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ARTICLE

Accelerated Apoptosis and Low Bcl-2 Expression Associated with Neuroendocrine Differentiation Predict Shortened Survival in Operated Large Cell Carcinoma of the Lung

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In order to test the hypothesis that increased apoptotic activity is connected with neuroendocrine differentiation and low differentiation degree in large cell carcinoma (LCLC) and is regulated by bcl-2 family proteins, we analysed the extent of apoptosis and tumor necrosis and their relation to the expression of bcl-2, bax, bak and mcl-1 in 35 LCLCs, of which 20 were classified as large cell neuroendocrine lung carcinomas (LCNEC) and 15 as large cell non-neuroendocrine lung carcinomas (LCNNEC). The extent of apoptosis was determined by detecting and counting the relative and absolute numbers of apoptotic cells and bodies using in situ 3´-end labelling of the apoptotic DNA. The extent and intensity of expression of the bcl-2, bax, bak and mcl-1 proteins were studied by immunohistochemistry. Also the relative volume density of necrosis was evaluated and correlated with the other parameters. Finally, all the parameters were evaluated as prognostic markers and correlated with data on the survival of the patients. Relatively high apoptotic indices were seen in both tumor types (average for both 2.53%, range 0.09–27.01%). Significantly higher bcl-2 and bak indices were detected more often in LCNECs than in LCNNECs. Immunohistochemically detected bax, bcl-2 and bak expression was independent of apoptotic index in both tumor types, while there was a statistically significant positive association between mcl-1 expression and apoptotic index in LCNNEC but not in LCNEC. There was a statistically significant association between high apoptotic index and shortened survival in LCLC. However, no association was found between tumor stage and apoptosis. The patients with LCNEC and low bcl-2 protein expression had a significantly shorter survival time than those with high bcl-2 indices. There was also a clear association between shortened survival and necrotic LCNNEC. LCLCs show relatively high apoptotic activity, which is associated with shortened survival. The expression of bcl-2, bak and mcl-1 is associated with neuroendocrine differentiation in LCLC. Finally, our results support some previous reports suggesting that bcl-2 expression in combination with some other markers involved in apoptosis and/or proliferation may be of prognostic value in cases of lung carcinoma with neuroendocrine differentiation. (Pathology Oncology Research Vol 5, No 3, 179–186, 1999)

Keywords: Mcl-1, bax, bak, neuroendocrine differentiation, prognosis

Introduction

Apoptosis is an active form of cell death which, unlike mitosis, is characterised by a number of alterations in cell morphology involving endonuclear cleavage of the DNA into oligonucleosomal length fragments.[26] Disturbances in the homeostatic mechanisms that balance cell proliferation and cell death can contribute to the growth rate of a tumor. The accumulation of neoplastic cells, for instance, does not consistently result in a decreased rate of apoptosis. On the contrary, high apoptotic activity seems to be apparent particularly in aggressive tumors such as urinary bladder carcinoma.[14,18] We have shown previously a relatively high extent of apoptosis in operated small cell lung carcinoma.[6]

The expanding family of bcl-2 oncogenes are among the main regulators of apoptosis acting at the effector stage.[16]

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Some of its members, bcl-2, mcl-1 and bcl-X_L, function as blockers of cell death, while others, bax, bak and bcl-X_S, function as promoters of apoptosis.[16,26,31] Bcl-2 family proteins possess variable numbers of bcl-2 homology (BH) regions (BH1-BH4), which determine their capacity to interact with each other or with other unrelated proteins[22,31,43] The proportions of homodimers and heterodimers that bcl-2 family proteins can form with each other finally determine the fate of a cell to either survive or die.[22,31,43]

Bcl-2 protein expression has been associated with an enhanced growth rate in tumors, probably by blocked cell death.[11,12,14] Bcl-2 is expressed in 8–30% of cases of non-small cell lung carcinoma and up to 90% of cases of small cell lung carcinoma.[7, 10] According to a previous report, the expression of bax has been predominant in NE tumors of high grade and is inversely associated with bcl-2 expression.[3] Bax expression has also been previously detected widely in prostate carcinoma.[15] Bak (bcl-2 homologous antagonist /killer), like bax, primarily promotes apoptosis, and it has been suggested that its function may be mediated by cell death inhibitory factors, particularly in cell types with a high life span.[4, 13] Transfection of mcl-1 into Chinese hamster ovary cells partially blocks myc-induced apoptosis, and mcl-1 has been shown to block bax-mediated cell death in a yeast two-hybrid system.[28] In solid tumors, mcl-1 has been expressed widely in prostate carcinoma, especially in those of a high grade.[15]

In the present paper we analyse 35 cases of large cell lung carcinomas (LCLCs) including 20 large cell neuroendocrine (LCNEC) and 15 large cell non-neuroendocrine carcinomas (LCNNEC) for the extent of apoptosis as measured by apoptotic indices, and for the level of expression of bcl-2 and its related proteins bax, bak and mcl-1. The extent of apoptosis was determined by detecting and counting the relative and absolute numbers of apoptotic cells and bodies using in situ 3´-end labelling of the apoptotic DNA. The extent and intensity of expression of the bcl-2, bax, bak and mcl-1 proteins were studied by immunohistochemistry. Also the relative volume density of necrosis was evaluated and correlated with the other parameters. Finally, all the parameters were evaluated as prognostic markers and correlated with data on the survival of the patients.

Materials and Methods

Tumor material

A total of 35 large cell lung carcinomas from patients, 29 men and 6 women, age range 39–78 years, treated surgically at Oulu University Hospital during the years 1978–1995 were included in this series. The removed lungs or lobes containing the tumor were fixed intrabronchially with 10% buffered formalin overnight and tumor samples were embedded in paraffin. LCLC was diagnosed histologically according to the WHO International Histological Typing of Lung Tumors.[42] LCNEC was identified on the basis of the immunohistochemically detectable NE markers synaptophysin and chromogranin and the histological criteria presented by Travis et al.[37] Of the 35 LCLCs, there were 20 LCNECs and 15 LCNNECs. A representative section from each tumor trying to avoid necrosis, and, thus, usually representing the peripheral parts of a tumor, was selected for the labelling of apoptotic cells and for immunohistochemical staining. Clinical follow-up and other data such as smoking history and TNM status of the tumors was collected from the hospital records. Of the 35 cases there were 24 stage I-II and 11 stage III-IV tumors. Most of the stage III-IV tumors were LCNECs (8 out of 11). Sixteen out of 35 patients (46%) including 8 patients of both subgroups died of the disease during the 5-year follow-up.

3´-end labelling of DNA in apoptotic cells

The 3´-end labelling of apoptotic DNA was performed by using an ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD) following the manufacturer´s instructions, with a few modifications as previously described.[6,39] 3´-hydroxy-DNA strand breaks in permeabilized tissue sections were enzymatically labelled with digoxigenin nucleotides using terminal deoxynucleotidyl transferase (TdT), after which the sections were exposed to peroxidase-conjugated anti-digoxigenin antibody. The color reaction was developed with diaminobenzidine and hydrogen peroxide.

Determination of apoptotic indices

Two indices were used to indicate the extent of apoptosis, the first involving the number of apoptotic cells and bodies, counted separately, per high power field (HPF) as a mean of ten HPFs (40 x objective, diameter of the field 400 µm), and the second index expressing apoptotic cells and bodies given as a percentage of the total number of tumor cells within a HPF (% index). As a positive control for the recognition of apoptosis we used a hyperplastic lymph node sample showing an increased number of apoptotic cells within germinal centres, and as a negative control a tumor sample previously shown to exhibit a high apoptotic index[6] where the TdT-enzyme had been omitted.

Immunohistochemical stainings

4 µm sections were cut from the specimens and placed on poly-l-lysine coated glass slides (Sigma, St.Louis, MO, USA). The sections were then dewaxed in xylene and rehydrated in graded ethanol. The endogenous peroxidase activity was consumed by immersing the sections in 0.1% hydrogen peroxide, and non-specific binding was blocked with 20% fetal calf serum.

Polyclonal anti-human bax, bak and mcl-1 antibodies (all three antibodies used at a dilution of 1:1000) were obtained from Pharmingen (San Diego, CA, USA) and a mouse monoclonal anti-human bcl-2 antibody from Dako (clone 124; used at a dilution of 1:50) (Glostrup, Denmark). Prior to incubation with these primary antibodies, the sections were heated in 10 mM citric acid monohydrate (pH 6.0) in a microwave oven. The avidin-biotin-peroxidase complex method was used for immunohistochemical detection. The color was developed with diaminobenzidine and hydrogen peroxide. Sections of a hyperplastic lymph node were used as a positive control for immunohistochemical stainings.

For evaluation of bax, bak, bcl-2 and mcl-1 positivity the percentage of positive tumor cells was graded as follows: 0 = none; 1 = 1 to 24%; 2 = 25–50%; and 3 = 51–100% of the tumor cells positive. The proportion of positive cells was counted in ten HPFs (objective, x 40; diameter of the field, 400 μm), and the intensity of staining was evaluated in the whole tumor area and graded as 0 = negative; 1 = weak; 2 = moderate; and 3 = strong. Finally, a staining index for bax, bak, bcl-2 and mcl-1 was given in which the sum of the qualitative and quantitative scores was calculated for each tumor specimen.

Western blot analysis

In order to test the reliability of the antibodies for bcl-2, bax, bak and mcl-1, fresh-frozen tissue from 3 large cell lung carcinomas (one LCNEC and two LCNNEC) was used. Tissue samples were homogenised in PBS, centrifuged at 10,000 g for 15 min, and samples for electrophoresis were prepared from the supernatants. SDS-PAGE was performed essentially according to Laemmli.[17] Resolved proteins were transferred to nitro-cellulose membranes.[36] The membranes were incubated overnight at room temperature in TBS (50 mM Tris-HCl/200 mM NaCl, pH 7.4) containing 5% dried milk to block non-specific binding sites. Anti-bcl-2, anti-bax, anti-bak and anti-mcl-1 antibodies (1:250, 1:2,000, 1:2,000 and 1:2,000 dilutions in TBS, respectively) were incubated with the membranes for 60 min at room temperature. Biotinylated secondary antibodies were used, after which the membranes were incubated with streptavidin-horseradish-peroxidase complex (ECL Western blotting kit, Amersham, Amersham, UK). Between each step, the membranes were washed extensively with TBS containing 0.05% Tween-20.

Finally, chemiluminescence-based detection of proteins was performed according to the manufacturer's protocol (Amersham).

Morphometric analysis of tumor necrosis

The volume density of tumor necrosis was determined by a morphometric method as described previously.[24] Briefly, a Nikon Labophot light microscope was employed, connected through a video camera (Panasonic F10) to a TV monitor (Sony) equipped with a grid of 100 points adhering to the screen. The total magnification for counting the volume density was x 100 and the total number of counts 2000 in each case.

Statistical analysis

The statistical analyses were performed with the SPSS for Windows program package (Chicago, IL. USA). Data on the apoptotic indices are presented as means with range. The

Table 1. **Apoptotic indices (ApoInd) as percentage (%) and absolute number of apoptotic cells (C) and bodies (B) per high power field (HPF) in large cell neuroendocrine lung carcinoma (LCNEC) and in large cell non-neuroendocrine carcinoma (LCNNEC).**

	Cells / C		*Bodies / B*		*C + B*		
	Mean	Range	Mean	Range	Mean	Range	Cases
LCNEC							
ApoInd (%)	0.87	0.01–7.69	1.88	0.04–19.32	2.73	0.09–27.01	20
ApoInd (HPF)	2.83	0.09–15.84	6.10	0.20–39.80	8.92	0.60–55.60	20
LCNNEC							
ApoInd (%)	0.86	0–3.83	1.41	0–3.55	2.28	0.19–6.55	15
ApoInd (HPF)	1.56	0–5.01	2.85	0–5.92	4.41	0.30–9.44	15
Both tumor types							
ApoInd (%)	0.87	0–7.69	1.67	0–19.32	2.53	0.09–27.01	35
ApoInd (HPF)	2.27	0–15.84	4.67	0–39.80	6.93	0.30–55.60	35

ApoInd (%) = Apoptotic index as percentage (%) of apoptotic cells and bodies of all the tumor cells in a HPF as determined from ten HPFs. ApoInd (HPF) = Apoptotic index as a total number of apoptotic cells and bodies, counted separately, HPF as counted from ten HPFs (40 x objective, diameter of the field 400 μm).

significances of the associations were determined using Fisher's exact probability test, the two-tailed t-test and linear regression. Univariate and multivariate analyses of the survival data were performed using survival curves and applying the Kaplan–Meyer method with log rank analysis. A probability of $p < 0.05$ was considered statistically significant.

Results

Extent of apoptosis in LCLC

The extent of apoptosis in LCLC as measured by the percentage and number of apoptotic cells and bodies is given in *Table 1*, and examples of apoptosis in LCLC in *Figure 1*. Apoptotic indices are given in percentages and as the total number of apoptotic cells and bodies per high power field (HPF). Both the LCNECs and LCNNECs showed reasonably high apoptotic indices, the mean apoptotic index as a percentage per HPF, i.e. ApoInd (%) being 2.53, range 0.09–27.01.The number of apoptotic bodies was roughly twice as high as that of apoptotic cells (mean for apoptotic bodies 1.67 and for apoptotic cells 0.87, see *Table 1*). There were no significant differences in apoptotic indices between the LCNEC and LCNNEC (*Table 1*).

Bax immunoreactivity and its relation to apoptosis

Bax immunoreactivity was seen in 83% of the cases (29/35), comprising 18/20 LCNECs and 11/15 LCNNECs. The intensity of the bax labelling was considered weak in 23/29 cases. In over half of the bax-positive cases (19/29) the labelling covered more than 50% of the tumor area. The staining intensity was similar in distribution and quantity in both tumor types (*Table 2*). No association was found between bax index and the extent of apoptosis.

Mcl-1 immunoreactivity and its relation to apoptosis

80% of the cases (28/35) showed mcl-1 immunoreactivity, including 19/20 LCNECs and 9/15 LCNNECs. 74% of all cases expressed weak staining, and mcl-1 positive tumor cells in over 50% of the tumor area were detected in less than half of the cases (17/35). Strong mcl-1 staining intensity was found significantly more often in LCNEC than in LCNNEC ($p = 0.03$ by Fisher's exact probability test) while the quantity of staining did not differ significantly. There was a statistically significant positive association between mcl-1 expression and apoptotic index ($p = 0.04$ by Fisher's exact probability test) in LCNNEC but not in LCNEC.

Bcl-2 immunoreactivity and its relation to apoptosis

40% of the cases (14/35) showed positive bcl-2 immunostaining, including 11/20 of the LCNECs and 3/15 of the LCNNECs. In most of the cases the bcl-2 labelling was graded as weak (8/14; 57%) but labelling covered more than 50% of the tumor area in 11/14 cases (78.6%). A high bcl-2 index was found more often in LCNEC than in LCNNEC ($p = 0.05$ by Fisher's exact probability test, *Table 2*). There was no association between bcl-2 immunopositivity and the extent of apoptosis.

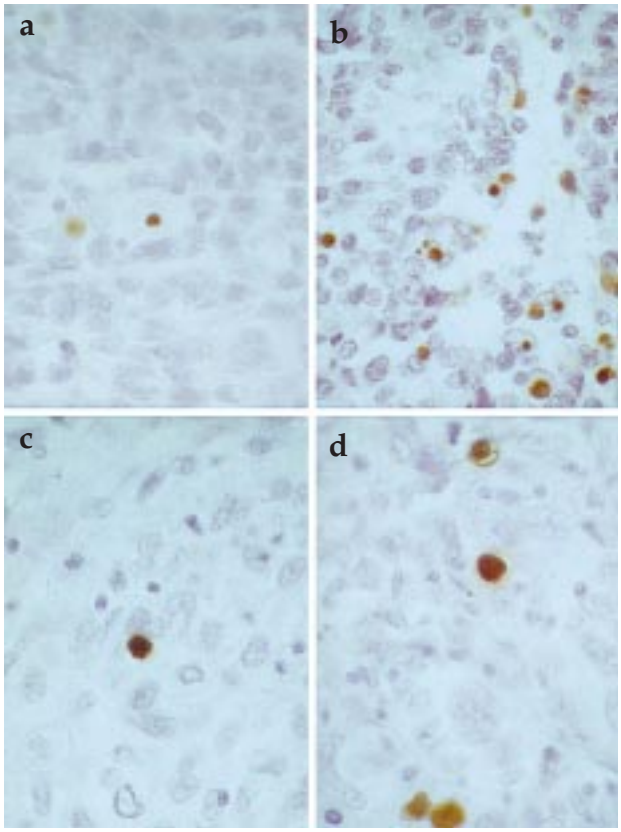

Figure 1. *Extent of Apoptosis as a Prognostic Marker in Large Cell Lung Carcinoma (a) Only one apoptotic cell visible in this large cell neuroendocrine lung carcinoma from a 71-year-old female patient, who was alive after five years since the radical operation. (b) Several apoptotic cell nuclei and smaller apoptotic bodies visible in this large cell neuroendocrine lung carcinoma from a 55-year-old female patient who died 29.5 months after radical operation. (c) Only one apoptotic cell visible in this large cell non-neuroendocrine lung carcinoma from a 71-year-old male who was alive after five years since the radical operation. (d) Four apoptotic cells can be seen in this large cell non-neuroendocrine lung carcinoma from a 60-year-old male patient who died 0.4 months after radical operation. (a-d) In situ 3´-end labelling, original magnification x 900.*

Bak immunoreactivity and its relation to apoptosis

Bak immunoreactivity was seen in 46% (16 /35) cases, of which 13/20 were LCNECs and 3/15 were LCNNECs. A weak bak labelling pattern was seen in all cases except one LCNEC, which expressed moderate bak labelling. In

half of the cases bak labelling covered over 50% of the tumor area. LCNEC showed high bak indices significantly more often than did LCNNEC (p = 0.02 by Fisher's exact probability test, *Table 2*). No statistically significant association was found between bak and the apoptotic indices.

Western blot analysis

Western blot analysis was performed from freshly frozen tissue samples from one LCNEC and two LCNNEC to validate the specificity of the antibodies used. In all 3 samples, anti-bax and anti-bak antibodies specifically detected their corresponding antigens (22 and 24 kDa) (*Figure 2*). In contrast, anti-mcl-1 antibody detected its antigen (40 kDa) in only one out of the three samples (*Figure 2*). The anti-bcl-2 antibody detected a faint 26-kDa band corresponding to the known molecular weight of bcl-2 protein (data not shown).

Other parameters and their relation to apoptosis

There was an association between the type and metastatic status of the tumors, LCNNECs having metastases more often than LCNECs (p = 0.06 by Fisher's exact probability test). There was a statistically significant inverse association between apoptotic index and the age of the patients, high apoptotic indices being detected more often in tumors from patients who were younger than 60 years (p = 0.01 by Fisher's exact probability test). There was no association between apoptosis and tumor stage in LCLC, nor when LCNEC and LCNNEC were analysed separately.

Table 2. **Bax, bak, bcl-2 and mcl-1 indices in large cell neuroendocrine carcinoma (LCNEC) and in large cell non-neuroendocrine carcinoma (LCNNEC).**

	LCNEC	*LCNNEC*	*Total*
	No of cases	*No of cases*	*No of cases*
Bax Index			
< 4	7	7	14
≥ 4	13	8	21
Bak Index			
< 2	7	12	19
≥ 2	13	3	16
Bcl-2 Index			
< 2	9	12	21
≥ 2	11	3	14
Mcl-1 Index			
< 4	8	10	18
≥ 4	12	5	17
Total No	20	15	35

High bak and bcl-2 indices are significantly more often found in LCNECs than in LCNNECs (p = 0.02 and p = 0.05 by Fisher's exact probability test).
Index is a sum of the qualitative and quantitative score determination of which is presented in Materials and Methods.

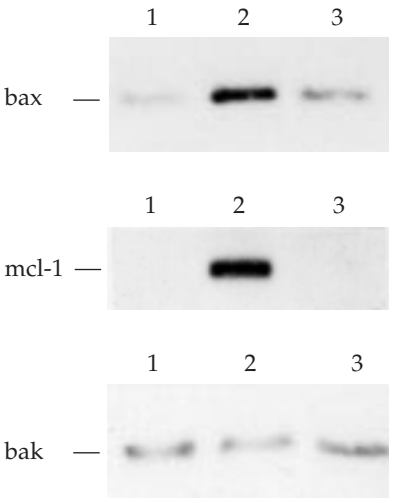

Figure 2. *Western Blot Analysis. Western blots from three cases of large cell lung carcinoma with antibodies to bax (top panel), mcl-1 (middle panel) and bak (lower panel). The bands correspond to the known molecular weights of bax (22 kDa), mcl-1 (40 kDa) and bak (24 kDa) proteins, respectively.*

The parameters as prognostic markers

The patients with LCLC showing high apoptotic indices (1.4%) had a significantly shorter survival time than those with apoptotic indices less than 1.4% (p = 0.01 by log rank; *Figures 1 and 3*). When both tumor types were analysed separately, shortened survival was associated with a high apoptotic index both in LCNEC and LCNNEC, but due to the small number of cases this association did not reach statistical significance (p = 0.10 and p = 0.09; *Figures 4 and 5*). There was a statistically significant association between low bcl-2 index and shortened survival in LCNEC but not in LCNNEC, patients with a low bcl-2 index (0-2) in their lung tumors having a significantly shorter survival time than those with a high bcl-2 index (3–6) (p = 0.02 by log rank; *Figure 6*). Furthermore, patients with necrotic LCNNEC (10% of the tumor area) had a shorter survival time than those with less necrotic tumors (p = 0.01 by log rank; *Figure 7*), while no such association was found in the LCNECs. The patients whose tumor diameter exceeded 4 cm had a significantly shorter survival time than those with a smaller tumor (p = 0.05 by log rank).

Discussion

Although apoptosis as a phenomenon was recognised two decades ago, it has only recently gained the attention it deserves among researchers. Apoptosis, along with cell proliferation, has an important role in the growth of a neo-

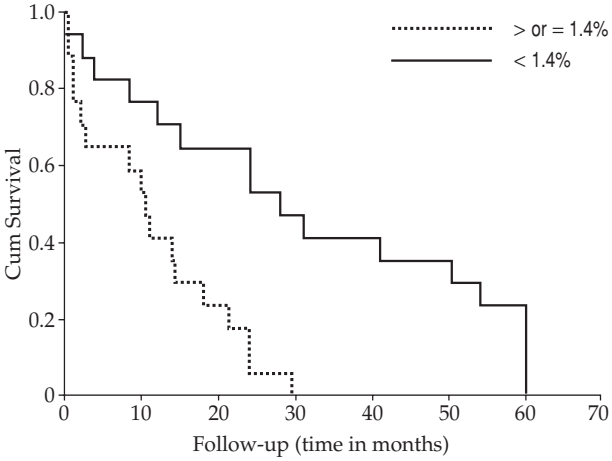

Figure 3. Survival in relation to apoptosis in LCLC. The patients with LCLC showing high apoptotic indices (1.4%) had shorter survival times than the patients with apoptotic indices less than 1.4% (p = 0.01 by log rank).

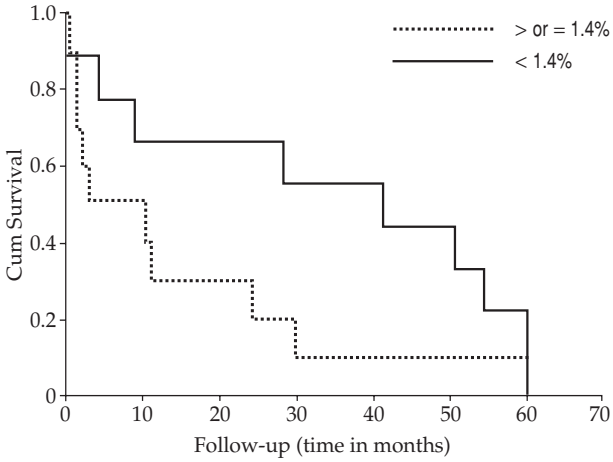

Figure 4. Survival in relation to apoptosis in LCNEC. The patients with LCNEC showing high apoptotic indices (1.4%) had shorter survival times than the patients with apoptotic indices less than 1.4% (p = 0.10 by log rank).

plasm. Since the development of techniques for measuring the extent of apoptosis in vivo, an increasing number of papers have been published reporting its extent in different tumors and in non-neoplastic tissues.[31] We have reported low apoptotic indices, i.e. less than 1%, in hepatocellular carcinoma,[33] pancreas carcinoma[40] and malignant salivary gland tumors,[32] while moderate to high rates of apoptosis (1.20–8.10%) have been reported previously in tumors sharing neuroendocrine features and a low degree of differentiation. Neuroendocrine differentiation has been associated with high apoptotic activity most distinctively in small cell lung carcinoma,[6,35] while a high apoptotic index and low differentiation degree has been demonstrated in breast carcinoma,[20] urogenital carcinoma[14] and thyroid carcinoma.[2] In this paper we hypothesised that there is increased apoptotic activity in large cell lung carcinomas, some of which show neuroendocrine differentiation,[37] whereas the rest are poorly differentiated and share a poor prognosis.[5,38] Accordingly, we were able to demonstrate high apoptotic activity in both LCNECs and LCNNECs.

One may assume that enhanced apoptosis will retard tumor growth and hence indicate a favourable prognosis compared with tumors showing low apoptotic activity. There are a few studies available on apoptosis and the prognosis for carcinomas, some of which are contradictory. In our previous report we demonstrated an association between a high rate of apoptosis and shortened survival in

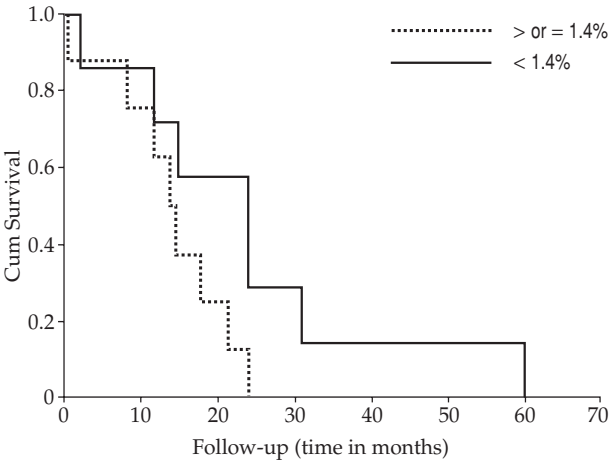

Figure 5. Patients survival in relation to apoptosis in LCNNEC. The patients with LCNNEC expressing high apoptotic indices (1.4%) had shorter survival time than the patients with apoptotic indices less than 1.4% (p = 0.09 by log rank).

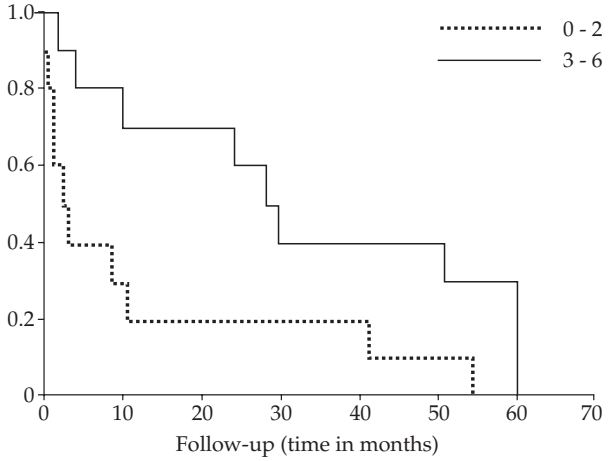

Figure 6. Survival in relation to Bcl-2 Index in LCNEC. The patients with LCNEC expressing low bcl-2 indices (0-2) had shorter survival time than the patients with high bcl-2 indices (3-6). (p = 0.03 by log rank).

non-small cell lung carcinoma,[39] and Stammler and Volm[34] reported a trend towards a similar association. High apoptotic activity also seems to be connected with a poor prognosis in hormone-dependent tumors such as breast carcinoma[30] and in prostate carcinoma,[1] whereas no association between apoptosis and the prognosis has been found in pancreatic carcinoma.[40] In hepatocellular carcinoma patients, tumors with a high growth index, i.e. tumors showing high proliferation activity and a low degree of apoptosis and necrosis, were associated with shortened survival.[33] In our present study we were able to demonstrate an association between enhanced apoptosis and poor prognosis in large cell carcinoma of the lung. It is well known that LCNECs show extensive high mitotic activity,[5,37,38] and hence proliferate rapidly. Further studies are needed to test the hypothesis that high apoptotic activity is connected with increased cell proliferation.

Our finding of more frequent bcl-2 expression in LCNECs (11/20) than in LCNNECs (3/15) is well in line with previous papers suggesting that bcl-2 is associated with neuroendocrine differentiation.[11,27,29] A high level of bcl-2 expression has previously been detected in small cell lung carcinoma cell lines.[10] We and others have also demonstrated a high frequency of bcl-2 positivity in small cell carcinomas in vivo.[6,41] Bcl-2 was not the only member of the bcl-2 family studied here which was associated with neuroendocrine differentiation, however, as bak and mcl-1 were also significantly more often expressed in LCNECs than in LCNNECs.

There are a few studies demonstrating that bcl-2 expression is connected with survival, most of which report that it is associated with a favourable prognosis. Bcl-2 expression has been linked with better survival in non-small cell lung carcinoma [8,23] and with a better short-term prognosis for breast carcinoma.[30] In contrast, Brambilla et al.[3] reported that bcl-2 overexpression correlates with a lower survival rate in cases of LCNEC and carcinoid tumors. In contrast with this we show here that low bcl-2 expression is associated with a shortened survival in LCNEC, while there are several studies demonstrating no association between bcl-2 expression and survival.[6,7,39] There seems to be controversy in the results concerning the association with bcl-2 expression and prognosis in lung carcinoma. However, our results suggest that bcl-2 expression may have a prognostic value in lung carcinoma, at least in tumors showing NE differentiation.

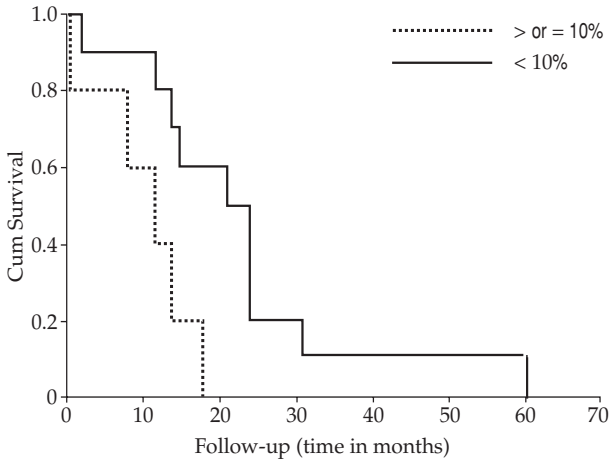

Figure 7. *Survival in relation to tumor necrosis in LCNNEC. The patients with LCNNEC showing largely necrotic tumors (10% of the tumor area) had shorter survival time than the patients with less necrotic tumors (p = 0.01 by log rank).*

Bcl-2 overexpression and bax down-regulation has previously been associated with a high apoptotic index in LCNEC,[3] but we were not able to find any association here between either bcl-2 or bax expression and the extent of apoptosis. Instead, mcl-1 showed an interesting association with high apoptotic activity in LCNNEC, but not in LCNEC. This is nevertheless contrary to the general expectation that mcl-1 expression is associated with low apoptotic activity.[16] Further research is needed to demonstrate whether mcl-1 plays any role in the regulation of apoptosis in large cell carcinoma of the lung.

Our results show that bax is widely expressed in LCLC regardless of NE differentiation. It has recently been demonstrated that certain somatic frameshift mutations in the bax gene take place in colon cancer of the microsatellite mutator phenotype,[25] suggesting that bax may be an important tumor suppressor gene in human cancer on a wider scale. Further research is needed to evaluate bcl-2 family genes as potential tumor suppressors in LCLC as well.

It is possible that there are also other pathways that are involved in the regulation of apoptotis in LCLC. One of these could be mediated by the Fas/Fas-ligand system and caspase activation.[19] In accordance with this idea, Hellquist et al.[9] were able to demonstrate the presence of Fas receptor in squamous cell lung carcinomas and Niehans et al.[21] pointed to the presence of Fas ligand in lung carcinoma cell lines. Activation of this pathway would presuppose the presence of intratumoral cytotoxic T-lymphocytes. At the moment, there are no reports available showing the presence of a Fas/Fas ligand or cytotoxic T-lymphocytes in LCLCs.

We conclude that LCLCs show relatively high apoptotic activity, and that patients whose tumors demonstrate enhanced apoptosis have a shortened survival time. Expression of bcl-2 and its related proteins bax, bak and mcl-1 is widely detected in LCLC, and bcl-2, bak and mcl-1 protein expression is preferentially associated with NE differentiation. Finally, our results support some previous reports suggesting that bcl-2 expression in combination with some other markers involved in apoptosis and/or proliferation may be of some prognostic value in lung carcinoma, at least in those cases with NE differentiation.

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