.

Johnson DG, Cress WD, Jakoi L, Nevins JR (1994) Oncogenic capacity of the E2F1 gene. Proc Natl Acad Sci USA 91: 12823-12827.

Kawahara E, Okada Y, Nakanishi I, Iwata K, Kojima S, Kumagai S, Yamamoto E. (1993) The expression of invasive behavior of differentiated squamous carcinoma cell line evaluated by an in vitro invasion model. Jpn J Cancer Res 84: 409-418.

Kaye FJ (2002) RB and cyclin dependent kinase pathways: defining a distinction between RB and p16 loss in lung cancer. Oncogene 21: 6908 – 6914.

Kurakawa E, Shimamoto T, Utsumi K, Hirano T, Kato H, Ohyashiki K (2001) Hypermethylation of p16 (INK4a) and p15 (INK4b) genes in non-small cell lung cancer. Int J Oncol 19:277-81.

Lee C, Chang JH, Lee HS, Cho Y (2002) Structural basis for the recognition of the E2F transactivation domain by the retinoblastoma tumor suppressor. Genes Dev 16: 3199-3212.

Lai R, Medeiros LJ, Coupl R, McCourty A, Brynes RK (1998) Immunohistochemical detection of E2F1 in non-Hodgkin's lymphomas a survey of 124 cases. Mod Pathol 11: 457-463.

Liu Y, Szekely L, Grandér D, Söderhäll S, Juliusson G, Gahrton G, Linder S, Einhorn S (1993) Chronic lymphocytic leukemia cells with allelic deletions at 13q14 commonly have one intact RB1 gene: Evidence for a role of an adjacent locus. Proc Natl Acad Sci

USA 90: 8697-8701.

Matsumura K, Kallioniemi A, Kallioniemi O, Chen L, Smith HS, Pinkel D, Gray J, Waldman FM (1992) Deletion of chromosome 17q loci in breast cancer cells detected by fluorescence in situ hybridization. Cancer Res 52: 3474-3477.

McGee TL, Cowley GS, Yandell DW, Dryja TP (1990) Detection of the XbaI RFLP within the retinoblastoma locus by PCR. Nucl Acids Res 18: 207.

Mori N, Yokota J, Akiyama T, Sameshima Y, Okamoto A, Mizoguchi H, Toyoshima K, Sugimura T, Terada M. (1990) Variable mutations of the RB gene in small-cell lung carcinoma. Oncogene 5:1713 – 1717.

Nevins JR (1990) E2F: a link between the Rb tumor suppressor protein and viral oncoprotein. Science 258: 424-429.

Noonan KE, Beck C, Holzmayer TA, Chin JE, Wunder JS, Andrulis IL, Gazdar AF, Willman CL, Griffith B, Von Hoff DD, Roninson IB (1990) Quantitative analysis of MDR1 (multidrug resistance) gene expression in human tumors by polymerase chain reaction. Proc Natl Acad Sci USA 87: 7160-7164.

Rabbani F, Richon VM, Orlow I, Lu M-L, Drobnjak M, Dudas M, Charytonowicz E, Dalbagni G, Cordon-Cardo C (1999) Prognostic significance of transcription factor E2F1 in bladder cancer genotypic and phenotypic characterization. J Natl Cancer Inst 91: 874-881.

Reissmann PT, Koga H, Takahashi R, Figlin RA, Holmes C, Piantadosi S, Cordon-Cardo C, Slamon DJ, the Lung Cancer Study Group (1993) Inactivation of the retinoblastoma susceptibility gene in non-small-cell lung cancer. Oncogene 8: 1913-1919.

Riedy MC, Timm EA, Stewart CC (1995) Quantitative RT-PCR for measuring gene expression. BioTechniques 18: 70-76.

Sachse R, Murakami Y, Shiraishi M, Hayashi K, Sekiya T (1994) DNA aberrations at the

retinoblastoma gene locus in human squamous cell carcinomas of the lung. Oncogene 9: 39-47.

Saito M, Helin K, Valentine MB, Griifffith BB, Willman CL, Harlow E, Look AT (1995) Amplification of the E2F1 transcription factor gene in the HEL erythroleukemia cell line. Genomics 25: 130-138.

Sellers WR, Rodgers JW, Kaelin WG (1995) A potent trans-repression domain in the retinoblastoma protein induces cell cycle arrest when bound to E2F sites. Proc Natl Acad Sci USA 92: 11544-11548.

Suzuki T, Yasui W, Yokozaki H, Naka K, Ishikawa T, Tahara E (1999) Expression of the E2F family in human gastrointestinal carcinomas. Int J Cancer 81: 535-538.

Tamura K, Zhang X, Murakami Y, Hirohashi S, Xu H-J, Hu S-X, Benedict WF, Sekiya T (1997) Deletion of three distinct regions on chromosome 13q in human non-small-cell lung cancer. Int J Cancer 74: 45-49.

Wadayama B, Toguchida J, Shimizu T, Ishizaki K, Sasaki MS, Kotoura Y, Yamamuro T (1994) Mutation spectrum of the retinoblastoma gene in osteosarcomas. Cancer Res 54: 3042-3048.

Weinberg RA (1995) The retinoblastoma protein and cell cycle control. Cell 81: 323-330.

Xu G, Livingston DM, Krek W (1995) Multiple members of the E2F transcription factor family are the products of oncogenes. Proc Natl Acad Sci USA 92: 1357-1361.

Xu H-J, Hu S-X, Cagle PT, Moore GE, Benedict WF (1991) Absence of retinoblastoma protein expression in primary non-small cell lung carcinoma. Cancer Res 51: 2735-2739.

Xu Q, Dyson N, Harlow E (1990) The regions of the retinoblastoma binding to adenovirus E1A or SV40 large T antigen are common sites for mutations. EMBO J 9: 1147-1155.

Zhang SY, Liu SC, Al-Saleem LF, Holloran D, Babb J, Guo X, Klein-Szanto AJ (2000) E2F1: a proliferative marker of breast neoplasia. Cancer Epidemiol Biomarkers Prev 9:

395-401.

Zöchbauer-Müller S, Gazdar AF, Minna JD (2002) Molecular pathogenesis of lung cancer. Ann Rev Physiol 64: 681-708.

			E2F1		RB						
			mRNA [a)]	Immuno	RFLP			VNTR	LOH	Immunostain	
Case	Age	Sex		-stain	Intron 1	Intron 17	Intron 25	Intron 20		RB	p-RB
Squamous cell carcinoma											
1	58	M	**13.9**	(+)	(+)	(+)	NI	(+)	(+)	(+)	(+)
2	76	M	**12**	(+)	(-)	(+)	NI	(+)	(+)	(+)	(-)
3	75	F	**12**	(+)	(+)	NI	NI	(+)	(+)	(+)	(+)
4	80	M	**10**	(+)	(-)	nd	NI	(-)	(-)	(+)	(+)
5	55	M	**9.5**	(+)	(-)	(+)	NI	(-)	(+)	(+)	(+)
6	61	M	**8.2**	(+)	(-)	nd	NI	NI	(-)	(+)	(+)
7	70	M	**6**	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(-)
8	50	F	**4**	(+)	NI	nd	NI	NI	NI	(+)	(-)
9	74	M	**2.2**	(-)	(-)	(-)	NI	(-)	(-)	(+)	(-)
10	70	F	1.4	(-)	(-)	nd	nd	nd	(-)	(+)	(-)
11	61	M	1.2	(-)	(-)	(+)	NI	(+)	(+)	(+)	(-)
12	75	F	1	(-)	(-)	nd	NI	NI	(-)	(+)	(-)
13	58	M	1	(-)	NI	NI	NI	(-)	(-)	(+)	(+)
14	76	M	0.8	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(-)
15	71	M	0	(-)	(-)	nd	nd	nd	(-)	(+)	(+)
16	71	M	0	(-)	NI	NI	(-)	(-)	(-)	(+)	(-)
17	74	M	0	(-)	NI	NI	NI	NI	NI	(+)	(-)
Adenocarcinoma											
18	72	F	**41.7**	(+)	(+)	(+)	(-)	(+)	(+)	(-)	(-)
19	37	M	**5.2**	(+)	(+)	NI	(+)	(+)	(+)	(+)	(+)
20	63	F	**5**	(+)	(-)	(+)	NI	(-)	(+)	(+)	(+)
21	75	F	**2.6**	(+)	(+)	(-)	NI	(-)	(+)	(+)	(-)
22	58	M	**2.2**	(+)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
23	71	F	**2**	(+)	(+)	NI	NI	NI	(+)	(+)	(-)
24	72	F	1.8	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(-)
25	80	F	1.7	(-)	NI	NI	NI	NI	NI	(-)	(-)
26	74	M	1.7	(-)	(-)	(+)	NI	NI	(+)	(+)	(-)
17	46	F	0.9	(-)	(-)	(-)	(-)	NI	(-)	(+)	(-)
28	80	F	0.7	(-)	NI	NI	NI	(-)	(-)	(-)	(-)
29	77	F	0.7	(-)	(-)	nd	NI	(-)	(-)	(+)	(-)
30	40	F	0.6	(-)	(-)	(-)	(-)	NI	(-)	(+)	(-)
31	83	M	0.6	(-)	(-)	(-)	NI	(-)	(-)	(+)	(-)
32	57	F	0	(-)	(-)	nd	NI	(-)	(-)	(+)	(-)
33	58	M	0	(-)	(-)	(-)	NI	NI	(-)	(-)	(-)

Table 1. Summary of expression of E2F1 and LOH of RB (RFLP restriction fragment length polymorphism, VNTR variable number tandem repeats, LOH loss of heterozygosity, NI not informative, nd not done, p-RB phosphorylated RB)

[a)] fg/ng total mRNA

Figure legends

Fig. 1. Amounts of E2F1 mRNA in tumor tissues and non-tumor tissues. The concentration of E2F1 was determined by quantitative RT-PCR using 5 ng of purified total RNA. Final evaluation was based on the amount of E2F1 mRNA extracted from 1 ng of total RNA.

Fig. 2. Immunohistochemistry for E2F1 and phosphorylated RB. E2F1-positive squamous cell carcinoma (A, case 1) and adenocarcinoma (B, case 21). Phosphorylated RB-positive squamous cell carcinoma (C, case 15) and adenocarcinoma (D, case 20)

Fig. 3. Loss of heterozygosity (LOH) at various loci in RB. LOH was detected on the basis of restriction fragment length polymorphism (RFLP) (A B C) and variable number of tandem repeats (VNTR) (D). A. BamHI polymorphism in intron 1 in case 18. B. XbaI polymorphism in intron 17 in case 1. C. DraI polymorphism in intron 25 in case 14. D. VNTR in intron 20 in case 7. When the tumor signal was reduced by more than 30% from the normal signal, the case was recorded as allelic loss, because normal cells were contained within the tumor. M indicates a size marker (M1 ØX174 DNA digested with Hae III; M2 ØX174 DNA digested with Hinf I); N non-tumor tissue; T tumor tissue; Numbers to the left or right side of fragments indicate fragment size (base pairs).

Fig. 1

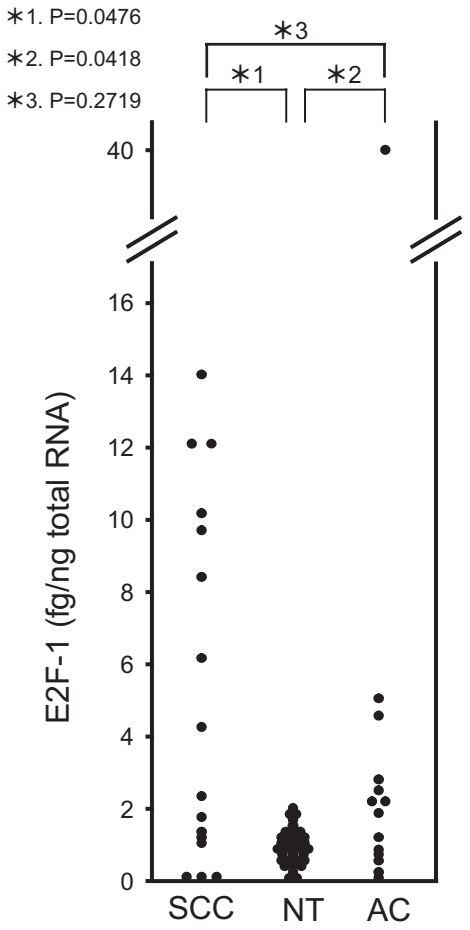

Fig. 3

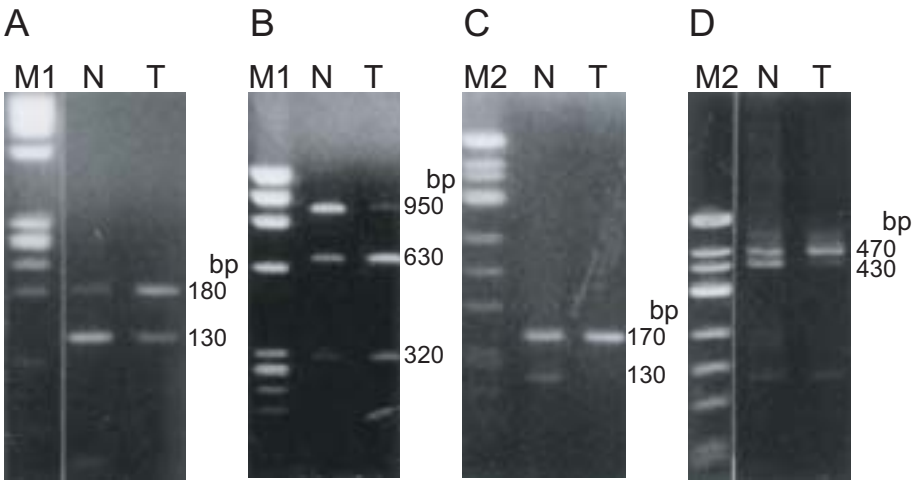

Fig. 2