Biological Characterization of Subgroups of Squamous Cell Lung Carcinomas

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ABSTRACT

Recently, Pezzella *et al.* (Am. J. Pathol., 1997, *151:* 1417–1423, 1997) reported on a subgroup of non-small cell lung carcinomas that had no morphological evidence of neoangiogenesis but appeared to grow and were highly aggressive. In this investigation, we subdivided 87 squamous cell lung carcinomas into four subgroups according to angiogenesis (low and high vessel density) and tumor growth (low and high tumor cell proliferation). The aim was to find differences, if any, in the angiogenic status and clinical behavior between these subgroups. We identified a group of tumors with low angiogenesis and high tumor cell proliferation that was characterized by high expression of vascular endothelial growth factor, low expression of basic fibroblast growth factor, reduced apoptosis, increased incidence of metastases, and short survival times. These data show that even squamous cell lung carcinomas are a heterogeneous group of tumors that can be subdivided in tumors with different biological properties and different clinical behaviors.

INTRODUCTION

In 1971, the hypothesis was formulated by Folkman (1) that tumor growth is angiogenesis dependent. He suggested that tumor cells and vascular endothelial cells within a neoplasm may constitute a highly integrated ecosystem and that endothelial cells may be triggered from a resting state to a rapid growth phase by a "diffusible" chemical signal from tumor cells. This hypothesis was soon supported by multiple animal experiments in which tumors implanted in animals were restricted in growth during the avascular phase but rapid growth and metastasis occurred shortly after vascularization. There is also considerable indirect and direct evidence that tumor cells can produce diffusible angiogenic regulatory molecules and that angiogenesis inhibitors can slow or prevent tumor growth (2).

Multiple clinical observations in human cancer have provided additional support to the hypothesis that tumors are angiogenesis dependent. The number of vessels in a tumor specimen correlates with the disease stage and has prognostic value for survival of patients. The majority of published reports have shown a significant correlation between intratumoral microvessel density and incidence of metastases and/or patient survival (3). However, the association between high microvessel density and poor prognosis has not been demonstrated in all studies. In squamous cell carcinoma of the tongue (4), breast carcinoma (5), and non-small cell lung carcinoma (6), no association has been found with survival or metastasis.

Recently, Pezzella *et al.* (7) demonstrated that tumors may be able to grow without neovascularization. They identified a subgroup of non-small cell lung carcinomas that have no morphological evidence of neoangiogenesis but appear to grow by exploiting the vessels present in the normal tissue. These putative nonangiogenic tumors were highly aggressive and were associated with poor survival. The observations of Pezzella *et al.* (7) prompted us to investigate a collective of 87 squamous cell lung carcinomas to determine whether subgroups with different biological potential exist in this tumor type. For this purpose, we subdivided the tumors into four subgroups according to angiogenesis (low and high vessel density) and to tumor growth (low and high tumor cell proliferation), and the biological characteristics of these tumors were investigated. The aim of the study was to find differences, if any, in the biological status and clinical behavior of these subgroups.

MATERIALS AND METHODS

Patients and Tumors. A total of 87 cases of previously untreated squamous cell lung carcinomas were examined. All patients were surgically treated at the Chest Hospital Heidelberg-Rohrbach (Heidelberg, Germany). Morphological classification of the carcinomas was performed according to WHO (8). Tumor classifications were carried out by two pathologists. All patients were staged at the time of surgery according to the guidelines of the American Joint Committee on Cancer (9). Of the 87 patients, 14 had stage I, 9 had stage II, and 64 had stage IIIa tumors. The mean age of the patients (80 men and 7 women) was 58 years (range, 37–75 years). Follow-up data were obtained from hospital charts and by correspondence with the referring physicians. Only patients who were alive >3 weeks after surgery were included in our investigation.

Immunohistochemistry. The biotin-streptavidin method was used to detect the angiogenic factors (10, 11). The following primary antibodies were tested: antibody to VEGF[2] (dilution

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[2] The abbreviations used are: VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; PCNA, proliferating cell nuclear antigen.

1:10; Ab-2, Dianova, Hamburg, Germany), antibody to bFGF (dilution 1:200), and antibody to PDGF-A (dilution 1:50; Santa Cruz Biotechnology, Heidelberg, Germany). The immunohistochemical procedures were carried out at room temperature unless otherwise stated.

Briefly, formalin-fixed, paraffin-embedded sections were deparaffinized using standard histological procedures. After preincubation with H_2O_2, unlabeled streptavidin, and nonimmunized normal serum, sections were incubated overnight at 4°C in a moist chamber with the primary antibodies. Biotinylated sheep antimouse immunoglobulin or goat antirabbit immunoglobulin were used as secondary antibodies (with 5% normal human serum). Subsequently, the streptavidin-biotinylated peroxidase complex (Amersham, Braunschweig, Germany) was added, and the peroxidase activity was visualized with 3-amino-9-ethylcarbazole, which gives a reddish reaction product. Counterstaining was performed with hematoxylin.

Tumor sections with documented positivity were used as positive controls. Negative controls were carried out by omitting the primary antibody and by replacing the primary antibody with an irrelevant antibody. Endogenous peroxidase activity was quenched using H_2O_2. Nonspecific binding sites were blocked by incubation with normal serum. Sections were also preincubated with unlabeled streptavidin to suppress endogenous biotin activity.

Assessment of Expression. Three observers independently evaluated the results from the immunohistochemical staining without any prior knowledge of each patient's clinical data. The few cases with discrepancies among the investigators were reevaluated and then classified according to the classification given most frequently by the observers. For evaluation of angiogenic factor expression, a semiquantitative scoring system was used that was based on both the staining intensity (0, negative; 1, weak; 2, intermediate; 3, strong) and the percentage of positive cells (0, 0% cells; 1, ≤25% positive cells; 2, 26–50% positive cells; 3, >50% positive cells). The two scores were added, with a maximum score of 6. A score of >2 represented a positive immunohistochemical identification of a marker.

Determination of Tumor Cell Proliferation. Nuclei of proliferating cells were stained with the antibody for the PCNA (Dianova, Hamburg, Germany; clone PC10) at a dilution of 1:10. Tumor cell proliferation was scored by selecting the maximally immunostained areas and counting PCNA-positive and -negative tumor cells at ×400 magnification and with an eyepiece grid. All reactive cells were counted as positive regardless of the intensity of staining. In each case, a minimum of 500 cells were counted, and the fraction of positive cells was determined. The cases were scored without knowledge of other clinical parameters.

Evaluation of Tumor Vascularity. Intratumoral blood vessels were highlighted by staining of endothelial cells with antihuman factor VIII antibody (Dako Diagnostika, Hamburg, Germany) at a dilution of 1:20. Microvessel density was determined in the area of most intense vascularization (hot spot) of each tumor. Individual microvessel counts were made on a ×250 field (×25 objective and ×10 ocular, corresponding to an area of 0.363 mm^2) by three independent observers. The average count from the three observers was used as the final score.

Assessment of Apoptosis. Apoptotic cell death was detected with a nonradioactive 3′ end DNA-labeling technique using the *in situ* cell death detection kit (Boehringer Mannheim). First, the paraffin-embedded specimens were dewaxed by washing in xylene, rehydrated through a graded series of ethanol and H_2O, and finally incubated in PBS. The tissue sections were treated with proteinase K and rinsed with PBS. Endogenous peroxidase was blocked with 0.03% hydrogen peroxide in PBS and subsequently rinsed with PBS. Cells were permeabilized in 0.1% Triton X-100–0.1% sodium citrate for 2 min on ice. Then, they were rinsed with PBS and incubated with the labeling-reaction-mixture containing fluorescein-labeled nucleotides and terminal deoxynucleotidyl transferase. Slides were rinsed again with PBS and treated with antifluorescein antibody conjugated with peroxidase, diluted 1:2. After washing with PBS, peroxidase activity was made visible with 3-amino-9-ethylcarbazole, which gives a reddish reaction product. Counterstaining was performed with hematoxylin. For negative controls, terminal deoxynucleotidyl transferase was omitted. As positive controls, DNase-treated specimens were used. The apoptotic indices were calculated as the ratio of terminal deoxynucleotidyl transferase-mediated nick end labeling-positive cancer cells to the total number of cancer cells, for at least 1,000 cells.

Xenotransplantation into Nude Mice. Of the 87 squamous cell lung carcinomas, 61 tumors could be heterotransplanted into nude mice. For transplantation, the tumor specimens were finely minced with scissors and suspended in Hanks' balanced salt solution. Enough medium was added to reach a tissue:medium ratio of 1:3 (v/v). Three hundred μl of each suspension ($>10^7$ cells/mouse) were injected s.c. into the flanks of three nude mice each with a 1.4-mm trochar needle. Tumor take was assumed when the presence of growing nodule(s) was noted within 3 months and tumor histology was confirmed (12, 13).

Statistical Analysis. The statistical analyses were performed with the ADAM for Windows program package (DKFZ, Heidelberg, Germany). Differences among apoptosis, PCNA index, and microvessel count were analyzed by Wilcoxon rank sum test. Correlation between clinical parameters and angiogenic growth factor expression were examined by the χ^2 test. Analyses of survival data were undertaken by using survival curves and applying the Kaplan-Meier method with log-rank analysis. The results were regarded as statistically significant if $P \leq 0.05$.

RESULTS

Frequency of Marker Expression. Eighty-seven squamous cell lung carcinomas were analyzed for expression of several angiogenic factors, tumor cell proliferation, and vascularity. Table 1 lists the frequency of positivity for the various markers investigated. Overall, the lowest positivity was observed for PDGF (48%), followed by VEGF (58%) and bFGF (62%). The median number of vessels was 6 (range, 0–64), and the median PCNA labeling index was 24.1% (range, 1.3–72.1%).

Stratification into Different Subgroups. In Fig. 1, for 87 squamous cell lung carcinomas, the percentages of proliferating tumor cells were plotted against the number of blood

Table 1 Biomarker expression in squamous cell lung carcinomas

	Positive		Negative		Not assessable	
	No.	%	No.	%	No.	%
VEGF	51	58	36	42	0	0
bFGF	54	62	26	30	7	8
PDGF	42	48	35	40	10	12

vessels. There was no relationship between PCNA labeling index and vascularity. To find subgroups with different biological potential, we subdivided the lung carcinomas into four subgroups, according to median values of vessel density and PCNA labeling index. The cutoff levels of vessel density and PCNA labeling index are marked with dashed lines in Fig. 1 and divide the tumors as follows: tumors with low vascularization and high proliferation (group *A*), tumors with high vascularization and high proliferation (group *B*), tumors with low vascularization and low proliferation (group *C*), and tumors with high vascularization and low proliferation (group *D*).

Expression of the Biological Markers in the Subgroups. The results of the expression of different angiogenic factors in the four subgroups are given in Table 2. The tumors of group A differ in many aspects from the other subgroups. Group A tumors (tumors with low vascularization and high proliferation) expressed VEGF at a higher frequency than did group C and D tumors. bFGF was less frequently expressed in group A as compared to the other groups. PDGF expression was not significantly different among the four subgroups. Apoptotic indices were lower in group A than in groups B and D. Xenotransplantability in immune-deficient mice was higher in group A than in group B and D.

Association with Clinical Factors. No differences were found when patients in different groups based on sex, age, and stage were compared (data not shown). However, there were significant differences in survival in the different subgroups defined by PCNA labeling index and vessel density ($P = 0.04$, log-rank analysis; Fig. 2 and Table 2). Patients of group A had lower median survival times (28 weeks) than patients of group B (88 weeks), group C (111 weeks), or group D (117 weeks). When we looked at metastases, a slightly higher incidence in formation of metastases (regional lymph node and distant metastases) was seen in patients of group A in comparison to the other groups, although statistical significance was not reached.

When lung carcinomas were divided in only two subgroups according to high (groups B and D) and low (groups A and C) vessel density (Table 3), tumors with low vessel density had lower apoptotic indices ($P = 0.01$) and showed better growth in nude mice ($P = 0.01$), and patients with such tumors had shorter median survival times (64 weeks *versus* 111 weeks), but the differences did not reach statistical significance.

In summary, the analysis shows that the various biological markers were quite differently expressed in the different subgroups of squamous cell lung carcinomas and that tumors of group A, characterized by low vessel density and high tumor cell proliferation, exhibited especially high expression of VEGF, low expression of bFGF, reduced apoptosis, and increased xenotransplantability in nude mice; patients with group A tumors also had increased incidence of metastases and shorter survival times.

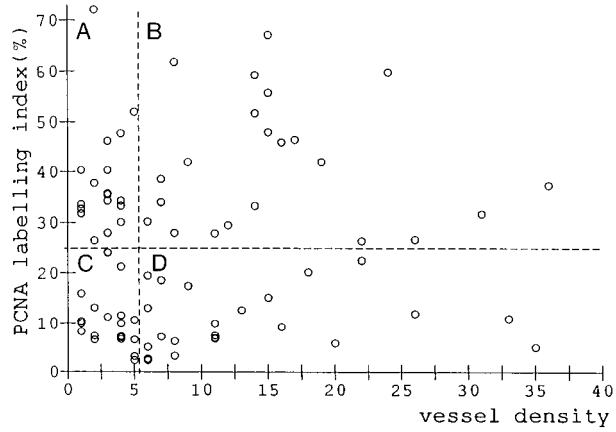

Fig. 1 Relationship between vessel density and PCNA labeling index of squamous cell lung carcinomas. The median values (- - - - -) divide the figure into four areas: tumors with low vascularization and high proliferation (group *A*), tumors with high vascularization and high proliferation (group *B*), tumors with low vascularization and low proliferation (group *C*), and tumors with high vascularization and low proliferation (group *D*).

DISCUSSION

It is well known that tumors of different origin differ in many respects, including their biological properties and their responses to treatment. But diversity also occurs among neoplasms within one tumor type. Lung tumors illustrate quite well the diversity that can be found among cancers of one type of carcinoma. The majority of bronchogenic carcinomas can be classified histologically into four major types: squamous cell carcinoma, adenocarcinoma, large cell carcinoma, and small cell carcinoma. Histological features, ultrastructure, clinical course, and response to therapy indicate, however, that small cell lung carcinoma is a separate entity. In this study, we demonstrate that even squamous cell lung carcinomas are a heterogeneous group of tumors that can be subdivided in tumors with different properties and different clinical behavior.

In a recent paper, Pezzella *et al.* (7) reported on a subgroup of lung carcinomas that had no morphological evidence of neoangiogenesis but appeared to grow. This group of tumors was highly aggressive and was associated with poor survival. In our study with squamous cell lung carcinomas, we subdivided the tumors into four subgroups on the basis of median values for vessel density and tumor cell proliferation. By dividing the tumors this way, we identified a group of tumors (group A) that is characterized by low angiogenesis and high tumor cell proliferation. This group probably corresponds to the alveolar or putative nonangiogenic tumors described by Pezzella *et al.* (7). We also found that this group of tumors is highly aggressive. The high take rate in nude mice and the short survival times of patients are indicators of this highly aggressive nature.

Here, the tumors of group A differed in some aspects from the other subgroups. Despite low microvessel density, these

Table 2 Characteristics of squamous cell lung carcinomas subdivided according to PCNA index and vessel density

	Group A	Group B	Group C	Group D
PCNA index (%)	37.7 ± 10.4	42.1 ± 12.6	10.3 ± 6.4	11.0 ± 5.6
Vessel density	2.7 ± 1.5	18.3 ± 12.7	3.4 ± 1.9	15.6 ± 8.5
Apoptosis	0.31 ± 0.21	0.47 ± 0.35	0.28 ± 0.26	0.40 ± 0.34
P[a]		0.07	0.14	0.27
VEGF				
Positive	19 (95%)	21 (95%)	5 (18%)	6 (33%)
Negative	1	1	22	12
P		0.94	<0.0001	<0.0001
bFGF				
Positive	8 (44%)	15 (71%)	19 (76%)	12 (75%)
Negative	10	6	6	4
P		0.08	0.03	0.07
PDGF				
Positive	14 (70%)	10 (55%)	10 (45%)	8 (47%)
Negative	6	8	12	9
P		0.35	0.10	0.15
Growth in mice				
Yes	8 (53%)	4 (27%)	12 (60%)	3 (27%)
No	7	11	8	8
P		0.13	0.69	0.18
Metastasis[b]				
Positive	14 (74%)	15 (68%)	14 (52%)	12 (66%)
Negative	5	7	13	6
P		0.69	0.13	0.64
Survival (weeks)	28	88	111	117
P		0.13	0.03	0.009

[a] All *P*s were calculated *vs.* group A.
[b] One case could not be defined.

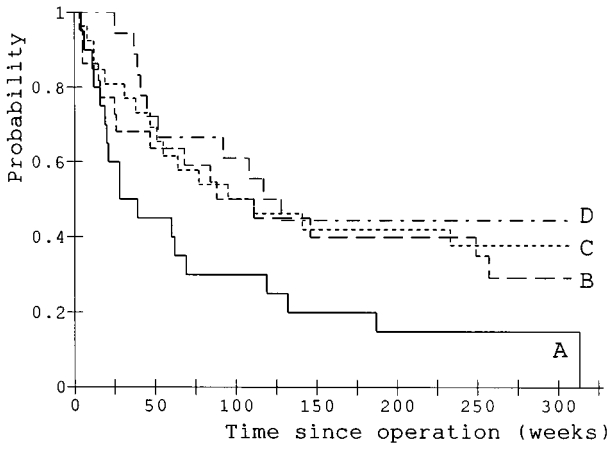

Fig. 2 Survival curves of patients with squamous cell lung carcinomas (Kaplan-Meier estimates), subdivided according to different groups *A–D* (see legend to Fig. 1). $P = 0.04$, by log-rank analysis.

Table 3 Characteristics of the tumors according to high (groups B and D) or low (groups A and C) vessel density

	Groups B and D	Groups A and C	*P*
Vessel density	17.1 ± 11.0	3.1 ± 1.7	<0.0001
PCNA index (%)	28.1 ± 18.5	21.9 ± 16.0	0.07
Apoptosis	0.44 ± 0.34	0.29 ± 0.24	0.01
VEGF			
Positive	27 (67%)	24 (51%)	0.12
Negative	13	23	
bFGF			
Positive	27 (73%)	27 (63%)	0.33
Negative	10	16	
PDGF			
Positive	18 (51%)	24 (57%)	0.61
Negative	17	18	
Growth in mice			
Yes	7 (27%)	20 (57%)	0.01
No	19	15	
Metastasis[a]			
Positive	27 (67%)	28 (61%)	0.52
Negative	13	18	
Survival (weeks)	111	64	0.20

[a] One case could not be defined.

tumors expressed VEGF to a high degree. This suggests that perhaps neoangiogenesis in these tumors is inhibited by inhibitory factors rather than stimulated by VEGF. Angiogenesis is suggested to be regulated by the balance between stimulators and inhibitors (14). Either reducing the inhibitor concentration or increasing the activator levels can each change the balance and activate the angiogenic switch, leading to growth of new blood vessels. When the levels of inhibitors are sufficiently high, the signals of the positive activator(s) can be overruled, keeping the angiogenic switch off. This hypothesis is supported, *e.g.*, by the pancreatic islet model, in which VEGF and aFGF are expressed constitutively in normal islet cells, but remarkably, there is no new blood vessel growth in normal pancreatic islets, despite the expression of these inducers (15, 16). On the other hand, the fact that tumors of group B also express VEGF to a high degree suggests that VEGF correlates with high PCNA rather than with vessel density in these tumors.

In our study, bFGF was less frequently expressed in group A as compared to the other groups and negatively correlated to VEGF. If all tumors are considered, no significant relationship was found between intratumoral expression of VEGF, bFGF, or PDGF, suggesting that these three factors might be differentially regulated in lung tumors.

In previous studies with non-small cell lung carcinoma (17) and ovarian carcinoma (18), we found a close correlation of VEGF expression with tumor cell proliferation, suggesting that VEGF might act as an autocrine growth factor in these tumor types. This stimulation of tumor cell proliferation by VEGF, together with a reduced incidence of apoptosis, probably accounts for the fact that tumors of group A are highly aggressive, indicated by the higher take rate in nude mice, the higher incidence of metastases, and the shorter survival times.

Differences in marker expression are also evident when the tumors are subdivided according to low or high vessel density alone. Tumors with low vessel density have a significantly lower incidence of apoptosis than tumors with high vessel density. It should be noted that low vascularity alone was also associated with a worse prognosis in this study; however, the difference did not reach statistical significance. This result is consistent with two other reports showing that patients whose lung tumors exhibited low vascularity had shorter survival times (6, 19).

In conclusion, we identified a subgroup of squamous cell lung tumors with low angiogenesis and high tumor cell proliferation that is characterized by high expression of VEGF, low expression of bFGF, reduced apoptosis, enhanced xenotransplantability in nude mice, increased incidence of metastases, and shorter survival times. However, the alterations of the parameters investigated in this study do not fully explain the worse prognosis of this subgroup. Further investigations in prognostic factors of lung carcinomas are necessary to clarify their roles and to make more precise predictions concerning the outcome of patients. The identification and biological characterization of different subgroups of lung carcinomas will be of importance for better understanding of the biological behavior of these tumors and for better planning of treatment leading to a more rational, perhaps individualized choice of therapy. Perhaps in the future, the combination of clinical and tumor-related parameters in lung tumors will lead to a grading system with a better prognostic value.

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