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Neural Cell Adhesion Molecule Expression, Neuroendocrine Differentiation and Prognosis in Lung Carcinoma

Robby E. Kibbelaar, Kitty E.C. Moolenaar, Rob J.A.M. Michalides, Peter C. Van Bodegom, R.G.J.R.A. Vanderschueren, Sjoerd S. Wagenaar, Koert P. Dingemans, Dieter Bitter-Suermann, Otilia Dalesio, Nico Van Zandwijk and Wolter J. Mooi

We investigated the expression of the neural cell ahesion molecule (NCAM) in a series of surgically resected lung carcinomas of various histological subtypes by means of a panel of monoclonal antibodies recognising different N-CAM epitopes. In a subgroup of 56 tumours, the results of immunostaining with MAb 123C3—the antibody studied most extensively in our material—were compared to the ultrastructure, and in 231 radically resected non-small cell carcinomas, with histological tumour type and with clinical follow-up data. N-CAM expression was not limited to neuroendocrine tumours, as assessed ultrastructurally. Non-small cell lung carcinomas positive for MAb 123C3 showed post-operative overall and disease-free survival times significantly shorter than 123C3-negative non-small cell carcinomas.
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INTRODUCTION

THE PATHOLOGICAL classification of lung carcinomas is largely based on morphological and histochemical detection of squamous, glandular and neuroendocrine (NE) differentiation [1]. Important differences in clinical behaviour are seen between the lung carcinoma subtypes defined in this way, the clinically most distinctive tumour type being small cell lung carcinoma (SCLC). Within each histological subtype, however, there is still a marked variability in clinical behaviour between individual tumours of the same histological subtype.

The elucidation at the molecular level of the factors responsible for differences in tumour growth rate, metastatic potential and sensitivity to chemotherapy and radiotherapy are currently providing important new opportunities for clinicopathological correlation studies aiming to identify new prognostic parameters and refining lung tumour typing. Alterations of normal cellular adhesiveness caused by abnormal patterns or levels of expression of adhesion molecules may play an important role in tumour invasion and metastasis, so that immunostaining for various adhesion molecules is of considerable interest in the search for such additional prognostic markers.

We recently found that monoclonal antibody (MAb) 123C3, one of the "small cell lung carcinoma (SCLC)-cluster I" antibodies [2] raised by us, recognises the polypeptide backbone of the neural cell adhesion molecule, NCAM [3]; this finding led us directly to conduct the present study. MAb 123C3 was previously found positive in all neuroendocrine lung tumours tested, but also in 20% of NSCLC of various histological types [4]. MAb 735, which in contrast to MAb 123C3 can be used on paraffin sections, recognises an alpha (2 → 8) linked polysialic acid polymer which in mammals has only been found on NCAM [5, 6]. MAb anti-Leu 7 also recognises a carbohydrate epitope present on some subtypes of NCAM, but has limited specificity [7, 8].

For the immunohistochemical detection of NE differentiation in lung tumours, anti-neuron specific enolase (NSE), anti-chromogranin A, and anti-synaptophysin were used. Because the specificity and/or sensitivity of these antibodies is limited, we also investigated the ultrastructure of some tumours in order to ascertain more precisely whether NCAM expression in lung carcinomas is limited to neuroendocrine tumours.

MATERIALS AND METHODS

Tumours and cell line

Deep frozen specimens of 308 lung tumours resected between 1982 and 1986 at the St Antonius Hospital, Nieuwegein, were used for immunostaining with MAb 123C3. The ultrastructure of a subgroup of 59 tumours of various histological types was investigated, in order to assess whether NCAM expression could occur in tumours without ultrastructural evidence on NE differentiation. Paraffin sections of 119 of these lung tumours, including 18 resected SCLC, 5 resected atypical carcinoids, 54 of the ultrastructurally investigated tumours and a number of randomly selected 123C3 positive and negative non-small cell carcinomas (NSCLC), were immunostained for MAbs 735 and anti-Leu 7 to investigate the association with 123C3 immunoreactivity. In addition, in order to enlarge the group of tumours

Correspondence to W.J. Mooi, Department of Pathology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.
R.E. Kibbelaar, K.E.C. Moolenaar, R.J.A.M. Michalides, P.C. Van Bodegom, N. Van Zandwijk, O. Dalesio and W.J. Mooi are at the Divisions of Clinical Oncology and Tumour Biology, The Netherlands Cancer Institute, Amsterdam; R.G.J.R.A. Vanderschueren and S.S. Wagenaar are at the Departments of Pathology and Pulmonology, St Antonius Hospital, Nieuwegein; K. P. Dingemans is at the Department of Pathology, University of Amsterdam, Amsterdam, the Netherlands; and D. Bitter-Suerman is at the Institute for Medical Microbiology, Medizinische Hochschule Hannover, Hannover, Germany.
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with neuroendocrine differentiation studied, paraffin embedded material of 18 typical carcinoids was investigated.

All tumours were histologically typed according to WHO criteria [1] and surgicopathological staging (pTNM) was performed after routine mediastinal lymph node mapping in all cases [9].

For the immunoprecipitation study (see below), SCLC cell line H69 [10] was obtained from the American Type Culture Collection (Rockville, Maryland) and was grown in Dulbecco's modified Eagles medium (DMEM), obtained from Gibco, supplemented with 10% fetal calf serum.

Antibodies

For immunohistochemistry, MAb 123C3, an IgG1 antibody raised at our institute against a membrane fraction of a fresh SCLC specimen, was used in a dilution of 1:200. Polyclonal antiserum directed against NCAM was a generous gift of Dr E. Bock [11]. MAb 735, of the IgG2a subtype, was raised against live meningococci group B by one of us (D. B.-S. and Ref. 12). It was used in a dilution of 1:4000 for immunohistochemistry. MAb anti-Leu 7 was purchased from Becton Dickinson and used as prescribed by the manufacturer. Polyclonal antiserum against NSE was from DAKO, and used in a dilution of 1:7000. Anti-chromogranin A was from Hybritech and used in a 1:4000 dilution. Anti-synaptophysin was from Boehringer Mannheim and used in a dilution of 1:10. MAb BA-1, of the IgM subtype, was purchased from Boehringer Mannheim. MAb MOC 32, of the IgM subtype, was kindly provided by Dr L. De Ley [13]. MAb 66IG10 (IgG1) was generated at the Netherlands Cancer Institute against the transferrin receptor. MAb NKI-nbl was raised at our institute against a neuroblastoma cell line and recognises NCAM [3].

Immunohistochemistry

For immunohistochemistry on frozen sections, an indirect alkaline phosphatase technique was used as previously described [4]. For formalin fixed paraffin-embedded material, the avidin-biotin complex (ABC) method was used as previously described [14]. Internal positive controls were provided by the nerves present within the sections, and by control slides of a carcinoid. Negative controls consisted of omission of the primary antibody, and in the case of MAb 735, of a preincubation with neuraminidase (*Vibrio cholerae*), (Calbiochem), 5 mU in 50 μl of 0.05 mol/l NaAc, pH 5.5, applied during 3 h at 37°C before the incubation with the primary antibody. Only those lung tumours were scored positive for MAb 735 which showed distinct cell membrane staining which could be abolished by pretreatment with neuraminidase.

Biochemical analyses

Full data showing that 123C3 recognises NCAM in SCLC are given elsewhere [3]; MAbs NKI-nbl-3, 123C3 and MOC-32 were found to recognise the same epitope on NCAM. Here, we only illustrate that in SCLC cell line H69, NCAM is recognised by several SCLC-cluster I MAbs. H69 cells were lysed in 25 mM Tris containing 100 mmol/l NaCl, 2 mmol/l EDTA and 1% Nonidet P-40, in the presence of protease inhibitors phenylmethylsulphonyl-fluoride, trypsin inhibitor and aprotinin. Immunoprecipitations were carried out with monoclonal antibodies NKI-nbl-3, a MAB belonging to SCLC-cluster I [15], and 66IG10, (directed against the transferrin receptor); and Protein A–Sepharose CL-4B (Pharmacia). Immunoprecipitated material was dissolved in 30 μl 62 mmol/l Tris, 20% glycerol, 4% sodium dodecyl sulphate (SDS), 0.05% bromophenol blue, 10% 2-mercaptoethanol, pH = 6.8. After boiling for 5 min, the samples were clarified by centrifugation (5 min, 10 000 *g*) and analysed on a 5% SDS polyacrylamide gel according to Laemmli [16]. High molecular weight markers from Bio-Rad were used as reference. After electrophoresis, antigens were blotted onto nitrocellulose filters and immunostained with monoclonal antibodies NKI-nbl-3 (1/1000 ascites dilution), 66IG10 (1/100 ascites dilution), MOC-32 (1/100 ascites dilution), Leu-7 (1.2 μg/ml) and BA-1 (1.2 ug/ml; 18) as previously described [17].

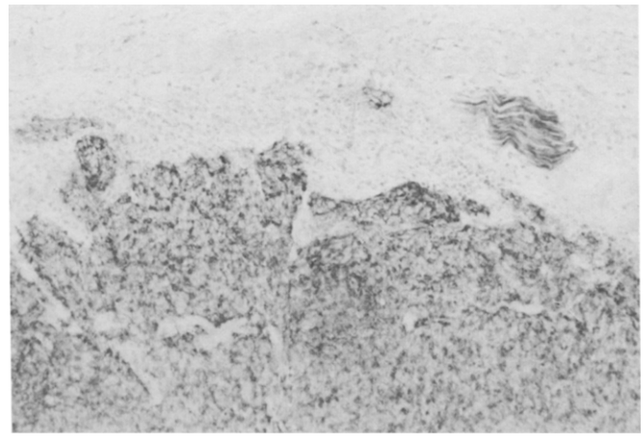

Fig. 1. SCLC, immunostained with MAb 123C3. Strong positivity throughout the tumour; negative stroma, in which a positive nerve is seen (bottom left). Immunoperoxidase (× 140).

Ultrastructural analysis

Karnovsky-fixed material of 59 tumours was available and used in order to compare 123C3 immunoreactivity with the presence of dense-core granules (DCG), which up till now appears to remain the most sensitive way to assess small traces of neuroendocrine differentiation in lung tumours [18]. Intracytoplasmic granules were considered to represent neuroendocrine granules when they were 50–200 nm in diameter, rounded or faintly hexagonal and surrounded by a smooth membrane, and contained a central, rounded electron-dense core separated from the surrounding membrane by a thin electron-lucent halo [19]. These granules were present preferentially in cytoplasmic processes or at the cell periphery.

Clinicopathological correlations

Of the 308 patients operated, 226 radically resected NSCLC were evaluable for prognostic studies. The remaining 82 cases were excluded because of postoperative complications (death within 30 days due to surgically related complications), insufficient follow-up data, the presence of synchronous double tumours, metastasis detected at operation, or SCLC histology.

Data were analysed with respect to overall survival (all causes of death), disease-free survival (time to recurrence or death) and disease-free interval (time to tumour recurrence, with cases of death of causes other than tumour or unknown being censored at that date).

RESULTS

MAb 123C3, 735 and anti-Leu 7 reactivity vs. histological tumour type

MAb 123C3 in SCLC characteristically yielded strong membrane staining (Fig. 1). MAb 123C3 was positive in all NE

Table 1. Immunoreactivity (Numbers positive/total number analyzed) of 123C3, 735 and anti LEU-7 in lung tumours

Histology	123C3	735	Leu-7
SCLC	20/20	38/39	17/39
Atypical carcinoid	5/5	4/6	5/6
Typical carcinoid	1/1	9/18	18/18
NSCLC, various types	42/91	18/91	18/91

tumours tested (Table 1); in this study frozen material of only one typical carcinoid was available, but in a previous study we found positivity in all 15 typical carcinoids tested [17].

MAb 735 was positive in all SCLC except one, in 4 out of 6 atypical carcinoids, and in 9 of 18 typical carcinoids. Staining was usually strong but mostly focal, and was often accentuated in thin and irregular strands of tumour cells as opposed to large compact tumour nodules (Fig. 2).

MAb anti-Leu 7 was positive in 17 of 39 SCLC, 5 of 6 atypical carcinoids, and all 18 typical carcinoids.

MAb 123C3 positivity was found in 53 out of 278 NSCLC tested. As indicated above, material of only 91 NSCLC was available for additional immunostaining with the other antibodies. Of these tumours, 42 were positive for MAb 123C3, 18 for 735 and 18 for anti-Leu 7. Positivity was usually focal, and varied in intensity (Fig. 3). Only 15 tumours positive for MAb 123C3 were also positive for MAb 735. Some NSCLC, especially adenocarcinomas, occasionally showed cytoplasmic granular staining, which resembled granular positivity seen in pre-existent bronchial goblet cells. No positivity of the cell membrane was detected in these cases; they were scored negative. Anti-Leu 7 frequently showed a granular immunostaining similar to MAb 735 in NSCLC, especially in adenocarcinomas.

NCAM markers vs. NE immunomarkers and ultrastructure

MAb 123C3 was positive in all but one carcinoma with DCG (Table 2); however, in many 123C3 positive tumours, DCG were absent, so that 123C3 positivity is not an indication of NE differentiation. 51 of these 59 tumours (including all 10 cases with DCG on electron microscopy) were immunostained with anti-NSE, anti-chromogranin A and anti-synaptophysin: anti-NSE failed to recognise 4 out of 10 NE tumours, and was positive in 13 out of 41 tumours without DCG. Anti-chromogranin A and anti-synaptophysin were more specific but also lacked sufficient sensitivity.

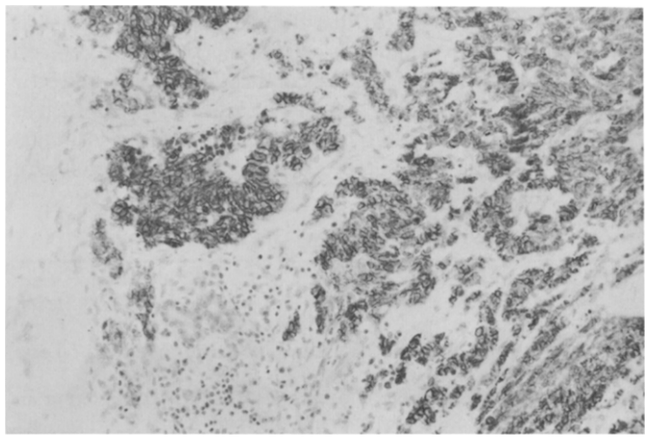

Fig. 2. SCLC, immunostained with MAb 735. Strong membrane-staining of tumour cells, infiltrating in thin, irregular strands between negative stroma. Immunoperoxidase (× 140).

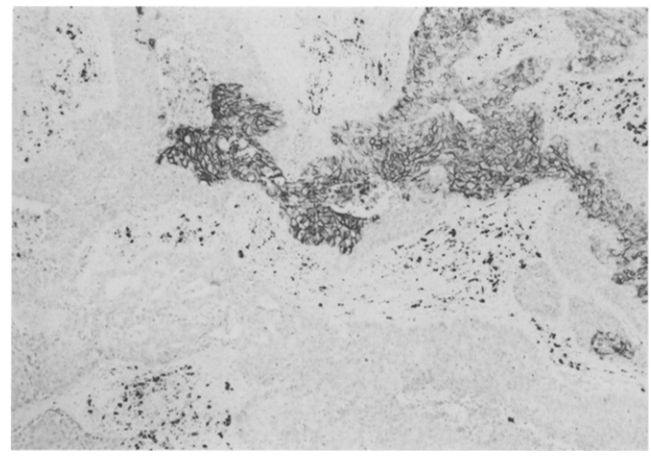

Fig. 3. Focal MAb 735 positivity in squamous carcinoma. Areas of strong positivity intermingled with totally negative areas. Immunoperoxidase (× 80).

Table 2. 123C3 positivity and presence of dense core granules

	DCG +	DCG −
123C3 +	8	23
123C3 −	1	27

N = 59 tumours of various histological types (3 SCLC, 2 atypical carcinoids, 25 squamous carcinomas, 14 adenocarcinomas, 4 adenosquamous carcinomas, 11 large cell carcinomas).

Immunoprecipitation

In the immunoprecipitation studies on the SCLC cell line H69 and frozen tumour material, MAbs 123C3, 735 and polyclonal NCAM antiserum precipitated the same protein bands: one major band of 145 kDa and one of 185 kDa [3, 14]. By indirect

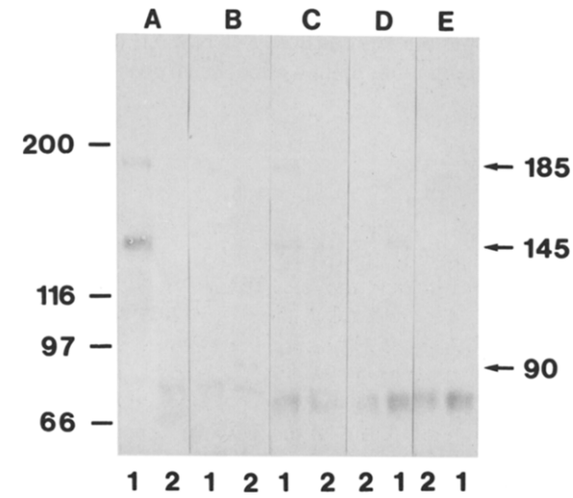

Fig. 4. Immunoprecipitations of SCLC cell line H69 with NKI-nbl 3 (lane 1) and 66IG10 (lane 2) were immunostained with MAb NKI-nbl-3 (A), 66IG10 (B), MOC-32 (C), Leu-7 (D) and a negative control BA-1 (E).

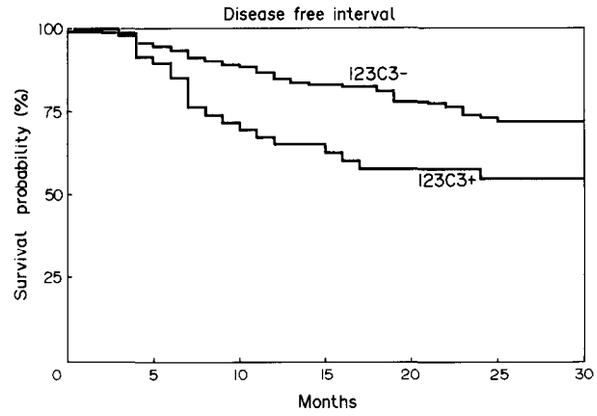

Fig. 5. Overall survival of 226 NSCLC according to 123C3 immunoreactivity.

immunoprecipitation, MAb anti-Leu 7 recognised the same proteins as MAbs 123C3, 735 and the polyclonal anti-NCAM (Fig. 4), although the Leu-7 epitope is hardly detectable on the larger NCAM isoform.

Prognostic correlations

Overall survival, relapse-free survival and relapse-free interval was assessed of the whole group of tumours, broken down according to stage, histological type and positivity for all individual markers tested. The strongest prognostic factor was stage. Within the group of 226 radically resected NSCLC, tumours positive for MAb 123C3 showed a significantly shorter overall survival and disease-free survival, also when corrected for differences in tumour stage (overall survival: $P = 0.046$, disease-free survival: $P = 0.044$; log-rank test, Figs 5 and 6). Other markers did not yield significant differences in survival. Survival curves of MoAB 735 were similar to those of MAb 123C3, but since the numbers tested were small, no definitive conclusion could be drawn. We are currently enlarging that series in order to ascertain whether MAb 735, which can be used on paraffin-embedded material, can yield prognostic information similar to MAb 123C3.

DISCUSSION

Based on the fact that SCLC are poorly differentiated NE tumours, while the large majority of NSCLC shows no signs of NE differentiation, it has often been presumed that NE differentiation in lung carcinomas is directly related to the virulent tumour behaviour of SCLC. Since the distinction between SCLC and NSCLC can sometimes be difficult histologically, immunohistochemical or ultrastructural assessment of NE differentiation has been used to predict this virulent type of tumour behaviour [19]. However, the presently used immunomarkers of neuroendocrine differentiation are not as sensitive and specific as electron microscopy, and cannot be considered optimal markers to recognise the small traces of neuroendocrine differentiation which may be relevant in this context. Also, and perhaps even more importantly, the fact that SCLC shows NE differentiation does not provide an answer to the question, why SCLC grows and metastasises so rapidly; bronchial carcinoid is also a NE tumour, but shows a far less malignant behaviour.

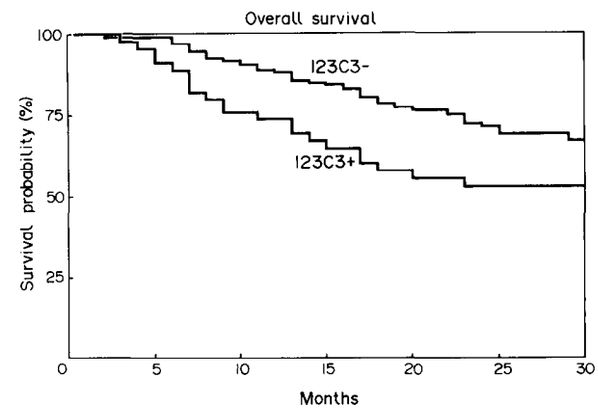

Fig. 6. Disease free interval of 226 NSCLC according to 123C3 immunoreactivity.

MAb 123C3, although it is not specific for NE differentiation (Refs 4 and 17 and this study) does recognise the polypeptide backbone of NCAM [3]. NCAM is known to have a wider tissue distribution than just the neural and neuroendocrine cells [6]. In view of the hypothesis that cellular adhesion molecules may be directly involved in differences in behaviour between various types of lung cancer, this finding makes it an interesting antibody to use for clinicopathological correlation studies. We previously reported that 20% of NSCLC are positive for MAb 123C3 [4]. We now included other antibodies recognising NCAM, such as MAb 735, which recognises alpha ($2 \rightarrow 8$) sialic acid polymers of at least 8 residues. These are present on an NCAM isoform which is characteristic of an early, "embryonal" form of NCAM [6]. Staining intensity for MAb 735 is proportional with the amount of sialic acid present on NCAM [6]. It has previously been demonstrated that extensively sialylated NCAM results in weak adhesive properties, as compared to desialylated NCAM [20]. It is tempting to speculate on the significance of our findings of MAb 735 positivity in all SCLC, two thirds of the atypical carcinoids, and half of the typical carcinoids studied, all of which are positive for 123C3, which recognises both highly and poorly sialylated forms of NCAM (data obtained after neuraminidase treatment, not shown).

Our finding of a significantly shorter overall and disease-free survival on MAb 123C3 positive NSCLC may have clinical consequences: one could contemplate possible benefits from adjuvant chemotherapy in these patients. Whether the same would apply for MAb 735, which in contrast to 123C3 can be used on paraffin sections, could not be assessed with the small numbers of cases thusfar stained for MAb 735.

The prognostic significance of MAb 123C3 reactivity, which is not specific for any particular differentiation type, supports the idea that we may not require better markers of the classical differentiation pathways, but instead, markers of factors directly involved in one of the aspects of malignant behaviour, such as growth rate, invasion and metastasis, and their distribution which may cross the borders defining classical histological tumour subtypes.

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Listeria monocytogenes Brain Abscesses in a Girl with Acute Lymphoblastic Leukaemia after Late Central Nervous System Relapse

Claudio Viscoli, Alberto Garaventa, Giuseppe Ferrea, Graziana Manno, Agostino Taccone and Alberto Terragna

A case of *Listeria monocytogenes* bacteraemia and meningitis with intracerebral abscesses in a girl with acute lymphoblastic leukaemia in relapse is reported. The clinical features included subacute onset with fever and marked irritability followed by seizures, meningism and confusion. The pathogen was isolated from blood and cerebrospinal fluid. Computerised tomography of the brain showed two intracerebral parenchymal localisations, in the left frontal lobe and in the right occipital lobe, respectively. The patient survived this severe infection without neurological sequelae. 2 months later she underwent allogeneic bone marrow transplantation without major complications. This case report should alert pediatric oncologists about the possible occurrence of severe intracerebral listerial infections in the immunocompromised child and suggests that this infection can be treated successfully and should not necessarily preclude continuation of antineoplastic treatments.
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INTRODUCTION

MENINGITIS IS the most common clinical form of infection caused by *Listeria monocytogenes*, accounting for about 50% of cases [1]. Nevertheless, this intracellular pathogen has never been associated with brain abscesses in immunocompromised children younger than 15 years of age [2, 3]. We describe the case of a young girl with acute lymphoblastic leukaemia in relapse, who developed listerial bacteraemia and meningitis with intracerebral parenchymal localisations.

CASE REPORT

A 6-year-old girl with a 4-year history of acute lymphoblastic leukaemia had a central nervous system relapse in December 1987. She received intensive chemotherapy with vincristine, prednisone, doxorubicin, L-asparaginase and weekly intrathecal methotrexate. On 14 January 1988 she was admitted to G. Gaslini Children's Hospital, Genova, Italy, with high fever and generalised seizures. The mother reported a 2-week history of aspecific behavioral abnormalities. The physical examination