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Expression of Hypoxia-inducible Carbonic Anhydrase-9 Relates to Angiogenic Pathways and Independently to Poor Outcome in Non-Small Cell Lung Cancer

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ABSTRACT

Carbonic anhydrase-9 (CA9), a transmembrane enzyme with an extracellular active site, is involved in the reversible metabolism of the carbon dioxide to carbonic acid. Up-regulation of CA by hypoxia and the hypoxia-inducible factor (HIF) pathway has been recently postulated (Wykoff *et al.* Cancer Res., *60:* 7075–7083, 2000). In the present study we examined the expression of this enzyme in non-small cell lung cancer. Of 107 cases analyzed, 39 (36.4%) had strong membrane/cytoplasmic expression of CA9 and were grouped as positive. The staining was confined around areas of necrosis, and a significant association of CA9 expression with the extent of necrosis was noted ($P = 0.004$). Nevertheless, 38 of 74 cases with focal or extensive necrosis did not express CA9. CA9 expression was more frequent in the squamous cell histology ($P = 0.001$) and with advanced T stage ($P = 0.009$). A significant coexpression of CA9 with platelet-derived endothelial cell growth factor and basic fibroblast growth factor receptor expression was noted. Double staining of CA9 with anti-CD31 monoclonal antibody revealed an overall higher microvessel density in the areas expressing CA9 than in negative areas ($P = 0.0005$). Thirty-one of 38 CA9-positive cases were positive for HIF1a/HIF2a, but HIF positivity was a more common event (68 of 107) and their patterns of expression were diffuse (not confined in the necrotic areas). A direct association of CA9 expression with epidermal growth factor receptor, c-erbB-2, and MUC1 expression was also noted ($P < 0.04$). Survival analysis showed that CA9 expression is related to poor prognosis. CA9 expression in tumors with low vascularization defined a prognosis similar to the one of patients with highly angiogenic tumors. Multivariate analysis revealed that CA9 expression is a significant prognostic factor independent of angiogenesis. We conclude that CA9 is an important molecule in non-small cell lung cancer, the up-regulation of which occurs in highly hypoxic/necrotic regions of the tumors. The expression of CA9 is linked to the expression of a constellation of proteins involved in angiogenesis, apoptosis inhibition, and cell-cell adhesion disruption, which explains the strong association of CA9 with poor outcome.

INTRODUCTION

CA9,[2] or MN protein, consists of a signal peptide, a proteoglycan-related sequence, an enzymatically active extracellular CA domain, a transmembrane segment, and a short intracellular tail (1). This protein is absent from most of normal tissues, but is induced constitutively in some tumor types, such as clear cell renal carcinoma (2). Mutations of the VHL protein are believed to be responsible for the constitutive expression of CA9 in renal malignancies, and indeed, introduction of the wild-type *VHL* gene in renal cell carcinomas suppressed the expression of CA9 and CA12 (3).

Recently, Wykoff *et al.* (4) showed that, apart from its constitutive expression in some tumors, CA9 is also induced by hypoxia. The HIF pathway up-regulated by hypoxia or by VHL mutations (5) is therefore suggested as the major pathway of CA9 up-regulation in human carcinomas. A strongly perinecrotic localization of CA9 in several tumor types (6) confirms the up-regulation of CA9 under severely hypoxic (necrosis-inducing) conditions.

The enzymatic activity of CA9 involves the hydration of carbon dioxide to carbonic acid, which suggests a putative role of the enzyme in the acidification of the tumor environment (6). Because both hypoxia and acidic pH are linked to aggressive tumor behavior and to resistance of cancer to radiotherapy and chemotherapy (7–10), the use of CA9 as a prognostic and predictive marker in oncology may be useful and also define a therapy target.

In this study we investigated the patterns of CA9 expression in squamous cell lung cancer and lung adenocarcinomas and their correlation with histopathological variables, with angiogenesis and angiogenic factor expression, and with the activation of other molecular pathways, such as the erbB, episialin, bcl-2, and p53 proteins. Survival analysis was also performed to identify subgroups of patients in whom CA9 expression may define prognosis.

MATERIALS AND METHODS

Archival paraffin-embedded biopsy materials from 70 primary squamous cell lung carcinomas and 37 lung adenocarcinomas were retrieved, and 4-μm tissue sections were cut on slides. All patients had early operable cancer ($T_{1,2}$-$N_{0,1}$ stage) and were treated with surgery alone. Histological diagnosis, grading and N-stage analysis was done on H&E-stained sections.

Eighty-five patients were male and 22 were female; their ages ranged from 35 to 74 years (median, 63 years). Survival data were available for all 107 patients. Patients dying within 60 days after operation were excluded to avoid bias from perioperative death. The median follow-up of surviving patients at the time of analysis was 56 months (range, 22–83 months).

Immunohistochemistry for CA9 and Tumor Endothelium. For the detection of CA9, we used the APAAP procedure and the mouse monoclonal antihuman CA9 antibody M75 (4). Briefly, sections were dewaxed and rehydrated. After sections were microwaved (twice for 4 min each), the primary antibody (dilution 1:50) was applied at room temperature for 90 min, and slides were washed in TBS. Rabbit antimouse antibody 1:50 (v/v) was applied for 30 min, followed by application of APAAP complex (1:1, v/v; Dako, Copenhagen, Denmark) for 30 min. After the slides were washed in TBS, the last two steps were repeated for 10 min each. The color was developed by a 15-min incubation with new fuchsin solution. The specimens were scanned at low optical power (×40 and ×100), and the percentage of cells with positive CA9 reactivity was assessed on all ×200 fields (three to seven ×200 fields per case).

The JC70 MAb (Dako), which recognizes CD31 (platelet/endothelial cell adhesion molecule), was used for microvessel staining by the APAAP method, as described previously. The specimens were scanned at low optical power

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[2] The abbreviations used are: CA9, carbonic anhydrase-9; VHL, von Hippel-Lindau; HIF, hypoxia-inducible factor; APAAP, alkaline phosphatase-antialkaline phosphatase; TBS, Tris-buffered saline; MAb, monoclonal antibody; MVD, microvessel density; PD-ECGF, platelet-derived endothelial cell growth factor; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; bFGFR, bFGF receptor; NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor.

(×40 and ×100), and microvessel counting was performed on ×200 fields. Three areas (per case) of high vascularization were chosen for microvessel counting. The final MVD for a case was the mean value of the three appraised fields. Microvessels adjacent to normal lung were excluded from the appraisal. Vessels with a clearly defined lumen or well-defined linear vessel shape but not single endothelial cells were taken into account for microvessel counting. Cases were divided in three groups, low, medium, and high MVD (<15, 16–45, and >45 vessels per ×200 optical field, respectively), as reported previously (11).

Thirty tissue sections with CA9-positive and -negative areas were double-stained with a mixture of M75 and JC70 (1:50). After the peroxidase was quenched and the sections were microwaved, they were stained using a modified streptavidin technique. The mixture of primary antibodies was applied for 90 min. After being washed with TBS, sections were incubated with a secondary rabbit antimouse antibody (Kwik Biotinylated Secondary, 0.69A Shandon-Upshaw) for 15 min and washed in TBS. Kwik Streptavidin peroxidase reagent (039A Shandon-Upshaw) was applied for 15 min, and sections were again washed in TBS. The color was developed by a 15-min incubation with 3,3′-diaminobenzidine solution, and sections were weakly counterstained with hematoxylin. The CA9 expression and the MVD assessed in these double-stained slides was compared with the results recorded for the original single-stained slides. The staining results were identical (data not shown). The MVD was assessed in two optical fields in the stroma adjacent to CA9-expressing cancer areas and in two optical fields in the stroma of CA9-negative tumor areas.

Assessment of Angiogenic Factor Expression. Thymidine phosphorylase (PD-ECGF) expression was assessed with the P-GF.44C MAb. Staining was performed with the streptavidin-biotin-peroxidase (Dako, Glostrup, Denmark) technique as described previously (12). Tumors were assessed for PD-ECGF expression by the intensity and extent of staining. Two staining groups were considered according to a previous study by our group (12): low/medium reactivity (0–50% of cells stained or weak diffuse staining intensity) and high reactivity (strong intensity in >50% of cells).

VEGF expression was assessed with the VG1 MAb, which recognizes the 121, 165, and 189 isoforms of VEGF. Staining was performed with the horseradish peroxidase technique as described previously (13). The percentage of VEGF-positive cancer cells (0–100%) was assessed by three observers. Taking into account the extent of positive staining, we divided our cases into two groups: low/medium reactivity (0–69% positive cells) and high reactivity (70–100% positive cells). These cutoff points were chosen according to a previous study (13).

Expression of cytoplasmic bFGF and its "bek" receptor (FGFR-2) was assessed in cancer cells by the APAAP technique. We used the FGF-2 (147)-G and the Bek(C-17)-G MAbs, respectively (Santa Cruz Biotechnology). The mean percentage of positive cells was used as a cutoff point to define two groups of low and high reactivity (66 and 17% for bFGF and FGFR-2, respectively), as described previously (14).

Assessment of HIF Expression. The HIF1a and HIF2a proteins were detected by the ESEE 122 (IgG1 MAb; 1:20 dilution) and the EP190b (IgG1 MAb; undiluted) as we have described previously (15). Sections were deparaffinized, and peroxidase was quenched with methanol and 3% H_2O_2 for 15 min. For antigen retrieval, sections were microwaved (three times for 4 min each). The primary antibodies were applied for 90 min. After being washed with TBS, sections were incubated with a secondary anti rabbit antimouse antibody (Kwik Biotinylated Secondary, 0.69A Shandon-Upshaw) for 15 min and washed in TBS. Kwik Streptavidin peroxidase reagent (039A Shandon-Upshaw) was applied for 15 min, and sections were again washed in TBS. The color was developed by a 15-min incubation with 3,3′-diaminobenzidine solution, and sections were weakly counterstained with hematoxylin. Breast cancer tissue sections with strong nuclear HIF1a and HIF2a expression were used as positive controls. Normal rabbit IgG was substituted for primary antibody as the negative control (same concentration as the test antibody). Tumor samples with strong cytoplasmic and/or nuclear expression were considered positive (16, 17).

Immunohistochemistry for Adhesion-disrupting Proteins. C-erbB-2 oncoprotein expression was assessed with the MAb NCL-CB11 (Novocastra Laboratories, Newcastle upon Tyne, United Kingdom), which recognizes the internal domain of the c-erbB-2 protein amino acid sequence. Staining was done with an indirect immunoperoxidase technique, as described previously (18). In a previous study we showed that membrane staining was impossible to assess in NSCLC, in the context of strong cytoplasmic reactivity (18). Cytoplasmic staining intensity was scored as negative (−), moderate (+), and strong (++), and the extent of staining as negative (<30% of cells), focal (30–70%), and diffuse (>70%). Two groups were identified for cytoplasmic staining: positive staining (strong intensity and diffuse/focal staining) and negative/weak staining (all other staining combinations).

EGFR1 murine MAb was used to identify EGFR by means of an indirect immunoperoxidase technique (19) on 5-μm cryostat sections. Samples with negative (−) or weak (+) staining intensity were considered negative, whereas samples with moderate (++) or strong (+++) staining were considered positive.

The glycosylated form of episialin MUC1 expression was assessed on paraffin-embedded material with the Ma695 (IgG1) MAb (YLEM, Rome, Italy), which recognizes a carbohydrate epitope of the MUC1 glycoprotein. The avidin-biotin complex immunoperoxidase technique was used as described previously (14). Overexpression of the episialin results in a circumferential cytoplasmic and membrane immunoreactivity, which is never seen in normal cells. The percentage of cancer cells with episialin overexpression was recorded. The mean value of the percentage of cells with positive reactivity (27%) was used as a cutoff point to distinguish cases of negative and positive reactivity, as described previously (14).

Other Immunohistochemistry. Bcl-2 and p53 expression was examined on 8-μm cryostat sections with the APAAP technique. For bcl-2 detection, we used the MAb 124 (dilution 1:80; Dako) and the horseradish peroxidase technique. Strong cytoplasmic-perinuclear reactivity in >10% of cells was required to characterize a sample as positive for bcl-2 protein expression. The D07 and CM11 antibodies (dilution 1:30; Dako) were used to detect nuclear p53 accumulation. These antibodies are considered as markers of mutant p53 activity, although wild-type p53 expression may also be detected (20). Cytoplasmic-perinuclear p53 was assessed in cryostat sections by the pAb248 MAb. This antibody recognizes the wild-type cytoplasmic p53 protein (21). Positivity in 10% of cancer cells was used as a cutoff point to define p53-positive *versus* -negative cases.

Assessment of Necrosis. Necrosis was assessed on both H&E sections and sections stained with CA9. According to the patterns of necrosis, we distinguished three groups: (*a*) cases with extensive necrosis, where large areas of necrosis were present in >50% of the optical fields; (*b*) cases with no apparent necrosis; and (*c*) all other cases were considered to have focal necrosis.

Statistical Analysis. Statistical analysis was performed and graphs were constructed using the GraphPad (San Diego CA)[3] Prism 2.01 and the Instat 3.0 packages. The Fisher exact *t* test or the Yates continuity corrected χ^2 test was used for testing relationships between categorical tumor variables as appropriate. Survival curves were plotted using the method of Kaplan and Meier, and the log-rank test was used to determine statistical differences between life tables. A Cox proportional hazard model was used to assess the effects of patient and tumor variables on overall survival. All *P*s are two sided, and $P < 0.05$ was considered significant.

RESULTS

Necrosis and Patterns of CA9 Expression. Three different patterns of cancer cell CA9 expression that could coexist in the same tissue sample were recognized: negative, weak cytoplasmic, and strong membrane/cytoplasmic staining (Fig. 1*a*). Tissue samples showing no or weak cytoplasmic CA9 reactivity were considered negative for CA9, whereas cases with strong membrane/cytoplasmic reactivity were considered positive. CA9 expression was confined around areas of necrosis. Even small clusters of some cells undergoing necrosis were often accompanied by intense activation of CA9 in the surrounding cells (Fig. 1*b*). CA9 expression was confined in a layer 1–5 cells in thickness away from necrosis, whereas more distant cellular layers (close to the stroma) did not express CA9 (Fig. 1*a*). Extensive CA9 expression with no apparent areas of necrosis was rarely noted (two squamous cell carcinomas and one adenocarcinoma).

[3] www.graphpad.com.

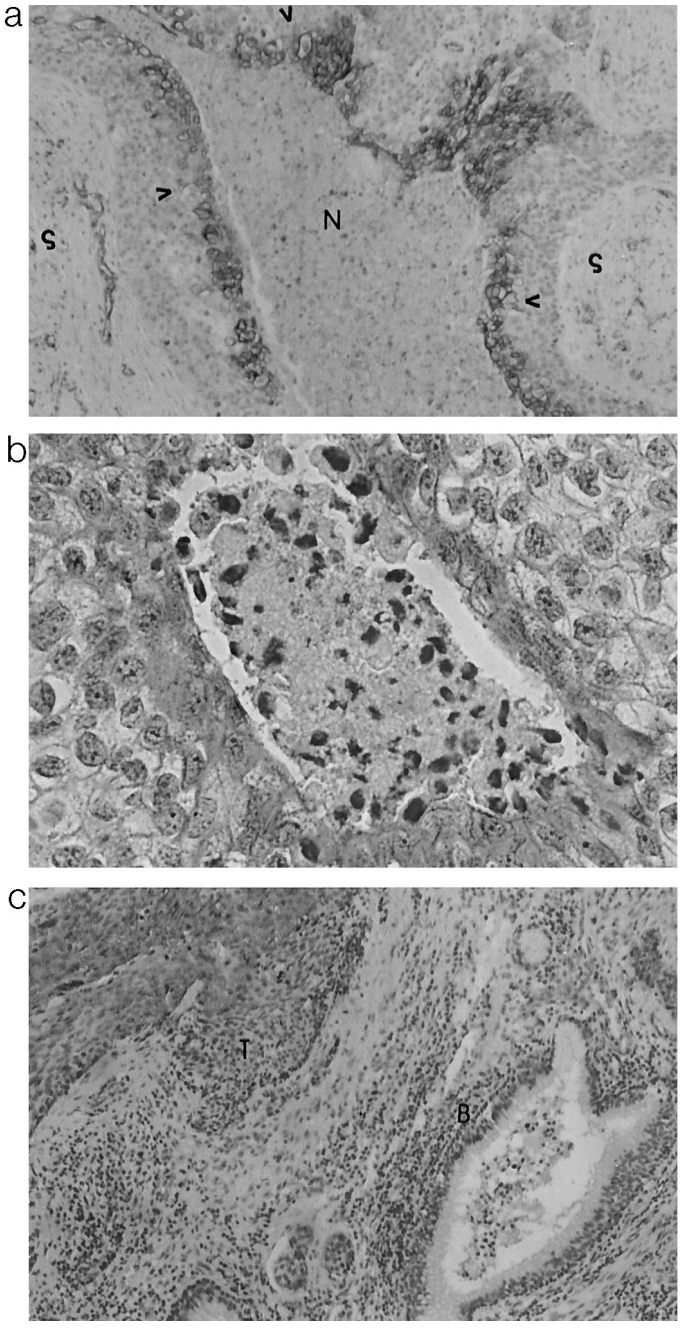

Fig. 1. *a*, double staining of a squamous cell lung cancer with anti-CD31 and anti-CA9 (×100), showing the typical pattern of CA9 expression by cancer cell (*arrowheads*) around the area of necrosis (*N*) but not in the area proximal to the vascularized stroma (*S*). *b*, typical CA9 membrane/cytoplasmic staining of cancer cells around a small necrotic area (×200). *c*, occasionally, weak cytoplasmic expression of CA9 in the normal bronchi (*B*) proximal to the invading tumor (*T*) was noted (×100).

Fibroblasts and vessels were occasionally positive in the tumoral stroma in 14 of 107 (13%) and 22 of 107 (20.5%) of cases, respectively. The staining, when present, was weak cytoplasmic, whereas strong membrane staining was never observed. There was no association of weak vessel staining with the presence of CA9+ cancer cell areas.

In lung tissue samples obtained during autopsy or thoracic surgery for reasons other than lung cancer, the bronchial and alveolar mucosa were persistently negative. Although the normal bronchial mucosa in lung tissues from patients with lung cancer was similarly negative, a rather weak cytoplasmic positivity was occasionally noted in the bronchial epithelium and submucosa vessels found in close proximity to the invading tumor (Fig. 1*c*). Chondrocytes were persistently positive for CA9. Alveolar macrophages were negative, and impressively, tumor-infiltrating macrophages around areas of necrosis were only occasionally positive regardless of the cancer cell reactivity.

Of 107 cases analyzed, 39 (33 squamous cell carcinomas and 6 adenocarcinomas; 36.4%) had strong CA9 expression and were grouped as positive, whereas 68 (63.6%) had no or weak cytoplasmic staining and were grouped as CA9-negative cases. Overall, a strong association of CA9 expression with necrosis was noted. Only 3 of 25 (12%) tumor sections (2 squamous cell carcinomas and 1 adenocarcinoma) with no apparent necrosis had positive CA9 expression *versus* 13 of 28 (46%) and 23 of 46 (50%) specimens with focal and extensive necrosis, respectively ($P = 0.004$). Assessment of the patterns of necrosis on H&E sections and on CA9-stained sections was quite similar. However, 12 cases with focal expression on H&E sections were grouped as bearing extensive necrosis in CA9-stained sections, and 11 cases with no apparent necrosis in H&E sections were grouped as bearing focal necrosis in CA9 sections. The grading of necrosis used for subsequent correlation analysis with other variables was the one appraised on the CA9-stained slide.

CA9 Expression and Histopathological Variables. CA9 expression in the two different histology groups showed a significant association with the squamous cell cases. Only 6 of 37 (16%) adenocarcinomas were positive for CA9 *versus* 33 of 70 (47%) of the squamous cell carcinomas ($P = 0.001$). We further analyzed the patterns of necrosis between the two histology groups to assess whether this could explain the difference. Indeed, extensive necrosis (but not focal) was more frequent in squamous cell carcinomas, but the difference was not significant (11 of 37 *versus* 33 of 70; $P = 0.10$).

Given the very low number of CA9+ adenocarcinomas, further analysis of CA9 correlation with histopathological variables was feasible only in squamous cell carcinomas. Table 1 shows the association of CA9 expression with other histopathological features. Significant associations between CA9 with more advanced T stage and the patterns of necrosis were noted ($P = 0.009$ and 0.003, respectively). No association of CA9 with N stage, histology grade, and Ki67 proliferation index was observed.

CA9 Coactivation with the PD-ECGF and bFGF Pathway. Table 2 shows the relationships between CA9 expression with angiogenesis parameters. Significant direct associations with PD-ECGF and with the bFGF-bek receptor were noted. Squamous cell carcinomas with CA9 reactivity had a 2-fold increased incidence of PD-ECGF

Table 1 *Correlation of CA9 expression in squamous cell lung cancer with histopathological variables*

Parameter	CA9		
	Negative ($n = 37$)	Positive ($n = 33$)	*P*
T stage			
T_1 ($n = 33$)	23	10	0.009
T_2 ($n = 37$)	14	23	
N stage			
N_0 ($n = 44$)	25	19	0.46
N_1 ($n = 26$)	12	14	
Grade			
1/2 ($n = 26$)	12	14	0.46
3 ($n = 44$)	25	19	
Ki67			
L/M[a] ($n = 55$)	27	28	0.25
H ($n = 15$)	10	5	
Necrosis			
No ($n = 15$)	13	2	
Focal ($n = 22$)	12	10	0.003
Extensive ($n = 33$)	12	21	

[a] L/M, low/moderate; H, high.

Table 2 *Correlation of CA9 expression with angiogenesis and with angiogenic factor expression*

	CA9 expression								
	All cases			Squamous cell carcinoma			Adenocarcinoma		
Parameter	Negative (n = 68)	Positive (n = 39)	P	Negative (n = 37)	Positive (n = 33)	P	Negative (n = 31)	Positive (n = 6)	P
MVD									
Low/Medium	46	28		27	23		19	5	
High	22	11	0.82	10	10	0.79	12	1	0.58
PD-ECGF									
Low	50	22		30	19		20	3	
High	18	17	0.08	7	14	0.03	11	3	0.83
VEGF									
Low	32	15		19	15		13	0	
High	36	24	0.42	18	18	0.81	18	6	0.13
bFGF									
Low	39	19		21	16		18	3	
High	29	20	0.42	16	17	0.63	13	3	0.93
bFGFR									
Low	48	17		25	15		23	2	
High	20	22	0.03	12	18	0.09	8	4	0.13

overexpression (42% *versus* 23%; $P = 0.03$), whereas an overall 2-fold higher incidence of bFGFR expression was noted in CA9-expressing cases (56% *versus* 29%; $P = 0.03$).

Although CA9 was not associated with MVD when all tumors were compared, analysis of the MVD in sections double-stained for CA9 and CD31 showed that, in half of the cases examined, the stroma adjacent to areas expressing CA9 had a higher MVD compared with the MVD in CA9-negative cancer areas within the same sample. Overall, the mean MVD in CA9-positive areas was significantly higher than in CA9-negative areas within the same tissue sample (23 ± 10 *versus* 16 ± 12; $P = 0.0005$; Fig. 2).

CA9 and HIF Expression. Table 3 shows the distribution of CA9-positive cases according to HIF1a and 2a reactivity. A marginal association of HIF1a expression with CA9 expression was noted ($P = 0.09$). Only 8 of 39 cases with high CA9 reactivity did not show high HIF1a/2a reactivity. All six adenocarcinomas positive for CA9 were also positive for HIF1a/2a (five for HIF1a and five for HIF2a). Nevertheless, quite a high number of cases with high HIF reactivity (22 of 68) did not show any CA9 expression.

CA9 Is Coexpressed with EGFR, C-erbB-2, and MUC1. All three proteins (EGFR, c-erbB-2, and MUC1) were significantly co-expressed in tumors with strong CA9 reactivity (Table 4). The association of c-erbB-2 with CA9 expression was more evident in adenocarcinomas ($P = 0.03$), whereas in squamous cell carcinomas this association was marginal ($P = 0.12$). For EGFR and MUC1, a significant direct relationship with CA9 was noted only in squamous cell carcinomas. No association of the bcl-2 and p53 proteins with the CA9 expression was noted (data not shown).

CA9 Expression, MVD, and Survival. Survival analysis showed that CA9 expression was significantly associated with poor prognosis in squamous cell carcinomas (Fig. 3*a*). Double stratification for MVD and CA9 showed that although high MVD was related to poor prognosis compared with poorly vascularized cases ($P = 0.009$), CA9 expression defined a subgroup in this latter group with particularly poor outcome ($P = 0.007$; Fig. 3*b*). A similar analysis in adenocarcinomas (despite the very low number of patients in the CA9-positive category) showed the same prognostic results (Fig. 3*c*).

Indeed, in a bivariate analysis (all cases), both MVD and CA9 were revealed as significant and independent prognostic variables ($P = 0.007$; t ratio, 2.72; and $P = 0.03$; t ratio, 2.18, respectively). When we excluded highly vascularized cases and used a multivariate model that included CA9 expression together with the standard histopathological variables (T and N stage, histological grade, and histology), CA9 expression was the most important and independent prognostic factor ($P = 0.02$; t ratio, 2.2) followed by T stage ($P = 0.03$; t ratio, 2.1).

Necrosis *per se* showed a marginal association with poor outcome (extensive *versus* no necrosis, $P = 0.08$; data not shown). However, double stratification for necrosis and CA9 expression showed that in the group with extensive necrosis, CA9-positive cases had a poorer overall survival than CA9-negative cases ($P = 0.01$; Fig. 4).

DISCUSSION

CA9 is expressed by many tumor types, but although a detailed study of CA9 expression in normal tissues has not been published, it seems to be rarely expressed in normal tissues. Constitutive expression of CA9, as a result of VHL protein mutations (3) and HIF

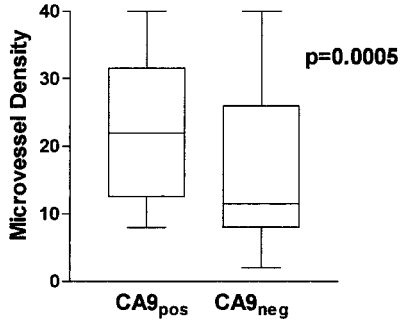

Fig. 2. Stroma around areas of positive CA9 expression ($CA9_{pos}$) showed an overall higher MVD than the MVD in areas of CA9 negativity ($CA9_{neg}$) in the same tissue sample.

Table 3 *Correlation of CA9 expression with angiogenesis and with HIF1a and HIF2a expression*

	CA9		
Parameter	Negative (n = 68)	Positive (n = 39)	P
HIF1a			
Low (n = 39)	29	10	
High (n = 68)	39	29	0.09
HIF2a			
Low (n = 52)	35	17	
High (n = 55)	33	22	0.54
HIF1a/2a			
Low/Low (n = 30)	22	8	
All other (n = 77)	46	31	0.26

Table 4 *Correlation of CA9 expression with the expression of erbB and MUC1 proteins*

	CA9 expression								
	All cases			Squamous cell carcinoma			Adenocarcinoma		
Parameter	Negative (n = 68)	Positive (n = 39)	P	Negative (n = 37)	Positive (n = 33)	p	Negative (n = 31)	Positive (n = 6)	P
EGFR									
Negative	20	5		11	2		9	3	0.59
Positive	48	34	0.06	26	31	0.01	22	3	
c-erbB-2									
Negative	59	26		33	24		26	2	0.03
Positive	9	13	0.04	4	9	0.12	5	4	
MUC1									
Negative	44	17		24	13		20	4	0.71
Positive	24	22	0.04	13	20	0.03	11	2	

pathway activation (4), is observed in renal carcinomas, where a diffuse, necrosis-independent pattern of expression is noted in 92% of clear cell carcinomas (2). Although focal expression of CA9 has been reported in cervical (22), esophageal (23), colorectal (24), and lung cancer (25), its pathological and clinical roles in human malignancies remain obscure.

In the present study, we examined the patterns of CA9 expression in squamous cell cancer and adenocarcinoma of the lung. Strong cytoplasmic and membrane staining of the protein was noted in half of squamous cell carcinomas, whereas its expression in adenocarcinomas was not frequent (16%). Normal bronchi and alveolar tissue did not express CA9, although rather weak expression was occasionally noted in normal bronchial epithelium in close proximity to the invading tumor and in chondrocytes, known to be hypoxic. Similar results have been reported in a recent study by Vermylen *et al.* (25), where strong expression of CA9 was noted in 69% of lung carcinomas but not in normal mucosa and premalignant lesions.

In our study, CA9 expression was strongly related to the extent of necrosis in both histology types, although cases with necrosis and absence of CA9 expression were also noted, showing that in some tumors CA9 induction is not feasible. CA9 localization, when present, was persistently noted in cancer cells around areas of necrosis. Even very small areas of necrosis or areas composed by some degenerated cells undergoing necrosis were enough to show protein expression in surrounding cells. The cellular layers of the cancer cell nests located at a longer distance from necrosis and close to the intact stromal vasculature did not express CA9 (a typical and persistent finding, shown in Fig. 1*a*), which reveals an oxygen perfusion-dependent sequence starting from the cancer cell layers proximal to the vessels (good oxygen perfusion and no CA9 expression), followed by cancer cell layers proximal to necrosis (poor oxygen perfusion and CA9 up-regulation), and finally necrosis (induced by the very low oxygen levels). These patterns of expression, also reported in a recent study by Wykoff *et al.* (4), show the strong association of CA9 expression with pronounced tissue hypoxia within the frame of hypoxic necrosis. Indeed, Wykoff *et al.* (4) in an experimental study showed that CA9 is strongly induced by hypoxia through activation of the HIF1a pathway (4). We also examined the correlation of CA9 expression with the expression of HIF1a and HIF2a in the same series of patients. Eighty percent of CA9-positive cases examined were also positive for HIF1a and/or HIF2a, suggesting that the HIF pathway may be involved in the CA9 induction in NSCLC. Nevertheless, one-third of HIF-positive cases did not express CA9.

Taking into account the diffuse patterns of HIF and of VEGF (HIF-inducible) expression occurring in the presence or absence of necrosis (4, 13, 17), as well as the lack of association of VEGF with CA9 expression, it could be suggested that different intensities of hypoxia are necessary for the induction of proteins downstream from HIF activation. Mild hypoxia may be enough for the induction of HIFs and VEGF, whereas CA9 is induced only in severely hypoxic regions. In addition, the duration of hypoxia is likely to relate to the patterns because the CA9 pattern correlates closely with chronic hypoxia and HIF has a half-life of only a few minutes in air and may reflect acute

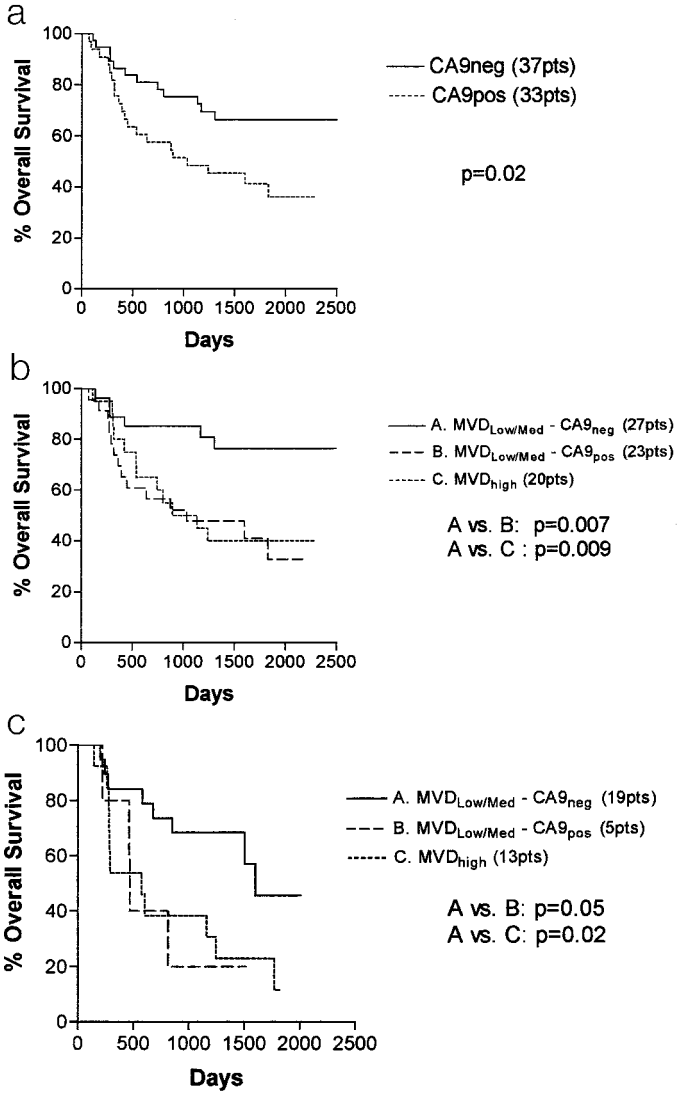

Fig. 3. *a*, Kaplan-Meier overall survival curves stratified for CA9 expression in squamous cell lung carcinomas. *b* and *c*, double stratification for CA9 and MVD revealed a subgroup of patients with poorly vascularized CA9-positive tumors and poor prognosis (*b*, squamous cell carcinoma; *c*, adenocarcinoma). *neg*, negative; *pos*, positive; *pts*, patients; *Med*, medium.

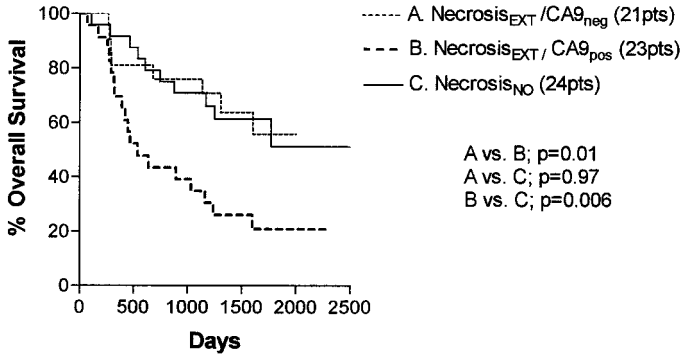

Fig. 4. Kaplan-Meier survival curves (all cases) based on double stratification for the extent of necrosis and CA9 expression reveal that extensive (*EXT*) necrosis is related to poor prognosis only when CA9 is up-regulated. *NO*, none; *neg*, negative; *pos*, positive.

hypoxia. CA9 was associated to PD-ECGF expression by cancer cells and to the bFGF-activated pathway. Different types of hypoxia may therefore account for the induction of different angiogenic factors in NSCLC, whereas the HIF pathway seems to maintain a key role in the whole process. This observation may be of clinical relevance because the development of simple immunohistochemical tests that predict for different levels of hypoxia may prove of importance in the practice of radiotherapy and chemotherapy. Nevertheless, cytokines released by necrotic cells may also account for the CA9 up-regulation, which should be further investigated in experimental studies.

It could be suggested that successful induction of angiogenesis after up-regulation of HIFs and VEGF by mild hypoxia may prevent the formation of necrosis and CA9 induction. Failure of VEGF-mediated angiogenesis [*e.g.*, presence of endogenous angiogenesis inhibitors such as angiostatin (26), irregular perfusion of the newly formed vasculature (27), or the consumption of oxygen by rapidly growing tumors] may account for the focal establishment of higher levels of hypoxia and induction of CA9 and of angiogenic factors such as PD-ECGF. The significant association of CA9 expression with bFGFR-2 may also show that activation of bFGFRs by bFGF released by dying cells would promote CA9 expression, which may be a critical event in achieving maximal CA9 expression *in vivo*. CA induces the formation of carbonic acid from the carbon dioxide produced by cellular metabolism. Acidification of the extracellular matrix is well known to induce expression of angiogenic factors such as interleukin-8 (10). Reactive angiogenesis may therefore follow this hypoxic/necrotic event, and indeed, double staining for CA9 and CD31 frequently showed intense neovascularization in the stroma adjacent to the necrotic/CA9-expressing region.

In the present study we also found a significant association of CA9 expression with prognosis, which was evident in patients with poorly angiogenic NSCLC. CA9 correlated with advanced T stage, showing that CA9-positive tumors may have a growth advantage over CA9-negative tumors. A growth advantage of CA9-expressing tumors has also been suggested by Saarnio *et al.* (24) in a study on colorectal cancer, where CA9-positive areas had a higher Ki67 proliferation index. Although in our study we did not confirm a significant association of Ki67 with CA9 expression, a field-by-field comparative analysis in parallel sections was not performed. The frequent coexpression of CA9 with the angiogenic factors PD-ECGF and bFGFR may also account for the prognostic significance of CA9 expression. PD-ECGF, apart from its angiogenic properties (28), is well known to inhibit the apoptotic process and to confer a proliferation advantage in carcinomas (29, 30). bFGF and bFGFR have also been shown to form an autocrine loop that enhances the proliferation of cancer cells (31).

Acidification of the extracellular matrix by carbonic acid formation may also account for the aggressive behavior of CA9-expressing tumors. Although the lactic acid produced by the glycolytic pathway of metabolism is considered the main cause of acidification of the extracellular environment in cancer (32), several experimental studies have suggested that lactate dehydrogenase-deficient cancer cells show extracellular pH levels as low as cells with normal lactate dehydrogenase activity (33). Experiments on ischemia-reperfusion tumor models showed that although accumulation of lactic acid in an initial phase accounts for the tissue acidification, in a subsequent phase a further decrease of the pH is not followed by increasing lactic acid accumulation, suggesting that additional metabolic pathways contribute to the extracellular acidification during hypoxia (34). Carbonic acid produced by the CA9-mediated catalytic transformation of carbon dioxide may well contribute to the appearance of extracellular acidosis. An acidic environment of approximately pH 6.4 protects mitochondria from both oxidative and hypoxic stress (35, 36), which may be an important pathway for the survival of cancer cells under poorly oxygenated conditions, especially in cases with low angiogenic ability. Indeed, tumoral pH measured by microelectrodes is <7 (9). This respiration-related survival advantage conferred by CA9-induced acidification may account for the poor outcome of patients with CA9-positive/poorly angiogenic tumors.

The effect of the acidic tumor environment on cellular immunity may also account for the aggressiveness of CA9-positive tumors. Non-MHC-restricted cytotoxicity is severely inhibited under acidic conditions; Fischer *et al.* (37) showed that tumor necrosis factor- and Fas receptor-mediated apoptosis by lymphokine-activated killer and mononuclear cells is completely abolished at pH 5.8 (although this was never achieved *in vivo*). Secretion of cytokines such as interleukin-10, transforming growth factor-β and IFN-γ is also down-regulated in acidic conditions. CA9-induced acidification of the tumor environment may therefore counteract cellular immunity, which further contributes to tumor aggressiveness. The striking lack of CA9 expression by tumor-infiltrating macrophages and lymphocytes within and around the areas of necrosis observed in the present study suggests that the cutoff level of hypoxia necessary for the induction of CA in these reactive cells is higher than the one of epithelial cells or that there is cell type specificity for this pathway. Cytokine or hypoxia activation of CA9 may be important to tumor cell expression, but such a pathway may be inactive in some cell types, such as macrophages and lymphocytes.

In the present study we also found a significant association of CA9 expression with the up-regulation of three proteins, EGFR, c-erbB-2, and MUC1, involved in the invasion, migration, and metastasis of cancer. All three proteins disrupt cell-cell adhesion by degrading the catenin-cadherin complex (38, 39) or the integrin-mediated cell adhesion system (40). The erbB proteins are well known to mediate cellular motility and migration (41), whereas MUC1 is involved in cell-endothelium adhesion and cancer cell metastasis (42). Indeed, in a recent study, treatment of renal cancer cells with acetazolamide, a CA inhibitor, resulted in suppression of the invasive capacity (43). Whether acidic environment or hypoxia *per se* directly favors overexpression of these proteins is unknown, but CA9 coexpression with cell-cell adhesion-disrupting molecules may well contribute to the aggressiveness of CA9-expressing tumors. In a previous study we reported that c-erbB-2 overexpression occurs in poorly vascularized NSCLC and is associated with poor prognosis (18), which is now further sustained by the significant coexpression of this molecule with CA9 and the important prognostic role of the latter in poorly angiogenic tumors.

The present study suggests that CA9 is an important molecule in NSCLC, the expression of which is triggered in highly hypoxic/necrotic regions. The expression of CA9 is linked to the expression of

a constellation of proteins involved in angiogenesis, apoptosis inhibition, growth intensification, and cell-cell adhesion disruption, which explains the strong association of CA9 with poor outcome in early stage NSCLC. The observation that CA9 expression is associated with poor survival mainly in poorly vascularized carcinomas shows that even a poorly angiogenic tumor growth may be lethal for a variety of reasons. CA9 analysis with angiogenesis provides an even more important (than angiogenesis alone) tool for the prognostic categorization of NSCLC patients. Further studies are required to investigate the role of CA9-mediated acidification of the tumor environment in the up-regulation of angiogenic and migratory pathways. This may be of therapeutic importance because CA inhibitors suppress tumor invasive growth (43) and, furthermore, enhance the cytotoxicity of several chemotherapeutic agents (44).

REFERENCES

1. Pastorekova, S., Zavadova, Z., Kostal, M., Babusikova, O., and Zavada, J. A novel, quasi viral agent, MaTu, is a two component system. Virology, *187:* 620–626, 1992.
2. Murakami, Y., Kanda, K., Tsuji, M., Kanayama, H., and Kagawa, S. MN/CA9 gene expression as a potential biomarker in renal cell carcinoma. BJU Int., *83:* 743–747, 1999.
3. Ivanov, S. V., Kuzmin, I., Wei, M. H., Pack, S., Geil, L., Johnson, B. E., Stanbridge, E. J., and Lerman, M. I. Down-regulation of transmembrane carbonic anhydrases in renal cell carcinoma cell lines by wild-type von Hippel-Lindau transgenes. Proc. Natl. Acad. Sci. USA, *95:* 12596–12601, 1998.
4. Wykoff, C. C., Beasley, N. J. P., Watson, P. H., Turner, K. J., Pastorek, J., Wilson, G. D., Turley, H., Maxwell, P. H., Pugh, C. W., Ratcliffe, P. J., and Harris, A. L. Hypoxia inducible regulation of tumor associated carbonic anhydrases. Cancer Res., *60:* 7075–7083, 2001.
5. Maxwell, P. H., Wiesener, M. S., Chang, G. W., Clifford, S. C., Vaux, E. C., Cockman, M. E., Wykoff, C. C., Pugh, C. W., Maher, E. R., and Ratcliffe, P. J. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature (Lond.), *399:* 271–275, 1999.
6. Opavsky, R., Pastorekova, S., Zelnik, V., Gibadulinova, A., Stanbridge, E. J., Zavada, J., Kettmann, R., and Pastorec, J. Human MN/CA9 gene, a novel member of the carbonic anhydrase family: structure and exon to protein domain relationships. Genomics, *33:* 480–487, 1996.
7. Gray, L. H., Conger, A. D., Ebert, M., Hornsby, S, and Scott, O. C. A. Concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. Br. J. Radiol., *26:* 638–648, 1953.
8. Brown, J. M. The hypoxic cell: a target for selective cancer therapy—eighteenth Bruce F. Cain Memorial Award Lecture. Cancer Res., *59:* 5863–5870, 1999.
9. Vaupel, P., Kallinowski, F., and Okunieff, P. Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. Cancer Res., *49:* 6469–6465, 1989.
10. Shi, Q., Abbruzzese, J. L., Huang, S., Fidler, I. J., Xiong, Q., and Xie, K. Constitutive and inducible interleukin 8 expression by hypoxia and acidosis renders human pancreatic cancer cells more tumorigenic and metastatic. Clin. Cancer Res., *5:* 3711–3721, 1999.
11. Giatromanolaki, A., Koukourakis, M., Theodossiou, D., Barbatis, C., Harris, A. L., and Gatter, K. C. Comparative evaluation of angiogenesis assessment with anti-factor VIII and anti-CD31 immunostaining in non small cell lung cancer. Clin. Cancer Res., *3:* 2493–2500, 1997.
12. Koukourakis, M. I., Giatromanolaki, A., O'Byrne, K., Comley, M., Whitehouse, R., Talbot, D., Gatter, K. C., and Harris, A. L. Platelet-derived endothelial cell growth factor expression correlates with tumor angiogenesis and prognosis in non-small cell lung cancer. Br. J. Cancer, *4:* 477–481, 1997.
13. Giatromanolaki, A., Koukourakis, M. I., Kakolyris, S., Turley, H., O'Byrne, K., Scott, P. A. E., Pezzella, F., Georgoulias, V., Harris, A. L., and Gatter, K. C. Vascular endothelial growth factor, wild-type p53 and angiogenesis in early operable non-small cell lung cancer. Clin. Cancer Res., *4:* 3017–3024, 1998.
14. Giatromanolaki, A., Koukourakis, M. I., Sivridis, E., O'Byrne, K., Cox, G., Thorpe, P. E., Gatter, K. C., and Harris, A. L. Co-expression of MUC1 glycoprotein with multiple angiogenic factors in non-small cell lung cancer suggests co-activation of angiogenic and migratory pathways. Clin. Cancer Res., *6:* 1917–1921, 2000.
15. Talks, K. L., Turley, H., Gatter, K. C., Maxwell, P. H., Pugh, C. W., Ratcliffe, P. J., and Harris, A. L. The expression and distribution of the hypoxia inducible factors HIF-1a and HIF-2a in normal human tissues, cancers and tumor associated macrophages. Am. J. Pathol., *157:* 411–421, 2000.
16. Koukourakis, M. I., Giatromanolaki, A., Skarlatos, J., Corti, L., Blandamura, S., Piazza, M., Gatter, K. C., and Harris A. L. Hypoxia inducible factor (HIF-1a and HIF-2a) expression in early esophageal cancer and response to photodynamic therapy and radiotherapy. Cancer Res., *61:* 1830–1832, 2001.
17. Giatromanolaki, A., Koukourakis, M. I., Sivridis, E., Turley, H., Talks, K., Pezzela, F., Gatter, K. C., and Harris, A. L. Relation of hypoxia inducible factor 1a and 2a in operable non-small cell lung cancer to angiogenic/molecular profile of tumors and survival. Br. J. Cancer, *85:* 881–890, 2001.
18. Giatromanolaki, A., Koukourakis, M., O'Byrne, K., Kaklamanis, L., Dicoglou, C., Trichia, E., Whitehouse, R., Harris, A. L., and Gatter, K. C. Non small cell lung cancer: C-erbB-2 correlates with low angiogenesis and poor prognosis. Anticancer Res., *16:* 3819–3825, 1996.
19. Veale, D., Aschroft, T., Gibson, G. J., and Harris, A. L. Epidermal growth factor receptors in non-small cell lung cancer. Br. J. Cancer, *55:* 513–516, 1987.
20. McLaren, R., Kuzu, I., Dunnill, M., Harris, A. L., Lane, D., and Gatter, K. C. The relationship of p53 immunostaining to survival in carcinoma of the lung. Br. J. Cancer, 66:735–738, 1992.
21. Pezzella, F., Micklem, K., Turley, H., Jones, M., Kocialkowski, S., Delia, D., Aiello, A., Bickenell, R., Smith, K., Harris, A. L., Gatter, K. C., and Mason, D. Y. Antibody for detecting p53 protein by immunohistochemistry in normal tissues. J. Clin. Pathol., *47:* 592–596, 1994.
22. Liao, S. Y., Brewer, C., Zavada, J., Pastorek, J., Pastorekova, S., Manetta, A., Berman, M. L., DiSaia, P. J., and Stanbridge, E. J. Identification of the MN antigen as a diagnostic biomarker of cervical intraepithelial squamous and glandular neoplasia and cervical carcinomas. Am. J. Pathol., *145:* 598–609, 1994.
23. Turner, J. R., Odze, R. D., Crum, C. P., and Resnick, M. B. MN antigen expression in normal, preneoplastic and neoplastic esophagus: a clinicopathological study of a new cancer-associated biomarker. Hum. Pathol., *28:* 740–744, 1997.
24. Saarnio, J., Parkkila, S., Parkkila, A. K., Haukipuro, K., Pastorekova, S., Pastorek, J., Kairaluoma, M. I., and Karttunen, T. J. Immunohistochemical study of colorectal tumors for expression of a novel transmembrane carbonic anhydrase, MN/CA IX, with potential value as a marker of cell proliferation. Am. J. Pathol., *153:* 279–285, 1998.
25. Vermylen, P., Roufosse, C., Burny, A., Verhest, A., Bosschaerts, T., Pastorekova, S., Ninane, V., and Sculier, J. P. Carbonic anhydrase IX antigen differentiates between preneoplastic malignant lesions in non-small cell lung carcinoma. Eur. Respir. J., *14:* 806–811, 1999.
26. Volm, M., Mattern, J., and Koomagi, R. Angiostatin expression in non-small cell lung cancer. Clin. Cancer Res., *6:* 3236–3240, 2000.
27. Konerding, M. A., Malkusch, W., Klapthor, B., van Ackern, C., Fait, E., Hill, S. A., Parkins, C., Chaplin, D. J., Presta, M., and Denekamp, J. Evidence for characteristic vascular patterns in solid tumours: quantitative studies using corrosion casts. Br. J. Cancer, *80:* 724–732, 1999.
28. Moghaddam, A., Zhang, H. T., Fan, T. P. D., Hu, D. E., Lees, V. C., Turley, H., Fox, S. B., Gatter, K. C., Harris, A. L., and Bicknell, R. Thymidine phosphorylase is angiogenic and promotes tumor growth. Proc. Natl. Acad. Sci. USA, *92:* 998–1002, 1995.
29. Natsura, T., Kuratate, I., Teramchi, K., Osaki, M., Fukuda, Y., Ito, H. Thymidine phosphorylase expression is associated with both increase of intratumoral microvessels and decrease of apoptosis in human colorectal carcinomas. Cancer Res., *59:* 5037–5040, 1999.
30. Matsushita, S., Nitanda, T., Furukawa, T., Sumizawa, T., Tani, A., Nishimoto, K., Akiba, S., Miyadera, K., Fukushima, M., Yamada, Y., Yoshida, H., Kanzaki, T., and Akiyama, S. The effect of thymidine phosphorylase inhibitor on angiogenesis and apoptosis in tumors. Cancer Res., *59:* 1911–1916, 1999.
31. Berger, W., Setinek, U., Mohr, T., Kindas Mugge, I., Vetterlein, M., Dekan, G., Eckersberger, F., Caldas, C., and Micksche, M. Evidence for a role of FGF-2 and FGF receptors in the proliferation of non-small cell lung cancer cells. Int. J. Cancer, *83:* 415–423, 1999.
32. Mueller-Klieser, W., Walneta, S., Paschen, W., Kallinowski, F., and Vaupel, P. Metabolic imaging in microregions of tumors and normal tissues with bioluminescence and photon counting. J. Natl. Cancer Inst. (Bethesda), *80:* 842–848, 1988.
33. Yamagata, M., Hasuda, K., Stamato, T., and Tannock, I. F. The contribution of lactic acid to acidification of tumours: studies of variant cells lacking lactate dehydrogenase. Br. J. Cancer, *77:* 1726–1731, 1998.
34. Parkins, C. S., Strattford, M. R. L., Dennis, M. F., Stubbs, M., and Chaplin, D. J. The relationship between extracellular lactate and tumour pH in a murine tumour model of ischaemia-reperfusion. Br. J. Cancer, *75:* 319–323, 1997.
35. Bronk, S. F., and Gores, G. J. Acidosis protects against lethal oxidative injury of liver sinusoidal endothelial cells. Hepatology, *14:* 150–157, 1991.
36. Nemoto, S., Takeda, K., Yu, Z. X., Ferrans, V. J., and Finkel, T. Role for mitochondrial oxidants as regulators of cellular metabolism. Mol. Cell. Biol., *20:* 7311–7318, 2000.
37. Fischer, B., Muller, B., Fischer, K. G., Baur, N., and Kreutz, W. Acidic pH inhibits non-MHC-restricted killer cell functions. Clin. Immunol., *96:* 252–263, 2000.
38. Ochiai, A., Akimoto, S., Kanai, Y., Shibata, T., Oyama, T., and Hirohashi, S. C-erbB-2 gene product associates with catenins in human cancer cells. Biochem. Biophys. Res. Commun., *205:* 73–78, 1994.
39. Wesseling, J., van der Valk, S. W., and Hilkens, J. A mechanism for inhibition of E-cadherin-mediated cell-cell adhesion by the membrane-associated mucin episialin/MUC1. Mol. Biol. Cell, *7:* 565–577, 1996.
40. Wesseling, J., van der Valk, S. W., Vos, H. L., Sonnenberg, A., and Hilkens, J. Episialin (MUC1) overexpression inhibits integrin-mediated cell adhesion to extracellular matrix components. J. Cell Biol., *129:* 255–265, 1995.
41. Wiechen, K., Karaaslan, S., and Dietel, M. Involvement of the c-erbB-2 oncogene product in the EGF-induced cell motility of Sk-OV-3 ovarian cancer cells. Int. J. Cancer, *83:* 409–414, 1999.
42. Regimbald, L. H., Pilarski, L. M., Longenecker, B. M., Reddish, M. A., Zimmermann, G., and Hugh, J. C. The breast mucin MUCI as a novel adhesion ligand for endothelial intercellular adhesion molecule 1 in breast cancer. Cancer Res., *56:* 4244–4249, 1996.
43. Parkkila, S., Rajaniemi, H., Parkkila, A. K., Kivela, J., Waheed, A., Pastorekova, S., Pastorek, J., and Sly, W. S. Carbonic anhydrase inhibitor suppresses invasion of renal cancer cells in vitro. Proc. Natl. Acad. Sci. USA, *97:* 2220–2224, 2000.
44. Teicher, B. A., Liu, S. D., Liu, J. T., Holden, S. A., and Herman, T. S. A carbonic anhydrase inhibitor as a potential modulator of cancer therapies. Anticancer Res., *13:* 1549–1556, 1993.