Evaluation of the Prognostic Role of a Panel of Biomarkers in Stage IB-IIIA Non-small Cell Lung Cancer Patients

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Abstract. *Background: Non-small cell lung cancer (NSCLC) remains a highly lethal disease worldwide and research for more effective treatment strategies is ongoing. Identification of molecular prognostic and predictive markers remains under investigation with results that are often conflicting. Patients and Methods: Seventy-three patients (n=73) with stage IB-IIIA completely resected NSCLC who were postoperatively treated with 6 cycles of paclitaxel and carboplatin from July 1998 to September 2002 took part in this study. Most stage IIIA patients subsequently received adjuvant radiotherapy. Cyclooxygenase-2 (COX-2), vascular endorhelial growth factor (VEGF), dihydrodiol dehydrogenase (DDH), receptor-binding cancer antigen expressed on SiSo cells (RCAS-1) and epidermic rrowth factor receptor (EGFR/HER-1) expression were assessed immunohistochemically; Heregulin family (HER1-4), VEGF and p53 were analysed by RT-PCR. Results: Totally, 61 (84%) men and 12 (16%) women with median age of 63 years and median PS of 0 were included in the study. There were 18 stage IB, 29 stage II and 26 stage IIIA patients. Sixty-seven samples were available for immunohistohemistry. COX-2 expression was detected in 24 patients (36%), VEGF in 14 (21%), RCAS1 in 31 (46%), DDH in 15 (22%). For EGFR, only 58 samples were evaluated, 13 of which were positive (22%). Messenger RNA expression data was only available for 60 patients; VEGF was detected in 32 (53%), p53 in 30 (50%), EGFR in 35 (58%), HER2 in 4 (7%) and HER3 in 19 (32%). HER4 was not detected in any sample. In the Cox analysis for overall survival (OS) and disease-free survival (DFS), none of the factors evaluated by IHC or RT-PCR reached statistical significance. Conclusion: Even though the biomarkers tested are expressed in a significant proportion of lung tumors, none of them was found to be of prognostic significance in patients with NSCLC.*

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Non-small cell lung cancer (NSCLC) remains one of the most challenging fields in oncology, as all therapeutic efforts to date, including surgery, chemotherapy and radiotherapy, have failed to achieve a major breakthrough in outcome.

The role of adjuvant chemotherapy has risen in recent years following the positive results of the International Adjuvant Lung Trial (IALT), which showed that postoperative treatment with platinum combined with vinca alkaloid or etoposide offers a survival benefit of 4.1% compared to surgery only (1).

The importance of identifying molecular prognostic factors has been emphasized with the development of targeted treatment, but for NSCLC the field remains open due to the large volume of conflicting data. Eastern Cooperative Oncology Group (ECOG) retrospectively evaluated the patients of the randomized trial INT 0115 for prognostic and

predictive significance of *p53* and *K-ras* mutations (2). A trend of borderline significance for *K-ras* mutations was noted, but no statistically significant association could be found between these markers and survival. The ALPI trial also evaluated molecular markers for prognostic significance, including *K-ras* mutation, *p53* and *Ki-67*, but again no statistical association was identified (3).

Based on this, the expression of selected molecular markers was evaluated in a population of previously untreated patients with completely resected stage I_B-III_A disease, who received adjuvant chemotherapy with paclitaxel and carboplatin and were regularly followed afterwards, so that relapse and survival data were available. The selected markers were cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF), receptor-binding cancer antigen expressed on SiSo cells (RCAS1), dihydrodiol dihydrogenase (DDH) and HER1 (EGFR) were assessed by immunohistochemistry (IHC). VEGF, p53, and Heregulin family (HER1-4) were analysed by reverse transcriptase polymerase chain reaction (RT-PCR).

We decided to study DDH and RCAS1 based on initial literature data supporting their prognostic significance in NSCLC. COX-2, VEGF and Heregulin family were selected for evaluation of prognostic significance and also for their potential role in the context of targeted treatment for this patient population.

Patients and Methods

Patient eligibility. Eligible patients for the protocol had completely resected histologically confirmed NSCLC, age ≥18 years, performance status (PS) ≤2 on the Eastern Cooperative Oncology Group (ECOG) scale and stage I_B (T_2 N_0), II_A (T_1 N_1), II_B ($T_{2\text{-}3}$ $N_{0\text{-}1}$) or III_A ($T_{1\text{-}3}N_{1\text{-}2}$) tumours. The protocol was approved by the Protocol Review Committee of the Hellenic Cooperative Oncology Group (HeCOG) and the Institutional Review Board of AHEPA University Hospital of Thessaloniki, Greece. All patients provided a study-specific written informed consent.

Paraffin tissue blocks or unstained slides for analysis of prognostic factors were requested before study entry. Pretreatment evaluation included medical history, a physical examination, contrast-enhanced computed tomography scans of the chest and abdomen, bone scan, electrocardiogram, full blood count (FBC) and routine biochemistry.

Study design. The study included all eligible patients who registered in the participating centres between July 1998 and September 2002 and received adjuvant chemotherapy with paclitaxel and carboplatin. The expression of the selected molecular markers was tested for their relationship with disease-free survival (DFS) and overall survival (OS).

Treatment plan. The chemotherapy regimen consisted of paclitaxel 175 mg/m^2 over 3-hour infusion, followed by carboplatin at an area under the curve (AUC) of 6 mg·min/ml (according to the Calvert formula), every 3 weeks for 6 cycles on an outpatient basis. Patients with stage III_A disease, after the completion of chemotherapy, could receive mediastinal irradiation according to the protocol of each center.

Follow-up schedule. After completion of treatment, patients were assessed with a post-treatment CT scan or X-ray and thereafter followed up every three months for disease status and long-term toxicity. Each visit included a physical examination, routine blood tests and imaging (X-ray or CT scan). After the first year, patients were seen every six months for five years or until disease progression. The same schedule also applied for patients who had prematurely interrupted treatment due to reasons other than disease progression.

Immunohistochemistry. Paraffin-embedded tissue blocks were collected from 67 of the 73 study patients after full surgical resection of the tumour. These were analysed for expression of COX-2, VEGF, RCAS1 and DDH (Figure 1). Sections (4 μm) were processed using the NeXES automated system (Ventana, Tucson AZ, USA). For DDH expression the Anti-Human DDH mouse IgG monoclonal antibody (Abcam plc, Cambridge, UK) was used (1:250 dilution) without pretreatment. COX-2 expression was assessed using the Anti-Human COX-2 rabbit IgG Affinity Purified polyclonal antibody (Assay Designs Inc., Ann Arbor, Michigan, USA), 1:40 dilution. VEGF was assessed with an Anti-Human VEGF mouse monoclonal antibody, (Neomarker, Medicorp Inc, Montreal, Canada; 1:250 dilution). RCAS1 was assessed using the Anti-Human RCAS1 mouse IgM monoclonal antibody (IBL, Hamburg, Germany; 1:40 dilution).

Samples having 10% or more cells positive for cytoplasmic staining were considered positive for COX-2 and DDH (Figure 1a, d). The following scoring system was applied for evaluation of staining for VEGF (Figure 1b)and RCAS1 (Figure 1c): a) Stain intensity on cell cytoplasm, marked as: negative, (0); mild, (1); moderate, (2) or strong, (3); b) Percentage of positively stained cells marked as: <5%: 0, 5-24%: 1, 25-49%: 2, ≥50%: 3. Samples with total scores of between 4 and 6 were considered positive. Antigen retrieval was performed in a microwave oven for 15 minutes with citric acid for RCAS1, VEGF and COX-2.

Immunohistochemistry for EGFR was conducted as previously described elsewhere (Figure 1e) (4). Anti-EGFR antibody clone 31G7 (Zymed Laboratories Inc., South San Francisco CA, USA) was used at a dilution of 1:50 and detected using the labeled streptavidin-avidin-biotin method. Positive control sections of breast carcinoma were included. Immunoreactivity for EGFR was evaluated using a four-tier score system (5). Tumors showing a score of 0-1 were marked as negative and 2-3 were marked as positive.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Sixty paraffin blocks were available for mRNA extraction and RT-PCR analysis. RNA was extracted using QIAamp® RNA Blood Mini Kit and purified using an Oligotex mRNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions following deparaffinization of paraffin embedded tumor biopsy samples consisting of >75% tumor cells as previously described elsewhere (6). Complementary DNA was transcribed from 2 μg of tRNA with 500 U M-MuLV Reverse Transcriptase (New England Biolabs Inc, Beverly, MA USA) according to the manufacturer's recommendations. All primer pairs used in PCR analysis are shown in Table I.

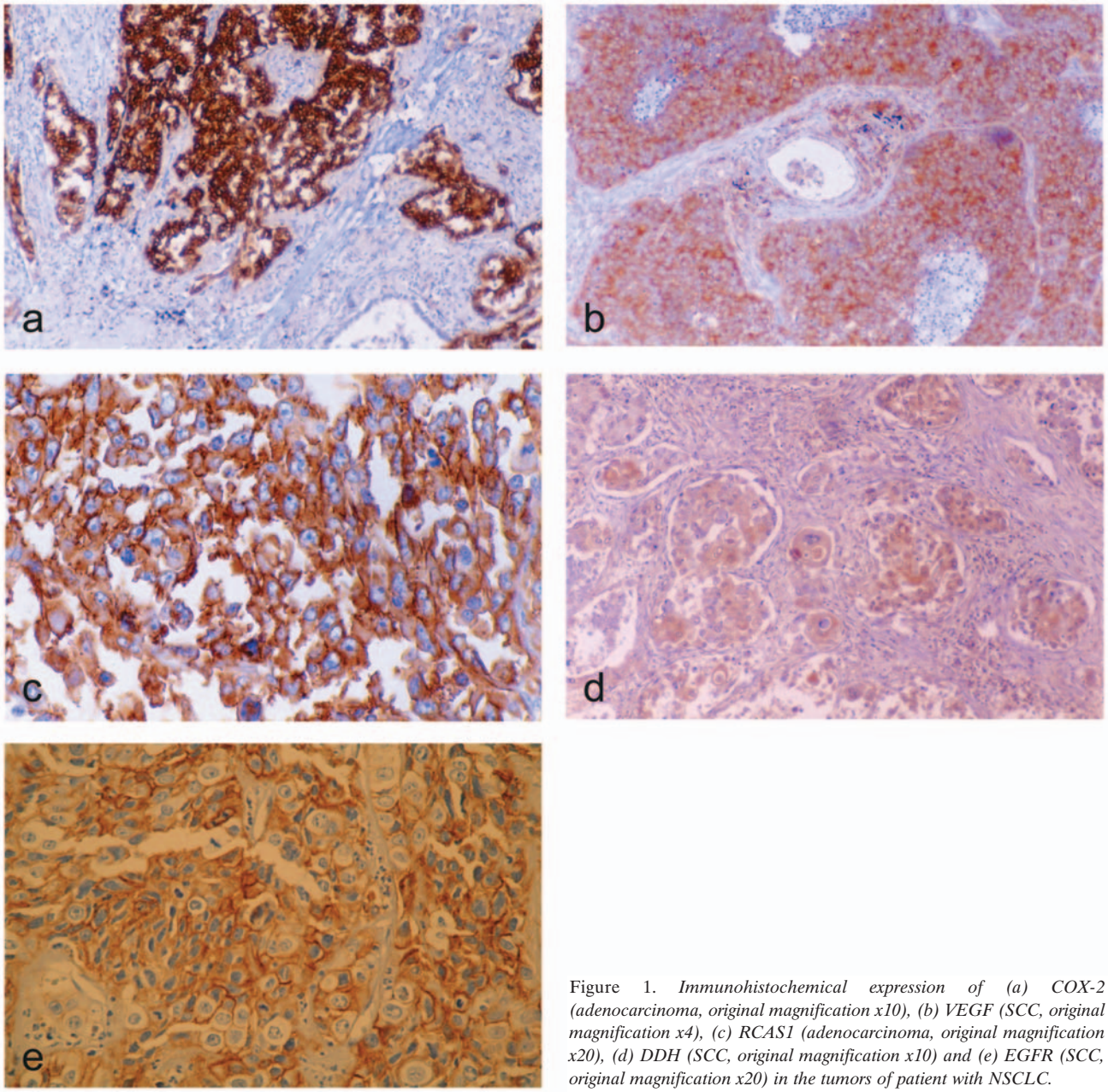

Figure 1. *Immunohistochemical expression of (a) COX-2 (adenocarcinoma, original magnification x10), (b) VEGF (SCC, original magnification x4), (c) RCAS1 (adenocarcinoma, original magnification x20), (d) DDH (SCC, original magnification x10) and (e) EGFR (SCC, original magnification x20) in the tumors of patient with NSCLC.*

EGFR (Acc No: NM 005228) and glyceraldehyde-3-phosphate dehydrogenase (GAPD) (Acc No: NM 002046) were amplified from cDNA using a nested PCR approach. Cycling conditions were 94˚C x 5 min; 30 cycles of 94˚C X 30 sec, 53˚C X 30 sec, 72˚C X 30 sec; followed by one cycle of 72˚C X 5 min. EGFR was scored on the basis of detectable expression of EGFR in the presence of GAPD.

PCR conditions for HER2, HER3, HER4, VEGF and p53 were as described for EGFR.

Statistical analysis. Overall survival (OS) was estimated from the registration date to the date of last follow-up or until the patient's death. DFS was deemed as the time between registration date and documentation of progression clinically and/or radiologically. Patients who discontinued treatment for any reason were considered at that time as having disease progression.

The Kaplan-Meier method (7) was used to calculate DFS, median follow up, and survival curves. The survival status of all patients was updated in November 2005. Fisher's exact test was used to compare the results from the immunohistochemical and RT-PCR analyses for each marker between patients with squamous cell carcinoma (SCC) and adenocarcinoma. Cox regression analysis was performed in order to identify the factors that had a significant effect on patients' survival and DFS (8). Variables included in this analysis were: age,

Table I. *Primer pairs used in PCR analysis.*

	Outer		Inner	
	Forward	Reverse	Forward	Reverse
EGFR	GATGTAGACGC AGATAGTCGC	CTATCAATGCAA GCCACGGTGG	GATGAGGTAC TCGTCGGCATC	GAGTTGATCAT CGAATTCTCCAA
GAPD	TTCTGGGTGGCA GTGATGGC	TTCTGGGTGGCA GTGATGGC	GCTTGTCATCAA TGGAAATCCC	CATGAGTCCTT CCACGATACC
HER-2	5'-CTGCTGAACTGG TGTATGCAG-3'	5'-TGCGGGAGAATT CAGACACCAA-3'	5'-GGCCGACATTCA GAGTCAATCA-3'	5'-TGGACATTGACG AGACAGAGTA-3'
HER- 3	5'-GGCTAGTATCC AGATGATGGAC-3'	5'-CTTGAGGAAC ATGGTATGGTGC-3'	5'-GTTGGGCGAATGTT CTCATCAA-3'	5'-CCTGATGATA AGCAGCTGCTATA-3'
HER- 4	5'-GGATGATTGATG CTGACAGTAG-3'	5'-CTACTGTCCTCTTG GACATGG-3'	5'-GATGGATGCTGAG GAGTACTTG-3'	5'-CACAGACACTC CTTGTTCAGCA-3'
VEGF	5'-CAGCACATAG GAGAGATGAGC-3'	5'-CGATCGTTCAGTAT CAGTCTTTC-3'	5'-CAGCACAACAAAT GTGAATGCAG-3'	5'-CAAGTACGTTC GTTCGTTTAACTC-3'
p53	5'-CTACAAGCAGTC ACAGCACATG-3'	5'-CTGGGCATCCT TGAGTTCCAA-3'	5'-CATTCAGCTCTCGGA ACATCTCGAA-3'	5'-TGTGCCTGTCC TGGGAGAGA-3'

tumour grade (I + II *vs.* III), stage (I_B + II *vs.* III_A), histological type (SCC *vs.* adenocarcinoma + large cell + neuroendocrine), COX-2^{IHC} (negative *vs.* positive), $RCAS1^{IHC}$ (negative *vs.* positive), DDH^{IHC} (negative *vs.* positive), VEGF (negative *vs.* positive), $EGFR^{IHC}$ (negative *vs.* positive), $VEGF^{RT\text{-}PCR}$ (negative *vs.* positive), $p53^{RT\text{-}PCR}$ (negative *vs.* positive), Heregulin $family^{RT\text{-}PCR}$ independently and combined (negative *vs.* positive).

Results

Patient characteristics. Seventy-three patients were enrolled in the clinical study, 61 men and 12 women, with median age 63 years (range 44-76). Fifty-seven had been smokers. Squamous cell carcinoma was the most common histological type (41 of 73, 56%). Surgical procedures for complete resection included lobectomy or pneumonectomy and mediastinal lymph node sampling. The majority had a performance status of 0 (ECOG) and their post-surgical staging was as follows: 18 stage I_B, 29 stage II and 26 stage III_A patients. Detailed patient characteristics are shown in Table II.

Treatment. Sixty patients completed treatment as per protocol. In the remaining 13 patients (pts), treatment was interrupted due to voluntary withdrawal (5 pts), non-fatal toxicity (3 pts), progressive disease (2 pts) and doctor's decision (1 pt), death following early progression (1 pt) and transfer of care to other hospital (1 pt). A total of 391 cycles was delivered, 94% of them in full dose (Table III). Twenty-two patients were irradiated to the mediastinum according to each centre's protocol after completion of chemotherapy. Most of them were stage IIIA.

There were no treatment-related deaths. The most common toxicities are shown in Table IV.

Immunohistochemistry. Immunohistohemically, COX-2 expression was detected in 24/67 patients (36%), VEGF in 14/67 (21%), RCAS1 in 31/67 (46%), DDH in 15/67 (22%) and EGFR in 13/58 (22%) patients. Results from the immunohistochemistry analysis for the molecular markers of squamous cell carcinomas *vs.* adenocarcinomas are shown in Table V. No significant differences were identified between the two groups.

RT-PCR. VEGF was detected in 32 (53%) patients, EGFR was positive in 35 patients (58%), HER2 in 4 (7%), HER3 in 19 (32%), HER4 in 0 (0%), and p53 in 30 (50%) patients. Results of the RT-PCR analysis for SCC and adenocarcinomas are shown in Table VI. Positive VEGF was more common in SCC tumors (68% *vs.* 34%, $p=0.030$). All other markers were similarly distributed between SCC and adenocarcinomas. Patients that had 2+/3+ score by $EGFR^{IHC}$ were all positive by RT-PCR.

With respect to VEGF, 32 patients (53%) were positive by RT-PCR. There was no direct association between $VEGF^{IHC}$ and $VEGF^{RT\text{-}PCR}$.

Survival. After a median follow-up of 60.2 months (range 2.2-86.5+), 47 (64%) patients had relapsed and 42 (58%) had died. Distribution of deaths by stage was 9 of the 18 stage I patients, 16 of 29 stage II and 17 of 26 stage III_A patients. Median survival by stage was 68.3 months (range 12.4-86.5+) for the I_B group, 43.2 months (range 2.6-84.4+) for the stage II group and 27.0 months (range 2.0-85.2+) for stage III_A patients.

Cause of death was NSCLC (34 pts), cardiac disease (6 pts), sudden death (1 pt) and unknown (1 pt). Three patients were lost to follow-up. The median OS was 40.0

Table II. *Selected patient and tumour characteristics.*

N	73	
Age (years)		
Median	63	
Range	44-76	
PS		
Median	0	
Range	0-2	
	N	%
Gender		
Men	61	84
Women	12	16
Stage		
I_B	18	25
II	29	40
III_A	26	35
T classification		
T1	6	8
T2	57	78
T3	10	14
N classification		
N0	20	27
N1	30	41
N2	23	31.5
Histologic type		
SCC	41	56
Adenocarcinoma	22	30
Large cell	9	12
Neuroendocrine	1	1
Type of surgery		
Pneumonectomy	37	51
Lobectomy	36	49
History of smoking		
Yes	57	78
No	14	19
Unknown	2	3
Grade		
I	5	7
II	36	49
III	32	44
Laterality		
Right lung	47	64
Left lung	26	36

PS: Performance status; SCC: squamous cell carcinoma.

Table III. *Selected treatment characteristics.*

N	73	
Number of cycles delivered	391	
% of cycles at full dose[a]	94	
Median duration between cycles	21	
% cycles delayed[b]	17	
	P	Cp
Cumulative dose (mg/m^2)		
Planned	1050	
Median delivered	1042	3305
DI		
Planned	58	
Median delivered	58	
Median relative DI	1.0	

[a]≥90% of the dose defined in the protocol; [b]>21 days. P, paclitaxel; Cp, carboplatin; DI, dose intensity (mg/m^2/week).

Table IV. *Incidence (%) of various toxicities (WHO) (39).*

	Grade (N=73)			
	1	2	3	4
Anemia	15 (20.5)	5 (7)	2 (3)	
Neutropenia	1 (1)	7 (10)	10 (14)	5 (7)
Thrombocytopenia	5 (7)	1 (1)	1 (1)	
Nausea/vomiting	14 (19)	10 (14)	1 (1)	
Stomatitis	2 (3)	1 (1)		
Diarrhea	2 (3)	1 (1)		
Infection	3 (4)	8 (11)		
Myalgias/arthralgias	18 (25)	13 (18)		1 (1)
HSR[a]	8(11)	8 (11)		
Constipation	7 (10)	7 (10)		
Fatigue	11 (15)	5 (7)		
Peripheral neuropathy	26 (36)	28 (38)	2 (3)	
Anorexia	3 (4)	2 (3)		
Gastritis	3 (4)			
Dizziness	4 (5.5)	1 (1)		

Alopecia was universal; [a]hypersensitivity reactions.

months (range, 2.0-86.5+), while the median DFS was 21.3 months (range, 1.5-86.5) (Figure 2). Survival rates at 1, 2 and 3 years were 83%, 67% and 57% respectively.

No statistically significant differences were seen with respect to OS for Heregulin family positive patients, or for patients classified as positive by combining data on $EGFR^{IHC}$ and $EGFR^{RT\text{-}PCR}$.

In the Cox analysis for OS and DFS, none of the factors evaluated by IHC or RT-PCR reached statistical significance.

For patients positive for VEGF by either or both methods the OS was poorer than those negative for expression, indicating a non-significant trend for worse prognosis for patients expressing VEGF.

Discussion

We performed this study to analyse selected molecular markers for prognostic significance in a group of patients enrolled in a clinical protocol for adjuvant treatment, who were planned to have regular follow-ups, so that relapse and

Table V. *Results from immunohistochemical analysis in squamous cell carcinomas (SCC) and adenocarcinomas.*

	SCC (N=39)		Adenocarcinoma (N=18)		
	N	%	N	%	*P*-value[a]
COX-2					
Negative	28	72	9	50	0.140
Positive	11	28	9	50	
VEGF					
Negative	30	77	15	83	0.734
Positive	9	23	3	17	
RCAS1					
Negative	24	62	9	50	0.565
Positive	15	38	9	50	
DDH					
Negative	29	74	17	94	0.146
Positive	10	26	1	6	
EGFR					
Negative	23	70	12	80	0.739
Positive	10	30	3	20	

[a]Fisher's exact test.

Table VI. *Results from RT-PCR analysis in squamous cell carcinomas (SCC) and adenocarcinomas.*

	SCC (N=35)		Adenocarcinoma (N=15)		
	N	%	N	%	*P*-value[a]
EGFR					
Negative	13	37	7	47	0.140
Positive	22	63	8	53	
HER2					
Negative	33	94	14	93	0.999
Positive	2	6	1	7	
HER3					
Negative	25	71	9	60	0.514
Positive	10	29	6	40	
VEGF					
Negative	11	32	10	66	0.030
Positive	24	68	5	34	
P53					
Negative	14	40	9	60	0.228
Positive	21	60	6	40	

[a]Fisher's exact test.

survival data would be available for statistical processing. Literature data for prognostic markers in the early stage setting is still quite controversial, with patient numbers being relatively small and inadequate to reach statistical significance. Moreover, many studies focus on specific early stage NSCLC cases undergoing complete resection and no details of further

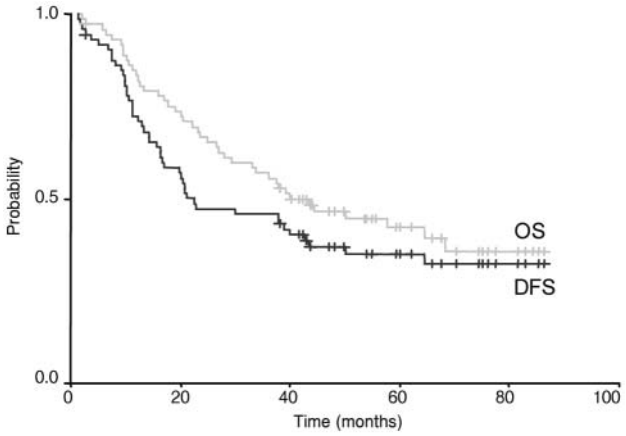

Figure 2. *Kaplan-Meier curve of disease-free survival (DFS, median: 21.3 months) and overall survival (OS, median: 40 months) of all patients included in the study.*

treatment, if any, are given. The development of targeted treatment strategies during the recent years has accentuated the importance of establishing molecular criteria to select patients more likely to benefit from certain agents. Furthermore, agents like the EGFR tyrosine kinase inhibitors gefitinib or erlotinib have been widely tested in advanced NSCLC with moderate results, while no data are available for their impact on early stage disease.

COX is a key enzyme involved in the prostanoid synthesis from arachidonic acid. It has significant role in many inflammatory processes and can be upregulated by several tumor promoters. Its prognostic value in completely resected non-small cell lung tumors has been widely investigated, with significant variability in reported results. It has been found to correlate strongly with shorter DFS and OS in patients with stage I disease (9, 10). Also, increased incidence in COX-2 expression has been reported in well- differentiated adenocarcinomas in contrast to poorly differentiated ones (11) and in invasive adenocarcinomas with positive lymph nodes, suggesting a possible role of COX-2 in enhancing the metastatic potential of primary tumors (12). In a series of 259 patients with completely resected NSCLC, COX-2 was identified as independent prognostic factor for overall survival in the stage I and II patients (13), while other investigators only reported borderline significance (14). Its participation in the angiogenic process has also been evaluated in a study showing a potential impact on survival in patients with co-expression of COX-2 and VEGF, implying a role for COX-2 inhibitors as antiangiogenic agents in the treatment of NSCLC (15). However, the detrimental effects of currently available COX-2 inhibitors on the cardiovascular system as seen in previous trials have placed serious limitations on any relevant clinical research (16, 17).

RCA-S1 is expressed on human cancer cells and acts as a ligand for a putative receptor on peripheral lymphocytes. It has been shown to inhibit the *in vitro* growth of receptor-expressing cells and to induce apoptosis, potentially contributing to the ability of tumour cells to evade host immune surveillance. It has been investigated in several types of human cancer and is indicated as an independent prognostic factor in gastric cancer (18), invasive ductal breast cancer (19) and lung adenocarcinoma (20). In the latter study, RCAS1-positive patients had significantly shorter survival compared to the negative ones. Another study identified a strong association with pathological staging, tumour differentiation and survival (21).

DDH is involved in the metabolism of polycyclic aromatic hydrocarbons in the liver and is rarely detected in normal lung tissue. This activity might suggest an association with carcinogenesis and disease progression. It has been evaluated in limited numbers of patients and the initial data seem to support further investigation. Low expression has been correlated with significantly lower incidence of early recurrence and distant metastases and longer overall survival (22). In another study, overexpression was significantly higher in male patients and in SCC histologies with low DDH expression correlating with a more favourable outcome (23).

VEGF is a multifunctional cytokine with potent angiogenic, mitogenic and vascular permeability-enhancing activity specific for endothelial cells (24, 25). By binding to specific tyrosine kinase receptors of the endothelial cells it promotes neovascularization. It has been investigated in several tumours and identified as significant prognostic factor in breast (26) and gastric carcinoma (27). In NSCLC patients (28), high VEGF expression has been found to correlate with significantly lower survival rate. Other studies have focused on early stage disease and identified VEGF as significant factor in co-expression with microvascular density (MVD) assessed by CD34 (29) or specifically assessed T1 adenocarcinomas and showed prognostic significance of VEGF-C expression (30).

The expression of the Heregulin family receptors (HER1-4) has been widely investigated in solid tumors including NSCLC and, following the development of targeted agents such as the monoclonal antibody trastuzumab and the small molecule TK inhibitors gefitinib and erlotinib, efforts subsequently focused mainly on HER- 1 and -2. There are conflicting data in the literature reporting HER- 2 expression as a significant prognostic factor (31), as non-significant (32) or as a sex-related prognostic factor in women with NSCLC (33). A 2005 meta-analysis concluded that overexpression is a significant adverse prognostic factor. However, the fact that several trials finding no significance were excluded as not fulfilling the meta-analysis criteria is pointed out by the authors as a potential confounding factor (34).

In contrast to breast cancer, there has been no association either between immunohistochemical overexpression and gene amplification assessed by fluorescent *in situ* hybridization (FISH), or between HER2 positivity and response to treatment with trastuzumab (35). The literature is very limited regarding the role of HER3 and -4, but there are older data reporting HER3 as adverse prognostic factor in advanced NSCLC (36). Among our patients we found high expression of EGFR (HER1) (63.3%), low HER2 (5%), HER3 in about one-third (33.3%) and no sample expressing HER4.

There was no significant correlation of any of the studied markers with rate of recurrence or overall survival.

Documentation of the predictive value of the EGFR mutations for response to gefitinib has triggered further research for more growth factor gene mutations and their potential clinical impact (37, 38). Our group has studied the incidence of EGFR mutations in this patient population and identified several new somatic ones, but they do not seem to confer any prognostic significance (4).

Conclusion

In contrast to the literature, we could not identify any significant correlation between the investigated molecular markers and survival. The limited number of patients and differences in patient selection, methodology or interpretation of the results might all account for this discrepancy. As both experience and literature data increase, it becomes more and more evident that tumor signaling pathways are rather complex and it is unlikely that tumor growth can be inhibited by blocking one pathway only. Identification of concurrent expression of large numbers of genes in the form of a tumor "profile" and assessment of their prognostic significance in this setting seems to be more promising as compared to study of isolated markers, in an effort to design more rational and effective treatments for the NSCLC patients.

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References

1 The International Adjuvant Lung Cancer Trial Collaborative Group: Cisplatin-based chemotherapy in patients with completely resected non-small cell lung cancer. N Engl J Med *350*: 351-360, 2004.

2 Schiller JH, Adak S, Feins RH, Keller SM, Fry WA, Livingston RB, Hammond ME, Wolf B, Sabatini L, Jett J, Kohman L and Johnson DH: Lack of prognostic significance of p53 and K-ras mutations in primary resected non-small-cell lung cancer on E4592: a Laboratory ancillary study on an Eastern Cooperative Oncology Group prospective randomized trial of postoperative adjuvant therapy. J Clin Oncol *19*: 448-457, 2001.

3 Scagliotti GV: Prognostic significance of K-ras, p53 and KI-67 in the ALPI randomized trial of adjuvant chemotherapy in completely resected non-small cell lung cancer (NSCLC) (on behalf of ALPI/EORTC-LCSG investigators). Ann Oncol *13(Suppl 5)*: (Abstr 470).

4 Murray S, Timotheadou E, Linardou H, Vrettou AV, Kostopoulos I, Skrickova , Papakostantinou C, Christodoulou C, Pectasides D, Samantas E, Papakostas P, Skarlos V, Kosmidis P and Fountzilas G: Mutations of the epidermal growth factor receptor tyrosine kinase domain and associations with clinicopathological features in non-small cell lung cancer patients. Lung Cancer *52*: 225-233, 2006.

5 Ooi A, Takehana T, Li X, Suzuki S, Kunitomo K, Iino H, Fujii H, Takeda Y and Dobashi Y: Protein overexpression and gene amplification of er-2 and EGFR in colorectal cancers: an immunohistochemical and fluorescent *in situ* hybridization study. Mod Pathol *17*: 895-904, 2004.

6 von Weizsacker F, Labeit S, Koch HK, Oehlert W, Gerok W and Blum HE: A simple and rapid method for the detection of RNA in formalin-fixed, paraffin embedded tissues by PCR amplification. Biochem Biophys Res Comm *174*: 176-180, 1991.

7 Kaplan E and Meier P: Nonparametric estimation from incomplete observations. J Am Stat Assoc *53*: 457-481, 1958.

8 Cox D: Regression models and life tables (with discussion). J Royal Stat Soc B *34*: 187-220, 1972.

9 Khuri FR, Wu H, Lee JJ, Kemp BL, Lotan R, Lippman SM, Feng L, Hong WK and Xu XC: Cyclooxygenase-2 overexpression is a marker of poor prognosis in stage I non-small cell lung cancer. Clin Cancer Res *7*: 861-867, 2001.

10 Achiwa H, Yatabe Y, Hida T, Kuroishi T, Kozaki K, Nakamura S, Ogawa M, Sugiura T, Mitsudomi T and Takahashi T: Prognostic significance of elevated cyclooxygenase-2 expression in primary resected lung adenocarcinomas. Clin Cancer Res *5*: 1001-1005, 1999.

11 Wolff H, Saukkonen K, Anttila S, Karjalainen A, Vainio H and Ristimaki A: Expression of cyclooxygenase-2 in human lung carcinoma. Cancer Res *58*: 4997-5001, 1998.

12 Hida T, Yatabe Y, Achiwa H, Muramatsu H, Kozaki K, Nakamura S, Ogawa M, Mitsudomi T, Sugiura T and Takahashi T: Increased expression of cyclooxygenase-2 occurs frequently in human lung cancers, specifically in adenocarcinoma. Cancer Res *58*: 3761-3764, 1998.

13 Laga AC, Zander DS and Cagle PT: Prognostic significance of cyclooxygenase 2 expression in 259 cases of non-small cell lung cancer. Arch Pathol Lab Med *129*: 1113-1117, 2005.

14 Lu C, Soria JC, Tang X, Xu XC, Wang L, Mao L, Lotan R, Kemp B, Bekele BN, Feng L, Hong WK and Khuri FR: Prognostic factors in resected stage I non-small-cell lung cancer: a multivariate analysis of six molecular markers. J Clin Oncol *22*: 4575-4583, 2004.

15 Kim HS, Youm HR, Lee JS, Min KW, Chung JH and Park CS: Correlation between cyclooxygenase-2 and tumor angiogenesis in non-small cell lung cancer. Lung Cancer *42(2)*: 163-170, 2003.

16 Solomon SD, McMurray JJ, Pfeffer MA, Wittes J, Fowler R, Finn P, Anderson WF, Zauber A, Hawk E and Bertagnolli M: Adenoma Prevention with Celecoxib (APC) Study Investigators. Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention. N Engl J Med *352(11)*: 1071-1080, 2005.

17 Bresalier RS, Sandler RS, Quan H, Bolognese JA, Oxenius B, Horgan K, Lines C, Riddell R, Morton D, Lanas A, Konstam MA and Baron JA: Adenomatous Polyp Prevention on Vioxx (APPROVe) Trial Investigators. Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. N Engl J Med *352(11)*: 1092-1102, 2005.

18 Enjoji M, Nakashima M, Nishi H, Choi I, Oimomi H, Sugimoto R, Kotoh K, Taguchi K, Nakamuta M, Nawata H and Watanabe T: The tumour-associated antigen, RCAS1 can be expressed in immune-mediated diseases as well as in carcinomas of the biliary tract. J Hepatol *36*: 786-792, 2002.

19 Rousseau J, Tetu B, Caron D, Malenfant P, Cattaruzzi P, Audette M, Doillon C, Tremblay JP and Guerette B: RCAS1 is associated with breast cancer progression. Biochem Biophys Res Commun *293*: 1544-1549, 2002.

20 Oizumi S, Yamazaki K, Nakashima M, Watanabe T, Hommura F, Ogura S, Nishimura M and Dosaka-Akita H: RCAS1 expression: A potential prognostic marker for adenocarcinomas of the lung. Oncology *62*: 333-339, 2002.

21 Izumi M, Nakanishi Y, Yoshino I, Nakashima M, Watanabe T and Hara N: Expression of tumor associated antigen RCAS1 correlates significantly with poor prognosis in non-small call lung carcinoma. Cancer *92*: 446-451, 2001.

22 Hsu NY, Ho HC, Chow KC, Lin TY, Shih CS, Wang LS and Tsai CM: Overexpression of dihydrodiol dehydrogenase as a prognostic marker of non-small cell lung cancer. Cancer Res *61*: 2727-2731, 2001.

23 Chen CY, Hsu CP, Hsu NY, Shih CS, Lin TY and Chow KC: Expression of dihydrodiol dehydrogenase in the resected stage I non-small cell lung cancer. Oncol Rep *9*: 515-519, 2002.

24 Leung DW, Cachianes G, Kuang WJ, Goeddel DV and Ferrara N: Vascular endothelial growth factor is a secreted angiogenic mitogen. Science *246*: 1306-1309, 1989.

25 Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J and Connolly DT: Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science *246*: 1309-1312, 1989.

26 Gasparini G, Toi M, Gion M, Verderio P, Dittadi R, Hanatani M, Matsubara I, Vinante O, Bonoldi E, Boracchi P, Gatti C, Suzuki H and Tominaga T: Prognostic significance of vascular endothelial growth factor protein in node-negative breast carcinoma. J Natl Cancer Inst *89*: 139-147, 1997.

27 Tomoda M, Maehara Y, Kakeji Y, Ohno S, Ichiyoshi Y and Sugimachi K: Intratumoral neovascularization and growth pattern in early gastric carcinoma. Cancer *85*: 2340-2346, 1999.

28 Han H, Silverman JF, Santucci TS, Macherey RS, d'Amato TA, Tung MY, Weyant RJ and Landreneau RJ: Vascular endothelial growth factor expression in stage I non-small cell lung cancer correlates with neoangiogenesis and a poor prognosis. Ann Surg Oncol *8*: 72-79, 2001.

29 Mineo TC, Ambrogi V, Baldi A, Rabitti C, Bollero P, Vincenzi B and Tonini G: Prognostic impact of VEGF, CD31, CD34, and CD105 expression and tumour vessel invasion after radical surgery for IB-IIA non-small cell lung cancer. J Clin Pathol *57(6)*: 591-597, 2004.

30 Kojima H, Shijubo N, Yamada G, Ichimiya S, Abe S, Satoh M and Sato N: Clinical significance of vascular endothelial growth factor-C and vascular endothelial growth factor receptor 3 in patients with T1 lung adenocarcinoma. Cancer *104(8)*: 1668-1677, 2005.
31 Meert AP, Martin B, Paesmans M, Berghmans T, Mascaux C, Verdebout JM, Delmotte P, Lafitte JJ and Sculier JP: The role of HER-2/neu expression on the survival of patients with lung cancer: a systematic review of the literature. Br J Cancer *89(6)*: 959-965, 2003.
32 Pelosi G, Del Curto B, Dell'Orto P, Pasini F, Veronesi G, Spaggiari L, Maisonneuve P, Iannucci A, Terzi A, Lonardoni A and Viale G: Lack of prognostic implications of HER-2/neu abnormalities in 345 stage I non-small cell carcinomas (NSCLC) and 207 stage I-III neuroendocrine tumours (NET) of the lung. Int J Cancer *113(1)*: 101-108, 2005.
33 Vallbohmer D, Brabender J, Yang DY, Danenberg K, Schneider PM, Metzger R, Holscher AH and Danenberg PV: Sex differences in the predictive power of the molecular prognostic factor HER2/neu in patients with non-small-cell lung cancer. Clin Lung Cancer *7(5)*: 332-337, 2006.
34 Nakamura H, Kawasaki N, Taguchi M and Kabasawa K: Association of HER-2 overexpression with prognosis in nonsmall cell lung carcinoma: a metaanalysis. Cancer *103(9)*: 1865-1873, 2005.
35 Swanton C, Futreal A and Eisen T: Her2-targeted therapies in non-small cell lung cancer. Clin Cancer Res *12(14 Pt 2)*: 4377s-4383s, 2006.
36 Yi ES, Harclerode D, Gondo M, Stephenson M, Brown RW, Younes M and Cagle PT: High c-erbB-3 protein expression is associated with shorter survival in advanced non-small cell lung carcinomas. Mod Pathol *10(2)*: 142-148, 1997.
37 Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris L, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J and Haber DA: Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med *350(21)*: 2129-2139, 2004.
38 Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE and Meyerson M: EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science *304(5676)*: 1497-500, 2004.
39 Miller AB, Hoogstraten B, Staquet M and Winkler A: Reporting results of cancer treatment. Cancer *47*: 207-214, 1981.

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