E2F1 Overexpression Correlates with Thymidylate Synthase and Survivin Gene Expressions and Tumor Proliferation in Non–Small-Cell Lung Cancer

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Abstract

Purpose: We investigated the clinical significance of *E2F1* gene expression in relation to its target genes, *thymidylate synthase* (*TS*) and *Survivin*, in case of non–small-cell lung cancer (NSCLC).

Experimental Design: One hundred twenty-seven cases of resected NSCLC were analyzed. Quantitative reverse transcription-PCR was done to evaluate the gene expression of *E2F1, TS*, and *Survivin*. Immunohistochemistry was done to investigate the protein expression of E2F1, TS, and Survivin. The Ki-67 proliferation index and the apoptotic index using the terminal deoxyribonucleotidyl transferase–mediated dUTP nick-end labeling method were also evaluated.

Results: *E2F1* gene expression significantly correlated with the Ki-67 proliferation index ($r = 0.487$; $P < 0.0001$), although no correlation was observed between *E2F1* gene expression and the apoptotic index. With regard to *E2F1* target genes, *E2F1* gene expression significantly correlated with *TS* gene expression ($r = 0.709$; $P < 0.0001$) and *Survivin* gene expression ($r = 0.403$; $P < 0.0001$). The overall survival rate was significantly lower in patients with high-*E2F1* tumors than in those with low-*E2F1* tumors ($P = 0.0027$), especially among patients with stage II to III NSCLCs ($P = 0.0188$). A Cox regression analysis showed that the *E2F1* status was a significant prognostic factor for NSCLC patients (hazard ratio, 2.052; $P = 0.0261$).

Conclusions: The present study revealed that *E2F1* gene expression correlates with *TS* and *Survivin* gene expressions and tumor proliferation. During the progression of NSCLC, *E2F1* overexpression could produce more aggressive tumors with a high proliferation rate and chemoresistance.

Lung cancer is a major cause of cancer-related deaths, and non–small-cell lung cancer (NSCLC) comprises ~75% of all lung cancers (1). It is important to design an optimal therapeutic strategy according to tumor biology to improve the treatment of NSCLC (2). The selection of effective chemotherapies based on the evaluation of biomarkers (i.e., "tailor-made chemotherapy") can improve the clinical outcome of NSCLC patients (3). For example, 5-fluorouracil (5-FU)–derived agents would be useful for tumors with a low expression of thymidylate synthase (TS; refs. 4, 5). Gefitinib and erlotinib would be effective for tumors with epidermal growth factor receptor (EGFR) mutations or increased *EGFR* gene copy numbers (6). Furthermore, the apoptotic index is also a useful indicator to predict the efficacy of chemotherapy for NSCLC patients (7).

The E2F1 transcription factor plays a key role in G_1-to-S phase transition by attracting numerous upstream signals (8). Experimental studies showed that E2F1 induces various genes encoding S phase–activating proteins, including TS (9, 10). Furthermore, a recent experimental study revealed that E2F1 induced the gene expression of *Survivin* (11), a member of the inhibitor of apoptosis protein family (12). It is important to clarify the molecular mechanisms of these biomarkers in NSCLCs to develop effective cancer treatments. Therefore, we conducted a clinical study in NSCLC patients to investigate *E2F1* gene expression in relation to *TS* and *Survivin* gene expressions.

Furthermore, E2F1 modulates diverse cellular functions such as DNA synthesis, mitosis, and apoptosis (8). With regard to tumorigenesis, *in vitro* studies have reported that *E2F1* can act either as an oncogene (13, 14) or as a tumor suppressor gene (15, 16). Previous clinical studies in human cancers also showed that E2F1 has a tumor-promoting effect in many cancers including lung cancer (17, 18), breast cancer (19), and thyroid cancer (20), whereas the opposite seems to be the case for other cancers such as colon cancer and bladder cancer (21, 22). Therefore, to investigate the clinical significance of

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E2F1 in NSCLCs, we also evaluated *E2F1* gene expression in relation to tumor proliferation using the Ki-67 index (23) and tumor apoptosis using the apoptotic index (7).

Materials and Methods

Patient characteristics. We studied consecutive NSCLC patients who underwent surgery at the Second Department of Surgery of Kagawa University from January 2000 to June 2003. This study was approved by the institutional review board of Kagawa University (14-7, a clinical study of biological markers in NSCLCs) and signed informed consent was obtained from each patient. Tumor-node-metastasis staging designations were made according to the postsurgical pathologic international staging system (24). The lymph node status was pathologically evaluated using specimens resected by either thoracotomy or mediastinoscopy. In total, 127 patients with lung cancer up to stage IIIB, including 63 patients with adenocarcinoma, 58 patients with squamous cell carcinoma, and 6 patients with large cell carcinoma, were investigated (Table 1). The patients' clinical records and histopathologic diagnoses were fully documented. This report includes follow-up data as of October 31, 2006. The median follow-up period for all patients was 62.2 months.

With regard to the methods of surgical resection, a pneumonectomy was done in 15 patients with stage II to III NSCLC. A lobectomy was done in 103 patients: 48 patients with stage I NSCLC, 25 patients with stage II NSCLC, and 30 patients with stage III NSCLC. A segmentectomy was done in five patients with stage I NSCLC, and a wedge resection was done in four patients with stage I NSCLC. Systemic chemotherapy using mitomycin/vinblastin/cisplatin or carboplatin/paclitaxel was done in all patients with stage II to III NSCLC: neoadjuvant chemotherapy in 36 patients and postoperative adjuvant chemotherapy in 34 patients with nodal metastases. Radiation therapy was done in 20 patients: 9 patients with T_3 or T_4 status and 11 patients with mediastinal lymph node metastases.

Table 1. Demographic and clinical characteristics of patients

Characteristics	No. patients (%)
Total no. patients	127 (100)
Age, y	
Median	68
Range	39-82
Gender	
Male	89 (70.1)
Female	38 (29.9)
Smoking status	
Nonsmoker	36 (28.3)
Smoker	91 (71.7)
Smoking pack-years	
Median	43
Range	0-146
ECOG performance status	
0	95 (74.8)
1	20 (15.7)
2	12 (9.5)
Histology	
Adenocarcinoma	63 (49.6)
Squamous cell carcinoma	58 (45.7)
Large-cell carcinoma	6 (4.7)
Pathologic stage	
I	57 (44.9)
II	29 (22.8)
III	41 (32.3)
Method of surgical resection	
Pneumonectomy	15 (11.8)
Lobectomy	103 (81.1)
Segmentectomy	5 (3.9)
Wedge resection	4 (3.2)
Chemotherapy	70 (55.1)
Neoadjuvant therapy	36 (28.3)
Postoperative adjuvant therapy	34 (26.8)
Radiotherapy	20 (15.7)

Abbreviation: ECOG, Eastern Cooperative Oncology Group.

Quantitative reverse transcription-PCR. Total RNA was extracted from frozen tissue specimens by the acid guanidinium thiocyanate procedure. First-strand cDNA synthesis was done with 5 μg of total RNA using a cDNA synthesis kit (Pharmacia). To quantify *E2F1* and *TS* gene expression, real-time quantitative PCR was done with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The primers and probes were from the Assays-on Demand Gene Expression Assay Mix (*E2F1* assay ID Hs00153451_m1, PCR product size 84 bp; *TS* assay ID Hs00426591_m1, PCR product size 87 bp; Applied Biosystems). The PCR cycling conditions were as follows: 50°C, 2 min for AmpErase UNG activation; 95°C, 10 min for AmpliTaq Gold activation; and 40 cycles for the melting (95°C, 15 s) and annealing/extension (60°C, 1 min) steps. Real-time PCR assays were conducted in triplicate for each sample, and each PCR experiment included three nontemplate control wells. The standard curves for serial dilutions of cDNA of the human lung cancer cell line A549, which had positive expressions of *E2F1* and *TS*, were similarly generated. The comparative threshold cycle method (Applied Biosystems) was used to calculate the gene expression ratio in each sample relative to the value observed in the control A549 cells, using *GAPDH* (Assays-on-Demand Gene Expression system, assay ID Hs99999905_m1, PCR product size 122 bp; Applied Biosystems) as a control for normalization among the samples.

Because *Survivin* has splice variants with different functions (25), real-time quantitative reverse transcription-PCR is not an appropriate method to discriminate wild-type *Survivin* from other splice variants. We carried out semiquantitative reverse transcription-PCR assays to evaluate the gene expression of wild-type *Survivin*, as described previously (26). For wild-type *Survivin* gene expression, the primers used were 5′-CCACCGCATCTCTACATTCA-3′ (sense) and 5′-TATGTTCCTC-TATGGGGTCG-3′ (antisense), and the PCR product size was 185 bp. The reaction mixture was subjected to 36 PCR amplification cycles of 60 s at 94°C, 60 s at 60°C, and 90 s at 72°C. *β-actin* DNA amplification was used as the internal PCR control. The primers used were 5′-GATATCGCCGCGCTCGTCGTCGAC-3′ (sense) and 5′-CAGGAAG-GAAGGCTGGAAGAGTGC-3′ (antisense), and the PCR product size was 792 bp. Preparations of A549 cells were used as positive controls. The amplified DNA samples were run on a 1% agarose gel with ethidium bromide, and the densitometric value obtained for a *Survivin* band in a given tumor sample was divided by the value of the *β-actin*, the internal control. Then the expression ratio for a given tumor sample was divided by the expression ratio of A549 to obtain the normalized *Survivin* gene expression ratio.

Immunohistochemistry. The following antibodies were used, along with isotype antibodies as negative controls: a mouse monoclonal antibody for E2F1 (KH95, Santa Cruz Biotechnology) diluted at 1:100, a rabbit polyclonal antibody for TS diluted at 1:500 (5), a mouse monoclonal antibody for Survivin (D-8, Santa Cruz Biotechnology) diluted at 1:50, and a mouse monoclonal antibody for the Ki-67 antigen (MIB-1, DAKO) diluted at 1:40. Formalin-fixed paraffin-embedded tissue was cut into 4-μm sections and mounted on poly-L-lysine–coated slides. After deparaffinization and rehydration, the slides were heated in a microwave for 30 min in a 10 μmol/L citrate buffer solution at pH 6.0 and were cooled to room temperature for 20 min. After quenching the endogenous peroxidase activity with 0.3% H_2O_2 (in absolute methanol) for 30 min, the sections were treated with 5% bovine serum albumin to block nonspecific staining. Duplicate sections were incubated overnight with the primary antibodies. The slides were

then incubated for 1 h with biotinylated secondary antibodies (Vector Laboratories). The sections were incubated with the avidin-biotin-peroxidase complex (Vector Laboratories) for 1 h, and the antibody binding was visualized with 3,3′-diaminobenzidine tetrahydrochloride. Finally, the sections were lightly counterstained with Mayer's hematoxylin. The sections of lung tumors known to express E2F1, TS, or Survivin were used as positive controls.

All of the immunostained sections were reviewed by two authors (C.H. and M.U.) without knowledge of the patients' characteristics. Cases with discrepancies were jointly reevaluated until a consensus was reached. With regard to E2F1 protein expression, only nuclear staining was considered positive, and the E2F1 status was assessed as the percentage of stained tumor nuclei (17). In the cytoplasmic staining for TS and the nuclear staining for Survivin, all sections were scored in a semiquantitative manner, which reflects both the intensity and the percentage of cells staining at each intensity. The intensity was classified as 0 (no staining), +1 (weak staining), +2 (distinct staining), or +3 (very strong staining). A value designated the "HSCORE" was obtained by using the following algorithm: HSCORE = $\Sigma(I \times PC)$, where I and PC represent staining intensity and the percentage of cells that stain at each intensity, respectively. The percentage of carcinoma cells with positive staining for Ki-67 in a given specimen was scored as the Ki-67 proliferation index (23).

Apoptosis. Apoptotic cells were detected with the terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) method using the In Situ Apoptosis Detection Kit (Takara Biomedicals). After deparaffinization and rehydration, the slides were treated for 15 min with 20 μg/mL proteinase K. After quenching the endogenous peroxidase activity with 3% H_2O_2 for 5 min, the sections were incubated for 90 min at 37°C with the TUNEL reaction mixture, including terminal deoxynucleotidyl transferase. The sections were subsequently incubated for 30 min at 37°C with anti-FITC-horseradish peroxidase conjugate. The staining was developed with 3,3′-diaminobenzidine tetrahydrochloride. Lastly, the sections were lightly counterstained with Mayer's hematoxylin. Sections incubated with the TUNEL reaction mixture without terminal deoxynucleotidyl transferase were used as negative controls. Apoptotic cells were determined based on observations of TUNEL-staining sections and serial H&E-staining sections. TUNEL-staining cells, if they represented the histologic features of necrosis in H&E-staining sections, were not considered to be apoptotic cells. In each case, a total of 10,000 tumor cells were evaluated at high magnification by two authors (J.N. and M.U.) independently without knowledge of the patients' characteristics. Cases with discrepancies were jointly reevaluated until a consensus was reached. The apoptotic index was defined as the number of apoptotic cells per 1,000 tumor cells.

Statistical analysis. Because the distributions of the values, including normalized *E2F1* gene expression ratio ($P = 0.1631$), normalized *TS* gene expression ratio ($P = 0.1606$), normalized *Survivin* gene expression ratio ($P = 0.4736$), Ki-67 proliferation index ($P = 0.9367$), and apoptotic index ($P = 0.3758$), all showed normal distributions (Kolmogorov-Smirnov analysis), the statistical significances of these values were assessed by the t test, ANOVA with Bonferroni/Dunn test, or Pearson's correlation coefficient. In addition, because each cutoff value showed the most significance in relation to the Ki-67 proliferation index, the sample was classified as a high-*E2F1* tumor when the normalized *E2F1* gene expression ratio was >0.25, a high-*TS* tumor when the normalized *TS* gene expression ratio was >0.25, and a high-*Survivin* tumor when the normalized *Survivin* gene expression ratio was >1.0. Overall survival was defined as the time from treatment initiation to the date of death from any cause. The Kaplan-Meier method was used to estimate the probability of overall survival as a function of time, and the differences in the survival of subgroups of patients were compared by using Mantel's log-rank test. A multivariate analysis was done using the Cox regression model to study the effects of different variables on survival. All P values were based on a two-tailed statistical analysis and $P < 0.05$ was considered statistically significant.

Results

E2F1 gene expression in NSCLCs. We initially studied *E2F1* gene expression in 10 noncancerous samples. The normalized *E2F1* gene expression ratio was low in normal lung tissues (mean, 0.0179 ± 0.0115). In contrast, the normalized *E2F1* gene expression ratio varied greatly among the 127 tumor tissues we studied (mean, 0.368 ± 0.547; Table 2). E2F1 protein expression exhibited a nuclear staining pattern with varied cytoplasmic staining (Fig. 1A). The normalized *E2F1* gene expression ratio significantly correlated with the percentage of tumor cells with E2F1 protein expression ($r = 0.708$; $P < 0.0001$). Seventy-six (59.8%) carcinomas were low-*E2F1* tumors and 51 (40.2%) carcinomas were high-*E2F1* tumors. With regard to tumor histology, the normalized *E2F1* gene expression ratio was 0.301 ± 0.508 in adenocarcinomas and 0.441 ± 0.605 in squamous cell carcinomas. There was no significant difference in *E2F1* gene expression according to tumor histology. However, for tumor differentiation, the normalized *E2F1* gene expression ratio was 0.119 ± 0.179 in well-differentiated tumors, 0.322 ± 0.430 in moderately differentiated tumors, and 0.559 ± 0.696 in poorly differentiated tumors. The normalized *E2F1* gene expression ratio was significantly higher in poorly differentiated tumors in comparison with well-differentiated and moderately differentiated tumors ($P = 0.0008$). Furthermore, for pathologic stage, the normalized *E2F1* gene expression ratio was 0.224 ± 0.363 in stage I tumors, 0.607 ± 0.808 in stage II tumors, and 0.400 ± 0.478 in stage III tumors. The normalized *E2F1* gene expression ratio was significantly lower in stage I tumors than in stage II or III tumors ($P = 0.0072$).

Tumor proliferation and apoptotic index of NSCLCs in relation to E2F1 gene expression. To investigate the biological function of *E2F1* expression in NSCLC, we evaluated the Ki-67 proliferation index and the apoptotic index in relation to the *E2F1* status (Fig. 1B). With regard to tumor proliferation, the normalized *E2F1* gene expression ratio showed a significant positive correlation with the Ki-67 proliferation index ($r = 0.487$; $P < 0.0001$; Fig. 2A). The Ki-67 proliferation index was significantly higher in high-*E2F1* tumors than in low-*E2F1* tumors (66.5 ± 22.2% versus 35.0 ± 26.0%; $P < 0.0001$). However, no correlation was observed between the normalized *E2F1* gene expression ratio and the apoptotic index ($r = 0.129$; $P = 0.1488$; Fig. 2B). The apoptotic index was 2.04 ± 0.67 in low-*E2F1* tumors and 2.02 ± 0.68 in high-*E2F1* tumors.

TS and Survivin gene expressions in relation to E2F1 gene expression. We subsequently studied the gene expressions of *TS* and *Survivin*, the tumor-associated *E2F1* target genes. With regard to *TS*, the normalized *TS* gene expression ratio varied greatly among the 127 tumor tissues we studied (mean, 0.668 ± 1.029). The TS protein expression exhibited a cytoplasmic staining pattern (Fig. 1C) and significantly correlated with the HSCORE of TS protein expression in tumor cells ($r = 0.603$; $P < 0.0001$). Furthermore, the normalized *E2F1* gene expression ratio showed a significant positive correlation with the normalized *TS* gene expression ratio ($r = 0.709$; $P < 0.0001$; Fig. 2C). The normalized *TS* gene expression ratio was significantly higher in high-*E2F1* tumors than in low-*E2F1* tumors (1.296 ± 1.369 versus 0.246 ± 0.282; $P < 0.0001$).

With regard to *Survivin*, the normalized *Survivin* gene expression ratio also varied greatly among the 127 tumor

Table 2. Distribution of *E2F1* gene expression in patients with NSCLC according to clinicopathologic characteristics

Characteristics	No. patients	*E2F1* gene expression	*P*
Smoking			
Nonsmoker	36	0.205 ± 0.311	0.0737
Smoker	91	0.433 ± 0.605	
Tumor status			
T_1	45	0.294 ± 0.565	0.6285
T_2	45	0.401 ± 0.573	
T_3	20	0.364 ± 0.359	
T_4	17	0.484 ± 0.622	
Nodal status			
N_0	81	0.258 ± 0.392	0.0022
N_1, N_2, N_3	46	0.562 ± 0.709	
Pathologic stage			
I	57	0.224 ± 0.363	0.0072
II	29	0.607 ± 0.808	
III	41	0.400 ± 0.478	
Differentiation			
Well	33	0.119 ± 0.179	0.0008
Moderate	41	0.322 ± 0.430	
Poor	53	0.559 ± 0.696	
Histology			
Adenocarcinoma	63	0.301 ± 0.508	0.3795
Squamous cell carcinoma	58	0.441 ± 0.605	
Large-cell carcinoma	6	0.369 ± 0.214	
Total no. patients	127	0.368 ± 0.547	

tissues we studied (mean, 1.327 ± 0.686). The Survivin protein expression exhibited a pattern of nuclear staining with varied cytoplasmic staining (Fig. 1D). The normalized *Survivin* gene expression ratio significantly correlated with the HSCORE of the nuclear staining of Survivin in tumor cells (r = 0.448; $P < 0.0001$). Furthermore, the normalized *E2F1* gene expression ratio showed a significant positive correlation with the normalized *Survivin* gene expression ratio (r = 0.403; $P < 0.0001$; Fig. 2D). The normalized *Survivin* gene expression ratio was significantly higher in high-*E2F1* tumors than in low-*E2F1* tumors (1.585 ± 0.694 versus 1.153 ± 0.626; P = 0.0004).

Clinical significance of TS expression in NSCLCs. With regard to tumor proliferation, the normalized *TS* gene expression ratio positively correlated with the Ki-67 proliferation index (r = 0.460; $P < 0.0001$; Fig. 2E). The Ki-67 proliferation index was significantly higher in high-*TS* tumors than in low-*TS* tumors (61.3 ± 23.4% versus 31.9 ± 26.8%; $P < 0.0001$). However, for tumor apoptosis, the apoptotic index was 2.14 ± 0.66 in low-*TS* tumors and 1.92 ± 0.67 in high-*TS* tumors. There was no difference in the apoptotic index in relation to *TS* status.

Clinical significance of Survivin expression in NSCLCs. The normalized *Survivin* gene expression ratio positively correlated with the Ki-67 proliferation index (r = 0.357; $P < 0.0001$; Fig. 2F). The Ki-67 proliferation index was significantly higher in high-*Survivin* tumors than in low-*Survivin* tumors (58.2 ± 24.8% versus 35.5 ± 28.8%; $P < 0.0001$).

Furthermore, there was a significant inverse relationship between the normalized *Survivin* gene expression ratio and the apoptotic index (r = -0.355; $P < 0.0001$; Fig. 2G). The apoptotic index was significantly lower in high-*Survivin* tumors than in low-*Survivin* tumors (1.80 ± 0.54 versus 2.29 ± 0.72; $P < 0.0001$).

Overall survival of NSCLC patients. With regard to *E2F1* status, among 127 NSCLC patients, the 3-year survival rate was significantly lower in patients with high-*E2F1* tumors than in those with low-*E2F1* tumors (50.0% versus 79.8%; P = 0.0027; Fig. 3A). Especially among patients with stage II to III NSCLCs, the 3-year survival rate was significantly lower in patients with high-*E2F1* tumors than in those with low-*E2F1* tumors (40.1% versus 71.6%; P = 0.0188; Fig. 3B). In contrast, no difference was observed on the survival of patients with stage I NSCLCs according to the *E2F1* status (Fig. 3C). As shown in Table 3, a multivariate analysis using the Cox regression model showed that the *E2F1* status (hazard ratio, 2.052; P = 0.0261) and the pathologic stage (hazard ratio, 2.001; P = 0.0008) were significant prognostic factors for NSCLC patients.

We subsequently studied the overall survival of NSCLC patients in relation to the *E2F1* target genes. With regard to *TS* status, the 3-year survival rate was significantly lower in patients with high-*TS* tumors than in those with low-*TS* tumors (59.9% versus 77.6%; P = 0.0356; Fig. 3D). Furthermore, for the *E2F1* and *TS* status, the 3-year survival rates were 83.6% in 53 patients with both low-*E2F1* and low-*TS* tumors, 61.8% in 28 patients with either high-*E2F1* or high-*TS* tumors, and 53.7% in 46 patients with both high-*E2F1* and high-*TS* tumors (Fig. 3E). The survival rate in patients with both low-*E2F1* and low-*TS* tumors was significantly the highest among all of these groups (P = 0.0098). On the other hand, for the *Survivin* status, no difference was observed between the 3-year survival rate in patients with high-*Survivin* tumors and that in patients with low-*Survivin* tumors (67.1% versus 68.8%).

Discussion

The E2F family plays a pivotal role in cell cycle control and apoptosis (8). E2F1 belongs to a subclass of E2F factors that are thought to act as transcriptional activators, and it is the "final frontier" of the G_1-to-S phase boundary (27). Many upstream

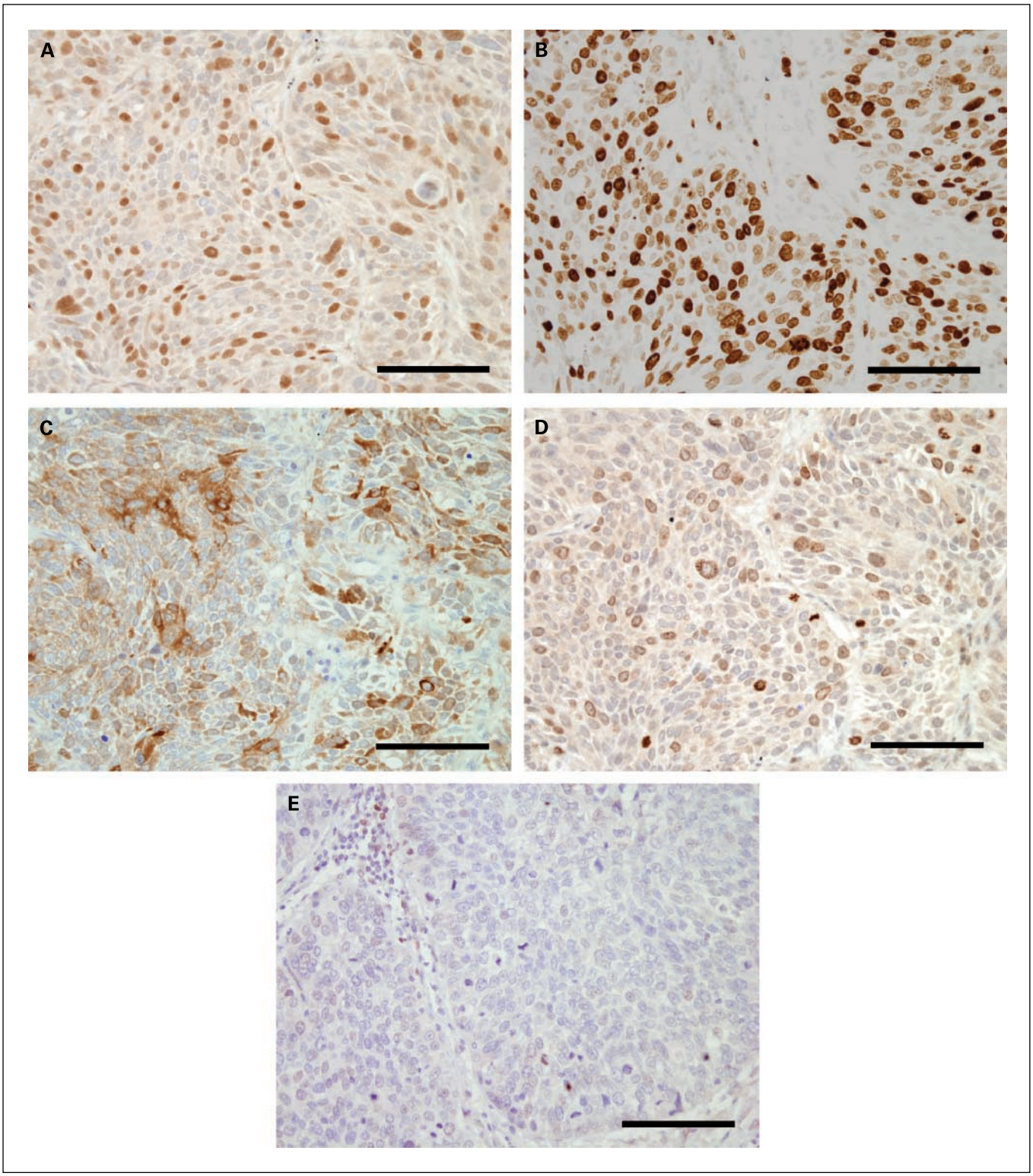

Fig. 1. Immunohistochemical staining of human NSCLC tissue using the avidin-biotin-peroxidase complex procedure. A carcinoma with high expression of E2F1 (*A*), high Ki-67 index (*B*), high expression of TS (*C*), and high expression of Survivin (*D*). *E,* a low-E2F1 carcinoma. Bar, 500 μm.

stimulatory and inhibitory signals converge on the E2F1-pRB (retinoblastoma gene product) complex (28). Inactive E2F1 is complexed to pRB. On hyperphosphorylation of pRB as part of the transition from the G_1 to S phase, the E2F1 protein is released from the E2F1-pRB complex, and the "free E2F1" becomes available to transcriptionally activate various target genes required for DNA synthesis.

However, because E2F1 modulates diverse cellular functions, such as mitosis and apoptosis (8), *in vitro* studies on E2F1 reported controversial results about its function associated with

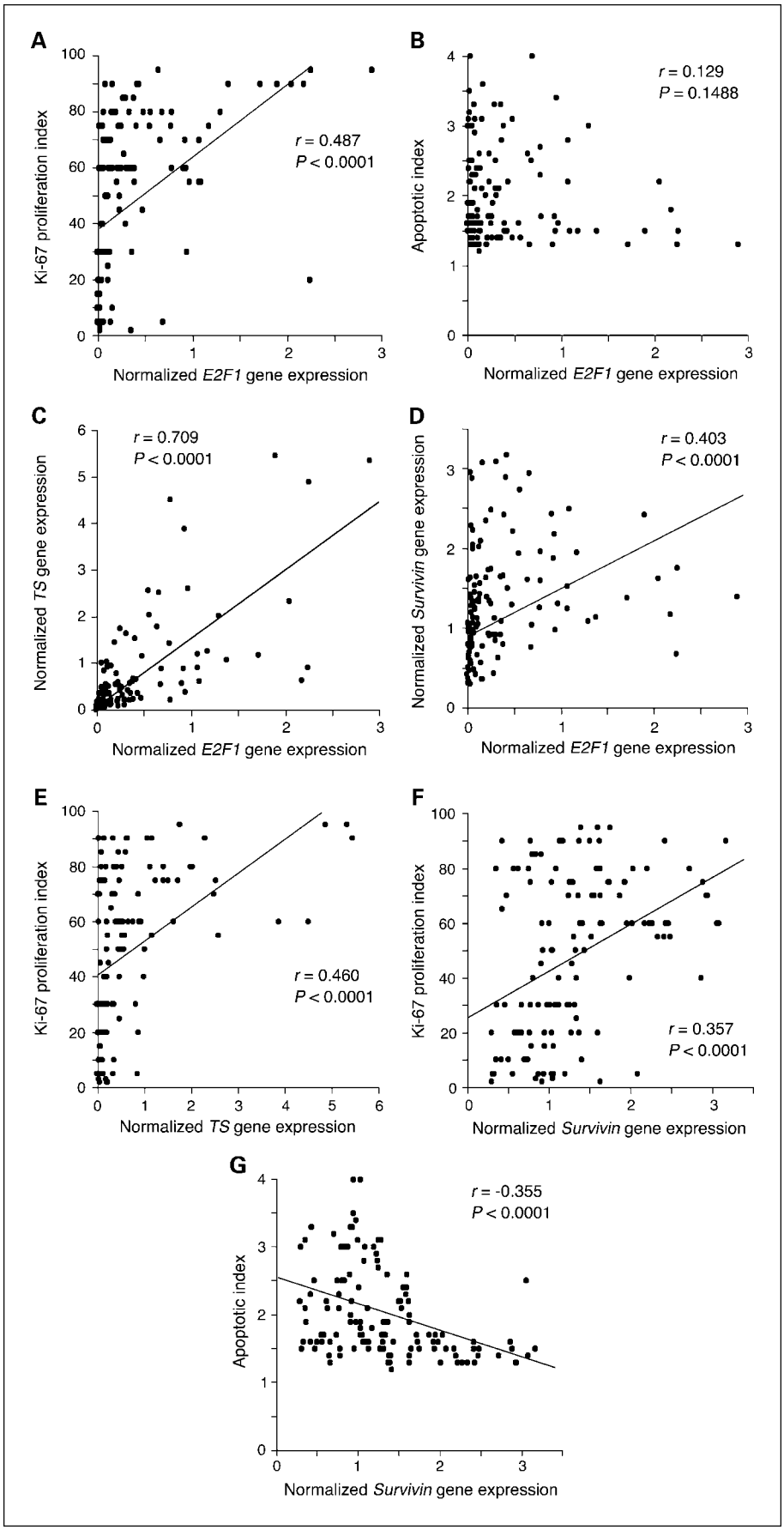

Fig. 2. *A,* the relationship between *E2F1* gene expression and the Ki-67 proliferation index. *B,* the relationship between *E2F1* gene expression and the apoptotic index. *C,* the relationship between *E2F1* gene expression and *TS* gene expression. *D,* the relationship between *E2F1* gene expression and *Survivin* gene expression. *E,* the relationship between *TS* gene expression and the Ki-67 proliferation index. *F,* the relationship between *Survivin* gene expression and the Ki-67 proliferation index. *G,* the relationship between *Survivin* gene expression and the apoptotic index.

tumorigenesis. Several studies have revealed that the *E2F1* gene can act as an oncogene by enhancing transformation activity, tumor proliferation, and invasive ability (13, 14). In contrast, other studies reported that the *E2F1* gene transfer induced apoptosis and suppressed tumor growth (15, 16). In fact, previous clinical studies also showed that E2F1 has a tumor-promoting effect in many cancers including lung cancer (17–21) whereas the opposite seems to be the case for other cancers (21, 22).

Therefore, to clarify the clinical significance of E2F1 in NSCLCs, we studied *E2F1* gene expression in relation to tumor proliferation and tumor apoptosis. The present study showed that *E2F1* gene expression positively correlates with tumor proliferation. However, no correlation was observed between *E2F1* gene expression and tumor apoptosis. Gorgoulis et al. (17) also reported similar results with E2F1 protein expression in NSCLC. These results in clinical studies might be partly because acquired gene mutations in the apoptotic pathway, such as in *p53*, can neutralize the apoptotic response of E2F1 (29). It is notable that 44% (56 of 127) of NSCLCs had *p53* mutations in the present study (data not shown). In addition, the present study revealed that *E2F1* overexpression is associated with a poor prognosis in NSCLC patients, as also reported by Gorgoulis et al. (17). These results revealed that *E2F1* overexpression has a tumor-promoting effect in NSCLCs.

Furthermore, the analyses of *E2F1* target genes associated with tumorigenesis are important for the clarification of its biological functions in NSCLC. We therefore carried out a

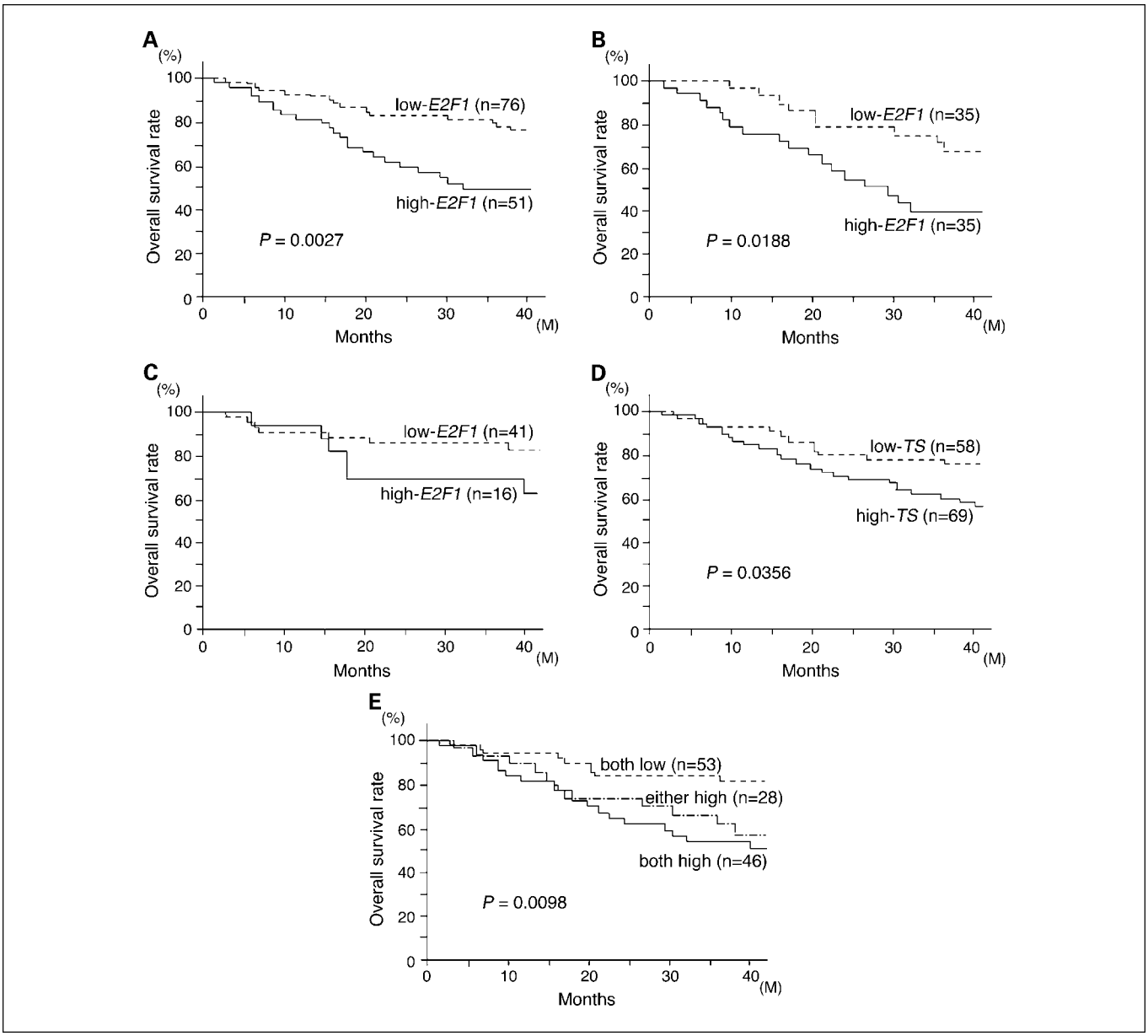

Fig. 3. *A,* overall survival of 127 NSCLC patients in relation to the *E2F1* status. *B,* overall survival of 70 patients with stage II to III NSCLC in relation to the *E2F1* status. *C,* overall survival of 57 patients with stage I NSCLC in relation to the *E2F1* status. *D,* overall survival of 127 NSCLC patients in relation to the *TS* status. *E,* overall survival of 127 NSCLC patients in relation to the *E2F1* and *TS* status.

Table 3. Multivariate regression analysis in predicting overall survival of 127 patients with NSCLC

Variables	Assigned score	Hazard ratio (95% CI)	*P*
E2F1 status			
Low	0	2.052 (1.089-3.865)	0.0261
High	1		
Pathologic stage			
I	1	2.001 (1.336-2.996)	0.0008
II	2		
III	3		
Age, y			
<60	0	1.752 (0.869-3.529)	0.1168
≥60	1		
Gender			
Female	0	1.245 (0.479-3.235)	0.6524
Male	1		
Smoking			
Nonsmoker	0	0.948 (0.355-2.536)	0.9160
Smoker	1		

Abbreviation: 95% CI, 95% confidence interval.

clinical study of *E2F1* gene expression in relation to its target genes, *TS* (9, 10) and *Survivin* (11). Consequently, the present study identified the novel fact that *E2F1* gene expression positively correlates with *TS* and *Survivin* expressions in NSCLC.

With regard to TS, it plays a central role in the biosynthesis of thymidylate, an essential precursor for DNA synthesis (30). Recent research has revealed that TS exhibits oncogene-like activity (31). Experimental studies showed that TS expression was associated with tumor cell proliferation (32). Clinical studies, including the present study, have also revealed that TS expression is positively associated with tumor proliferation (33). Furthermore, TS is a target molecule of 5-FU, one of the commonly prescribed antitumor drugs, and TS expression is related to 5-FU sensitivity (4). Many clinical studies involving human cancers, including NSCLC and gastrointestinal tumors, have shown that high TS expression is associated with 5-FU resistance and a poor outcome in cancer patients (5, 34, 35). The present study also revealed that high *TS* expression is associated with a poor prognosis in NSCLC patients.

It is also important to clarify the mechanisms of TS regulation. Previous experimental studies reported that *E2F1* induces DNA synthesis and G_1-S regulatory genes, including *TS* (9). In addition, *E2F1*-transfected cells associated with an up-regulation of *TS* were more resistant to 5-FU than the cells that had not been transfected with *E2F1* (10). Recent clinical studies also reported that *E2F1* gene expression correlates with *TS* gene expression in colon cancer and osteosarcoma (36–38). The present study is the first clinical study showing the positive association between *E2F1* gene expression and *TS* gene expression in NSCLC.

With regard to Survivin, it is the smallest member of the inhibitor of apoptosis protein family (12). Survivin has a single baculovirus inhibitor of apoptosis repeat domain that inhibits the caspase family, and the caspase family mediates apoptosis (39). Experimental studies have shown that the antisense RNA for *Survivin* can induce apoptosis in tumor cells (40). Furthermore, the nuclear localization of Survivin protein has been reported to affect cell mitosis (41, 42). Survivin expression is therefore predicted to promote tumorigenesis by regulating not only apoptosis but also mitosis. Previous clinical studies have revealed that Survivin is expressed in most human cancers (43–46), whereas its expression is undetectable in normal differentiated tissues (47). Furthermore, the Survivin expression is associated with apoptosis (43–45), tumor proliferation (45, 46), and a poor prognosis in cancer patients (44, 46). The present study has also showed that *Survivin* gene expression correlates with the enhancement of tumor proliferation and the inhibition of apoptosis in NSCLC. However, no difference was observed in the patient survival according to the *Survivin* status. This result might be due to the relatively small number of patients we studied.

Notably, a recent experimental study has shown that E2F1 can bind to the *Survivin* gene promoter and induce *Survivin* transcription (11). The present clinical study also revealed that *E2F1* gene expression correlates positively with *Survivin* gene expression in NSCLCs. To our knowledge, the present study is the first clinical report showing the association between *E2F1* gene expression and *Survivin* gene expression in human cancer. Furthermore, the up-regulation of *Survivin*, which can inhibit tumor apoptosis, might be partly responsible for the absence of a correlation between *E2F1* overexpression and tumor apoptosis in human cancers, as shown in the present study.

The present study revealed that *E2F1* gene expression was significantly higher in the advanced-stage tumors than in the early-stage tumors. Furthermore, *E2F1* expression in NSCLC correlates positively with *TS* expression, *Survivin* expression, and tumor proliferation. These results indicate that, during the progression of NSCLC, *E2F1* overexpression could result in the production of more aggressive tumor cells with a high proliferation rate and chemoresistance.

Although *E2F1* overexpression is frequently seen in many human cancers (17–21, 36–38, 48), its biological mechanism is still unclear. Gene amplification of *E2F1* has been reported to be a rare event in human cancers including NSCLC and gastrointestinal cancer (17, 18, 48). Suzuki et al. (48) reported that E2F1 protein expression was associated with the increased expression of its gene, rather than the amplification of its gene. The present study also showed that *E2F1* gene expression is positively correlated with E2F1 protein expression. Therefore, deregulated *E2F1* mRNA synthesis may be a chief mechanism of *E2F1* overexpression (49, 50). Additional investigations are required to develop new treatment strategies for patients with *E2F1*-overexpressing NSCLC.

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References

1. Cersosimo RJ. Lung cancer: a review. Am J Health Syst Pharm 2002;59:611–2.
2. Huang C, Liu D, Masuya D, et al. Clinical application of biological markers for treatments of resectable non-small-cell lung cancers. Br J Cancer 2005;92:1231–9.
3. Huang C, Yokomise H, Fukushima M, Kinoshita M. Tailor-made chemotherapy for non-small cell lung cancer patients. Future Oncol 2006;2:289–99.
4. Beck A, Etienne MC, Cheradame S, et al. A role for

dihydropyrimidine dehydrogenase and thymidylate synthase in tumour sensitivity to fluorouracil. Eur J Cancer 1994;30A:1517–22.
5. Nakano J, Huang C, Liu D, et al. Evaluations of biomarkers associated with 5-FU sensitivity for non-small-cell lung cancer patients postoperatively treated with UFT. Br J Cancer 2006;95:607–15.
6. Bell DW, Lynch TJ, Haserlat SM, et al. Epidermal growth factor receptor mutations and gene amplification in non-small-cell lung cancer: molecular analysis of the IDEAL/INTACT gefitinib trials. J Clin Oncol 2005;23:8081–92.
7. Tanaka F, Otake Y, Yanagihara K, et al. Apoptosis and p53 status predict the efficacy of postoperative administration of UFT in non-small cell lung cancer. Br J Cancer 2001;84:263–9.
8. Bell LA, Ryan KM. Life and death decisions by E2F-1. Cell Death Differ 2004;11:137–42.
9. DeGregori J, Kowalik T, Nevins JR. Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G_1/S-regulatory genes. Mol Cell Biol 1995;15:4215–24.
10. Banerjee D, Schnieders B, Fu JZ, Adhikari D, Zhao SC, Bertino JR. Role of E2F-1 in chemosensitivity. Cancer Res 1998;58:4292–6.
11. Jiang Y, Saavedra HI, Holloway MP, Leone G, Altura RA. Aberrant regulation of survivin by the RB/E2F family of proteins. J Biol Chem 2004;279:40511–20.
12. Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med 1997;3:917–21.
13. Johnson DG, Cress WD, Jakoi L, Nevins JR. Oncogenic capacity of the E2F1 gene. Proc Natl Acad Sci U S A 1994;91:12823–7.
14. Zhang SY, Liu SC, Johnson DG, Klein-Szanto AJ. E2F-1 gene transfer enhances invasiveness of human head and neck carcinoma cell lines. Cancer Res 2000;60:5972–6.
15. Fueyo J, Gomez-Manzano C, Yung WK, et al. Overexpression of E2F-1 in glioma triggers apoptosis and suppresses tumor growth *in vitro* and *in vivo*. Nat Med 1998;4:685–90.
16. Dong YB, Yang HL, Elliott MJ, et al. Adenovirus-mediated E2F-1 gene transfer efficiently induces apoptosis in melanoma cells. Cancer 1999;86:2021–33.
17. Gorgoulis VG, Zacharatos P, Mariatos G, et al. Transcriptional factor E2F-1 acts as a growth-promoting factor and is associated with adverse prognosis in non-small cell lung carcinomas. J Pathol 2002;198:142–56.
18. Eymin B, Gazzeri S, Brambilla C, Brambilla E. Distinct pattern of E2F1 expression in human lung tumours: E2F1 is up-regulated in small cell lung carcinoma. Oncogene 2001;20:1678–87.
19. Zhang SY, Liu SC, Al-Saleem LF, et al. E2F-1: a proliferative marker of breast neoplasia. Cancer Epidemiol Biomarkers Prev 2000;9:395–401.
20. Saiz AD, Olvera M, Rezk S, Florentine BA, McCourty A, Brynes RK. Immunohistochemical expression of cyclin D1, E2F-1, and Ki-67 in benign and malignant thyroid lesions. J Pathol 2002;198:157–62.
21. Zacharatos P, Kotsinas A, Evangelou K, et al. Distinct expression patterns of the transcription factor E2F-1 in relation to tumour growth parameters in common human carcinomas. J Pathol 2004;203:744–53.
22. Rabbani F, Richon VM, Orlow I, et al. Prognostic significance of transcription factor E2F-1 in bladder cancer: genotypic and phenotypic characterization. J Natl Cancer Inst 1999;91:874–81.
23. Scagliotti GV, Micela M, Gubetta L, et al. Prognostic significance of Ki67 labelling in resected non small cell lung cancer. Eur J Cancer 1993;29A:363–5.
24. Mountain CF. Revisions in the international system for staging lung cancer. Chest 1997;111:1710–7.
25. Mahotka C, Wenzel M, Springer E, Gabbert HE, Gerharz CD. Survivin-ΔEx3 and survivin-2B: two novel splice variants of the apoptosis inhibitor survivin with different antiapoptotic properties. Cancer Res 1999;59:6097–102.
26. Nakano J, Huang C, Liu D, Ueno M, Sumitomo S, Yokomise H. Survivin gene expression is negatively regulated by the p53 tumor suppressor gene in non-small cell lung cancer. Int J Oncol 2005;27:1215–21.
27. Wu L, Timmers C, Maiti B, et al. The E2F1-3 transcription factors are essential for cellular proliferation. Nature 2001;414:457–62.
28. Dyson N. The regulation of E2F by pRB-family proteins. Genes Dev 1998;12:2245–62.
29. Pan H, Yin C, Dyson NJ, Harlow E, Yamasaki L, Van Dyke T. Key roles for E2F1 in signaling p53-dependent apoptosis and in cell division within developing tumors. Mol Cell 1998;2:283–92.
30. Rustum YM, Harstrick A, Cao S, et al. Thymidylate synthase inhibitors in cancer therapy: direct and indirect inhibitors. J Clin Oncol 1997;15:389–400.
31. Rahman L, Voeller D, Rahman M, et al. Thymidylate synthase as an oncogene: a novel role for an essential DNA synthesis enzyme. Cancer Cell 2004;5:341–51.
32. Navalgund LG, Rossana C, Muench AJ, Johnson LF. Cell cycle regulation of thymidylate synthetase gene expression in cultured mouse fibroblasts. J Biol Chem 1980;255:7386–90.
33. Nakagawa T, Otake Y, Yanagihara K, et al. Expression of thymidylate synthase is correlated with proliferative activity in non-small cell lung cancer (NSCLC). Lung Cancer 2004;43:145–9.
34. Johnston PG, Lenz HJ, Leichman CG, et al. Thymidylate synthase gene and protein expression correlate and are associated with response to 5-fluorouracil in human colorectal and gastric tumors. Cancer Res 1995;55:1407–12.
35. Yeh KH, Shun CT, Chen CL, et al. High expression of thymidylate synthase is associated with the drug resistance of gastric carcinoma to high dose 5-fluorouracil-based systemic chemotherapy. Cancer 1998;82:1626–31.
36. Banerjee D, Gorlick R, Liefshitz A, et al. Levels of E2F-1 expression are higher in lung metastasis of colon cancer as compared with hepatic metastasis and correlate with levels of thymidylate synthase. Cancer Res 2000;60:2365–7.
37. Kasahara M, Takahashi Y, Nagata T, et al. Thymidylate synthase expression correlates closely with E2F1 expression in colon cancer. Clin Cancer Res 2000;6:2707–11.
38. Sowers R, Toguchida J, Qin J, et al. mRNA expression levels of E2F transcription factors correlate with dihydrofolate reductase, reduced folate carrier, and thymidylate synthase mRNA expression in osteosarcoma. Mol Cancer Ther 2003;2:535–41.
39. Deveraux QL, Reed JC. IAP family proteins-suppressors of apoptosis. Genes Dev 1999;13:239–52.
40. Ambrosini G, Adida C, Sirugo G, Altieri DC. Induction of apoptosis and inhibition of cell proliferation by survivin gene targeting. J Biol Chem 1998;273:11177–82.
41. Li F, Ambrosini G, Chu EY, et al. Control of apoptosis and mitotic spindle checkpoint by survivin. Nature 1998;396:580–4.
42. Adams RR, Carmena M, Earnshaw WC. Chromosomal passengers and the (aurora) ABCs of mitosis. Trends Cell Biol 2001;11:49–54.
43. Lu CD, Altieri DC, Tanigawa N. Expression of a novel antiapoptosis gene, survivin, correlated with tumor cell apoptosis and p53 accumulation in gastric carcinomas. Cancer Res 1998;58:1808–12.
44. Kawasaki H, Altieri DC, Lu CD, Toyoda M, Tenjo T, Tanigawa N. Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer. Cancer Res 1998;58:5071–4.
45. Morinaga S, Nakamura Y, Ishiwa N, et al. Expression of survivin mRNA associates with apoptosis, proliferation and histologically aggressive features in hepatocellular carcinoma. Oncol Rep 2004;12:1189–94.
46. Sui L, Dong Y, Ohno M, Watanabe Y, Sugimoto K, Tokuda M. Survivin expression and its correlation with cell proliferation and prognosis in epithelial ovarian tumors. Int J Oncol 2002;21:315–20.
47. Salvesen GS, Duckett CS. IAP proteins: blocking the road to death's door. Nat Rev Mol Cell Biol 2002;3:401–10.
48. Suzuki T, Yasui W, Yokozaki H, Naka K, Ishikawa T, Tahara E. Expression of the E2F family in human gastrointestinal carcinomas. Int J Cancer 1999;81:535–8.
49. Neuman E, Flemington EK, Sellers WR, Kaelin WG Jr. Transcription of the E2F-1 gene is rendered cell cycle dependent by E2F DNA-binding sites within its promoter. Mol Cell Biol 1994;14:6607–15.
50. Hsiao KM, McMahon SL, Farnham PJ. Multiple DNA elements are required for the growth regulation of the mouse E2F1 promoter. Genes Dev 1994;8:1526–37.