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Original article

Positive immunostaining with MLuC1 of bone marrow aspirate predicts poor outcome in patients with small-cell lung cancer

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Summary

Background: Immunocytochemistry has been proven able to identify tumor cells in bone marrow aspirate (BMA) of patients with SCLC. However, few data exist about the clinical significance of the procedure.

Patients and methods: 108 BMA taken from 60 patients were incubated with the MoAb MLuC1 (cluster 6) and stained by the APAAP (alkaline phosphatase-antialkaline phosphatase) method. The serum levels of LDH, TPA, NSE and CEA were also studied in relation to bone marrow involvement by means of discriminant analysis.

Results: Immunocytochemistry of the aspirate with MLuC1 detected positive cells in 23 patients (38%) (38 of 108 samples) vs. 13% of the conventional biopsies studied without MLuC1 ($P < 0.001$). With respect to bone marrow positivity, three groups of patients were identified: those with no positive cells in the aspirate and negative biopsy (group A); those with less than 10 positive cells in the aspirate and negative biopsy (group B); and those with more than 10 positive cells or clumps in the aspirate or positive biopsy (group C). Group C patients had poorer median survivals than those in the other two groups (5.5 vs. 11 months, respectively, $P = 0.01$). Discriminant analysis showed that the four serum markers were poor discriminators of the degree of bone marrow involvement, with only 55% of grouped cases being correctly classified.

Conclusions: These results show that detection of bone marrow involvement i) can be improved by the use of MLuC1 ii) is not predictable by conventional tumor markers, and iii) is related to poor outcome.

Key words: bone marrow, monoclonal antibodies, small-cell lung cancer, survival, tumor markers

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Introduction

The International Workshops on lung antigens have grouped many of the antibodies that react with small-cell lung cancer (SCLC) into clusters [1–3]. As SCLC frequently metastatises to bone marrow, MoAbs have been used to identify small numbers of tumor cells not otherwise identifiable. MLuC1 (IgG2), previously known as MOv15, recognizes the Le^y antigen [4] and has been grouped into cluster six. The Y hapten is a Lewis blood group determinant with the structure Fuc(α1–2)Gal(β1–4)[(Fuc(α1–3))]GlcNAc that is present on a variety of glycoproteins and glycolipids carried on the cell membrane of SCLC as well as on other tumors. It has also been demonstrated that SCLC produce higher amounts of glycolipid-bearing Le^y than do other tumors and normal lung cells [5, 6]. Since MLuC1 binds this saccharide epitope, this antibody seems suitable for targeting SCLC cells [7–9]; for this reason SCLC cell lines also have greater susceptibility to the immunotoxin-containing MLuC1 than do other Y hapten-bearing cell types [10]. In this study MLuC1 was used to identify tumor cells in bone marrow aspirates of patients with SCLC, and the results of immunostaining were correlated with survival and NSE, TPA, LDH, CEA in order to assess whether bone marrow involvement was predictable by tumor markers.

Patients and methods

Sixty unselected SCLC patients at diagnosis (52 males, 8 females) were prospectively collected from December 1991 to December 1995 at the Medical Oncology Unit of the University of Verona. According to standard criteria, patients were classified as having limited (LD) or extensive disease (ED). LD was defined as a tumor restricted to one hemithorax with regional metastasis to hilar, ipsilateral and controlateral and/or supraclavicular lymph nodes and ipsilateral pleural effusion; ED was defined as a tumor extended beyond these sites.

The study protocol included bilateral bone marrow aspiration and biopsy from the posterior iliac crest; however in some cases samples were taken unilaterally according to patient decision or physician judgment.

One hundred and eight bone marrow aspirates (3 to 5 ml each) were obtained, bilaterally in 48 patients and unilaterally in 12.

The preparation of the samples and the immunocytochemical procedures have already been described in detail [11].

Briefly: cytospin slides were incubated in a moist chamber with MLuC1 (kindly provided by Dr. Sylvie Ménard, National Cancer

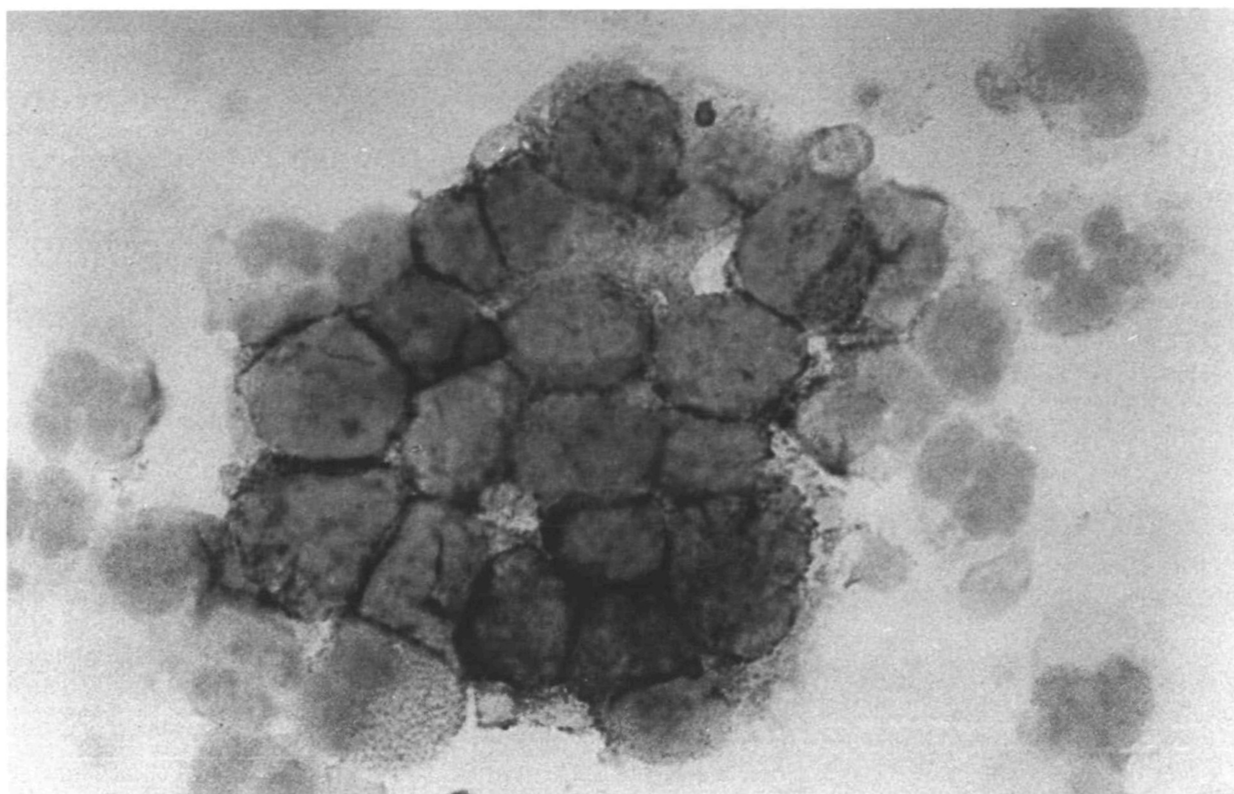

Figure 1. Immunostaining of SCLC cells in a bone marrow aspirate: a cluster of tumor cells shows a strong and continuous ring of staining around the cell membrane (APAAP method, ×1000, oil immersion).

Institute, Milan, Italy). Polyvalent rabbit anti-mouse immunoglobulin (Dakopatts, Glostrup, Denmark), and alkaline phosphatase-antialkaline phosphatase complex (APAAP; Dakopatts) were then added in sequence.

In eight cases with strong bone marrow positivity for SCLC cells, staining for the proliferation-associated nuclear antigen Ki-67 and double staining for Ki-67 and MLuCl were also performed as described [12].

Cytospins of the human SCLC cell line N592 were used as internal control material.

One hundred and three bone marrow core biopsies were obtained with the Jamshidi needle, bilaterally in 43 patients and unilaterally in 17. Biopsies, conventionally processed for paraffin embedding, were evaluated morphologically using hematoxylin-eosin and Giemsa stains and immunohistochemically for low molecular weight cytokeratins 8–18 using the MoAb CAM 5.2 (Becton-Dickinson, Mountain View, CA, USA); however MLuCl staining was not applied on bone marrow biopsies since in our experience it had provided poor immunostaining, even after antigen retrieval with microwave oven (not shown).

Scoring of immunostained cells

At least four slides were evaluated for each patient, and the number of cells labeled for MLuCl was assessed using a light microscope at a magnification of ×400. Taken into consideration were only cytospins with high cellularity and monolayered population of nucleated cells (Figure 1). Slides were considered as being positive if clumps or single cells showed a clearly visible, continuous, thick ring of immunostaining around the cell membrane, and the morphology was consistent with that of SCLC cells. Staining confined to the cytoplasm or discontinuous thin labeling of the cell membrane were not taken into consideration. A semiquantitative method was arbitrarily devised to assess bone marrow positivity: no positive cells in the aspirate and negative biopsy (group A); less than 10 positive cells in the aspirate and negative biopsy (group B); more than 10 positive cells or clumps in the aspirate or positive biopsy (group C).

Tumor markers

According to manufacturer instructions, the following serologic markers were determined in 58 patients: lactic dehydrogenase (LDH), neuron specific enolase (NSE), tissue polypeptide antigen (TPA) and carcinoembryonic antigen (CEA).

Statistical methods

The statistical tests used were the χ^2 test, the Fisher's exact test and the Mann–Whitney test; survival curves were calculated by the Kaplan–Meier method and compared by the log-rank test [13]. Discriminant analysis [14] was performed to verify whether the serum level of LDH, TPA, NSE and CEA could predict the positivity of b.m. involvement. Since these variables were not normally distributed according to the Kolmogorov–Smirnof test a preliminary logarithmic transformation was performed.

Results

Of the 60 patients, 23 (38%) were positive for MLuCl, while bone marrow biopsy was positive in eight patients (13.3%) (38 of 108 bone marrow aspirates vs. 12 of 103 bone marrow biopsies, $P < 0.001$). Myeloid cells were always negative for MLuCl, whereas plasma cells and megakaryocytes showed variable immunostaining. However, these cells were not confused with tumor cells

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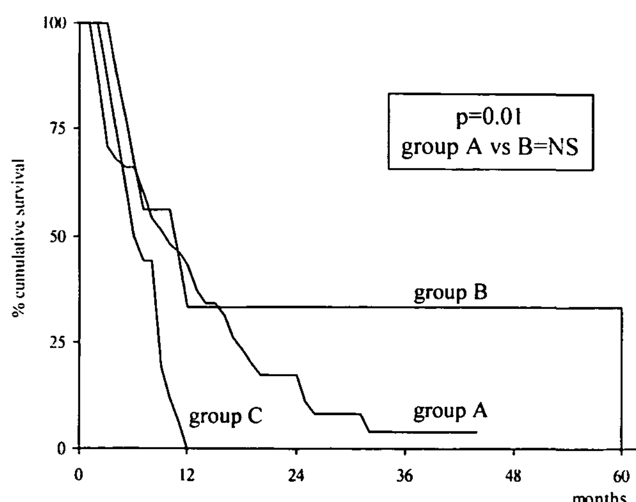

Figure 2. Survival of patients according to group.

since the positivity was confined to the cytoplasm and the morphology was not consistent with that of epithelial cells. Double staining procedures showed that in all of the patients examined, 30%–50% of MLuC1-positive cells also co-expressed immunostaining for the Ki-67 antigen.

Thirty-five patiens belonged to group A, nine to group B and 16 to group C. In the latter group two patients had positive bone marrow biopsies but negative aspirate.

According to standard staging procedures, including bone marrow biopsy, 27 patients had LD. Eight of the 27 patients with LD (30%) and 15 (45%) (six of 15 had also positive biopsy) of the 33 with ED (P = 0.3) were positive for MLuC1. Excluding the eight patients with positive biopsy, the immunocytochemistry of the aspirate alone increased the tumor cell detection rate by 36% (nine of 25 patients) and 30% in the ED and LD groups, respectively.

In two of the 48 patients (4%) with bilateral aspiration the antibody stained positively on one side of aspiration and negatively on the other side.

Regarding survival, the group C patients had lower median survivals than the other patients (5.5 vs. 11 months respectively, P = 0.01). Median survival was not statistically different in groups A and B (Figure 2).

Abnormal serum levels of LDH and TPA were statistically more frequent in group C, while the majority of the patients had normal levels of CEA. Moreover, the percentages of raised levels of NSE were similar in the three groups (Table 1).

Discriminant analysis showed that the combination including LDH, TPA and NSE allowed the best separation in relation to bone marrow involvement since with these three markers the percent of grouped cases correctly classified was 55.17% (Table 2). A slightly lower discriminant power was obtained combining only two variables: LDH-TPA (51.7%) and NSE-TPA (50%). In this series the inclusion of CEA in the model did not improve the results. Of note, the low discriminant power

Table 1. Distribution of abnormal serum levels of the markers in 58 patients.

	Group			*P*-value
	A No. (%)	B No. (%)	C No. (%)	
LDH	13 (38)	3 (33)	11 (73)	0.05
TPA	12 (35)	1 (11)	11 (73)	<0.01
NSE	22 (65)	6 (67)	11 (73)	0.8
CEA	9 (26)	2 (22)	3 (20)	0.9

Group A: negative; group B: positive with <10 cells and negative biopsy; group C: positive with >10 cells or clumps or positive biopsy.

Table 2. Discriminant analysis: prediction of bone marrow group membership in 58 patients according to the three serum markers (LDH, TPA, NSE) with the highest discriminating power.

Actual group	No. of cases	Predicted group membership		
		A No. (%)	B No. (%)	C No. (%)
A	34	18 (52.9)	8 (23.5)	8 (23.5)
B	9	2 (22.2)	6 (66.7)	1 (11.1)
C	15	3 (20)	4 (26.7)	8 (53.3)

Percent of 'grouped' cases correctly classified: 55.17%.

obtained was even optimistic, since it was assessed on the same data used to derive the discriminant functions. Thus, an even lower discriminant power is likely to be found when applying an established model to new data sets in a clinical setting.

Discussion

The aim of this study was to assess the incidence of bone marrow contamination and its clinical significance in a large series of patients with SCLC at diagnosis by means of immunocytochemistry with the MoAb MLuC1.

In our study MLuC1 identified tumor cells in 38% of the patients: similar data were reported by Ménard et al. [7] who, using immunofluorescence, found 10 positive samples out of 30. Moreover, our series showed bone marrow involvement in 30% of the patients with LD; these patients had lower median survivals than those with LD and negative aspirate (10 vs. 15 months, P = NS) (not shown); although not statistically significant, probably because of the small size of the groups, the finding confirms that monoclonal antibody staining of bone marrow aspirates reveals patients otherwise understaged [15, 16].

Regarding the methods of investigation, immunocytochemistry was preferred because of its superiority to other techniques [17] and probably also to immunofluorescence which may overestimate the positivity rate, since it does not allow direct morphologic examination of the stained cells. Moreover, the immunocytochemistry

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of the aspirate is reproducible, easier to perform [9] and less invasive than a biopsy.

To date the prognostic role of bone marrow involvement is still unclear. Regarding conventional bone marrow biopsy, large series have demonstrated poor survival for patients with positive bone marrow biopsy since positivity was almost always part of a widespread disease [18, 19]. On the other hand, only two studies have addressed the problem using the immunocytochemistry of the aspirate: in one the presence of residual marrow disease at clinical remission was correlated with earlier metastatic relapse [20] and in the other the absence of tumor cells at diagnosis, detected with an anti NCAM MoAb, was associated with a higher survival rate [21].

We found that survival of patients with a higher number of positive cells (group C) was very poor (median 5.5 months) and consistent with that of poor-prognosis patients with ED [22]. This finding shows that a clearly detectable bone marrow involvement at immunocytochemistry had prognostic significance, while median survival was comparable, when few or no positive cells (groups A and B) were detected. The prognostic implication of the presence of a few scattered cells (true metastatic cells or simply in-transit cells?) is unknown. In this study the clinical outcome of group B seems similar to that of negative patients, though the small size of the cohort does not allow us to draw conclusions. However, it appears reasonable, until further data are available, to examine this group separately since it represents an area of uncertainty. Moreover, with the double immunostaining technique, we found that many of the tumor cells co-expressed the associated proliferation antigen Ki-67, suggesting their clonogenic potentiality; this preliminary finding is bothersome since the leukapheresis product for the support of high-dose chemotherapy was shown to be contaminated in patients with bone marrow involvement [23].

It has been contended [9, 11] that immunocytochemistry with MLuCl stained less tumor cells than MoAbs recognizing the neural cell adhesion molecule; this may be due to different antigen expression as well as to problems of antigenicity damage by the fixation procedure, which we tried to minimize by using a mild fixation with acetone.

On the other hand, the potential usefulness of this MoAb in a clinical setting was suggested by *in vitro* as well as animal model studies. Bone marrow artificially contaminated *in vitro* by SCLC cells was effectively purged with a panel of 4 MoAbs, with MOC-1 (anti-NCAM) and MLuCl having similar effectiveness [24]. MLuCl- ricin A chain immunotoxin was potently and selectively toxic to SCLC cell lines in tissue culture [10] and immunoconjugates with doxorubicin successfully targeted a closely Le^y-related antigen with complete regression of xenografted human lung carcinomas [25].

In SCLC some serological markers are widely used in the clinical setting because they are associated with tumor bulk and prognosis [26–30]. In particular, normal levels of LDH were rarely associated with bone marrow involvement (2%–16%) [28, 31–33], but these studies did not use immunocytochemistry for the detection of micrometastases. In our series LDH rose in 73% (11 of 15) of the group C patients, apparently suggesting the usefulness of bone marrow examination only in LDH patients. However, the positive predictive value of the marker was only 41% (11 of 27) for group C and 52% (14 of 27) when groups B and C were combined (Table 1). By means of discriminant analysis, we tried to improve this rather low predictability, studying four serum markers (LDH, TPA, NSE and CEA) in relation to the presence of bone marrow involvement. Again, with at most 55% of the data correctly classified, discriminant analysis failed to demonstrate a clinical usefulness for the markers in predicting the degree of bone marrow involvement, which seems therefore not predictable by the standard laboratory tests.

In conclusion: these preliminary results provide further support for previous experiences [21, 34], showing the prognostic value of the immunocytochemistry of the aspirate, and indicate that this technique, along with other parameters, may have a role in the selection of patients suitable for new therapeutic strategies (i.e., high-dose chemotherapy and the MoAb targeted immunotherapy).

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