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CD117 immunoreactivity in stage I adenocarcinoma and squamous cell carcinoma of the lung: relevance to prognosis in a subset of adenocarcinoma patients

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CD117, a trans-membrane tyrosine kinase receptor, has been immunolocalized in a large variety of human neoplasms. Little, however, is known about the prevalence and clinical implications of CD117 in stage I adenocarcinoma and squamous cell carcinoma of the lung. We evaluated 201 consecutive stage I adenocarcinoma and squamous cell carcinoma of the lung for CD117 immunoreactivity (dichotomized as negative or positive if containing less than 5% or $\geq 5\%$ immunoreactive neoplastic cells, respectively), also taking into account the pattern (either membranous or cytoplasmic), and the intensity of immunostaining in comparison with intratumoral mast cells. The immunostaining results were then correlated with tumor biopathological characteristics and patients' survival. Membranous CD117 immunoreactivity was documented in 19 (22%) of 88 adenocarcinomas and 15 (13%) of 113 squamous cell carcinomas, whereas cytoplasmic labelling was seen in 28 (32%) adenocarcinomas and eight (7%) squamous cell carcinomas. In both tumor types, membranous or cytoplasmic CD117 immunoreactivity was associated with higher proliferative fraction and with features of more aggressive tumor behavior, including higher stage, size and grade, occurrence of clinical symptoms, high microvessel density and neuroendocrine differentiation. Furthermore, immunoreactive tumors exhibited increased levels of bcl-2, cyclin-E, Her-2, p27^{Kip1} and fascin, the latter being a marker of tumor cell metastatization in lung cancer. Membranous but not cytoplasmic labelling emerged as an independent risk factor for death and reduced time to progression in adenocarcinoma but not in squamous cell carcinoma patients, when singly adjusted for confounding factors. CD117 immunoreactivity identifies a peculiar subset of stage I adenocarcinoma and squamous cell carcinoma of the lung with highly proliferative tumors and may have prognostic relevance in adenocarcinoma patients. Targeting the CD117 pathway could be a novel therapeutic strategy in a subset of pulmonary carcinomas.
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Cell receptors with tyrosine kinase activity are important regulators mediating cell proliferation, differentiation and survival, and have been docu-

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mented to play a central role in the development and progression of several human malignancies, including lung cancer (reviewed by Maulik *et al*[1]). The proto-oncogene *c-Kit* mapping to the chromosome 4q11–q12 encodes for a 145–160 kDa type 3 trans-membrane tyrosine kinase receptor (CD117), which undergoes dimerization and activation by phosphorylation upon binding to its specific ligand, known as stem–cell factor, mast cell growth factor, steel factor or kit ligand.[2–4] Detailed information on

the *c-kit* gene structure and protein sequence is available online at web sites http://www.ncbi.nlm.nih.gov/UniGene/clust.cgi?ORG = Hs&CID = 81665 or http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l = 3815. CD117 is thought to be essential for normal gametogenesis, melanogenesis, hematopoiesis and brain[3–5] development, and it is expressed in a variety of normal epithelia, but not in either developing or adult lung tissue.[6,7] This molecule is also consistently expressed in a variety of neoplasms, such as gastrointestinal stromal tumors,[8] seminoma/dysgerminoma,[7] acute myelogenous leukemia,[9] neuroblastoma,[10] Ewing's sarcoma,[11,12] mast cell disorders,[13] and small-cell[14–19] or large-cell neuroendocrine carcinoma of the lung.[20] However, data on the prevalence and the clinical implications of CD117 in stage I adenocarcinoma and squamous cell carcinoma are still lacking, and most of the current knowledge on this subject is derived from experiments on cell cultures[21–23] or from expression studies of excised tumors, not correlated with clinicopathological features and survival analyses.[7,13,24–27] Another point of interest in assessing the CD117 status also in these carcinomas is that specific inhibitors of its tyrosine kinase activity, such as imatinib mesylate (Gleevec, Novartis Pharmaceuticals, Basel, Switzerland)—formerly referred to as STI571 or CGP57148B[28]—have recently been demonstrated to produce an antitumor effect in other subtypes of lung cancer, such as small-cell carcinoma.[14,29] Therefore, these drugs might thus constitute a novel targeted therapy also for those adenocarcinomas and squamous cell carcinomas of the lung strictly dependent on the c-kit kinase activity for growth.

Patients with pathological stage I adenocarcinoma or squamous cell carcinoma—who usually do not receive any adjuvant therapy—have the best life expectation because they lack major adverse prognostic factors at the time of diagnosis, such as incomplete tumor resection or presence of metastases. The long-term survival rate, however, still remains disappointing because 55–72% of these patients will experience relapsing disease,[30–32] mostly in the first 2–3 years after diagnosis.[33,34] Furthermore, patients free from disease after 5 years will still show an excess of lung cancer-related mortality as compared with the referring population.[35] Therefore, unveiling the causative role of growth factor receptors in the development of early-stage adenocarcinomas or squamous cell carcinomas might offer new insights into the mechanisms of local tumor growth and distant metastases, and allow the patients to be better stratified into different risk categories or to be offered novel options of targeted therapy.

The aim of this study was to evaluate the prevalence and the clinical implications of CD117 immunoreactivity in a large series of stage I adenocarcinoma and squamous cell carcinoma of the lung with long-term follow-up time (median 82 months), and to compare the results with the expression of several key gene products involved in cell cycle control and apoptosis (bcl-2, p53, cyclin-E, $p21^{Waf1}$, $p27^{Kip1}$), tumor growth (Her-2, proliferative fraction, neuroendocrine differentiation and neoangiogenesis) and lung tumor cell motility (fascin).[36] Our results indicate that CD117 represents a growth stimulator in a definite subset of these tumors, and its detection may be clinically relevant in patients with pulmonary adenocarcinoma.

Materials and methods

Patients

The study population consisted of 201 consecutive patients (182 males and 19 females) with newly diagnosed stage I adenocarcinoma or squamous cell carcinoma from 1345 patients with stage I–III primary lung carcinoma (201 out of 1342 patients: 15%), who were diagnosed and treated from 1987 to 1993 at the Verona City Major Hospital (Italy). For each case, all paraffin blocks were retrieved and archival hematoxylin–eosin sections were reviewed. As part of routine case, all patients had been studied preoperatively with clinical history, physical examination, respiratory tests, chest X-ray, total body CT-scan, bone scintigraphy and routine laboratory profile. To ensure accurate staging, all tumors were removed by radical surgery with extensive mediastinal lymph node dissection (median value: eight excised lymph nodes per patient). Only patients with a minimum 30-day postoperative survival were included in the study. Other inclusion criteria were pathological stage IA (pT1 N0 M0; 90 men and 10 women) and IB (pT2 N0 M0; 92 men and nine women),[31] no (neo)-adjuvant therapy and minimum follow-up of 5 years.

Clinical data were abstracted retrospectively from hospital charts, whereas follow-up data—recorded every 6 months until December 2002—were obtained by personal contact or by a questionnaire sent to the family doctor or to relatives of deceased patients.[35] Detailed information on the patients' cigarette smoking history was unavailable at the time of the present study. The patients' age ranged from 35 to 77 years for men (mean±s.d.: 62±8 years; median: 63 years) and from 47 to 76 years for women (mean±s.d.: 61±8 years; median: 62 years). In all, 76 patients presented with local symptoms (ie cough, chest pain, hemoptysis or dyspnea), and 45 with systemic symptoms (ie fever, weight loss, asthenia or digital osteopathy) at the time of diagnosis. In three patients with squamous cell carcinoma, the data on clinical symptoms were not available at the time of the present study. Complete clinical follow-up was available for all 201 patients with a mean duration of 79±44 months (median 82; range 2–159); during this period, 87 (43%) patients experienced relapses and 75 (37%) died of disease.

According to previously refined pathological criteria for lung tumors,[37] there were 113 (56%) squamous cell carcinomas and 88 (44%) adenocarcinomas. Subtyping of adenocarcinomas was performed according to the WHO classification.[37] In all, 11 (5%) carcinomas were well differentiated (10 adenocarcinomas and one squamous cell carcinoma), 117 (59%) moderately differentiated (75 squamous cell carcinomas and 42 adenocarcinomas) and 73 (36%) poorly differentiated (37 squamous cell carcinomas and 36 adenocarcinomas).

Immunohistochemistry and Evaluation of Data

Formalin-fixed and paraffin-embedded tissue samples obtained at surgery were investigated. Tumors up to 2 cm in size were entirely embedded and immunostained; at least two representative tissue blocks were investigated in larger neoplasms. Samples of normal pulmonary parenchyma from patients with nonmalignant lung diseases, and peritumoral lung tissue from the same cohort of adenocarcinoma and squamous cell carcinoma patients provided the control groups for noncarcinoma and carcinoma patients, respectively.

For the immunohistochemical experiments, after blocking endogenous peroxidase activity with 5% hydrogen peroxide for 12 min, de-waxed sections were reacted with a panel of commercially available primary antibodies (Table 1) for 30 min at room temperature using an automatic immunostainer (Autostainer, Dako, Glostrup, Denmark), and then incubated with a high-sensitivity detection kit (Dako EnVision Plus-HRP) according to the manufacturer's instructions. All primary antibodies were used according to the manufacturer's instructions with minor modifications in the pretreatment procedures for antigen retrieval, when appropriate. In particular

for CD117 immunostaining, tissue sections were reacted with two polyclonal antibodies, one raised against the region from 963 to 976 amino acid residues at the C terminus (Dako), the other against an amino acid sequence of the C terminal domain not otherwise specified (Santa Cruz Biotechnology, Santa Cruz, CA, USA), both recognizing a p145 kDa c-kit gene product of human origin. Peroxidase activity was then developed with 3-3'-diaminobenzidine-copper sulfate (Sigma Chemical Co, St Louis, MO, USA) to obtain a bright brown-black end product. The specificity of all immunoreactions was double-checked by substituting the primary antibody with a nonrelated isotypic mouse immunoglobulin or rabbit IgG, at a comparable dilution or with normal serum alone.[38] Appropriate internal positive controls represented by tumor-infiltrating mast cells were also checked in all reactions.

All immunostaining reactions were evaluated without any knowledge of the patients' identity or clinical outcome. The percentage of immunoreactive tumor cells for each marker (labelling index) was assessed scanning at least 1000 neoplastic cells in representative fields of immunostaining. For CD117, the immunostaining pattern was recorded as either membranous if staining was seen along the entire tumor cell membrane, or cytoplasmic if granular dotting was confined to the cytoplasm with no appreciable membrane reinforcement. Tumors were considered negative for CD117 if staining was either completely absent or observed in less than 5% of neoplastic cells. Also, the immunostaining intensity of either pattern was evaluated subjectively by using a two-tier scale: weak if it was less intense than that seen in tumor-infiltrating mast cells, or strong if it was of the same or greater intensity than the latter. Tumor cell proliferation was evaluated by Ki-67 immunostaining and tumor neoangiogenesis was inferred by microvessel density after CD34

Table 1 Antibody panel used in the study

Antibodies	m/p	Clone	Source	Dilution/ml (µg)	Pretreatment[a]
CD117	p	—	DAKO, Glostrup, Denmark	10	—
	p	—	Santa Cruz Biotechnology, Santa Cruz, CA, USA	10	—
Ki-67 antigen	m	MIB-1	Immunotech, Marseille, France	2.5	MWO-EDTA
CD34	m	QBEnd/10	Novocastra Laboratories, Newcastle upon Tyne, UK	2.5	MWO-CB
p21/Waf1	m	4D10	''	25	MWO-CB
Cyclin-E	m	13A3	''	20	MWO-EDTA
p53	m	DO7	''	1	MWO-CB
Fascin	m	IM20	''	2.5	MWO-EDTA
Her-2	p	—	DAKO, Glostrup, Denmark	0.6	MWO-EDTA
p27/Kip1	m	57	Transduction Laboratories, Lexington, KY, USA	0.4	MWO-CB
Chromogranin A	m	LK2H10	Signet Laboratories, Dedham, MA, USA	25	—
Synaptophysin	m	SY38	DAKO, Glostrup, Denmark	50	MWO-CB
Bcl-2	m	124	DAKO, Glostrup, Denmark	5	MWO-CB

m, monoclonal; p, polyclonal;
MWO-CB: microwave oven at 750 W for 10 min in citrate buffer, pH6; MWO-EDTA: microwave oven at 750 W for 12 min in ethylenediamine tetraacetic acid buffer, pH8.
[a]All pretreatments for antigen retrieval were optimized according to internal standarization of the author's laboratory.

immunostaining of endothelial cells as previously reported.[39]

Statistical Analysis

Qualitative data were presented as frequencies and/ or percentages and compared using χ^2 test or Fisher's exact test. Continuous data (ie age) were expressed using medians and contrasted employing the Wilcoxon or the Kruskal–Wallis test if medians were compared between two or more groups, respectively. All correlations were calculated using Spearman's rank test (*r*). In order to identify factors associated with CD117 expression, defined as a binary response (yes/no), either in the membrane or in the cytoplasm, multiple logistic regression models were built by including variables found significant when regressed individually. Logistic regression results were expressed using odds ratios (OR) and corresponding 95% confidence intervals (CI). Unadjusted survival estimates according to CD117 expression status, either overall or disease-free, were calculated using the Kaplan–Maier method and compared by the log-rank test. Cox proportional regression models were used to estimate the hazard ratios and 95% CI by adjusting known prognostic factors in lung cancer. Tables summarizing the results of the Cox regression models only display hazard ratios (HR) associated with CD117 expression either unadjusted or adjusted by individual variables and not the HRs associated with the remaining variables in the models. Multiple linear regression analysis was used to investigate if CD117/ ckit expression was an independent predictor of tumor diameter after taking into account additional explanatory variables. All analyses were carried out using SAS (SAS Institute, Cary, NC, USA). *P*-values were based on two-sided testing.

Results

CD117 Immunoreactivity is Lacking in Normal Epithelial Cells of the Lung, but Consistently Expressed in Mast Cells

Type I and II pneumocytes and Clara cells of the bronchioloalveolar epithelium in all samples of non-neoplastic peritumoral lung tissue from the cohort of adenocarcinoma and squamous cell carcinoma patients, as well as the normal pulmonary parenchyma from patients with nonmalignant lung diseases, were consistently unreactive for CD117. Likewise, surface ciliated and mucous cells of the bronchial epithelium and seromucous glands of the bronchial wall never showed CD117 immunostaining. Mast cells infiltrating the tumor tissue or scattered in the interstitium of all examined samples were the only cells consistently and strongly immunoreactive for this marker.

CD117 Immunoreactivity is Consistently Found in a Subset of Adenocarcinoma and Squamous Cell Carcinoma

Staining with the two primary antibodies to CD117 did not differ significantly in corresponding histological fields of adjacent sections, either as for the percentage of immunoreactive tumor cells, or the staining intensity and pattern. Representative features of CD117 immunoreactivity in lung carcinomas obtained with Dako antibody are depicted in Figure 1.

Overall, CD117 membrane immunoreactivity in 5% or more tumor cells was observed in 34/201 (17%) tumors, including 19 (22%) of 88 adenocarcinomas and 15 (13%) of 113 squamous cell carcinomas. The labelling index ranges from 5 to 90% (mean $\pm$ s.d.: 24 ± 23; median: 15) in the former, and from 5 to 100% (mean $\pm$ s.d.: 36 ± 31; median: 25) in the latter ($P = $ NS). Cytoplasmic decoration was seen in 36/201 (18%) tumors, including 28 (32%) adenocarcinomas and eight (7%) squamous cell carcinomas ($P < 0.0001$), with a labelling index ranging from 5 to 80% (mean $\pm$ s.d.: 27 ± 19; median: 20) in the former and from 5 to 37% (mean $\pm$ s.d.: 17 ± 12; median: 13) in the latter ($P = $ NS).

In adenocarcinomas, there was a prevalence of membrane-negative/cytoplasm-positive (17/21) or both positive (11/15) tumors, whereas in squamous cell carcinoma a prevalence of membrane-positive/ cytoplasm-negative (11/19) or both negative (94/154) tumors ($\chi^2 = 21.087$, $P < 0.001$) (Table 2). A close correlation was observed between CD117 labelling index and immunostaining intensity in both adenocarcinoma and squamous cell carcinoma for either membrane ($r = 0.899$ and 0.886, respectively) or cytoplasmic ($r = 0.899$ and 0.947, respectively) pattern ($P < 0.001$). In particular, tumors exhibiting 30% or more CD117 immunoreactive cells showed a strong intensity in both immunostaining patterns (14/34 for the membranous and 14/36 for the cytoplasmic), whereas tumors with less than 30% of immunoreactive cells mostly exhibited weak staining (20/34 and 22/36, respectively) ($P < 0.001$). No preferential distribution of CD117 immunoreactive cells was seen within individual cases in perivascular, peripheral or central viable areas of the tumors.

Relationship Between CD17/c-kit Immunoreactivity and Biopathological Variables

In adenocarcinomas, CD117 membrane immunoreactivity in 5% or more tumor cells was associated with lower age (median: 56 years), occurrence of systemic clinical symptoms, highly proliferating Her-2 expressing tumors, more advanced stage and greater size (Table 3). Also, there was a tendency for less differentiated and bcl-2-upregulating tumors with features of neuroendocrine differentiation to exhibit CD117 membrane decoration, even though
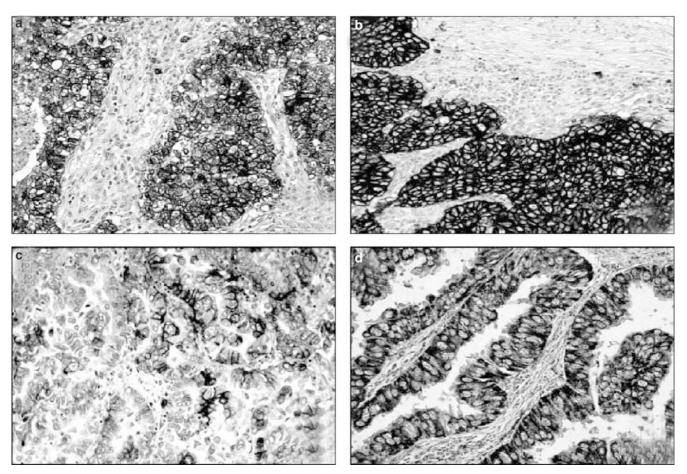
Figure 1 CD117/c-kit immunoreactivity patterns in different samples of lung carcinomas. The membrane pattern was characterized by moderate to strong decoration of the cell membrane of either adenocarcinoma (**a,c**) or squamous cell carcinoma (**b**) A few tumors (in this case an adenocarcinoma) presented cell membrane labelling in more scattered neoplastic cells, sometimes with baso-lateral immunostaining pattern (**c**) A strong immunoreactivity for CD117/c-kit in the cytoplasm of tumor cells is depicted in this case of pulmonary adenocarcinoma (**d**) (× 250. All immunoperoxidase staining was performed with diaminobenzidine, and counterstained with hematoxylin).

Table 2 Distribution of CD117 immunoreactivity in stage I adenocarcinoma and squamous cell carcinoma according to histology and immunostaining pattern

CD117/c-kit immunostaining pattern	Adenocarcinoma n = 88 (%)	Squamous n = 113 (%)	P-value
Both negative	52 (59)	94 (83)	
Membrane negative/cytoplasm positive	17 (19)	4 (3)	
Membrane positive/cytoplasm negative	8 (9)	11 (10)	
Both positive	11 (13)	4 (4)	$P < 0.001$

Positive immunostaining, either membrane or cytoplasmic, was defined as 5% or more tumor cells being reactive.

this did not achieve significance (Table 3). Cytoplasmic immunostaining in 5% or more tumor cells was preferentially distributed into adenocarcinoma patients complaining of systemic clinical symptoms, or bearing high proliferating tumors with greater size, or higher grade, or increased bcl-2 and cyclin-E expression or occurrence of neuroendocrine differentiation (Table 3). In squamous cell carcinoma, CD117 membrane decoration in 5% or more tumor cells was more prevalent in less differentiated tumors and in increased bcl-2,

p27[Kip1] and fascin expression and greater microvessel density, whereas cytoplasmic immunostaining was significantly associated with increased tumor grade and cyclin-E expression (Table 4). When regressing simultaneously, only pT and Her-2 expression in adenocarcinomas, and tumor grade, bcl-2 and p27[Kip1] in squamous cell carcinoma emerged as independently associated with CD117 membrane labelling (Table 5). In squamous cell carcinoma, but not in adenocarcinoma patients, tumor grade and cyclin-E expression were the only

Table 3 Relationship between CD117 and bio-pathological variables in 88 adenocarcinoma patients

Variable	Category	Membrane		P	Cytoplasmic		P
		0–4.99%	*≥5%*		*0–4.99%*	*≥5%*	
Age (year)	Median	62	56	0.02	61.5	59.5	NS
Gender	Male	56 (81%)	17 (89%)	NS	47 (78%)	26 (93%)	NS
	Female	13 (19%)	2 (11%)		13 (22%)	2 (7%)	
Symptoms at diagnosis	Absent	36 (52%)	6 (32%)	0.05	27 (45%)	15 (54%)	0.05
	Local	24 (35%)	6 (32%)		25 (42%)	5 (18%)	
	Systemic	9 (13%)	7 (37%)		8 (13%)	8 (29%)	
Vascular invasion	Yes	45 (65%)	12 (63%)	NS	38 (63%)	19 (68%)	NS
	No	24 (35%)	7 (37%)		22 (37%)	9 (32%)	
pT	1	38 (55%)	5 (26%)	0.03	32 (53%)	11 (39%)	NS
	2	31 (45%)	14 (74%)		28 (47%)	17 (61%)	
Tumor grade	1	10 (14%)	0	0.12	10 (17%)	0	0.01
	2	34 (49%)	8 (42%)		30 (50%)	12 (43%)	
	3	25 (36%)	11 (58%)		20 (33%)	16 (57%)	
Size (cm)	Median	2.5	5.0	0.002	2.55	3.55	0.04
Ki-67 (%)	Median	18	29	0.02	18	28	0.02
Synaptophysin	Neg	60 (87%)	13 (68%)	0.08	52 (87%)	21 (75%)	NS
	Pos	9 (13%)	6 (32%)		8 (13%)	7 (25%)	
Chromogranin A	Neg	66 (96%)	18 (95%)	NS	59 (98%)	24 (86%)	0.01
	Pos	3 (4%)	1 (5%)		1 (2%)	4 (14%)	
Fascin	Neg	42 (61%)	14 (74%)	NS	15 (25%)	5 (18%)	NS
	Pos	27 (39%)	5 (26%)		15 (25%)	8 (29%)	
Her-2	Neg	62 (90%)	13 (68%)	0.03	53 (88%)	22 (78%)	NS
	Pos	7 (10%)	6 (32%)		7 (12%)	6 (22%)	
Bcl-2 (%)	Median	0.8	6.6	0.09	0.3	2.1	0.04
Cyclin-E (%)	Median	2.5	5.9	NS	1.2	10.8	0.04
p53 (%)	Median	1.0	2.0	NS	0	12.0	NS
p21Waf1(%)	Median	4.1	5.5	NS	5.4	3.9	NS
p27Kip1 (%)	Median	26.0	28.0	NS	25.1	31.6	NS
Microvessel density	Median	117	108	NS	112	118	NS

independent predictors of cytoplasmic immunostaining (Table 5).

CD117 Membrane Immunoreactivity Is an Independent Correlate of Tumor Size in Adenocarcinoma

As either membrane or cytoplasmic CD117 immunostaining was correlated with several parameters of tumor growth, we carried out multiple linear regression analysis modelling tumor size. Interestingly, we found that only the occurrence of CD117 membrane ($P = 0.016$) and bcl-2-immunoreactive tumor cells ($P = 0.047$) emerged as independent

factors associated with increasing tumor diameter in a model including all variables significantly correlated with CD117 expression (Table 3). Expression in the cytoplasm was not independently associated with tumor size after adjusting for other variables significantly correlated with CD117 immunoreactivity (Table 4).

CD117 Membrane Immunoreactivity is a Predictor of Shorter Survival in Adenocarcinoma

Adenocarcinoma patients with positive ($≥5\%$) membrane but not cytoplasmic decoration had significantly lower OS and DFS rates compared to

Table 4 Relationship between CD117 and bio-pathological variables in 113 squamous cell carcinoma patients

Variable	Category	Membrane		P	Cytoplasmic		P
		0–4.99%	≥5%		0–4.99%	≥5%	
Age (year)	Median	63	64	NS	63	66	NS
Gender	Male	95 (97%)	14 (93%)	NS	101 (96%)	8 (100%)	NS
	Female	3 (3%)	1 (7%)		4 (4%)	0	
Symptoms at diagnosis[a]	Absent	27 (28%)	8 (53%)	NS	33 (32%)	2 (25%)	NS
	Local	41 (43%)	5 (33%)		43 (42%)	3 (37%)	
	Systemic	27 (28%)	2 (13%)		26 (26%)	3 (37%)	
Vascular invasion	Yes	71 (72%)	13 (87%)	NS	77 (73%)	7 (87%)	NS
	No	27 (28%)	2 (13%)		28 (27%)	1 (13%)	
pT	1	52 (53%)	5 (33%)	NS	53 (50%)	4 (50%)	NS
	2	46 (47%)	10 (67%)		52 (49%)	4 (50%)	
Tumor grade	1	1 (1%)	0	0.001	1 (1%)	0	0.03
	2	71 (72%)	4 (27%)		73 (70%)	2 (25%)	
	3	26 (27%)	11 (73%)		31 (29%)	6 (75%)	
Size (cm)	Median	3.0	4.0	NS	3.0	3.3	NS
Ki-67 (%)	Median	42	37	NS	42	32.5	NS
Synaptophysin	Neg	89 (91%)	12 (80%)	NS	94 (90%)	7 (87%)	NS
	Pos	9 (9%)	3 (20%)		11 (10%)	1 (12%)	
Chromogranin A	Neg	89 (91%)	14 (93%)	NS	95 (90%)	8 (100%)	NS
	Pos	9 (9%)	1 (7%)		10 (9%)	0	
Fascin	Neg	0	2 (13%)	0.009	1 (1%)	1 (12%)	NS
	Pos	98 (100)	13 (87%)		104 (99%)	7 (87%)	
Her-2	Neg	65 (66%)	7 (47%)	NS	67 (64%)	5 (62%)	NS
	Pos	33 (34%)	8 (53%)		38 (34%)	3 (37%)	
Bcl-2 (%)	Median	1.9	78.5	<0.001	3.0	3.3	NS
Cyclin-E (%)	Median	29.8	17.3	NS	2.2	30.5	0.007
p53 (%)	Median	6.0	24.0	NS	31.0	0	NS
p21Waf1(%)	Median	14.5	10.8	NS	14.3	11.5	NS
p27Kip1 (%)	Median	9.3	41.4	0.001	10.4	26.2	NS
Microvessel density	Median	90	108	0.04	92	94	NS

[a]In three patients the data on symptoms were not available.

patients with negative tumors (Figure 2). Moreover, evaluation of OS and DFS by immunostaining pattern suggested a trend for decreasing survival in patients with tumors expressing both patterns as compared with patients having either or both negative patterns (Figure 2). In squamous cell carcinoma, CD117 expression, regardless of the type or the combination of staining patterns, was not associated with patient's survival.

Cox's regression was performed including the same biopathological variables found to be significant ($P \leq 0.05$) or marginally ($P \leq 0.10$) associated with either membrane or cytoplasmic pattern (Tables 3 and 4). In adenocarcinoma, the risk of death and time to progression in patients with CD117 membrane expression persisted after singly adjusting for factors shown in Table 6. However, when simultaneously adjusting for all these variables, the differences in risk according to CD117 expression status vanished in this full model. Cytoplasmic immunostaining in adenocarcinoma was not predictive of survival. Neither cytoplasmic nor membrane CD117 expression in patients with SCC was associated with survival.

Discussion

The current investigation documents that a minor subset of stage I adenocarcinoma and squamous cell

Table 5 Multiple logistic regression of membrane and of cytoplasmic CD117 expression in adenocarcinoma and squamous cell carcinoma

Variable	Category	No. of patients	No. of c-kit positive	OR	CI	P
(a) Membrane expression of CD117 in adenocarcinoma patients (n = 88)[a]						
HER-2	Pos	13	6	6	1.16–30.8	0.032
	Neg	75	13	1		
pT	2	45	14	3.3	0.81–13.2	0.096
	1	43	5	1		
(b) Membrane expression of CD117 in squamous cell carcinoma patients (n = 113)[b]						
Grade	3	37	11	8	1.8–36	0.007
	1/2	76	4	1		
Bcl-2	Pos	72	14	27	1.9–400	0.015
	Neg	41	1	1		
p27^{kip1}	Continuous	—	—	1.05[c]	1.01–1.08	0.005
(c) Cytoplasmic expression of CD117 in squamous cell carcinoma patients (n = 113)[d]						
Grade	3	37	6	9.3	1.6–54	0.013
	1/2	76	2	1		
Cyclin-E	Pos	98	5	0.14	0.02–0.83	0.031
	Neg	15	3	1		

CI: 95% confidence intervals for odds ratios (OR).
[a]Other variables included in the model but not statistically significant were age, symptoms at diagnosis, Ki-67 labelling index, tumor grade and bcl-2 and synaptophysin expression;
[b]Other variables included in the model but not statistically significant were fascin expression and microvessel density;
[c]Odds ratio (OR) per CD117/c-kit membrane expression per 1% unit increase in p27^{Kip1}.
[d]No other variable was included in this model.

carcinoma are immunoreactive for CD117 showing either a membranous and/or a cytoplasmic labelling. CD117 immunoreactivity correlates with indicators of aggressiveness in lung cancer, such as the occurrence of clinical symptoms, higher tumor stage and larger size, neuroendocrine differentiation and fascin overexpression. Finally, CD117 membrane but not cytoplasmic stain increases the risk of death and reduces the time to progression in patients with pulmonary adenocarcinoma.

The reported prevalence of CD117 immunoreactivity in adenocarcinoma or squamous cell carcinoma of the lung is in keeping with previously published data documenting immunoreactivity in 6–74% of the cases.[7,13,24–26] Despite the lack of quantitative analyses of CD117 mRNA expression, the current results have been obtained using two different immunoreagents and comparing the staining of tumor cells with that of infiltrating mast cells, to minimize the bias of the variability in the immunostaining procedure or in the fixation time. Our data suggest that CD117 expression in stage I adenocarcinoma or squamous cell carcinoma is actually low, but it may have potential clinical and therapeutical implications.

CD117 immunoreactivity in tumor cells, either membranous or cytoplasmic, was significantly ($P \leq 0.05$) or marginally ($P \leq 0.10$) higher in pT2 stage patients complaining of systemic clinical symptoms and bearing less differentiated and highly proliferating tumors of greater size, showing upre-

gulation of Her-2, bcl-2, p27^{Kip1} and cyclin-E and increased microvessel density. All these findings point to a possible role of CD117 in the development and/or progression of this subset of stage I lung carcinomas. Furthermore, multiple linear regression analyses according to tumor size confirmed that the increase of tumor diameter was independently associated with CD117 and bcl-2 expression. As a matter of fact, tumor diameter is the net result of the interaction of many variables, including the rates of tumor cell replication and apoptosis, the effectiveness of neoangiogenesis and the preclinical duration of disease that in turn may affect the occurrence of clinical symptoms.

Furthermore, our study provides some hints into the molecular mechanisms independently involved in CD117 immunoreactive early-stage lung carcinomas, as assessed by odds ratio analysis (Table 5). Although the precise downstream pathway of CD117 in tumor cells is poorly understood, there is some evidence that an increased expression of bcl-2 or cyclin E *in vitro* eventually occurs after receptor activation upon stem-cell factor binding, leading to progression into cell cycle with increased proliferative activity, and to reduction of apoptosis with prolonged cell survival.[40,41] Also, the increased levels of p27^{Kip1} detected in CD117 overexpressing stage I lung carcinomas could facilitate the assembly of cyclin–cyclin-dependent kinase complexes, thereby promoting cell cycle progression, as previously suggested in pulmonary adenocarcinoma.[42]
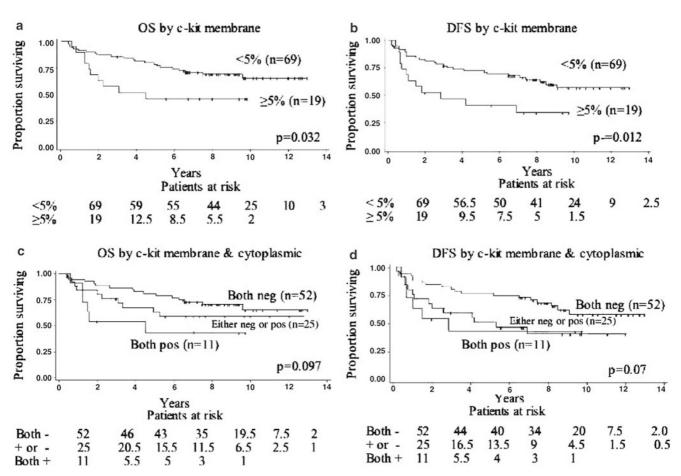
Figure 2 OS and DFS curve analysis performed according to CD117/c-kit immunoreactivity in 88 adenocarcinoma of the lung. Patients with positive ($\geq 5\%$) membrane decoration exhibited significantly lower OS (**a**) and DFS (**b**) rates as compared to patients with negative ($<5\%$) tumors. Moreover, evaluation of survival by immunostaining pattern suggested a trend for decreasing OS (**c**) and DFS (**d**) in patients with tumors expressing both positive ($+$) patterns as compared with patients having either or both negative ($-$) patterns.

Coexpression of CD117 and Her-2 has been found in human malignant myeloma,[43] but further larger studies are needed to determine if either one (CD117 or HER-2) may lead to overexpression of the other oncoprotein in stage I adenocarcinoma or squamous cell carcinoma.

As CD117 is likely relevant to the growth of a subset of stage I adenocarcinoma and squamous cell carcinoma, the use of specific inhibitors of its tyrosine kinase activity could be potentially helpful as adjuvant targeted therapy after immunohistochemical screening for receptor expression. In fact, tumors must be strictly dependent on the stimulus of CD117 for specific inhibitors to produce an antitumor effect.[23,44] Clinical trials aimed at targeting CD117 (eg Gleevec) in immunoreactive early-stage non-small-cell lung carcinomas, including adenocarcinoma and squamous cell carcinoma, are clearly warranted.

The evaluation of OS and DFS by immunostaining pattern showed a trend for decreasing survival in adenocarcinoma patients with tumors expressing both patterns, as compared with those showing either or both negative (Figure 2). In mutivariate analysis, only CD117 membrane decoration emerged as a significant and partially independent risk factor for death and reduced time to progression in adenocarcinoma patients when adjusting for single confounding factors (Table 6), whereas either cytoplasmic immunostaining in adenocarcinoma or both patterns in squamous cell carcinoma did not affect the patients' survival. When simultaneously adjusting for all variables modifying the magnitude of the HR for CD117 expression, the differences in risk for OS and DFS vanished even in the adenocarcinoma group probably because of the low statistical power of the full model dealing with a very small number of CD117-reactive cases rather than the lack of a predictive value for this molecule. Although we cannot draw clear-cut conclusions on the independent prognostic role of CD117 in this subset of adenocarcinoma patients, we indicate, however, that the molecule strictly parallels the tumor development and is likely to influence the tumor progression and the patients' survival. Further studies on a greater number of CD117-expressing

Table 6 Multivariate survival analysis by CD117 membrane in adenocarcinoma patients

	HR	95% cl	P-value
(a) Risk of death (OS)			
Unadjusted c-kit	2.3	1.1–4.9	0.032
Adjusted by age	2.2	1.0–4.8	0.05
Adjusted by diameter	2	0.9–4.3	0.093
Adjusted by pT	2	0.9–4.4	0.07
Adjusted by Ki-67 labelling index	2.2	1.0–5.0	0.05
Adjusred by HER-2	2.2	0.9–4.9	0.057
Adjusted by tumor grade	2.5	1.1–5.4	0.025
Adjusted by symptoms	2	0.9–4.3	0.07
Adjusted by bcl-2	2.4	1.1–5.1	0.025
Adjusted by synaptophysin	2.2	1.0–4.8	0.048
Full model[a]	1.7	0.6–4.6	0.3
(b) Risk of time to progression (DFS)			
Unadjusted c-kit	2.3	1.2–4.7	0.015
Adjusted by age	2	0.9–4.1	0.056
Adjusted by diameter	2.1	1.0–4.3	0.042
Adjusted by pT	2.2	1.1–4.4	0.029
Adjusted by Ki-67 labelling index	2.3	1.1–4.8	0.027
Adjusted by HER-2	2.3	1.1–4.8	0.03
Adjusted by tumor grade	2.6	1.3–5.3	0.01
Adjusted by symptoms	2.2	1.1–4.4	0.028
Adjusted by bcl-2	2.4	1.2–4.8	0.012
Adjusted by synaptophysin	2.2	1.1–4.5	0.026
Full model[b]	1.5	0.6–3.6	0.36

[a]In this model, which included the simultaneous modelling of the above-listed variables, only symptoms ($P = 0.016$) was significant.
[b]In this model, which included the simultaneous modelling of the above-listed variables, only age ($P = 0.048$) was significant.
Variable definitions: age and diameter used as continuous; pT2 *vs* pT1; Ki-67: $\geq 32\%$ *vs* $< 32\%$ (median value); HER-2, bcl-2 and synaptophysin: positive *vs* negative; tumor grade: G3 *vs* G1/G2; clinical symptoms: local/systemic *vs* none.

stage I adenocarcinomas and squamous cell carcinomas are clearly warranted for elucidating this point.

In conclusion, our investigation supports the view that CD117 immunoreactivity identifies a peculiar subset of stage I non-small-cell lung carcinoma patients, with rapidly growing adenocarcinoma or squamous cell carcinoma, and that its membrane expression is a partially independent risk factor of poor prognosis in adenocarcinoma patients.

References

1 Maulik G, Kijima T, Salgia R. Role of receptor tyrosine kinases in lung cancer. Methods Mol Med 2003;74:113–125.
2 Ashman LK. The biology of stem cell factor and its receptor C-kit. Int J Biochem Cell Biol 1999;31:1037–1051.
3 Witte O. Steel locus defines new multipotent growth factor. Cell 1990;63:5–6.
4 Yarden Y, Kuang WJ, Yang-Feng T, *et al.* Human proto-oncogene *c-kit*: a new cell surface receptor tyrosine kinase for an undefinited ligand. EMBO J 1987;6:3341–3351.
5 Ashman LK, Cambareri AC, To LB, *et al.* Expression of the YB5.B8 antigen (c-kit proto-oncogene product) in normal human bone marrow. Blood 1991;78:30.
6 Lammie A, Drobnjak M, Gerald W, *et al.* Expression of c-kit and kit ligand proteins in normal human tissues. J Histochem Cytochem 1994;42:1417–1425.
7 Tsuura Y, Hiraki H, Watanabe K, *et al.* Preferential localization of c-kit product in tissue mast cells, basal cells of skin, epithelial cells of breast, small cell lung carcinoma and seminoma/dysgerminoma in human: immunohistochemical study on formalin-fixed, paraffin-embedded tissues. Virchows Arch 1994;424:135–141.
8 Noguchi T, Sato T, Takeno S, *et al.* Biological analysis of gastrointestinal stromal tumors. Oncol Rep 2002;9:1277–1282.
9 Gadd SJ, Ashman LK. A murine monoclonal antibody specific for a cell-surface antigen expressed by a subgroup of human myeloid leukaemias. Leuk Res 1985;9:1329.
10 Cohen PS, Chan JP, Lipkunskaya M, *et al.* Expression of stem cell factor and c-kit in human neuroblastoma. Blood 1994;84:3465–3472.
11 Merchant MS, Woo C-W, Mackall CL, *et al.* Potential use of Imatinib in Ewing's sarcoma: evidence for *in vitro* and *in vivo* activity. J Natl Cancer Inst 2002;94:1673–1679.
12 Landuzzi L, De Giovanni C, Nicoletti G, *et al.* The metastatic ability of Ewing's sarcoma cells is modulated by stem cell factor and by its receptor c-kit. Am J Pathol 2000;157:2123–2131.
13 Arber DA, Tamayo R, Weiss LM. Paraffin section detection of the c-kit gene product (CD117) in human tissues: value in the diagnosis of mast cell disorders. Hum Pathol 1998;29:498–504.
14 Wang WL, Healy ME, Sattler M, *et al.* Growth inhibition and modulation of kinase pathways of small cell lung cancer cell lines by the novel tyrosine kinase inhibitor STI 571. Oncogene 2000;19:3521–3528.
15 Naeem M, Dahiya M, Clark JI, *et al.* Analysis of c-kit protein expression in small-cell lung carcinoma and its implication for prognosis. Hum Pathol 2002;33:1182–1187.
16 Krystal GW, Hines SJ, Organ CP. Autocrine growth of small cell lung cancer mediated by coexpression of c-kit and stem cell factor. Cancer Res 1996;56:370–376.
17 Hida T, Ueda R, Sekido Y, *et al.* Ectopic expression of c-kit in small-cell lung cancer. Int J Cancer Suppl 1994;8:108–109.
18 Sekido Y, Obata Y, Ueda R, *et al.* Preferential expression of c-kit protooncogene transcripts in small cell lung cancer. Cancer Res 1991;51:2416–2419.
19 Micke P, Basrai M, Faldum A, *et al.* Characterization of c-kit expression in small cell lung cancer: prognostic and therapeutic implications. Clin Cancer Res 2003;18–19.
20 Araki K, Ishii G, Yokose T, *et al.* Frequent over-expression of the c-kit protein in large cell neuroendocrine carcinoma of the lung. Lung Cancer 2003;40:173–180.
21 DiPaola RS, Kuczynski WI, Onodera K, *et al.* Evidence for a functional kit receptor in melanoma, breast, and lung carcinoma cells. Cancer Gene Ther 1997;4:176–182.
22 Papadimitriou CA, Topp MS, Serve H, *et al.* Recombinant human stem cell factor does exert minor stimula-

tion of growth in small cell lung cancer and melanoma cell lines. Eur J Cancer 1995;31A:2371–2378.

23 Zhang P, Gao WY, Turner S, *et al.* Gleevec (STI-571) inhibits lung cancer cell growth (A549) and potentiates the cisplatin effect *in vitro*. Mol Cancer 2003;2 http://www.molecular-cancer.com/content/2/1/.

24 Natali PG, Nicotra MR, Sures I, *et al.* Expression of c-kit receptor in normal and transformed human nonlymphoid tissues. Cancer Res 1992;52:6139–6143.

25 Matsuda R, Takahashi T, Nakamura S, *et al.* Expression of the c-kit protein in human solid tumors and in corresponding fetal and adult normal tissues. Am J Pathol 1993;142:339–346.

26 Pietsch T, Nicotra MR, Fraioli R, *et al.* Expression of the c-Kit receptor and its ligand SCF in non-small-cell lung carcinomas. Int J Cancer 1998;75:171–175.

27 Tang WZ, Zhang P. Expression of CD117 (c-kit) in non-small cell lung cancer is not associated with neuroendocrine differentiation (abstract #1439). Mod Pathol 2003;315A.

28 Radford IR. Imatinib. Novartis. Curr Opin Investig Drugs 2002;3:492–499.

29 Krystal GW, Honsawek S, Litz J, *et al.* The selective tyrosine kinase inhibitor STI571 inhibits small cell lung cancer growth. Clin Cancer Res 2000;6:3319–3326.

30 D'Amico TA, Massey M, Herndon II JE, *et al.* A biologic risk model for stage I lung cancer: immunohistochemical analysis of 408 patients with the use of ten molecular markers. J Thorac Cardiovasc Surg 1999;117:736–743.

31 Mountain CF. Revisions in the international system for staging for lung cancer. Chest 1997;111:1710–1717.

32 Nesbitt JC, Putnam Jr JB, Walsh GL, *et al.* Survival in early-stage non-small cell lung cancer. Ann Thorac Surg 1995;6:466–472.

33 Ichinose Y, Hara N, Ohta M, *et al.* Is T factor of the TNM staging system a predominant prognostic factor in pathologic stage I non-small-cell lung cancer? A multivariate prognostic factor analysis of 151 patients. J Thorac Cardiovasc Surg 1993;106:90–94.

34 Harpole Jr DH, Herndon II JE, Wolfe WG, *et al.* A prognostic model of recurrence and death in stage I non-small cell lung cancer utilizing presentation, histopathology, and oncoprotein expression. Cancer Res 1995;55:51–56.

35 Pasini F, Verlato G, Durante E, *et al.* Persistent excess mortality from lung cancer in patients with stage I non-small cell lung cancer, disease-free after 5 years. Br J Cancer 2003;88:1666–1668.

36 Pelosi G, Pastorino U, Pasini F, *et al.* Independent prognostic value of fascin-1 immunoreactivity in stage I non-small cell lung cancer. Brit J Cancer 2003;88:537–547.

37 Travis WD, Colby TV, Corrin B, *et al.* Histological Typing of Lung and Pleural Tumours. Springer Verlag: Berlin, 1999.

38 Burry RW. Specificity controls for immunocytochemical methods. J Histochem Cytochem 2000;48:163–166.

39 Pelosi G, Fraggetta F, Pasini F, *et al.* Immunoreactivity for thyroid transcription factor-1 in stage I non-small cell lung carcinomas of the lung. Am J Surg Pathol 2001;25:363–372.

40 Baghestanian M, Jordan JH, Kiener HP, *et al.* Activation of human mast cells through stem cell factor receptor (KIT) is associated with expression of bcl-2. Int Arch Allergy Immunol 2002;129:228–236.

41 Dolci S, Pellegrini M, Di Agostino S, *et al.* Signaling through extracellular signal-regulated kinase is required for spermatogonial proliferative response to stem cell factor. J Biol Chem 2001;276:40225–40233.

42 Shoji M, Dobashi Y, Morinaga S, *et al.* Tumor extension and cell proliferation in adenocarcinomas of the lung. Am J Pathol 1999;154:909–918.

43 Potti A, Ganti AK, Koch M, *et al.* Immunohistochemical identification of HER-2/neu overexpression and CD117 (c-kit) expression in multiple myeloma. Leuk Lymphoma 2002;43:2427–2430.

44 Krystal GW, Honsawek S, Kiewlich D, *et al.* Indolinone tyrosine kinase inhibitors block Kit activation and growth of small cell lung cancer cells. Cancer Res 2001;61:3660–3668.