Decreased Immunoreactivity of CD99 Is an Independent Predictor of Regional Lymph Node Metastases in Pulmonary Carcinoid Tumors

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Background: Few data are available on the prevalence and clinicopathological meaning of CD99, the transmembrane product of the pseudoautosomal MIC2 gene, in pulmonary neuroendocrine tumors.
Methods: We evaluated CD99 immunoreactivity in lung tissues, pulmonary neuroendocrine hyperplasias, and 136 consecutive pulmonary neuroendocrine tumors of diverse histological types.
Results: By immunohistochemistry, a membranous and/or cytoplasmic immunoreactivity was seen in 60 of 136 (44%) tumors, whereas both normal and hyperplastic neuroendocrine cells of the lung were consistently nonreactive. A steady decrease of the CD99 labeling index was observed from better to poorly differentiated tumors, with a prevalence of the membranous pattern in typical carcinoids (TCs), and of the cytoplasmic pattern in atypical carcinoids (ACs) and large cell neuroendocrine carcinoma/small cell lung carcinoma ($P < 0.0001$), independent of tumor stage. In TCs/ACs, increased levels of CD99 labeling index or the membranous pattern were associated with low proliferative fraction ($P = 0.0011$) and smaller tumor size ($P = 0.0054$) and with lack of regional lymph node metastases ($P = 0.0078$). Moreover, CD99 expression decreased according to the pN0-2 classes ($P = 0.0016$), with an inverse relationship between the number of positive lymph nodes, the labeling index ($P = 0.013$) and the nonmembranous pattern ($P = 0.016$). At multivariate analysis, both the decreased CD99 labeling index and the negative/cytoplasmic staining were independent risk indicators for lymph node metastases in the subset of TC/AC patients. No relevant relationships were found in large cell neuroendocrine carcinoma/small cell lung carcinoma.
Conclusion: CD99 is especially present in low- to intermediate-grade neuroendocrine tumors of the lung, and loss of the marker correlates with the occurrence of nodal metastases in TC/AC patients.

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ISSN: 1556-0864/06/0105-0468

Key Words: CD99, Carcinoid, Lung, Metastasis.

(*J Thorac Oncol.* 2006;1: 468–477)

The cell surface molecule CD99, also known as E2, 12E7, HuLy-m6, and FMC29,[1–4] is a heavily O-glycosylated M_r 32,000 glycoprotein encoded by the MIC-2 gene, which is mapped to the pseudoautosomal regions of both human Xp and Yp chromosomes.[5–9] The MIC-2 gene includes 10 exons encoding for an integral membrane protein with a single transmembrane region, an extracellular amino terminus, and a cytosolic carboxy terminus[8,10–12] that is constitutively expressed on the erythrocyte surface in most individuals.[13] The MIC-2 locus on chromosome Xp is not subject to inactivation,[14] and its gene product shares no homology with any known protein, with the exception of the XG (also called PBDX) blood group gene product (48% homologous to CD99) mapping to the same chromosomal region.[10,15–18]

Although originally thought to be a specific marker of Ewing's sarcoma/peripheral primitive neuroectodermal tumor (PNET),[18–21] CD99 expression has now been demonstrated in a wider range of normal[1,3,10,15,22–28] and neoplastic[9,29–41] human tissues, including epithelial tumors of the ovary,[42] breast,[43] stomach,[44] and some gastrointestinal[9,45] and pulmonary[2,45] neuroendocrine tumors.

The specific functions of CD99 still remain largely unknown, although this protein has been implicated in several biological activities such as homophilic cell-cell adhesion during hematopoiesis,[1,46] binding of leukocytes to vascular endothelial cells during the extravasation cascade,[24,47,48] apoptosis of T lymphocytes[49,50] and Ewing's family tumor cells,[51] expression of several transmembrane and cytoplasmic proteins,[23–25,28,32,46,52,53] regulation of cell cycle and differentiation,[27,34,43,54] and modulation of the insulinlike growth factor-I, insulin, and human growth hormone action.[9,55]

Neuroendocrine tumors of the lung are most commonly classified according to a four-tiered classification system, ranging from relatively indolent tumors with longer life expectation (including typical [TC] and atypical [AC] carcinoids) to very aggressive tumors with dismal prognosis (including large cell neuroendocrine carcinoma [LCNEC] and small cell lung carcinoma [SCLC]).[56,57] The World Health Organization/International Association of the Study of Lung

Cancer provided in 2004 histological criteria for classifying all the diverse histological types of neuroendocrine tumors,[56] even though other different classification schemes have been proposed.[58,59] The greatest difficulty in all these proposals, however, is to separate the subsets of patients with different biological behavior within each diagnostic category, especially in the case of low- to intermediate-grade tumors that collectively show a fairly better prognosis as compared with the poorly differentiated tumors.

In the past, many investigations dealing with low- to intermediate-grade tumors usually considered patient survival as the main clinical outcome,[60–70] whereas the role of factors implicated in the development of metastasis to regional lymph nodes has not been extensively investigated in these tumors. In a small series of 31 neuroendocrine neoplasms of the lung, we found that CD99 down-regulation was significantly associated with increased risk of lymph node metastases and, marginally, with reduced tumor cell proliferative activity.[45] However, the actual prevalence of CD99 expression and its clinicopathological implications have not been widely investigated thus far in a large series of neuroendocrine proliferations including tumors of various histological types.

This study was aimed at evaluating CD99 immunoreactivity in 136 pulmonary tumors encompassing the whole spectrum of neuroendocrine neoplasms, as well as in normal neuroendocrine cells of the respiratory epithelium and hyperplastic neuroendocrine tumorlets. Our results document that CD99 down-regulation is an independent predictor of lymph node metastasis only in patients with TC and AC.

PATIENTS AND METHODS

Patients

The study population includes 136 consecutive patients (89 males and 47 females) with stage I to IIIB neuroendocrine tumors (41 TCs, 26 ACs, 42 LCNECs, and 27 SCLCs) surgically treated at the City Hospital in Verona and the European Institute of Oncology in Milan, Italy, between 1987 and 2001. All tumors were pathologically staged according to the international system for staging lung cancer.[56] To ensure accurate staging, all tumors were radically excised along with an adequate number of local (N1) and mediastinal (N2) lymph node (median value: nine lymph nodes per patient). The paraffin sections of all cases were retrieved, and the original hematoxylin and eosin–stained sections reviewed. The relative prevalence of carcinoids, either typical or atypical, and of LCNECs over SCLCs (the latter usually accounting for at least 20% of all lung carcinomas considered as a whole)[56] is due to the fact that only surgically excised specimens were used in the study.

The diagnosis of neuroendocrine tumor was based on established morphological and immunohistochemical criteria (immunoreactivity for synaptophysin, chromogranin A, and a variety of respiratory tract–related hormones, including gastrin-related peptide, calcitonin, adrenocorticotropic hormone, serotonin and α-human chorionic gonadotropin.[45,56,57,59] Because a large body of evidence supports that TCs and ACs are genetically, pathologically, and clinically related and are not precursors of poorly differentiated neuroendocrine tumors,[60–66,71–73] clinicopathological comparisons in this study included two groups, i.e., patients having TC/AC and patients having LCNEC/SCLC, respectively. TCs and ACs were considered together in the analysis because of the small number of patients in each tumor group (41 for TCs and 26 for ACs, respectively). Relevant information on the main differences in the two tumor groups is provided in Table 1.

Follow-up information was available for 126 of 136 patients: 31 patients (24.6%) had recurrent disease and 25 (19.8%) of them died of disease. The follow-up time, however, was too short (mean ± SD: 20.5 ± 27.2 months; median value: 11 months) at the time of the analysis to allow a reliable survival analysis to be carried out. No patient presented with endocrine symptoms due to hormone hyperproduction.

Immunocytochemical Assay

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. Ten samples of normal pulmonary parenchyma and bronchial tree taken at different levels from patients with nonmalignant lung diseases, and 10 samples of pulmonary neuroendocrine hyperplasias and nonneoplastic peritumoural lung tissue from the study patients were used as control groups for CD99 immunoreactivity.

Immunohistochemical reactions were performed using the primary antibodies listed in Table 2. In particular, CD99 was immunolocalized with the monoclonal antibody H036-1.1, raised against purified E-rosette–forming cells from human peripheral blood lymphocytes (Novocastra Laboratories,

TABLE 1. Differential Clinicopathological Features

Variable	Category	TC/AC (n = 67)	LCNEC/SCLC (n = 69)	P value
Age (yr)	Median	55	64	<0.0001
Gender (%)	Male	32 (47.8)	57 (82.6)	
	Female	35 (52.2)	12 (17.4)	<0.0001
Size (cm)	Median	2.5	3.5	0.0005
Tumor stage (%)	I	47 (70.2)	34 (49.3)	
	II–IIIB	20 (29.8)	35 (50.7)	0.0151
pT (%)	T1	49 (73.1)	24 (34.8)	
	T2–T4	18 (26.9)	45 (65.2)	<0.0001
LN metastasis (%)	Absent	46 (68.7)	34 (49.28)	
	Present	21 (31.3)	35 (50.7)	0.0245
LN metastasis site (%)	pN0	46 (68.7)	34 (49.3)	
	pN1	12 (17.9)	18 (26.1)	
	pN2	9 (13.4)	17 (24.6)	NS
No. of positive LN	Median	0	1	0.0053
Chromogranin A	Median	95	15	<0.0001
Synaptophysin	Median	100	39	<0.0001

Categorical variables are compared with Fisher's exact t test or χ^2 test; continuous variables with Wilcoxon's two-sample test.

TC, typical carcinoid; AC, atypical carcinoid; LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma; NS, not significant; LN, lymph node.

TABLE 2. Antibody Panel Used in the Study

Antibodies	m/p	Clone	Source	Dilution	Pretreatment
CD99	m	HO36-1.1	Novocastra Laboratories, Newcastle upon Tyne, UK	1:500	MWO-CB
Chromogranin A	m	LK2H10	Signet Laboratories, Dedham, MA	1:40	None
α-Human chorionic gonadotropin	p	—	‘	1:200	MWO-CB
Synaptophysin	m	SY 38	DAKO, Glostrup, Denmark	1:20	MWO-CB
Gastrin-releasing peptide	p	—	‘	1:400	None
Calcitonin	p	—	‘	1:20	None
Serotonin	m	5HT-H209	‘	1:10	None
ACTH	m	02A3	‘	1:100	None
Ki-67 antigen	m	MIB-1	Immunotech, Marseille, France	1:400	MWO-EDTA

m/p, monoclonal/polyclonal; MWO-CB, microwave oven at 750 W for 10 minutes in citrate buffer, pH6; MWO-EDTA, microwave oven at 750 W for 12 minutes in ethylenediamine tetraacetic acid buffer, pH 8; ACTH, adrenocorticotropic hormone.

Newcastle upon Tyne, UK). The specificity of this CD99 antibody was determined on a panel of normal and tumor tissues, and results were compared with those of other published studies using other CD99 clones (Novocastra Laboratories, personal communication). For the immunohistochemical staining, after blocking endogenous peroxidase activity with hydrogen peroxide and microwave antigen retrieval with citrate buffer at pH6, the sections were reacted overnight at 4°C with the anti-CD99 antibody diluted 1:500 in buffer or the other primary monoclonal antibodies at their optimal dilution (Table 2), and then incubated with a commercially available, highly sensitive detection kit (DAKO EnVision Plus-HRP, Dakopatts, Glostrup, Denmark) following the manufacturer’s instructions. Peroxidase activity was developed with 3,3′-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) and copper sulfate to obtain a brown-black end product. The sections were eventually counterstained with Harris’ hematoxylin. Paraffin sections of normal thymus and a case of Ewing’s sarcoma, both known to be strongly immunoreactive for CD99, were stained in parallel as external positive controls. The specificity of all immunoreactions was also double-checked, either substituting the primary antibody with a nonrelated isotypic mouse immunoglobulin at a comparable dilution or with normal serum alone.[74]

Evaluation of the Results

All cases were evaluated by an experienced lung cancer pathologist (G.P.) blind to patient identity and stage of disease. The percentage of CD99-immunoreactive tumor cells (labeling index = number of CD99 immunoreactive cells/total of counted cells) was assessed for every case scanning at least 2,000 neoplastic cells in representative fields of immunostaining. Also, the immunostaining pattern was recorded, indicating whether pattern was either membranous if a moderate to strong cytoplasmic decoration with variable membrane reinforcement was seen in tumor cells or cytoplasmic if a granular dotting was confined to the cytoplasm without appreciable membrane decoration. Tumors were considered negative if staining was either completely absent or observed in 5% or less of neoplastic cells, independent of the immunostaining pattern. Proliferative activity of tumor cells was assessed by Ki-67 antigen immunoreactivity on consecutive paraffin sections, as described elsewhere in detail.[45,75,76]

Statistical Analysis

Qualitative data are presented as frequencies and percentages and compared using a χ^2 test (applying Yates’ correction for continuity) or Fisher’s exact test. Continuous data were expressed using median values and contrasted employing the Wilcoxon signed-rank test for pairs or the Kruskal-Wallis test if medians were analyzed between two or more groups. All correlation tests were performed using Spearman’s rank test (r). The risk for regional lymph node metastasis associated with CD99 expression, adjusting for potential confounders (i.e., age, gender, and pathologic T [pT] class), was analyzed using logistic regression (SAS, Cary, NC) and described by the odds ratios (ORs) and corresponding 95% confidence intervals (CIs). ORs were calculated for CD99% divided into two groups by the median. Any statistical test was considered significant if the corresponding P value was ≤ 0.05.

RESULTS

CD99 Immunoreactivity Is Consistently Lacking in Normal and Hyperplastic Neuroendocrine Cells of the Lung

All samples of normal pulmonary parenchyma and bronchial tree from patients with nonmalignant lung diseases and all samples of pulmonary neuroendocrine tumorlets and nonneoplastic peritumoural lung tissue from the study patients were consistently nonreactive for CD99. Likewise, surface ciliated and mucous cells of the bronchial epithelium, seromucous glands of the bronchial wall, and pneumocytes and Clara cells did not show any immunostaining.

CD99 Immunoreactivity Is Most Prevalent in TCs/ACs

Overall, CD99 immunoreactive cells were found in 60 of 136 (44%) tumors, including 31 of 41 (75.6%) TCs, 14 of 27 (51.8%) ACs, 10 of 42 (23.8%) LCNECs, and five of 27 (18.5%) SCLCs ($P < 0.0001$). A steady decrease of CD99 labeling index was observed from better to less differentiated tumors (Table 3). In TCs, there was a higher prevalence of moderate to strong membranous labeling in tumor cells, whereas in immunoreactive ACs and LCNECs/SCLCs, the

TABLE 3. Distribution of CD99 Immunoreactivity in 136 Neuroendocrine Tumors of the Lung

		CD99 labelling index		CD99 immunostaining pattern = no. (%)			
Tumor type	Cases (pos)	Median	P value	Neg	Cyto	Mem	P value
TC	41 (31)	58.0%		10 (24.4)	4 (9.7)	27 (65.9)	
AC	26 (14)	28.5%		12 (46.1)	14 (53.9)	0	
LCNEC	42 (10)	25.0%		32 (76.2)	8 (19.0)	2 (4.8)	
SCLC	27 (5)	20.0%	0.0068*	22 (81.5)	5 (18.5)	0	<0.0001*

*These differences persisted after adjusting for tumor stage I (37 pIA versus 10 pIB).

pos, positive; Neg, negative; Cyto, cytoplasmic; mem, membranous; TC, typical carcinoid; AC, atypical carcinoid; LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma.

prevalent pattern was exclusively cytoplasmic (Table 3). These differences in both CD99 labeling index and immunostaining pattern persisted after adjusting tumors for stage I ($P < 0.01$). Frequency distributions of CD99 labeling index and immunostaining patterns in the whole series as a function of tumor types (not stratified for stage of disease) are provided in Figure 1. Representative features of CD99 immunostaining in diverse tumor types are shown in Figure 2.

A strong correlation was found between CD99 labeling index and immunostaining pattern in TCs ($r = 0.75$), *ACs* ($r = 0.71$), LCNEC (0.92), and SCLC (0.77) ($P < 0.0001$). In particular, tumors expressing more than 45% CD99 immunoreactive cells (threshold corresponding to the median of the positives in the whole series of tumors) showed a sharp prevalence of membranous pattern (72%), whereas tumors with 45% or less of positive cells mostly exhibited cytoplasmic pattern (81%) ($P < 0.0001$). No preferential distribution of CD99-immunoreactive cells was seen within individual cases in perivascular, peripheral, or central viable areas of tumor.

CD99 Expression Is Associated with Reduced Proliferative Fraction, Small Tumor Size, and Lack of Regional Lymph Node Metastases in TCs/ACs

In TCs/ACs, increased (>27% median value) CD99 labeling indexes or the membranous staining patterns were preferentially observed in low proliferating stage I tumors of small size, with low prevalence of regional lymph node metastases (Table 4). On the contrary, either lower CD99 labeling index or negative to cytoplasmic immunostaining patterns correlated significantly with the pN class and the total number of positive lymph nodes as well as with a decreased immunoreactivity for synaptophysin (Table 4). These differences, however, disappeared after adjusting for stage I of disease (37 cases I-A versus 10 cases I-B). Moreover, no association was noted with age, gender, pT class, and respiratory tract-related hormone content in the same tumor group, even after adjusting for tumor stage.

In LCNEC/SCLC, no correlations were found with any of the tested clinicopathological variables, apart from a statistically significant reduced chromogranin A content in tumors lacking CD99 expression ($P = 0.033$).

CD99 Immunoreactivity Is an Independent Predictor of Lymph Node Metastases in TC/AC

Lymph node metastases were significantly more common in LCNEC/SCLC (50.7%) than in TCs/ACs (31.3%) (Table 1). This difference, however, did not occur in both genders: in males, it disappeared (42% versus 28%, respectively, $P = 0.19$), and in females was actually accentuated (92% versus 34%, $P < 0.001$). Moreover, AC patients had more lymph node metastases (16 of 26) than patients with TC (six of 41) ($P < 0.001$). Metastatic carcinoids, either typical or atypical, however, did not show statistically significant differences in CD99 labeling index (20% for TCs and 15% for ACs) and in immunostaining pattern (four tumors with cytoplasmic immunostaining and two nonreactive in TCs, and seven tumors with cytoplasmic immunostaining and nine nonreactive in ACs).

In TCs/ACs, the median value of CD99 labeling index in tumors without lymph node metastases was significantly higher than in tumors with lymph node involvement (45% versus 0%, respectively, $P = 0.0017$), whereas in LCNEC/SCLC, this difference disappeared (Fig. 3). In the former, the median CD99 labeling index steadily decreased according to the pN class, ranging from 45% in pN0 to 19.5% in pN1 to 0% in pN2 tumors ($P = 0.0016$). Also a close inverse relationship was found between the number of positive regional lymph nodes and both the CD99 labeling index ($r = -0.30$, $P = 0.013$) and the cytoplasmic immunostaining pattern ($r = -0.29$, $P = 0.016$).

Univariate regression analysis showed that patients with a CD99 labeling index less than 27.5% (median value), a negative or cytoplasmic immunostaining pattern, a younger age, decreased chromogranin A expression, and a higher proliferative fraction were more likely to have regional lymph node metastases (Fig. 4). Because of the high colinearity between CD99 index and immunostaining patterns, two separate models were built to assess the risk of metastases. In multivariate analyses adjusting for the significant variables (i.e., age, chromogranin A and Ki-67) and a potential confounder (i.e., pT class), low CD99 labeling index (OR = 7.43; CI: 1.83–30.06) and nonmembranous immunostaining pattern (OR = 5.55; CI: 1.22–25.33) were independent risk indicators for lymph node metastases in TC/AC patients (Fig. 4). In the two models, a younger age of the patients (OR =

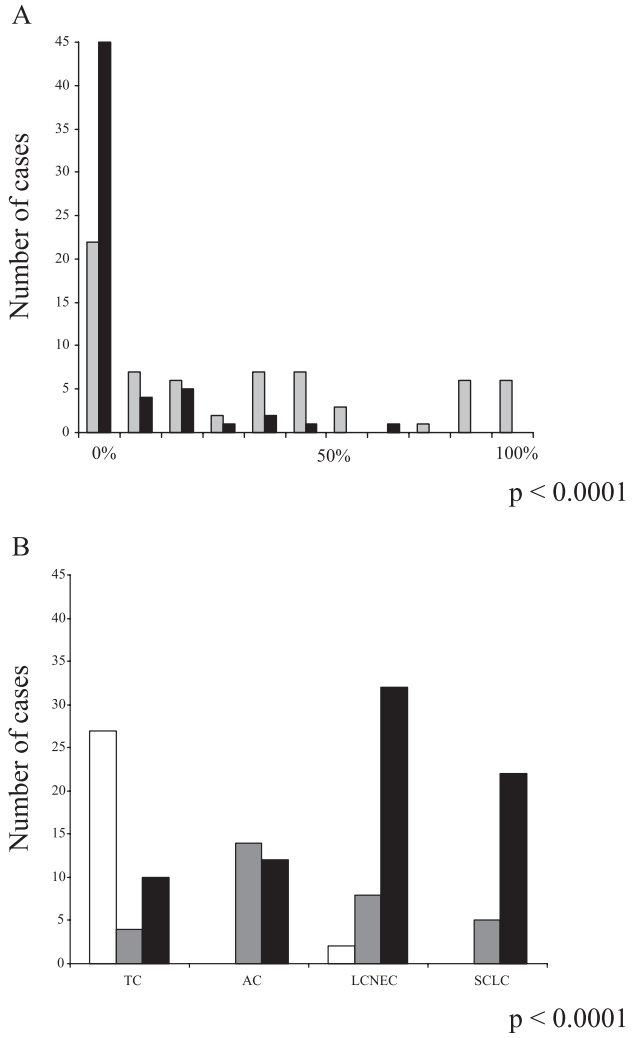


FIGURE 1. Frequency distributions of either CD99 labeling index (*A*) or immunostaining patterns (*B*) in the whole series of tumors as a function of tumor type, without stratifying them for stage. *A*: In patients with typical carcinoid (TC) and atypical carcinoid (TC) (shaded columns), the median values of CD99 labeling index in the whole tumor series ($n = 67$) and in the positive cases only ($n = 45$) are 27% and 45%, respectively. In large cell neuroendocrine carcinoma/small cell lung carcinoma (LCNEC/SCLC) (solid columns), the corresponding values in the total samples ($n = 69$) and among positive observations ($n = 15$) are 0% and 25%, respectively (in both series, $P < 0.0001$). *B*: The membranous pattern (open columns) prevails in TCs ($n = 27$), the cytoplasmic pattern (shaded columns) in ACs ($n = 14$), and a complete lack of any immunoreactivity (solid columns) in either LCNEC ($n = 32$) or SCLC ($n = 22$) ($P < 0.0001$).

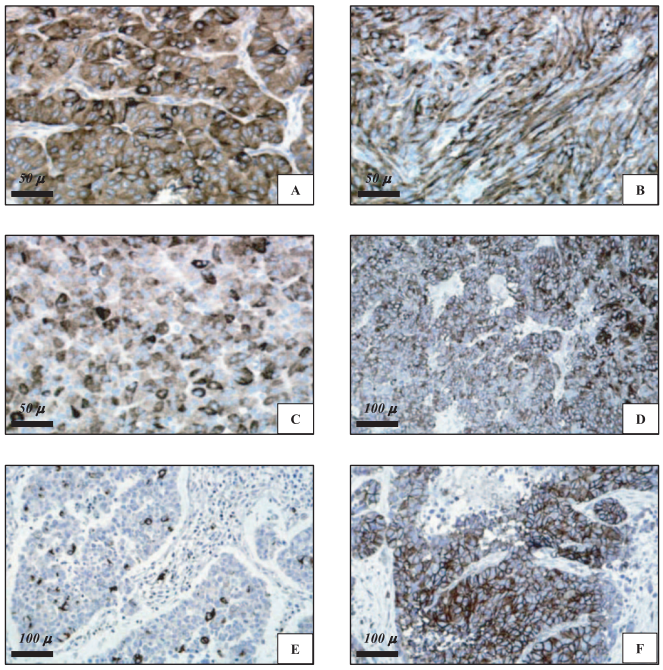


FIGURE 2. Distribution of CD99 immunoreactivity in different neuroendocrine tumors of the lung. Most typical carcinoids, with either solid trabecular (*A*) or spindled (*B*) features, usually show strong and diffuse reactivity for CD99, with a typical membranous labeling of tumor cells. On the contrary, only a fraction of atypical carcinoids is reactive for CD99, with cytoplasmic labeling (*C*) in fewer tumor cells than typical carcinoids (*D*). Poorly differentiated large cell neuroendocrine carcinoma (LCNEC), just like small cell lung carcinoma (SCLC), are usually devoid of any CD99 reactivity or show only cytoplasmic labeling in few tumor cells (*E*). LCNEC but not SCLC, however, may rarely exhibit membranous pattern of immunoreactivity (*F*). All immunostains were developed with diaminobenzidine and then counterstained with hematoxylin. Scale bars are reported in the bottom on the left of each panel.

0.95; CI: 0.91–0.99 and OR = 0.96; CI: 0.92–0.99, respectively) and decreased chromogranin A immunoreactivity (OR = 4.89; 95% CI: 1.21–19.78 and OR = 3.66 CI: 0.99–13.52, respectively) also increased the risk of lymph node metastases. On the contrary, in LCNEC/SCLC, none of the above parameters significantly correlated with the occurrence of lymph node metastases.

DISCUSSION

This investigation documents a different prevalence of CD99 immunoreactivity and diverse staining patterns in pulmonary neuroendocrine tumors, according to the tumor grade, stage, and proliferative fraction. Moreover, in low- to intermediate-grade tumors, CD99 immunoreactivity is an independent predictor of uninvolved regional lymph nodes.

CD99 is predominantly expressed in low- to intermediate-grade neuroendocrine neoplasms (75.6% and 51.8%, respectively), whereas either normal or hyperplastic endocrine cells of the lower respiratory tract are consistently nonreactive for this marker. Although it is unclear why CD99 is expressed in the low-grade part of the neuroendocrine tumor spectrum and not in the normal and hyperplastic neuroendocrine cells, this finding is in agreement with the general lack of CD99 immunoreactivity in several cell types of the normal diffuse neuroendocrine system, with the possible exception of pancreatic islet cells.[45] Also, CD99 is expressed in almost 25% of well-differentiated carcinoids of

TABLE 4. Clinicopathological Association of CD99 Labeling Index and Immunostaining Patterns in 67 Typical and Atypical Carcinoids

Variable	Category	≤ 27%*(%) (n = 34)	>27% (%) (n = 33)	P value
Tumor stage	I	18 (52.9)	29 (87.9)	
	II–III	16 (47.1)	4 (12.1)	0.0029
LN metastasis (%)	Absent	18 (52.9)	28 (84.5)	
	Present	16 (47.1)	5 (15.1)	0.0078
LN metastasis site (%)	pN0	18 (52.9)	28 (84.5)	
	pN1	7 (20.6)	5 (15.1)	
	pN2	9 (26.5)	0	0.0032
No. of positive LNs	Median	1	0	0.0025
Ki-67 index	Median	6.5	3	0.0134†
Calcitonin	Negative	27 (79.4)	17 (51.5)	
	Positive	7 (20.6)	16 (48.5)	0.0214†

		Neg (%) (n = 22)	Cyto (%) (n = 18)	Mem (%) (n = 27)	
Size (cm)	Median	2.4	3.5	2	0.0054
Tumor stage	I	11 (50.0)	11 (61.1)	25 (92.3)	
	II–III	11 (50.0)	7 (38.9)	2 (7.4)	0.0032
LN metastasis (%)	Absent	11 (50.0)	11 (61.1)	24 (88.9)	
	Present	11 (50.0)	7 (38.9)	3 (11.1)	0.0102
LN metastasis site (%)	pN0	11 (50.0)	11 (61.1)	24 (88.9)	
	pN1	4 (18.2)	5 (27.8)	3 (11.1)	
	pN2	7 (31.8)	2 (11.1)	0	0.0081
No. of positive LNs	Median	0.5	0	0	0.0055
Ki-67 index	Median	6.9	6	2.3	0.0011†
Synaptophysin	Median	80	90	100	0.0235†
Calcitonin	Negative	18 (81.8)	13 (72.2)	13 (48.1)	
	Positive	4 (18.2)	5 (27.8)	14 (51.9)	0.0375†

Categorical variables are compared with χ^2 test; continuous variables with Wilcoxon's two-sample test or Kruskal-Wallis test.
*Cutoff point corresponding to the median value of negatives and positives.
†These differences disappeared after adjusting for stage I of disease (37 cases I-A versus 10 cases I-B).
LN, lymph node; Neg, negative; Cyto, cytoplasmic; Mem, membranous.

the gastroenteropancreatic tract,[45] supporting the view that its occurrence may be considered a feature of low-grade neuroendocrine tumors independently of their anatomical site. In pulmonary TCs/ACs, the relationship between an increased CD99 labeling index of tumor cells with membranous immunostaining pattern and their grade of neuroendocrine differentiation are further confirmation that CD99 expression may be actually correlated with cell differentiation in this tumor group (Table 4). Although there is a statistical difference between carcinoids, either typical or atypical, and poorly differentiated neuroendocrine carcinomas as far as CD99 labeling index and immunostaining pattern are concerned, this marker cannot be reliably used alone in this difficult differential diagnosis, especially in case of small tissue fragments. In fact, high-grade neuroendocrine tumors of the lung may consistently display CD99 expression, and a labeling index dealing with membranous or cytoplasmic immunostaining pattern could not be easily assessable on small crushed biopsy fragments. Therefore, the combined use of a nuclear marker of cell proliferation, such as Ki-67 antigen labeling index, in addition to CD99 could assist in solving these difficult problems of differential diagnosis on small crushed biopsies obtained during bronchoscopy.[77]

Although the precise functions of CD99 in pulmonary neuroendocrine tumors remain largely unknown, a possible role in homophilic cell-to-cell adhesion has been proposed in other cellular systems, including normal hematopoiesis[1,46] and human thymocytes,[6,22] some Ewing's sarcoma cell lines,[53] and in the binding of leukocytes to vascular endothelial cells during the extravasation cascade.[24,47,48] The significant inverse correlation of CD99 expression with the occurrence of regional lymph node metastases, increasing pN class, the number of involved lymph nodes, and more advanced tumor stage may well indicate for this protein a role against the progression of neuroendocrine tumors, especially in cases of TCs and ACs. In multivariate analysis, patients with either low CD99 immunoreactivity (equal to or below the median value) or purely cytoplasmic staining pattern were 7.4 and 5.5 times more likely to have lymph node metastases, respectively (Fig. 4). Moreover, in the TC/AC group, the median CD99 labeling index progressively decreased according to the pN class, suggesting this marker could serve as a suitable indicator of subset of patients with different risk of metastases. The significant association of CD99 expression with tumor stage, however, disappeared after stratification for stage I. Because low- to intermediate-grade neuroendocrine

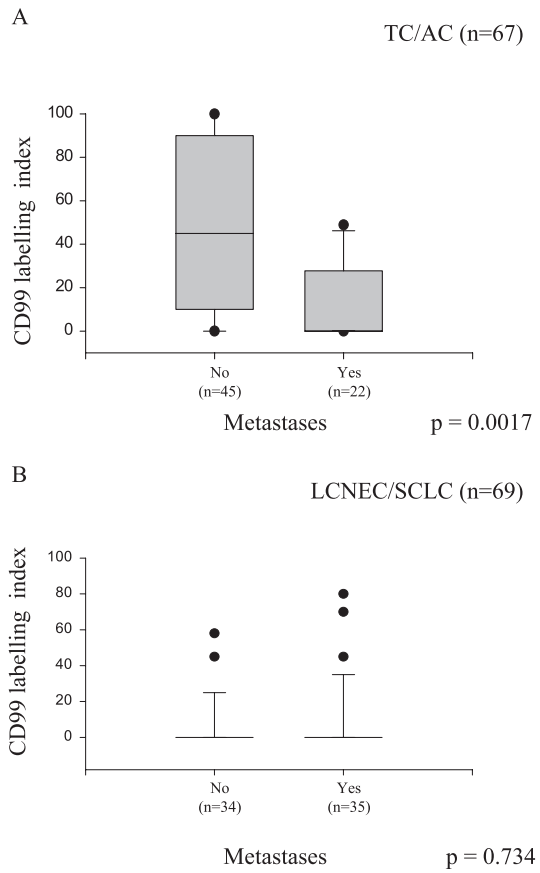
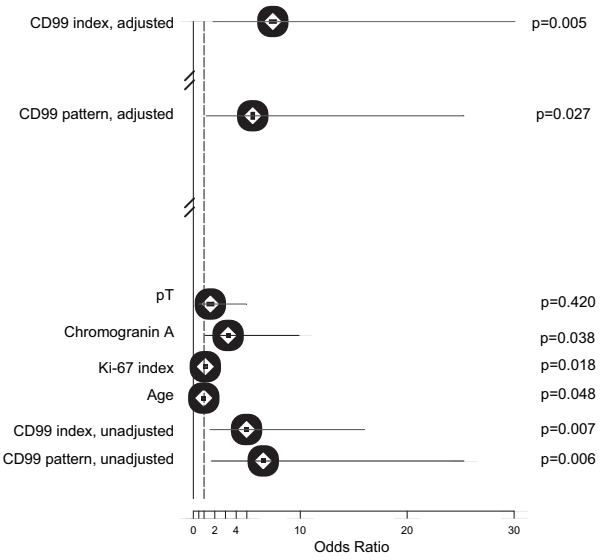


FIGURE 3. Box plots of CD99 labeling index by the occurrence of regional lymph node metastases in 67 TCs/ACs (*A*) and 69 LCNEC/SCLC (*B*) patients. All stage I patients (47 TCs/ACs and 34 LCNEC/SCLC) had by definition no lymph node metastases, whereas those with stage I to III (20 TCs/ACs and 35 LCNEC/SCLC) showed lymph node metastases in all tumors but two cases of LCNEC with stage IIB corresponding to pT3N0. The box is bounded below and above by the 25% and 75% percentiles, the median is the solid line in the box, and the lower and upper error bars indicate the 5% and 95% percentiles, respectively. The flatness of the boxes in the series of LCNEC/SCLC (*B*) is explained by the skewness of the data toward nonexpression because approximately 78% of values are equal to zero.



			Lymph Node Metastasis	
Variable	**Category**	**No. of cases**	No	Yes
CD99 index	² 27.5%	34	18	16
(baseline)	> 27.5%	33	28	5
CD99 pattern	negative/cytoplasm	40	22	18
(baseline)	membrane	27	24	3
pT	T2-T3	18	11	7
(baseline)	T1	49	35	14
Chromogranin A	² 95	35	20	15
(baseline)	> 95 %	34	26	6
Ki-67 index	mean (sd)	-	5.2 (4.8)	9.3 (7.3)
Age (years)	mean (sd)	-	54.8 (16.2)	45.7 (18.1)

FIGURE 4. Magnitude of odds ratios for lymph node metastasis and corresponding 95% confidence intervals are shown along the horizontal axis of the figure for each of the potential risk factors assessed. Odds ratios, 95% confidence intervals, and *P* values are generated using logistic regression. Adjusted estimates for CD99 index and CD99 pattern were derived from separated models including pT, chromogranin A, Ki-67 index, and age. The six odds ratios at the bottom of the figure represent the odds of metastasis associated with each of the six variables when regressed alone. Variables were treated as continuous or categorical as shown on the accompanying table.

neoplasms are actually no considered precursors to either LCNEC or SCLC, but separate and histogenetically unrelated neoplasms, this peculiar distribution indicates that CD99 is more likely to be involved in the metastatic spread (N factor) of TCs/ACs rather than in the local growth of tumors (T factor). Whether decreased CD99 immunoreactivity may also favor distant metastases and thus represent a negative predictor of survival cannot be derived from our data because of the lack of adequate follow-up time. Interestingly, however, we found that tumor emboli within vascular channels were consistently nonreactive for CD99, independent of tumor type, confirming a potential role for down-regulation of this molecule also in the development of distant metastases of pulmonary neuroendocrine tumors (data not shown).

Recently, two isoforms of CD99 have been identified that derive from alternative splicing of the same gene transcript: an M_r 32,000 long form (type I) corresponding to the full-length protein, and a M_r 29,000 short form harboring a deletion in the intracytoplasmic segment[8,46] and recognized by many of the antibodies reacting with the complete transmembrane form.[9] The coordinate expression of the two isoforms may lead to distinct functional outcomes, thus explaining the apparently contradictory properties of this molecule in different cell models. Although distinguishing between the two CD99 isoforms was not an aim of the current study, the different pattern of immunostaining (either membranous or cytoplasmic), as previously reported for gastroenteropancreatic carcinoids,[45] suggests that there exists complex CD99 expression in different types of neuroendocrine neoplasms, independent of their anatomical site. A definitive relationship, however, between CD99 isoform distribution and immunostaining pattern cannot be derived from the data of this study.

We found a strong positive correlation between the CD99 labeling index and the immunostaining pattern in neuroendocrine tumors of the lung, according to tumor type in which the neoplasms characterized by higher (>45%) CD99 labeling index (typically TCs) showed a definite prevalence of the membranous pattern of staining, and the tumors with 45% or less immunoreactive cells (typically ACs and LCNEC/SCLC) consistently exhibited a cytoplasmic staining pattern. This may suggest a deregulated expression of CD99 by neuroendocrine tumor cells according to their dedifferentiation and malignant potential, with a down-regulation of membrane expression and up-regulation of the cytoplasmic expression. Our finding of increased proliferative activity in TCs/ACs expressing cytoplasmic CD99 supports the hypothesis that cytoplasmic protein may parallel the cell cycle activity. There is evidence that CD99 may interfere with the action of insulinlike growth factor I on cellular proliferation,[55,78] as seen in several types of neuroendocrine tumors including SCLC cell lines.[79–82] Although the precise mechanism of this functional interaction is still elusive, the loss of cell-cell adhesion and contact inhibition due to either down-regulation of membrane CD99 or up-regulation of its cytoplasmic form may eventually promote cell cycle activation leading to increased proliferative status and larger tumor size.

Worth mentioning is that the cytoplasmic pattern of CD99 immunostaining in TCs/ACs was significantly associated with down-regulation of a neuroendocrine granule-related marker, such as calcitonin. As in lymphoblastoid cell lines, the membrane expression of CD99 has been associated with the post-Golgi trafficking machinery to cell surface,[53] it could be speculated that the membrane CD99 expression could also be involved in granule accumulation and/or hormone secretion.

In conclusion, our investigation documents that decreased expression of CD99 is an independent predictor of lymph node metastasis in patients with low- to intermediate-grade neuroendocrine tumors of the lung. Targeting the CD99 pathway could be a novel therapeutic strategy in this tumor group in which conventional radio-/chemotherapy is largely ineffective in the case of not radically operable disease.

ACKNOWLEDGMENTS

Supported by Associazione Italiana Ricerca sul Cancro.

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