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Cyclin E is the Only Cyclin-dependent Kinase 2-associated Cyclin that Predicts Metastasis and Survival in Early Stage Non-Small Cell Lung Cancer[1]

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ABSTRACT

Progression through G_1-S transition and S phase of the cell cycle is mediated by cyclin-dependent kinase 2 (cdk2), which interacts with several cyclins. Two of these, cyclin E and cyclin A2 (also known as cyclin A), are overexpressed in many cancers. Cyclin E2 and cyclin A1 are recently discovered cdk2-interacting cyclins that are found in malignant tumor cell lines and in acute myeloid leukemia, respectively. Expression and prognostic role of these cyclins in solid tumors is unknown. Here, we have analyzed expression and prognostic relevance of the cdk2-associated cyclins in non-small cell lung cancer (NSCLC). Fresh-frozen biopsies ($n = 70$) from completely resected tumors with stage I to IIIA NSCLC were studied. Gene expression was analyzed by quantitative real-time reverse transcription-PCR. Expression levels of *cyclin E* ($P = 0.04$) and *cyclin A2* ($P = 0.004$) were significantly higher in the tumor samples than in normal controls. *Cyclin A1*, *cyclin A2*, and *cyclin E2* expression levels did not have prognostic relevance for survival. The mean survival time associated with low and high levels of *cyclin E* was 69.4 and 47.2 months, respectively, which was statistically significant ($P = 0.03$). Differences in survival were particularly pronounced in stages I and II. *Cyclin E* was also closely associated with the development of distant metastasis ($P = 0.01$). Finally, we confirmed by immunohistochemistry analyses that *cyclin E* mRNA expression was closely associated with cyclin E protein expression. In conclusion, *cyclin E* is a strong independent prognostic indicator in patients with early-stage NSCLC, whereas *cyclin E2*, *cyclin A1*, and *cyclin A2* do not have a prognostic role in NSCLC.

INTRODUCTION

Dysregulation of the cell cycle is a prerequisite for the formation of most if not all malignant tumors (1). The relevant mechanisms that drive the cell from the G_0/G_1 phase into S phase have been discovered in recent years (2, 3). The importance of G_1-S progression in the formation of tumors has been highlighted by a high incidence of aberrations in involved genes in a wide variety of tumors (1). The central gene that controls S phase entry is *RB*.[3] *RB* and its related family members *p107* and *p130* are important for the control of DNA synthesis and S phase progression (4). RB in its under-phosphorylated form binds tightly to E2F, recruiting a repressor complex to responsive genes by binding to E2F sites in their promoters. These repressive effects are overcome during G_1-S progression by consecutive phosphorylation of *RB* at different sites by cyclin-cdk complexes (5). During the G_1 phase, *RB* phosphorylation is mediated by D-type cyclins complexed with cdk4 and cdk6. These phosphorylations appear to be growth factor-dependent and constitute the final messenger in signal transduction pathways originating from transmembrane receptors. In addition, steroid hormones such as estrogen have been shown to interact directly with D-type cyclins (6, 7). After passing G_1 phase, the cell arrives at the G_1-S checkpoint, which is critically controlled by cdk2 that associates with E- and A-type cyclins (8). Cyclin E-cdk2 complexes induce *RB* phosphorylation at sites distinct from cyclin D (5). Finally, complete phosphorylation of *RB* triggers the initiation of S phase. Overexpression of *cyclin D1*, *cyclin E*, and *cyclin A* have been demonstrated to promote S phase entry (9, 10). Physiologically, cyclin E levels determine the time point when cells enter S phase (11, 12), and activation of *cyclin E* appears to be a main function of *cyclin D1* (13). In addition, the cyclin E promoter is transactivated by the E2F transcription factor, and cyclin E protein is targeted for destruction by ubiquitination (14, 15).

The important role of *cyclin E* has also been demonstrated by reports that *cyclin E* is overexpressed in several types of cancer (16–19). Immunohistochemistry studies demonstrated that high levels of *cyclin E* and low levels of *p27* predicted a poor prognosis in young breast cancer patients (12). In addition, *cyclin E* expression in cancer is often deregulated with regard to the cell cycle (20). In two recent papers, the expression of *cyclin E* has been studied in lung cancer patients by immunohistochemistry (21, 22). The findings obtained suggested that a high labeling index for cyclin E protein might be associated with a poorer prognosis for patients with early-stage NSCLC (21, 22). However, there are no data regarding the important question of whether this effect is confined to *cyclin E*, or whether expression levels of other cdk2-associated cyclins are associated with a poor prognosis in NSCLC as well.

Recently, a new mammalian cyclin, *cyclin E2*, has been cloned that shows a high degree of homology to *cyclin E* (23, 24). This cyclin was expressed in lung cancer cell lines but not in their nontransformed counterparts (25). The unknown importance of E-type cyclins in early stage NSCLC prompted us to study the level of expression and the prognostic role of cyclin E and cyclin E2 in early-stage NSCLC. Furthermore, A-type cyclins may drive cells into S phase, and levels of cyclin A2 (formerly cyclin A) are associated with the percentage of tumor cells in S phase (26). Cyclin A1 is a recently cloned A-type cyclin that is highly expressed in acute leukemias (27).

To study expression of the cdk2-interacting cyclins, we developed real-time quantitative RT-PCR assays that are based on the 5′ nuclease assay (28, 29). Quantitative real-time PCR allows for an accurate quantitation of gene expression that is less error-prone and much more accurate than immunohistochemistry. In addition, quantitative RT-PCR can be performed on very small amounts of tumor tissue.

In this study, we demonstrate that expression of E-type and A-type cyclins in NSCLC is independent of tumor stage and is not associated with size or grade of the tumor. Overexpression of *cyclin E* and *cyclin A2*, but neither *cyclin E2* nor *cyclin A1*, was found in a high percentage of tumor samples. In addition, high expression levels of cyclin E

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[3] RB, retinoblastoma gene product; cdk, cyclin-dependent kinase; NSCLC, non-small cell lung cancer; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TAMRA, 6-carboxytetramethylrhodamine; PCNA, proliferating cell nuclear antigen.

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were associated with the subsequent development of metastasis and a poor prognosis. In summary, we show that in stage I and II NSCLC, *cyclin E* constitutes an important independent prognostic parameter for survival.

MATERIALS AND METHODS

Tumor Specimens and Survival Data. Primary tumor specimens were obtained at the time of initial surgery for NSCLCs at a university hospital in Germany. Samples were snap-frozen in liquid nitrogen and stored at −80°C. Only samples from individuals with stages I to IIIA that were resected without pathological evidence for remaining tumor (R0 resection) were included into this study. In addition, patients who survived for <90 days after surgery were excluded. Patient characteristics are presented in Table 1. Patients with stage IIIA tumors received radiation therapy after surgery. All patients were followed-up for a minimum period of 5 years.

RNA Isolation and cDNA Preparation. The tumor samples were checked for the percentage of tumor cells by histology, and only tumor biopsies with at least 70% cancer cells were used for subsequent analyses. Similarly, cancer-free control samples were confirmed by histological examination. For RNA preparation, samples were disrupted into small pieces, and RNA was isolated from tumor samples using Trizol reagent (Life Technologies, Inc.). A total of 1 μg of RNA from each sample was reverse-transcribed using an oligo d(T) primer and MMLV reverse transcriptase according to the protocol of the manufacturer (Clontech, Palo Alto, CA). The cDNA was diluted to give a total volume of 200 μl, and 5 μl of this dilution was used for each PCR reaction. The quality of the cDNA was confirmed by amplification of GAPDH (see below), and only samples with consistent and strong amplification of GAPDH were included in the final analyses.

Analyses of Gene Expression by Real-time Quantitative RT-PCR. The quantitation of cyclin mRNA levels was carried out using a real-time fluorescence detection method (28, 30). The cDNA was prepared as described above and amplified by PCR in the ABI Prism 7700 sequence detector (PE Biosystems, Foster City, CA). The following primers and probes were used (all 5′to 3′ direction, the sequence in the middle indicates the probe): Cyclin E: CTC CAG GAA GAG GAA GGC AA, FAM-CGT GAC CGT TTT TTT GCA GGA TCC-TAMRA, and TCG ATT TTG GCC ATT TCT TCA; Cyclin E2: TGT TGG CCA CCT GTA TTA TCT GG, FAM-CAC TCA TGT TGA GAC TTA ATC CCT AAT GTG GCA-TAMRA, ATC TGG AGA AAT CAC TTG TTC CTA TTT C; Cyclin A2: AGC TGC CTT TCA TTT AGC ACT CTA C, FAM-TCA CGG GAC AAA GCT GGC CTG AA-TAMRA, TTA AGA CTT TCC AGG GTA TAT CCA GTC; and PCNA: ATC ATT ACA CTA AGG GCC GAA GAT AAC, FAM- CCT TGG CGC TAG TAT TTG AAG CAC CAA ACC-TAMRA, TCA TTT CAT AGT CTG AAA CTT TCT CCT G. The cyclin A1 and GAPDH primers and probes have been described previously (29). Primer and probe combinations were positioned to span an exon-exon junction. When genomic DNA was used as a template, no bands were seen after PCR amplification. The probes were labeled at the 5′ end with VIC (GAPDH probe) or with FAM (all others) and at the 3′ end with TAMRA, which served as a quencher. The 5′ to 3′ nuclease activity of the Taq polymerase cleaved the probe and released the fluorescent dyes (VIC or FAM), which were detected by the laser detector of the sequence detector (29). After the detection threshold was reached, the fluorescence signal was proportional to the amount of PCR product generated. Initial template concentration could be calculated from the cycle number when the amount of PCR product passed a threshold set in the exponential phase of the PCR reaction. Relative gene expression levels were calculated using standard curves generated by serial dilutions of U937 cDNA. The relative amounts of gene expression were calculated by using the expression of GAPDH as an internal standard. At least two independent analyses were performed for each sample and for each gene. Analyses of gene expression data were performed without the knowledge of patient data.

Statistical data analyses were performed using SPSS 9.0 and the statistical tests indicated in "Results." The following parameters were included into the Cox regression analysis for individuals with stages I and II NSCLC: (*a*) cyclin E; (*b*) cyclin E2; (*c*) cyclin A1; (*d*) cyclin A2, (*e*) p53 mutation status; (*f*) sex; (*g*) smoker/nonsmoker; (*h*) tumor diameter; (*i*) histological subtype; (*j*) stage (I or II); (*k*) tumor grade; and (*l*) patient age. A $P < 0.05$ was considered as significant.

Immunohistochemistry. Anti-cyclin E monoclonal antibody (PharMingen, San Diego, CA) was used for immunohistochemical analysis. Frozen sections from corresponding tumor material were cut at a thickness of 5–6 μm and mounted on poly-L-lysine-coated glass slides and fixed in an ice-cold methanol/aceton mix (1:1). Sections were digested with proteinase K (0.1 μg/ml) in 1 M Tris-buffer (pH 7.5) at 37° for 5 min. Reaction was stopped in 70% ethanol, and slides were rinsed in PBS. A 1:50 dilution of the primary antibody was applied for 45 min at room temperature, and then a rabbit-antimouse (RPMI 1640, 1:30; 30 min at room temperature; DAKO, Copenhagen, Denmark) and a mouse-antirabbit phosphatase-anti-alkaline phosphatase complex (RPMI 1640, 1:100; 60 min at room temperature; Dako). Finally, the sections were rinsed in destilled water for 10 min and counterstained with hematoxylin and mounted in Kayser's glycerine gelatin. Omission of the primary antibody served as negative control. Slides were examined at ×100 and ×200 by an experienced pathologist, who was not informed of the results obtained from mRNA measurements. They were judged as "negative," "positive," or "strongly positive," depending on the percentage of the cells showing a nuclear staining pattern. At least five visual fields at ×200 were examined on at least two slides per section.

Table 1 *Patient characterization*

Included into the study were patients with pathologically confirmed R0 resection of NSCLC stage I to IIIA who survived for at least 90 days after surgery.

Age	63.51 (34–63)
Sex	
Male	54 (77.1%)
Female	16 (22.9%)
Tumor stage	
Stage I	37 (52.9%)
Stage II	13 (18.6%)
Stage IIIA	20 (28.6%)
Smoking history	
Nonsmoker	7 (10%)
Smoker	63 (90%)
Nodal involvement	
N0	42 (60%)
N1	17 (24.3%)
N2	11 (15.7%)
Tumor size	
T1	13 (18.6%)
T2	46 (65.7%)
T3	11 (15.7%)
Grading	
Grade 1	1 (1.4%)
Grade 2	15 (21.4%)
Grade 3	54 (77.1%)
Histology	
Squamous cell carcinoma	34 (48.6%)
Adenocarcinoma	26 (37.1%)
Large cell carcinoma	10 (14.3%)
Local recurrence	
No	56 (80%)
Yes	14 (20%)
Development of metastasis	
No	44 (62.9%)
Yes	26 (37.1%)
Survival	
Alive	38 (54.3%)
Death (not tumor related)	1 (1.4%)
Tumor related death	31 (44.3%)
Tumor localization	
Peripheral carcinoma	52 (74.3%)
Central carcinoma	18 (25.7%)

RESULTS

Tumors from 70 patients with early NSCLC were surgically resected, and patients with stage IIIA disease subsequently received radiation therapy. This study included only patients with stages I to IIIA disease who were resected without pathological evidence of remaining tumor. Clinical characteristics are shown in Table 1. Parts of the surgically resected tumors were immediately shock-frozen and stored at −70°C until analysis. All specimens were confirmed to contain a high percentage of tumor cells (>70%). Lung tissue without

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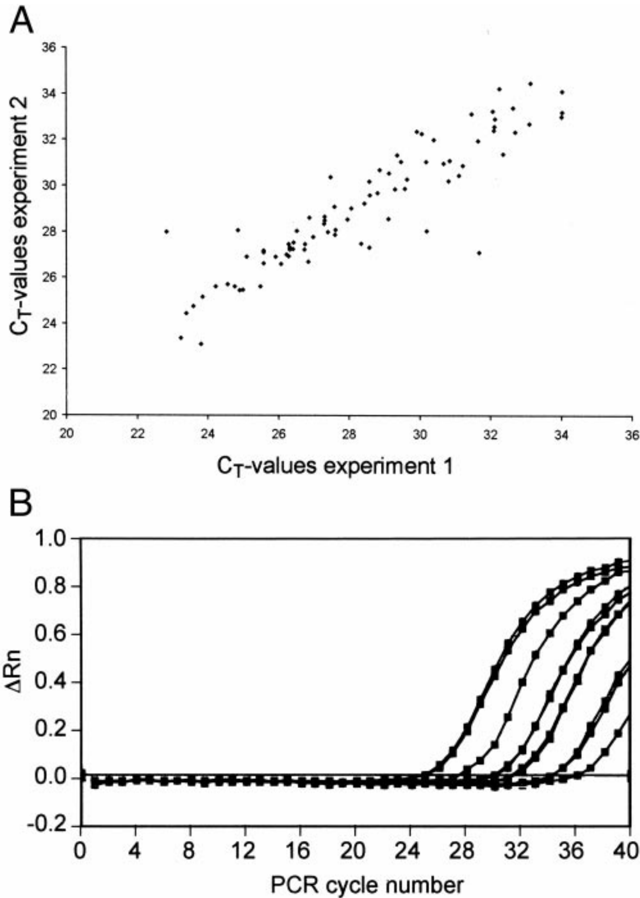

Fig. 1. Quantitative real-time RT-PCR analyses. *A*, cDNA from patients and controls was subjected to two independent PCR reactions in a SDS 7700 Sequence detection system. Fluorescent probes were used to analyze the amount of the specific PCR product during each cycle (see "Materials and Methods"). The cycle number where the fluorescence reached the detection threshold was recorded (*C_T-value*). Shown are the C_T-values for two independent analyses of cyclin E expression. In almost all cases, the degree of correlation between the first and second analyses was very high. Only very few samples amplified differently between the two experiments. These samples were independently analyzed a third time. *B*, a standard curve derived from serial (4-fold) dilutions of a tumor cell line cDNA (U937) was used to calculate the actual concentration in the patient sample. Each curve shows amplification of a standard sample. Duplicates of each standard were run on each plate, and the overlapping curves represent the duplicates. The determined amount of the gene of interest was then divided by the amounts of GAPDH detected in the sample to standardize for differences in reverse transcription efficiency. Relative gene expression was calculated as the amount of target gene expression (compared with standard cDNA)/the amount of GAPDH (compared with standard cDNA).

histological evidence of tumor infiltration was isolated from 12 of the individuals to serve as a control. RNA preparation and reverse transcription were carried out as described in "Material and Methods." Subsequently, expression levels for the different cyclins and GAPDH were analyzed using real-time quantitative RT-PCR. At least two independent analyses of each cDNA sample were performed for each gene and comparisons of these results showed a very high degree of reproducibility (Fig. 1*A*). For relative quantitation of gene expression, standard curves with serial dilutions of cDNA were established (Fig. 1*B*). The amount of gene expression was standardized to expression levels of the housekeeping gene *GAPDH*.

Twelve lung tissue samples without evidence of tumor infiltration were used as controls. The cutoff value for normal *versus* elevated cyclin expression was set at the mean of controls plus twice the SD (Table 2). Levels of cyclin mRNA of tumor samples and controls are shown in Fig. 2. Interestingly, expression of *cyclin E2* was lower in most tumor samples than in controls, and none of the tumor samples reached the cutoff value. For *cyclin A1*, one control and six tumor samples were above the cutoff value. In contrast to the low expression levels seen for *cyclin A1* and *cyclin E2*, a large fraction of patients presented with elevated levels of *cyclin E* (41%, 29 of 70) and *cyclin A2* (31%, 22 of 70). *Cyclin E* levels did not correlate significantly with the size of the tumor or the proliferating fraction of the tumor as indicated by PCNA expression (Table 3). *Cyclin A2* levels were significantly higher in tumor samples than in controls (Fig. 2). In contrast with *cyclin E*, a close association between *cyclin A2* expression and PCNA was noted (Table 3).

Survival of patients was closely associated with the stage of the disease. Those with stage I showed a mean survival of 77.7 months (95%CI 68.4–87.0 months), and stages II and IIIA patients had a mean survival of 63.9 months (95%CI 44.7–83.2 months), and 28.8 months (95%CI 17.7–39.8 months), respectively ($P < 0.0001$). No significant association existed between the stage of disease and expression levels of the various cyclins (data not shown).

To analyze the relationship between cyclin expression and progno-

Table 2 *Expression levels of cyclins in NSCLC*

The means and standard deviations of cyclin expression levels were calculated for control and tumor samples.

	Controls (n = 12)		Tumors (n = 70)	
	Mean	Standard Deviation	Mean	Standard Deviation
Cyclin A1	0.11	0.23	0.018	0.036
Cyclin A2	0.36	0.25	0.84	0.89
Cyclin E	2.71	1.55	9.16	15.67
Cyclin E2	2.92	2.53	0.63	1.13

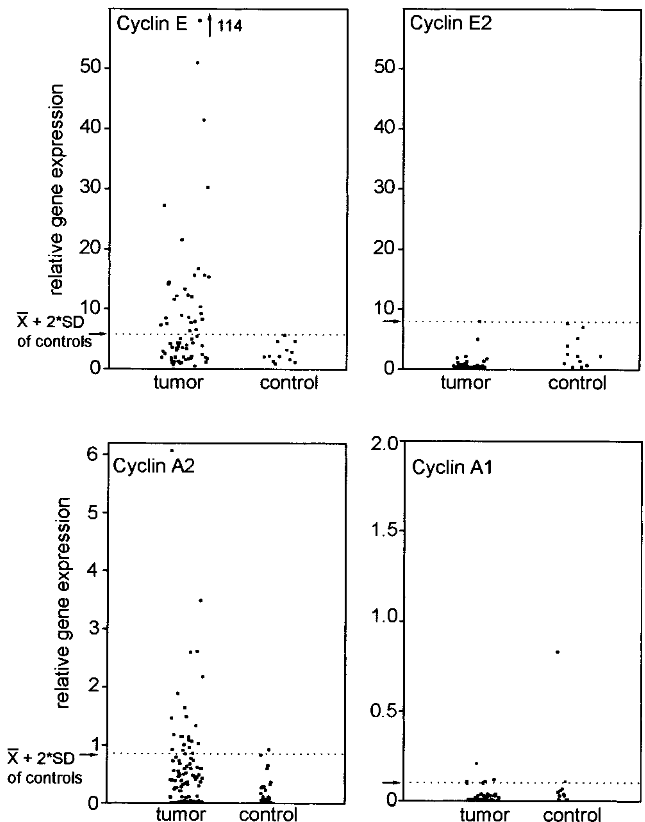

Fig. 2. Cyclin expression in NSCLC and control lung tissue. Cyclin expression in cancer-free lung tissue from 12 patients was used as a control. The mean + 2 × SD of controls (Table 2) was used to determine the cutoff value for overexpression. The cutoff values are shown as *dotted lines* and indicated by an *arrow*. For calculation of the cutoff value for cyclin A1, the one control sample with high cyclin A1 expression was excluded. Cyclin overexpression was detected in 41% (29 of 70) of tumors for cyclin E, in 31% (22 of 70) for cyclin A2, in 9% (6 of 70) for cyclin A1, and in 0% (0 of 70) for cyclin E2.

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Table 3 *Correlation coefficients*

This table shows the linear correlation coefficients between levels of gene expression in the tumor samples ($n = 70$). Significant correlation coefficients are indicated with the respective *P*. All other correlations were not significant.

	Cyclin A1	Cyclin A2	Cyclin E	Cyclin E2	PCNA
Cyclin A2	+0.2				
Cyclin E	+0.07	+0.215			
Cyclin E2	+0.02	−0.04	+0.136		
PCNA	+0.263 $P = 0.03$	+0.65 $P = 0.0001$	+0.012	+0.1	
Tumor diameter	+0.15	+0.003	+0.034	−0.047	−0.035

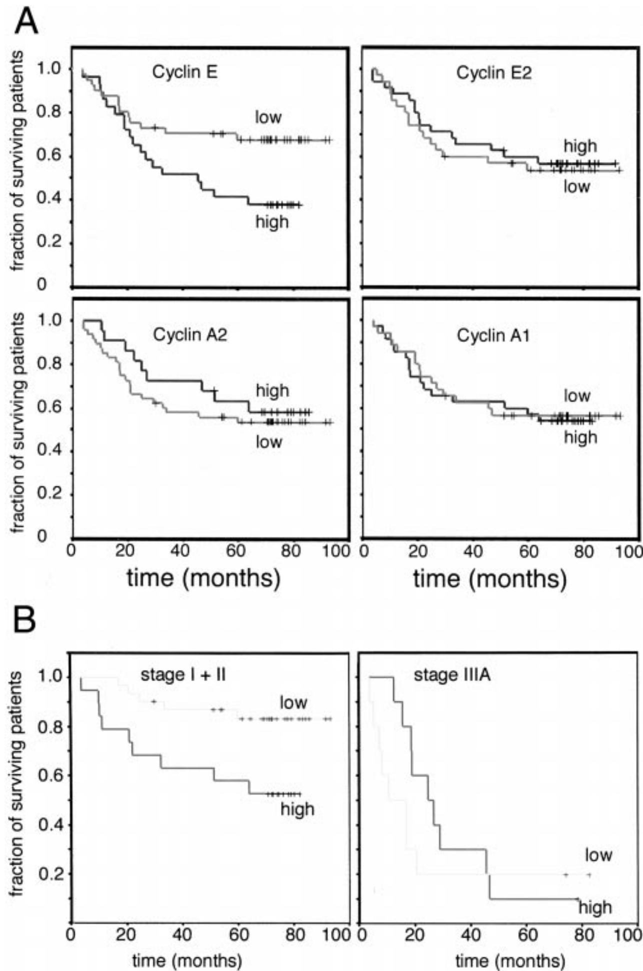

Fig. 3. Cyclin E determines survival in stage I-IIIA NSCLC. *A*, Kaplan-Meier plots of the survival of patients with high *versus* low expression of the different cyclins. High expression of cyclin E and cyclin A2 was defined as expression above the cutoff value (Table 2 and Fig. 2). A different approach was used for cyclin A1 and cyclin E2. Expression either above or below the median was considered as "high" and "low," respectively. Patients with high cyclin E levels had significantly shortened survival ($P = 0.03$), whereas no difference was seen for the other cyclins. *B*, the stage of the disease is the main determinant for survival in NSCLC. We analyzed by Kaplan-Meier plots the importance of cyclin E in early-stage NSCLC I-II and in stage IIIA. Cyclin E was a strong predictor for survival in stages I and II (mean survival 82.9 *versus* 55.1 months; $P = 0.01$) but not in stage IIIA (mean survival 25.4 *versus* 31.7 months, $P = 0.4$).

sis, patients were grouped according to high and low expression. For *cyclin E* and *cyclin A2*, the cutoff values as defined in Table 2 were used. Concerning *cyclin A1* and *cyclin E2*, because few or none of the samples showed increased expression, the tumor samples were split into two groups at the median of the expression level.

Patients with low levels of *cyclin E* at diagnosis showed a mean survival time of 71.7 months (95%CI 60.2–83.2 months), whereas the mean survival time for patients with high *cyclin E* levels was only 47.7 months (95%CI 37.6–57.8 months). This difference was statistically significant ($P = 0.03$, log rank test; Fig. 3*A*). Differences in the expression levels of either *cyclin E2*, *cyclin A1*, or *cyclin A2* did not predict survival (Fig. 3*A*). We further analyzed the predictive role of low *versus* high *cyclin E* expression in different stages of NSCLC. Strong predictive values of *cyclin E* expression were detected for stage I (mean survival 59.0 *versus* 90.9 months) and stage II disease (mean survival 40.6 *versus* 74.8 months; Fig. 3*B*). No significant difference could be detected in stage IIIA patients (mean survival 30.3 *versus* 26.4 months).

Most patients with early-stage NSCLC died because of the subsequent development of distant metastasis. Because all tumors were completely resected, occult metastasis had to be present at the time of surgery. We analyzed whether cyclin expression was directly associated with the subsequent development of distant metastasis (Fig. 4). The percentage of all patients who developed distant metastasis was 37.1% (26 of 70). Fewer than 25% of patients with low *cyclin E* levels (10 of 41) developed distant metastasis. However, 55% (16 of 29) of patients with high *cyclin E* levels developed distant metastasis ($P = 0.01$). In contrast to the predictive value of *cyclin E* levels, no relationship was found between the development of metastasis and expression levels for *cyclin E2* ($P = 1.0$) and *cyclin A1* ($P = 0.8$). The small differences seen for *cyclin A2* did not reach statistical significance ($P = 0.18$; Fig. 4).

To confirm whether the *cyclin E* mRNA-levels measured by real-time RT-PCR correlated with the expression of *cyclin E* protein levels, we performed immunohistochemistry on tumors from 17 randomly selected patients. These tumor samples contained different expression levels of *cyclin E* mRNA as judged by real-time RT-PCR (Fig. 5). We found a strong association between *cyclin E* expression in immunohistochemistry and the quantitative expression data obtained by real-time RT-PCR (Fig. 6). The mean *cyclin E* mRNA expression levels of tumors without immunohistochemistry staining (negative) was 2.52 ± 1.3 SD ($n = 8$), whereas mRNA expression of positive tumors was 9.59 ± 5.7 SD ($n = 9$). The differences in expression were statistically highly significant ($P = 0.01$; Mann-Whitney u-test).

Cox regression analysis was used to determine the independent prognostic value of E-type cyclins in early-stage NSCLC. The disease stage at presentation is known to be the major determinant of survival in NSCLC. Stage IIIA disease is characterized by the presence of metastatic lymph nodes in the mediastinum. These patients already

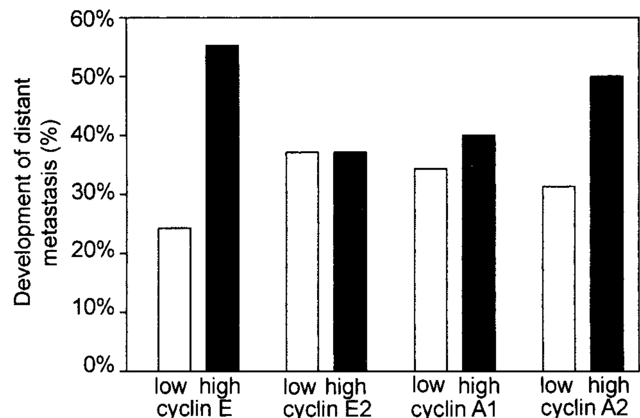

Fig. 4. Overexpression of cyclin E is closely associated with development of distant metastasis. Patients with low levels of cyclin E infrequently developed distant metastasis (24%, 10 of 41). However, the majority of patients with high levels of cyclin E subsequently presented with distant metastasis (55%, 16 of 29). This difference was statistically significant ($P = 0.01$, Fisher's exact test). No significant differences in the frequency of development of metastasis occurred between low and high expression levels of any of the other cyclins.

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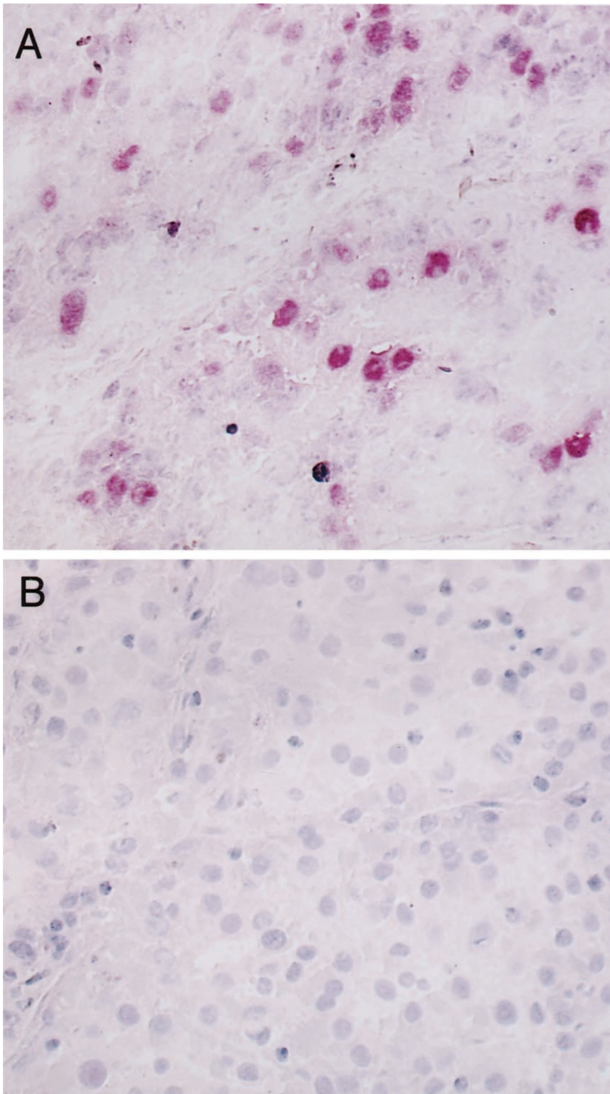

Fig. 5. Analyses of cyclin E expression by immunohistochemistry. To analyze whether cyclin E mRNA expression correlated with protein expression, frozen sections were stained with anti-cyclin E antibody as outlined in "Materials and Methods." *A*, strong nuclear staining for cyclin E was seen in the tumor. *B*, in contrast, no staining was detectable in sections from the tumor.

have a far less favorable prognosis than those with stage I/II disease and the likelihood of occult metastasis is high. Therefore, we limited the Cox regression analysis to stages I and II patients ($n = 50$), where the prognosis is difficult to predict. Within this group, tumor grade ($P = 0.01$), disease stage ($P = 0.02$), and high levels of *cyclin E* ($P = 0.02$) emerged as independent determinants of subsequent tumor-related death. Neither a patient's sex, smoking habits, tumor size, histological subtype, levels of expression of *cyclin E2*, *cyclin A1*, *cyclin A2*, or PCNA, mutational status of *p53*, or patient age at diagnosis were of prognostic importance.

DISCUSSION

The main findings of our study are as follows: (*a*) mRNA levels of *cyclin E* and *cyclin A2*, but not *cyclin A1* or *cyclin E2*, are elevated in a large fraction of patients with early-stage NSCLC; (*b*) expression of *cyclin A2* is associated with proliferation in NSCLC; (*c*) levels of *cyclin E* do not correlate with cellular proliferation in NSCLC; (*d*) elevated expression of *cyclin E* is closely associated with the development of distant metastasis; and (*e*) levels of *cyclin E* expression constitute an independent prognostic parameter for survival in early stage NSCLC.

To our knowledge, this is the first study to show that real-time quantitative RT-PCR might be of clinical use for the prediction of metastasis and survival of patients with solid tumors. Analyses of gene expression levels can be performed at the mRNA as well as on the protein level. The level of cyclin E protein, measured by immunohistochemistry, has been studied previously in NSCLC (21, 22). Analyses of expression levels by immunohistochemistry reveal information about protein stability and the spatial organization of its expression. On the other hand, immunohistochemistry usually results in semiquantitative data only, and the reliability depends on the antibody and detection system used and may be subject to bias. Western blotting, another method to measure protein levels, does not give any information about the cell type expressing the protein and is rather difficult to perform on a large number of patient samples. Measurements at the RNA level can overcome some of these obstacles. For clinical specimens, analyses by RT-PCR provide a rapid way of analyzing the presence or absence of specific transcripts. However, before the advent of real-time PCR, the accurate measurement of the amount of transcripts in the specimen was time-consuming and error prone. In our study, we have used the 5′ nuclease assay to determine quantitatively the level of expression of cdk2-associated cyclins in NSCLC specimens. Despite the convenience and accuracy of real-time PCR, several factors have to be taken into account to avoid potential pitfalls that might hamper the quality of the data. To minimize the problem of normal cell contamination of the tumor sample, we only used specimens that contained a high percentage (>70%) of tumor cells. To exclude analysis of genomic DNA that might contaminate the RNA preparation, the probes were designed to cover an exon-exon junction of the gene of interest. Because pseudogenes might be present in genomic DNA, we checked the samples for amplification in reverse transcription reactions in the absence of reverse transcriptase. In addition, all samples were quantitated according to a standard curve, which was run on every PCR plate. Furthermore, the same standard samples were used for each PCR plate to standardize results among different plates. All samples were analyzed at least twice on independently prepared and analyzed reaction plates. In our experience, this type of analysis results in highly reproducible data with a high degree of correlation of data points obtained at

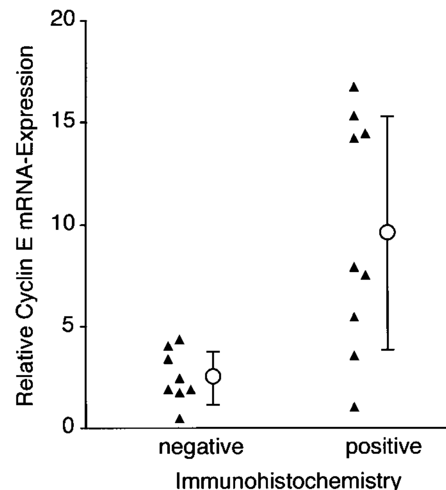

Fig. 6. Close association between *cyclin E* mRNA and protein levels. Seventeen randomly picked tumors were analyzed by immunohistochemistry and regarded as "positive" or "negative." The graph shows the quantitative mRNA values of *positive* and *negative* samples. Also indicated are means and standard deviations. The difference was statistically highly significant ($P = 0.01$).

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different occasions (Fig. 1*A*). Because of the exponential nature of PCR, the minimum difference in expression levels that can be detected by the 5′ nuclease assay is about 2-fold. In our analyses, we demonstrated that expression levels of cyclins, and in particular *cyclin E*, vary in a wide range. For example, high expressing samples can have more than 1000-fold higher expression than samples expressing low levels of *cyclin E*. We could also demonstrate, that the *cyclin E* mRNA levels measured in our assay correlated well with immunohistochemical staining patterns of the cyclin E protein (Fig. 6).

The cell cycle is a basic cellular mechanism that controls fundamental processes such as growth and proliferation as well as apoptosis (31). A large body of data evidences the pivotal role of the RB pathway in the G_1-S transition and its aberrations in tumor cells (1). Cdk2-containing complexes are critically involved in phosphorylating RB at the G_1-S transition. Cyclin genes are often amplified in cancers (32). Thus, elevated levels of *cyclin E* are not surprising in malignant tumors. However, it is unclear is whether *cyclin E* is merely reflecting the enhanced proliferation of the transformed cells, or whether elevated levels of *cyclin E* itself confer a more malignant phenotype. *Cyclin A2*, the main partner for cdk2 during S phase, was found to be elevated in a significant portion of the tumors. Its expression correlated well with levels of PCNA, suggesting that *cyclin A2* expression was a consequence rather than a cause of enhanced proliferation in these tumors. We also studied expression and significance of the cdk2 partners *cyclin A1* and *cyclin E2*, two cdk2 partners that are much less studied. *Cyclin A1* is 60% identical to *cyclin A2*, and it enhances the cdk2 kinase activity to phosphorylate RB (33). Its expression is regulated differently than the expression of *cyclin A2* (34, 35). Under physiological conditions, *cyclin A1* is highly expressed in testis, and it is essential for spermatogenesis (36, 37). In malignant disease, *cyclin A1* is highly expressed in some leukemic blasts derived from myeloid lineages (27, 37). The homozygous deletion of *cyclin A2* leads to embryonal lethality indicating *that cyclin A1* does not substitute for *cyclin A2* (38). In the current study, we found increased *cyclin A1* expression in six patient samples, whereas the majority of lung tumors expressed very low levels of *cyclin A1*. These findings show that *cyclin A1* expression occurs in some solid tumors but in general appears to be a rare event.

Cyclin E2 is a recently cloned homologue of *cyclin E* and its expression was reported previously in several lung cancer-derived cell lines (25). Our data show that expression of *cyclin E2* in primary NSCLC is very low, and it is not associated with levels of either PCNA or *cyclin E*. These findings indicate that functions of cyclin E2 in the cell cycle obviously differ from those of *cyclin E*. In addition, its low expression in primary tumors casts doubt onto a role for this cyclin in the pathogenesis of NSCLC. The discrepancy between the prominent expression found in cell lines with primary tumors needs additional investigation.

Cyclin E was overexpressed in the majority of tumor samples studied and closely associated with survival in early-stage NSCLC. Of all of the cdk2-associated cyclins studied, *cyclin E* was the only one to have prognostic relevance. Inasmuch as all tumors included into our study were pathologically confirmed to be completely resected, enhanced cellular proliferation by itself would not worsen the prognosis of the patient. In addition, tumor size did not correlate with levels of *cyclin E* in our study. Also, *cyclin E* levels in our study and in a previous study (21) did not correlate with levels of PCNA; and levels of *cyclin A2*, which we show to be closely associated with cellular proliferation, did not predict survival. It has been shown that cyclin E expression in cancer cells can be unrelated to the phase of the cell cycle (39). A possible explanation for the strong prognostic value of *cyclin E* may be suggested by our finding that elevated levels of *cyclin E* were closely associated with the development of distant metastasis. How could *cyclin E* be linked to metastasis? One possibility is that targets of cyclin E-cdk2 complexes are involved in the loss of cellular adherence, and the increased cellular mobility associated with mitosis strengthens this point of view. Another possible explanation is conveyed by recent findings that overexpression of *cyclin E* leads to increased chromosome instability (40). High levels of cyclin E lead to impaired S phase progression and the development of aneuploidy in the cell population. This phenomenon was observed solely when *cyclin E*, but not when *cyclin A2* or *cyclin D1*, were overexpressed. These findings in cell lines correspond well with the unique prognostic role of *cyclin E* levels in our study population. The induction of chromosomal instability by deregulation of *cyclin E* might be an important mechanism in the development of metastasis in early-stage NSCLC. To analyze this possibility in more detail, we used comparative genomic hybridization to examine overall genomic instability in high- and low-*cyclin E*-expressing tumors. However, we did not detect significant differences (data not shown). An alternative hypothesis might be that *cyclin E* is simply an indicator gene and does not have pathogenetic relevance. Even if this is the case, analyses of *cyclin E* expression levels might be clinically useful as an indicator for the likelihood of metastasis in NSCLC. This information may help to choose patients who are most likely to profit from adjuvant or neoadjuvant chemotherapeutic treatment strategies in the early stages of the disease.

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Cyclin E is the Only Cyclin-dependent Kinase 2-associated Cyclin that Predicts Metastasis and Survival in Early Stage Non-Small Cell Lung Cancer

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