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Examination of Bone Marrow Biopsy Specimens and Staging of Small Cell Lung Cancer

G.P.M. ten Velde, B.T.M.J. Kuypers-Engelen, A. Volovics and F.T. Bosman

Bone marrow biopsy specimens were evaluated retrospectively in 63 of 88 (72%) patients with small cell lung cancer (SCLC). Significant differences were not found between extensive disease (ED) patients with or without bone marrow metastases in survival nor in nadirs of leucocytes or platelets subsequent to chemotherapy. A panel of antibodies was used to investigate whether immunohistochemical analysis on routinely processed bone marrow biopsy specimens could detect marrow metastases more effectively than conventional microscopy. In histologically proven marrow metastases and in control SCLC sections a combination of an antibody against cytokeratin 8, 18 and 19 (NCL5D3) and an antibody against neurone specific enolase was validated for detection of metastases. In histologically negative marrow biopsy samples, however, this combination did not yield any additional tumour positive cases. Therefore, histological evaluation of a bone marrow biopsy specimen, even when analysed by immunohistochemistry, does not contribute information relevant for staging, therapy evaluation or prognosis in SCLC.
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INTRODUCTION

DESPITE SIGNS that the rate of lung cancer is falling in the USA and Europe [1] this disease constitutes an enormous problem, with as many as 9000 new cases in The Netherlands in 1988. About 20–25% of lung cancer cases are small cell lung cancer (SCLC), which is rapidly growing with early dissemination [2]. The staging of SCLC is different from non-SCLC; classification into limited disease (LD) or extensive disease (ED) is commonly accepted. LD comprises cases with tumour confined to one hemithorax and ED comprises the remaining patients [3]. In this staging, examination of bone marrow with conventional methods has been considered important, in addition to ultrasound or computed tomography of the liver, bone scan and neurological examination. However, the contribution of bone marrow examination to the staging results and its impact on prognosis in comparison with the other staging procedures is questionable [4, 5].

The significance of examination of bone marrow biopsy specimens for SCLC staging may be influenced by the effectiveness of the histological technique for detection of tumour cells in the tissue sections. In marrow aspirates, immunocytochemistry with monoclonal antibodies specific for SCLC cells has led to the detection of tumour cells in up to 72% of the tested samples [6, 7]. Against this background, we studied retrospectively a series of SCLC patients to examine the significance of routinely performed histological bone marrow biopsy in the staging of SCLC and whether the presence or absence of bone marrow metastases indicates the chance of developing severe marrow toxicity during chemotherapy. We also investigated whether the efficiency of detection of metastatic disease in routinely processed histological bone marrow biopsy samples can be improved by immunohistochemistry and, if so, whether this has any impact on the significance of marrow biopsy for clinical decisions in SCLC management.

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PATIENTS AND METHODS

From April 1980 to October 1986, 88 patients with histologically or cytologically confirmed SCLC were admitted and treated with essentially the same combination chemotherapy (cyclophosphamide 1000 mg/m^2 and doxorubicin 45 mg/m^2 on day 1 and etoposide 100 mg/m^2 on days 1, 3 and 5) (Table 1).

63 (72%) patients had bone marrow biopsy. The specimens were routinely fixed in 10% phosphate-buffered formalin, decalcified in Kristensen solution (8% sodium formate and 40% formic acid at room temperature for 1 h) and embedded in paraffin. 5 μm sections were stained with haematoxylin and eosin. Biopsy specimens were classified as tumour positive or negative on the basis of morphologically identifiable single or clustered tumour cells. For analysis of the prognostic value of the positive and negative findings, we calculated Kaplan–Meier [8]. Differences between curves were tested with the logrank test. For analysis of the clinical value of bone marrow findings, nadirs of leucocytes and platelets, after the first dose of chemotherapy, were compared with bone marrow results by t and F tests.

Table 1. Patients' characteristics

Not staged or not treated (n = 14)	
File lost	3
Not staged	1
Incompletely staged	6
Not treated (> 80 yr)	4
Staged (n = 74)	
Extensive disease	39
Limited disease	35
Total	88

Table 2. Immunoreactivity of positive primary tumour (n = 23) *and bone marrow biopsy specimens* (n = 6) *in SCLC*

Antibody	Antigen	Ref.	No. of primary tumours positive	No. of marrows positive
RCK102*	Cytokeratin 5, 8	10	NT	3 (2 focal)
NCL5D3*	Cytokeratin 8, 18, 19	11	8	3
67D11†	Human milk fat globule membrane antigen	12	NT	2
1042†	Collagen type IV	8	NT	1‖
A589‡	NSE	13	20	6 (1 focal)
K2H10‡	Chromogranin A	–	3	0
Leu 7§	Leu 7 antigen	14	0	0

*Eurodiagnostics, from † Dr J.H. Hilgers, ‡ DAKO and § Becton Dickinson.
‖ Staining of pericellular basement membrane.
NT = not tested.

Table 3. Results of bone marrow biopsy haematoxylin staining

No biopsy (n = 25) because of	
Osteomalacia (haemodialysis)	1
Age (> 80 yr)	4
Emergency treatment	2
Unknown	7
ED stage before biopsy	11
Biopsy (n = 63)	
LD (tumour positive)	2
LD (tumour negative)	35
ED (tumour positive)	8
ED (tumour negative)	18

For immunohistochemical detection of tumour cells several antibodies were used. Attempts to use monoclonal antibodies specific for SCLC were unsuccessful, due to their lack of reactivity with formalin-fixed and paraffin-embedded SCLC tissue. Therefore antibodies that react with routinely processed tissue were selected (Table 2). As general markers for epithelial cells we chose cytokeratins (antibodies reactive with cytokeratins 5, 8, 18 and 19) and epithelial membrane antigen. As markers for neuroendocrine differentiation, neurone specific enolase (NSE), chromogranin A and the Leu 7 antigen were used. Type IV collagen was selected as a basement membrane marker to detect deposition of basement membrane material, which is a characteristic of epithelial cells. 23 SCLC sections of primary tumours were used as controls.

Immunohistochemistry was done with an indirect peroxidase-labelled antibody technique [9]. The paraffin-embedded sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by incubation in 0.5% hydrogen peroxidase in methanol. First-step and second-step incubations were done for 30 min with antibodies diluted in phosphate-buffered saline (PBS) with 1% bovine serum albumin at room temperature and were followed by washing in PBS. For rabbit polyclonal antisera, a swine-antirabbit Ig-peroxidase conjugate and for mouse monoclonal antibodies, a rabbit-antimouse Ig-peroxidase conjugate were used (DAKO). To restore immunoreactivity, which might have been lost during processing, sections were pretreated in pepsin in 0.1 mol/l HCl at room temperature. Immunoreactivity was assessed with diaminobenzidine as chromogen and the sections were counterstained with haematoxylin. Staining of tumour cells in bone marrow, which occurred isolated and single or in groups, was assessed as either positive or negative.

RESULTS

Of the 63 patients who had bone marrow biopsy, histology was positive in 10 (16%). 2 of these cases would have been staged as LD, if the biopsy specimen had not contained metastases. In 25 cases a sample was not obtained (Table 3). No significant differences in survival occurred between ED patients with negative or positive biopsy specimens or in patients in whom a biopsy was not done (Fig. 1).

The clinical significance of bone marrow biopsy was evaluated by calculating differences in leucocyte and platelet nadirs after the first chemotherapy course in the ED patients. No significant differences were found in the leucocyte nadir between the ED patients with a positive or negative biopsy sample. Comparison of ED patients in whom no biopsy was done with those with a positive or a negative specimen did not yield significant differences. The same was true for the platelet nadir.

Only 6 of 10 tumour positive biopsy specimens contained abundant metastatic tumour tissue by routine histological evaluation and sufficient adequately preserved material for immunohistochemical analysis. In 3 of these cases material of the primary tumour (bronchial biopsy) was available. These specimens with an additional 20 samples from cases of primary SCLC, were also stained with the antibodies. The pattern of immunoreactivity with the selected antibodies is summarised in Table 2. The antibody against NSE stained tumour cells in most primary SCLC (20/23) and in all bone marrow biopsy specimens. The NCL5D3 anti-cytokeratin antibody stained only some of the specimens (8/23 and 3/6). The other antibodies stained tumour cells infrequently or not at all. Basement membranes were rarely

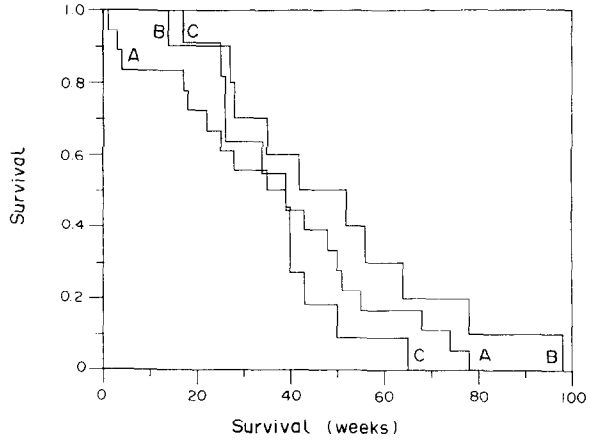

Fig. 1. Kaplan–Meier survival curve for ED patients. A = bone marrow tumor negative patients (n = 18); B = bone marrow tumor positive patients (n = 8); C = no bone marrow biopsy performed patients (n = 11).

deposited by tumour cells, as indicated by the lack of type IV collagen immunoreactivity. We therefore decided to use NCL5D3 and anti-NSE to test for the presence of morphologically undetected tumour cells in the negative biopsy specimens. Of 53 of the 63 biopsies sufficient adequately preserved material was available for immunohistochemical studies. However, in none of these specimens were immunoreactive tumour cells identified in five serial sections.

DISCUSSION

The overall frequency of tumour metastasis to the bone marrow in SCLC varies in different studies from 16% to 50% [15–17]. In our material a positive bone marrow biopsy occurred in 16% of the cases.

Tumour metastasis to the bone marrow is particularly associated with ED, because bone marrow is rarely the only site of extrathoracic metastasis [5]. We also found only 2 tumour positive bone marrow biopsy specimens in 37 patients who did not show metastatic disease. Therefore, the question as to whether or not a bone marrow biopsy in SCLC patients with ED provides essential information on prognosis and/or treatment-related toxicity is relevant. In addition it is important to address the question as to whether or not the presence of marrow metastasis is correlated with survival in patients that would have been classified as LD without a bone marrow biopsy.

Our results, confirming those of the Toronto study [4], showed no differences in survival or treatment-related toxicity between cases with or without bone marrow metastasis as assessed by routine morphology. Other investigations [17, 18] found a slightly shorter survival for marrow positive patients. In none of the reported studies was the treatment toxicity more severe in marrow positive than in marrow negative patients. This leads to the conclusion that for patients with ED, bone marrow biopsy does not provide additional clinically relevant information. For patients with LD the presence or absence of marrow metastases could be important, especially if more aggressive chemotherapy with autologous bone marrow transplantation is considered. However, the results of this approach [19] have so far not been encouraging.

Our rather negative conclusions on the significance of bone marrow biopsy results in the clinical management of SCLC are based upon routine histological examination of a single specimen. Could multiple biopsies or the use of more sophisticated labelling techniques that detect tumour cells significantly alter these conclusions? Neither marrow aspiration [17, 20] nor bilateral bone marrow biopsy [17] has increased the frequency of detection of bone marrow metastases, although repeated marrow aspirates in patients with breast cancer have yielded a much higher frequency of marrow metastases than a single aspiration [21]. On the whole, the yield of marrow metastases in histological bone marrow biopsy specimens appears to be at least as high as that in marrow aspirates [17]. With regard to the use of labelling techniques for identification of tumour cells in marrow biopsy samples, some studies yielded a higher frequency of metastases in marrow aspirates by immunohistochemistry with monoclonal antibodies directed against cellular antigens in SCLC [6, 7, 22, 23]. We therefore expected that immunohistochemistry might detect additional SCLC metastases in bone marrow biopsy specimens. Our results, however, indicated that staining for NSE and cytokeratins 8, 18 and 19 does not increase the frequency of tumour positive specimens.

This contrasts sharply with the results of immunocytochemical studies on marrow aspirates [7, 22, 23]. Evidently, the combination of marrow aspiration and immunostaining is sensitive, conceivably due to sampling advantage of the aspiration biopsy procedure and more successful immunocytochemistry with monoclonal antibodies against antigens that would be destroyed during tissue fixation and embedding. The clinical importance of these immunocytologically detected marrow metastases remains to be established in prospective studies. It could be argued that this technique detects circulating tumour cells, as has been noted in peripheral blood after surgical intervention [24].

Although our study was limited by the small sample size of the tumour positive bone marrows and the impossibility of using suitable antibodies on unfixed material, our results indicate that histological examination of a bone marrow biopsy specimen in SCLC does not yield information that is relevant for staging of the disease, for tolerance of chemotherapy or determination of prognosis. Immunohistochemistry with antibodies against cytokeratins or NSE does not yield a higher frequency of marrow metastases than conventional microscopy.

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Relation between Apocrine Differentiation and Receptor Status, Prognosis and Hormonal Response in Breast Cancer

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The release of a gross cystic disease fluid protein (GCDFP 15) by tumour explants grown in tissue culture was used to measure apocrine differentiation in 117 women with breast carcinoma. GCDFP 15 was detected by radioimmunoassay in the media from 90% of tumours (range 2–2100 ng/ml, mean 41). Tumour secretion of GCDFP 15 was higher in oestrogen receptor rich (over 20 fmol/mg) tumours ($P < 0.05$) but did not correlate with any other prognostic factors or with survival. Response to hormonal therapy was assessable by UICC criteria in 33 women (6 partial responses, 8 stable disease, 19 progression). Responders had significantly higher tumour oestrogen receptor levels ($P < 0.005$) but a lower GCDFP 15 secretion than non-responders ($P < 0.02$). Apocrine differentiation in breast cancer may be a marker for oestrogen receptor positive tumours that do not respond to hormonal therapy.
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INTRODUCTION

THE CLINICAL significance of apocrine differentiation in carcinoma of the breast is not well defined. Problems with assessing apocrine change in breast cancer have led to estimates of frequency ranging from 0.3% to 57% [1–3] depending on the histological criteria employed. Gross cystic disease fluid protein 15 (GCDFP 15) is a major protein component of human breast cyst fluid [4]. Expression of the protein in breast cancers correlates closely with apocrine differentiation [5, 6]. Using the presence of GCDFP 15 in the media from cultured tumour explants, we have measured apocrine differentiation in women with breast cancer in relation to long-term follow-up and factors of prognostic importance.

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PATIENTS AND METHODS

Tumour was obtained at biopsy or mastectomy from 117 women with carcinoma of the breast diagnosed in the Department of Clinical Surgery, Edinburgh University. The tissue was cut into explants measuring 4 × 1 × 1 mm. Four weighed explants were placed on lens paper mounted on stainless steel grids in each of three petri dishes. 2 ml Waymouth's MB752/1 medium containing N-glutamine and 20 mmol/l HEPES was added to each dish which were incubated in 95% oxygen and 5% carbon dioxide at 37°C. Medium was removed and assayed for GCDFP 15 at 24 and 48 h. Culture fluids assayed at 48 h always had low levels of GCDFP 15 and most release had occurred by 24 h; we therefore took values at 24 h as representative of tumour content. Explants were also analysed histologically to confirm presence of tumour and to ascertain cellularity.

GCDFP 15 was measured by radioimmunoassay [5] with purified antigen and antibody donated by D. E. Haagensen. Tumour material adjacent to that taken for culture was also used for histological assessment and oestrogen receptor (ER) measurements [7]. Tumours were considered positive if they contained greater than 20 fmol/ER per mg tissue [7, 8].