

Diagnostic value of neurotrophin expression in malignant pleural effusions

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Abstract. Neurotrophins (NTs) modulate the growth of human malignancies, including lung cancers. Our prospective study evaluated the accuracy of pleural NTs [nerve growth factor, brain-derived neurotrophic factor (BDNF), neurotrophin 3 (nT3) and 4 (nT4)] levels for differentiating benign from malignant pleural exudates. Levels of NTs were measured by ELISA in 170 patients with non-neutrophilic (<50%) exudative benign or malignant pleurisies diagnosed by pleuroscopy. Fifty-nine benign (9 infections and 50 inflammatory diseases) and 111 malignant (50 extrathoracic tumors, 51 lung cancers and 10 mesotheliomas) pleural exudates were diagnosed by thoracoscopy. Levels of BDNF were significantly higher in malignant than in benign effusions [17 pg/ml (0-367) vs. 8 pg/ml (0-51), $p<0.05$]. ROC analysis showed an area under the curve of 0.609 ($p=0.012$; best threshold 44 pg/ml). Pleural BDNF levels were significantly higher in pleural metastasis of pulmonary tumors and in mesothelioma than in pleural benign effusions. Finally, a higher proportion of pleural nT3 was detected in squamous cell lung carcinoma in comparison to that in non-squamous cell lung carcinoma (72.7 vs. 10%, $p<0.0001$). NTs and particularly BDNF may play a role in the pathogenesis of malignant pleural effusions.

Introduction

Pleural effusion is a common problem which manifests in a wide range of local and systemic potentially life-threatening diseases. Accurate diagnosis is difficult without resorting to invasive procedures. In particular, diagnosis of malignant pleural effusion presents a challenge since its differentiation from benign effusion is often difficult using currently available parameters derived from thoracosynthesis. Although essential for distinguishing between a transudate and exudate

(1), biochemical, microbiological and cytological pleural fluid analyses have poor value for identifying the cause of a pleural lymphocytic exudate (2). The sensitivity of cytological examination of pleural fluid and blind needle biopsy, even when combined together, is generally less than 75% (3-7). Although there is a huge interest in biomarkers (cytokines, matrix metalloproteinases, growth factors and tumor markers) for the diagnosis of pleural effusions, their lack of sensitivity and specificity limits their use. Moreover, there is a lack of accepted and reliable diagnostic criteria particularly for malignancy based on morphological imaging (CT and MR imaging) (8-10). The development of inflammation in the pleura results in an increased vascular permeability, and pleural liquid accumulation is the result of increased fluid production and/or reduced lymph drainage. This pleural fluid is enriched in proteins, inflammatory cells and mediators (5,11). Cytokine-producing cells and cytokines, such as the vascular endothelial growth factor (VEGF) which is able to increase angiogenesis and enhance the permeability of vascular endothelial cells (12,13), are thought to be important in malignant pleural fluid formation (14,15).

In malignant pleural effusion, neoplastic cells infiltrate the pleural layer and growth progressively in the pleural cavity.

Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (nT3) and neurotrophin 4 (nT4) comprise the mammalian neurotrophins (NTs), a family of structurally related growth factors that play a crucial role in the survival, development, differentiation, neurite outgrowth and maintenance of a specific neuronal population in the nervous system (16,17). They belong to a class of growth factors, secreted proteins, which are capable of signaling particular cells to survive, differentiate or grow mediated by two classes of receptors, p75 and the 'Trk' family of tyrosine kinase receptors.

Although classically known for their effects on neurons, NTs are multifunctional growth factors and exert numerous effects including differentiation of B-lymphocytes (18), histamine release from mast cells (19), formation of intramyocardial blood vessels (20) and growth of follicles in the ovaries (21) on non-neuronal cells, particularly in immunocompetent cells and lymphoid organs (22). Interest in the NTs system has grown particularly in regards to several lung diseases (23-26). Under normal conditions, Trk B (the physiological high-affinity

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receptor for BDNF) has been found to be involved in the development and maintenance of the normal structure of the lung (27,28). In addition, NTs play a role in the modulation of certain human malignancies (29,30), such as myeloma (31), fibrosarcoma (32), hepatocellular (33) and pancreatic (34) carcinoma as well as lung cancer (35,36). More importantly, there is *in vitro* evidence that compounds blocking NT signaling, such as k252a, are able to block lung cancer cell progression (37). Yet, to date, the role of NTs in malignant pleural effusion (38) and in mesothelioma (39) has been poorly investigated.

In the present study, we aimed to ascertain whether determination of levels of four NTs (NGF, BDNF, nT3 and nT4) in pleural fluid aids in identifying the etiology of non-neutrophilic pleural effusions and, in particular, in differentiating malignant from benign effusions.

Materials and methods

Patient selection. We conducted a prospective study, including 170 consecutive patients (mean age 66.4 ± 13.4 years; range 18-96; 98 males and 72 females) who were treated at the Pneumology Department, CHU, Liège, between 2004 and 2009. All patients presented with an exudative pleural effusion for whom the combination of chest X-ray, thoracic CT scanning (PQ 2000 4th generation; Picker, Cleveland, OH, USA) and thoracocentesis failed to provide an etiologic diagnosis. Therefore, the indication for thoracoscopy was justified along with pleural biopsy. In the chemical analysis, pleural effusion was considered to be an exudate according to Light's criteria (1). Thus, the pleural effusion had to meet at least one of the following criteria: ratio of pleural fluid protein to serum protein >0.5 ; ratio of pleural fluid lactic dehydrogenase (LDH) to serum lactic dehydrogenase >0.6 ; pleural fluid lactic dehydrogenase level greater than two-thirds of the upper limit of the serum normal value. Neutrophilic pleurisy ($>50\%$ neutrophils) (2) and empyema were excluded from this study. In our series thoracoscopy procedure allowed establishment of a diagnosis in each case.

Pleural fluid analysis. A diagnostic thoracocentesis of the pleural fluid (10 ml) was performed on each subject before thoracoscopy was carried out. A first sample of 5 ml was subjected to routine biochemical analysis, including tests for pleural protein, glucose, LDH and amylase levels. A second sample of 5 ml was added to a tube containing ethylenediamine-tetraic-potassium anticoagulant for differential cell counting.

For NTs measurements, 20 ml of pleural fluid was centrifuged at $400 \times g$ for 10 min at 4°C . The supernatant was separated from the cell pellet. The supernatant was immediately stored at -70°C until the ELISA was performed.

NTs (BDNF, NGF, nT3 and nT4) levels were determined according to the following commercially available enzyme-linked immunosorbent assay (ELISA) kit (Duoset; R&D Systems Europe, Abingdon, UK). The ELISA was validated by determination of the assay sensitivity and spiking recovery. Assay sensitivity was determined by calculating the mean response of 10 sets of blanks and evaluating the mean plus 2 standard deviations on the standard curve. The limit of detection was 5 pg/ml for BDNF, NGF and nT3 and 10 pg/ml for nT4. Spiking recovery was determined by adding 0, 7.5, 15.6,

Table I. Demographic characteristics and etiologies of the patients with malignant or benign pleural effusions.

Benign pleural effusions	
Age (years)	68 ± 13
Gender (M/F)	38/21
Etiology	
Infectious pleurisies (n=9)	
6 parapneumonic pleurisies	
3 tuberculosis	
Inflammatory pleurisies (n=50)	
1 Dressler's syndrome	
2 chronic pancreatitis	
3 heart failures	
1 post-radic	
6 benign asbestos	
1 uremic	
1 drug-induced	
1 post-traumatic	
3 connective tissue diseases	
2 rheumatoid arthritis	
1 scleroderma	
40 non-specific chronic inflammatory changes	
Malignant pleural effusions	
Age (years)	65 ± 14
Gender (M/F)	61/50
Etiology	
Pleural metastases of extrathoracic tumors (n=50)	
18 breast cancers	
5 ovarian cancers	
5 kidney cancers	
7 pancreatic cancers	
4 colic tumors	
2 rectal carcinomas	
2 prostatic carcinomas	
2 lymphomas	
1 skin cancer	
1 genital carcinoma	
1 acute leukemia	
1 laryngeal cancer	
1 unknown primary	
Pleural metastases of lung cancer (n=51)	
11 squamous non-small-cell carcinomas	
31 adenocarcinomas	
5 large-cell carcinomas	
2 sarcomas	
2 small-cell lung cancers	
Mesothelioma (n=10)	

31.2, 62.5, 125, 250 and 500 pg/ml of NTs to a pool of 10 pleural liquids. The recovery for BDNF, NGF, nT3 and nT4, at a concentration of 62.5 pg/ml, was 99, 61, >96 and 80%, respectively.

Table II. Pleural cell counts and biochemical parameters in the malignant and benign pleural effusions.

	Benign pleural effusions (n=59)	Malignant pleural effusions (n=111)
RBC/mm ³	2,515 (40-720,000)	4,640 (0-1,840,000)
WBC/mm ³	710 (85-14,920)	600 (4-10,000)
Neutrophils (%)	6 (0-47)	11 (0-49)
Lymphocytes (%)	62 (3-98)	52 (0-100)
Reticulo/monocytes (%)	15 (0-83)	22 (0-94)
Eosinophils (%)	2 (0-73)	1 (0-43)
Proteins (g/l)	42 (20-62.9)	41.3 (10-68.3)
LDH (UI/l)	420 (286-1,735)	551 (120-6,512)
Amylase (UI/l)	39 (12-22,540)	37.5 (4-903)
Glucose (g/l)	0.90 (0.02-1.94)	0.84 (0.14-2.73)
Protein PI/blood ratio	0.60 (0.28-0.88)	0.62 (0-1.16)
LDH PI/blood ratio	0.94 (0.15-33)	1.40 (0-27) ^a

Values are expressed as median (range); ^ap<0.05.

Etiologic diagnosis of pleural exudate. The final diagnosis of the pleural effusion was obtained by invasive pleural biopsy during a thoracoscopy. When a diagnosis of benign disease was established, based on histopathology, the patients were followed up for at least 18 months to ensure absence of a malignant pleural process. Benign pleural effusions included both infectious (parapneumonic and tuberculosis) and inflammatory pleural effusions. Malignant pleural effusions were divided into three groups: i) pleural metastasis of an extra-thoracic cancer, ii) pleural metastasis of a primary lung cancer and iii) mesothelioma.

The size of the pleural effusion was estimated in each patient by the total pleural fluid volume aspirated when starting the thoracoscopy procedure.

The protocol was approved by the local ethics committee, and informed consent was obtained from each subject prior to the study.

Statistical analysis. All data were expressed as the median (range) levels for pleural cell counts, pleural biochemical parameters and NTs. Characteristics of pleural fluid and pleural cell counts in malignant vs. benign pleural effusion were compared using the non-parametric Mann-Whitney test. A Kruskal-Wallis analysis (Dunn's multiple comparisons post-test) was used to compare pleural neurotrophin levels in subgroups of malignant vs. benign pleural effusions. To calculate correlations between variables, the Spearman rank coefficient of correlation was used. The accuracy of each pleural NT to distinguish malignant from benign pleural lesions was calculated with receiver operating characteristic (ROC) analyses. A p-value <0.05 was considered statistically significant.

Results

Clinical diagnosis. Thoracoscopic biopsies indicated benign pleural lesions in 59 patients and malignant pleural effusions in 111. Demographic characteristics and pleural effusion etiologies are provided in Table I. The gender ratio was different

with females accounting for 45% of the malignant group, while only representing 36% of the benign group (NS).

Pleural cell counts and biochemical parameters. Biochemical and cytological characteristics of the pleural effusions are provided in Table II. There was no significant difference in pleural protein, LDH, glucose and amylase level and in protein Light's ratio between the malignant and benign pleural effusions. However, LDH Light's ratio was significantly higher in the malignant effusions (p<0.05) (Table II). There was no significant difference in pleural cell counts between the benign and malignant effusions (Table II).

None of the pleural samples showed a positive bacterial growth during the culture, even when the pleural effusion was deemed to be of infectious origin.

Determination of neurotrophins in pleural effusions. The median level of pleural BDNF was 17 pg/ml (0-367) in patients with malignant pleural effusions, which was significantly higher compared to the value of 8 pg/ml (0-51) found in benign effusions (p<0.05) (Table III). By contrast, no significant difference was found in NGF and nT3 pleural levels between malignant and benign effusions (Table III). nT3 was only observed in 13.5% of the 170 pleural effusions, including 8 and 16% of benign and malignant effusions, respectively (NS). nT4 was undetectable with the exception of 1 patient with pleural metastasis of squamous lung carcinoma.

Pleural BDNF levels were positively correlated with pleural red cell counts (r=0.29, p<0.001), pleural neutrophil counts (r=0.16, p<0.05) and with pleural effusion volume (r=0.19, p<0.05). BDNF was negatively correlated with pleural eosinophil counts (r=-0.22, p<0.05) and pleural glucose (r=-0.22, p<0.01). No correlation was found with total pleural protein levels (r=-0.10, p>0.05).

ROC curve of BDNF for distinguishing between malignant and benign effusions is presented in Fig. 1. Only the measurement of BDNF showed significant value in identifying malignant effusions with an area under the curve (AUC) of

Table III. Comparison of pleural neurotrophin levels in benign and malignant pleural effusions.

Pleural neurotrophins	Benign pleurisy (n=59)	Malignant pleurisy (n=111)
BDNF (pg/ml)	8 (0-51)	17 (0-367) ^a
NGF (pg/ml)	0 (0-120.3)	0 (0-376)
nT3 (pg/ml)	0 (0-43)	0 (0-137)
nT4 (pg/ml)	0 (0-0)	0 (0-28)

Values are expressed as median (range); ^ap<0.05.

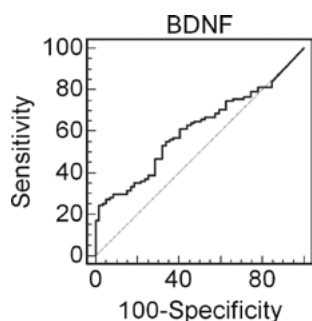


Figure 1. Receiving operating characteristic (ROC) curves of BDNF for differential diagnosis (malignant vs. benign) of the pleural effusions.

0.609 ($p<0.05$). Derived from this curve, the best cut-off point was found to be 44 pg/ml which gave a sensitivity, a specificity and an accuracy of 24, 99 and 50%, respectively. Sensitivities and specificities for different cut-off values for distinguishing benign from malignant pleurisies are presented in Table IV.

Regarding different subgroups of malignant and benign pleural effusions, a significant difference in pleural BDNF levels was noted, which was significantly higher in pleural metastasis of lung cancer and in mesothelioma when compared to benign effusions [19 pg/ml (0-232) and 27 pg/ml (0-367) vs. 8 pg/ml (0-51), respectively, $p<0.05$] (Fig. 2). ROC analysis showed BDNF may help to distinguish between pleural metastasis of lung cancer and benign effusion ($AUC=0.664$, $p<0.01$; 72 and 59% for sensitivity and specificity, respectively, for best threshold of 11 pg/ml), and between pleural metastasis of lung and extrathoracic cancers ($AUC=0.613$, $p<0.05$; 69 and 56% for sensitivity and specificity, respectively, for best threshold of 12 pg/ml) (Table V). Finally, there was a strong trend for BDNF to identifying mesothelioma from benign effusion ($AUC=0.703$, $p=0.055$; 40 and 100% for sensitivity and specificity, respectively, for best threshold of 51 pg/ml). We found no significant difference in the four neurotrophin levels according to the etiology of the benign pleural effusions [infectious ($n=9$) vs. inflammatory ($n=50$)].

In malignant pleural effusions, pleural nT3 concentrations were observed in 10% of pleural metastasis of extrathoracic cancer, in 10% of mesothelioma and in 23.5% of pleural metastasis of primary lung cancer (NS). In this last subgroup, a higher proportion of pleural nT3 was detected in squamous cell carcinoma in comparison to that in non-squamous cell carcinoma (72.7 vs. 10%, $p<0.0001$) (Fig. 3).

Table IV. Sensitivities, specificities, negative predictive value (NPV) and positive predictive value (PPV) for different cut-off values derived from ROC curves of BDNF for distinguishing benign from malignant pleurisies.

Cut-off (pg/ml)	Sensitivity (%)	Specificity (%)	NPV (%)	PPV (%)
10	63	54	44	72
15	53	68	43	76
20	47	71	39	73
25	39	71	39	73
30	39	75	39	73
35	33	81	39	77
40	28	91	41	86
45	24	98	41	96
50	17	98	39	95

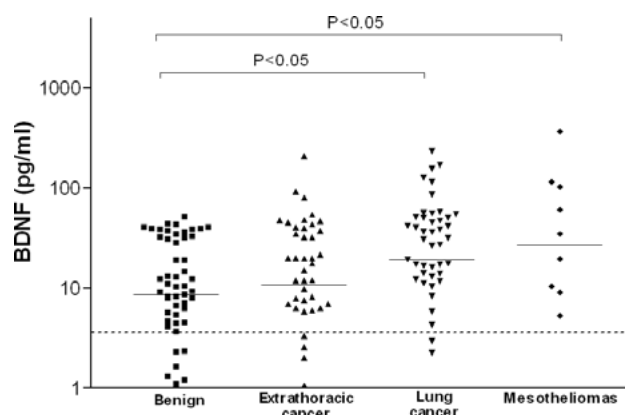


Figure 2. Comparison of pleural BDNF levels in different subgroups of pleurisy (benign effusion, pleural metastasis of extrathoracic tumor, pleural metastasis of lung cancer and mesothelioma). Dashed line represents the limit of the detection of our assay. Solid line represents the median value in the different pleurisies.

Discussion

The aim of the present study was to assess the levels of NTs in pleural effusion where etiology was not obvious. The present study demonstrated for the first time in a large and well-characterized population of patients with non-neutrophilic exudates that, even though the pleural BDNF level was higher in malignant pleural effusions, it exhibited a rather limited clinical value in distinguishing between malignant and benign pleural effusions.

ROC curve analysis indicated that BDNF at a concentration of 44 pg/ml yields the best compromise between sensitivity and specificity, although we recognize it to be fairly modest as a diagnostic tool due to a low sensitivity (24%). However, this can be compared to what is usually found with chest CT scanner which has a sensitivity that may range from 22 to 35% according to the morphological chosen criteria (40), while specificity is around 80%.

Even though several NTs were reported to influence extra-thoracic neoplastic cell differentiation and growth (29-32,41), in our study only BDNF levels were found to be significantly

Table V. Receiving operating characteristics (ROC) curves of BDNF for distinguishing between the subgroups of pleural effusions.

	AUC	p-value	Cut-off (pg/ml)	Sensitivity	Specificity
Lung cancer pleural metastasis vs. benign pleurisy	0.664	0.002	11	72	59
Lung cancer vs. extrathoracic tumor pleural metastasis	0.613	0.040	12	69	56
Lung cancer pleural metastasis vs. mesothelioma	0.516	0.590	57	40	88
Extrathoracic tumor pleural metastasis vs. benign pleurisy	0.534	0.550	43	18	97
Extrathoracic tumor pleural metastasis vs. mesothelioma	0.661	0.130	55	40	94
Mesothelioma vs. benign pleurisy	0.703	0.055	51	40	100

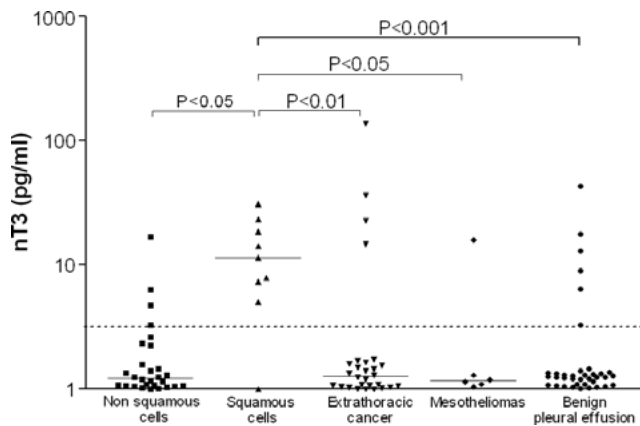


Figure 3. Comparison of pleural nT3 levels according to pleural metastasis of primary lung tumor histology (squamous cell carcinoma vs. non-squamous), pleural metastasis of extrathoracic tumor, mesothelioma and benign pleural effusions. Dashed line represents the limit of the detection of our assay. Solid line represents the median value in the different pleurisy.

increased in malignant effusions. Moreover, these increased levels were essentially due to lung cancer pleural metastasis and mesothelioma.

Our study confirms in a larger series of patients the results of Ricci *et al* who reported significantly higher concentrations of BDNF in malignant pleural effusions in comparison to inflammatory exudates and transudates (38).

The reason why BDNF is increased in malignant effusions is not clear. Although we cannot exclude that increased BDNF levels may partly be related to increased pleural endothelial permeability, the lack of correlation between pleural BDNF and protein levels suggests that there may be additional mechanisms involved. Local production of BDNF by mesothelial cells, recruited inflammatory and malignant cells is likely to contribute to the increased levels found in malignant effusions and points to the existence of an autocrine and/or paracrine mechanism (35,36,38). Although we excluded pleural fluids with high neutrophil counts (>50%), we found a significant correlation between neutrophil counts and BDNF suggesting that recruited neutrophils may contribute together with tumoral cells to local BDNF production.

In contrast, the absence of a correlation between pleural LDH, a marker of tumor activity, and BDNF supports the hypothesis that pleural BDNF levels may not be directly related to tumor growth itself, but rather to an interaction between malignant cells and local mesenchymal or inflammatory cells. The significant relationship between the volume of pleural effusion and BDNF is in keeping with the role of this NT in controlling pleural fluid homeostasis (35,38). Implication of BDNF in vascular permeability regulation is further supported by the recognition of its involvement in cerebral edema and subsequent neuronal tissue damage (42).

Immunohistochemical analysis showed that Trk B receptors, the BDNF receptors, were expressed in several neoplastic cells, fibroblasts and blood vessels (21,35). Activation of Trk B receptors may be a key mechanism in tumor cell survival after detachment from extracellular matrix (a process called 'anoikis') (43). The particularly elevated levels of BDNF in malignant pleural fluid from lung cancer cells known to express high level of Trk B receptors (36) may cause a more aggressive pleural invasion as compared to extrathoracic tumors. Notably, an inverse relationship was found between pleural glucose and BDNF levels. This supports the idea that BDNF is increased when metabolic activity of the pleura is intense as in tumor cell proliferation (44,45). Additionally, a strong correlation was found between pleural red cell counts and BDNF levels which is in line with the role of BDNF in pleural neoangiogenesis, a phenomenon critical for tumor proliferation.

In contrast to BDNF, neither NGF nor nT3 were found to be significantly increased in pleural effusion of malignancies. Furthermore, nT3 pleural levels were only detected in 13.5% of all pleurisy, with a trend to a higher percentage of detection in malignant pleurisy (16%) vs. benign effusion (8%). Pleural nT3 level was observed in the majority of squamous cell lung carcinoma (73% of cases) which sharply contrasts to what was observed in other types of lung cancer histology (~10%). Our data corroborate those reported by Ricci *et al* for lung surgical samples (36).

NGF has been reported to be more commonly a regulator of differentiation and/or survival than a cancer cell growth factor (38). Down-regulation of NGF has even been demonstrated in aggressive human malignancies (46). In light of this view, we

find no surprise that NGF levels were not increased in the malignant pleural effusions.

In conclusion, albeit of a limited diagnosis value, our results demonstrated in a large patient series that malignant pleural effusions display increased BDNF levels which may contribute to the local growth and invasiveness of tumors. This may offer a novel target in the treatment strategy for malignant pleural diseases.

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