

## Imbalance of Cell Proliferation and Apoptosis During Progression of Lung Carcinomas

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**Abstract.** To evaluate the relationship between cell proliferation and apoptosis during progression of lung carcinomas, immunohistochemistry for proliferating cell nuclear antigen (PCNA) and the *in situ* end labelling (TUNEL) method for identifying apoptotic bodies were performed on paraffin sections from 135 lung carcinomas. These results were correlated with the corresponding tumor volumes as a model of disease progression in lung tumors. We found that, with increasing tumor volume, the proliferation rate decreased significantly, whereas the apoptotic rate increased. There was no relationship between apoptotic and proliferative indices except in carcinomas with a tumor volume between 51 and 100 cm<sup>3</sup>. These data suggest that progression of lung carcinomas, i.e. the increase in tumor volume, is accompanied by an increase in apoptosis rather than an increase in cell proliferation.

The balance of proliferation and apoptosis plays an important role in the control of tumor growth. In general, progression of tumor growth is characterized by a net increase in the number of tumor cells. This could be due to increased proliferation and/or decreased apoptosis, or both. There is a lot of evidence in the literature that cell proliferation and apoptosis are often related to each other (1-8). However, several studies did not confirm this positive association (9-14). No study has yet systematically evaluated proliferation and apoptosis during progression of tumor growth. To evaluate the role of proliferation and apoptosis in the development of lung carcinomas, 135 lung carcinomas were quantitatively analyzed by immunohistochemistry for the proliferating cell nuclear antigen (PCNA) and by *in situ* end labelling for apoptotic cells and correlated with the

corresponding tumor volume at the time of operation as a model of disease progression in lung cancer.

### Material and Methods

**Patients.** One hundred and thirty-five patients with histologically proven resectable non-small cell lung cancer (NSCLC) were recruited for evaluation of tumor size and for determination of tumor cell proliferation and apoptosis. All patients had been surgically treated at the Heidelberg-Rohrbach Chest Hospital, Germany and had received no post-operative therapy. The tumor diameter and the calculation of the tumor size was done intra-operatively by the resection of the whole tumor.

**Determination of the tumor cell proliferation.** Nuclei of proliferating tumor cells were stained with the antibody for PCNA (Dianova, Hamburg, Germany; clone PC10) at a dilution of 1:10. The tumor cell proliferation was scored by selecting the maximally immunostained areas and counting PCNA-positive and -negative tumor cells at x400 magnification and with an eyepiece grid. All reactive cells were counted as positive regardless of the intensity of staining. In each case, a minimum of 500 cells were counted, and the fraction of positive cells was determined. The cases were scored without knowledge of other clinical parameters.

**Assessment of apoptosis.** Apoptotic cell death was detected with a nonradioactive 3'end DNA-labelling technique (TUNEL reaction) using the *in situ* cell death detection kit (Boehringer, Mannheim, Germany). First, the paraffin-embedded specimens were dewaxed by washing in xylene, rehydrated through a graded series of ethanol and H<sub>2</sub>O, and finally incubated in PBS. The tissue sections were treated with proteinase K and rinsed with PBS. Endogenous peroxidase was blocked with 0.03% hydrogen peroxide in PBS and subsequently rinsed with PBS. Cells were permeabilized in 0.1% Triton X-100 - 0.01% sodium citrate for 2 min on ice. Then, they were rinsed with PBS and incubated with the labelling-reaction-mixture containing fluorescein-labelled nucleotides and terminal deoxynucleotidyl transferase. The slides were rinsed again with PBS and treated with anti-fluorescein antibody conjugated with peroxidase, diluted 1:2. After washing with PBS, peroxidase activity was made visible with 3-amino-9-ethylcarbazole, which gives a reddish reaction product. Counterstaining was performed with hematoxylin. For negative controls, terminal deoxynucleotidyl transferase was omitted. As positive control, DNase-treated specimens were used. The apoptotic indices were calculated as the

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Table I. Proliferation and apoptosis in human lung carcinomas subdivided according to tumor size.

Tumor volume (cm <sup>3</sup> )	n	PCNA index <sup>a</sup> (%)	(range)	Apoptotic index <sup>a</sup> (%)	(range)
A <50	49	22.3	(0-72)	9.1	(0-31)
B 51-100	28	25.2	(0-64)	11.3	(0-35)
C 101-200	13	19.5	(0-46)	10.3	(0-34)
D 201-250	24	15.5	(0-56)	13.1	(0-51)
E >250	21	17.6	(0-40)	12.5	(0-33)

<sup>a</sup>mean values

PCNA index: A vs E:  $p=0.004$ ; Apoptotic index: A vs E:  $p=0.02$  (Student's *t*-test)

ratio of TUNEL-positive cancer cells to the total number of cancer cells, for at least 1,000 cells.

## Results

In order to determine whether or not the rate of proliferation and apoptosis changed with the progression of lung cancer, we investigated tumor specimens of 135 patients that were obtained at the time of operation and were classified according to tumor volume. In Table I the proliferative and apoptotic indices in lung carcinomas subdivided according to tumor size are summarized. The PCNA index was higher in small tumors than in large tumors (22.3 vs 17.6;  $p=0.004$ ). On the other hand, the apoptotic index (AI) was lower in small tumors and high in large tumors (9.1 vs 12.5;  $p=0.02$ ). That means, with increasing tumor size, apoptotic tumor cell death overcomes tumor cell proliferation (Figure 1) and thus may be responsible for retardation of tumor growth. As expected, there was no correlation between PCNA index and AI for all tumors (Figure 2). However, there was a positive linear correlation between proliferation and apoptosis in carcinomas with a tumor volume between 51 and 100 cm<sup>3</sup> ( $r=0.51$ ,  $p<0.05$ ; data not shown). In conclusion, these data suggest that progression of lung carcinomas is accompanied by a decrease in cell proliferation and an increase in apoptosis.

## Discussion

Cellular proliferation and apoptosis have each been shown to play crucial roles in tumor development. However, the interrelationships and relative contributions of these processes to tumor progression have yet to be defined. In the present study, we compared tissues of lung carcinomas

of different tumor size at the time of operation as a model of disease progression in lung tumors. We found that indices of apoptosis and proliferation changed with tumor progression, however, at different rates. It must be borne in mind that all tumor specimens were taken from different patients. Our results showed that there was a significant increase in apoptosis and a decrease in proliferation in large tumors compared to tumors with a small tumor size. There was no relationship between apoptotic and proliferative indices, except in carcinomas with a tumor volume between 51 and 100 cm<sup>3</sup>.

The fact that the growth kinetics of tumors change with increasing tumor size is common for experimental tumors (15). As a tumor grows, changes occur in cell proliferation and cell loss. It is also common that, with increasing tumor size, tumors develop regions of necrosis. Using published data on six types of transplanted mouse tumors, it can be seen that the general tendency was that the retardation of tumor growth was due more to increase in cell loss than to decrease in growth fraction (15).

Data with human tumors are scarce. Vakkala *et al.* (16), using primary and recurrent tumors of the breast as model for tumor progression, found that apoptosis and proliferation are increased during breast cancer progression. Mommers *et al.* (17) found, for poorly-differentiated breast lesions, a significant increase in mitotic index (MI) and apoptotic index (AI) from hyperplasia to poorly-differentiated DCIS (ductal carcinoma *in situ*). From DCIS to poorly-differentiated invasive carcinoma, the MI increased significantly and the AI decreased. In renal carcinomas, with an increase of cytological tumor grade and proliferative activity, a decrease of apoptotic rate was found (11). Dixon *et al.* (18) explored the relationship of the size of leiomyomas to their proliferative index. They found that the mitotic count and the PCNA and Ki-67 labelling indices decreased in tumors over 6 cm in tumor diameter. However, this did not reach statistical significance, perhaps because of the small number of cases.

The relationship between apoptotic and proliferative indices are not clear. Several studies described a positive correlation between apoptosis and proliferation (1-8). However, many examples exist where AI was not found to be associated with proliferation (9-14). In our present study, there was also no relationship between apoptotic and proliferative indices, except in carcinomas with a tumor volume between 51 and 100 cm<sup>3</sup>. Various regulatory mechanisms operating in the cell cycle checkpoint as well as in the apoptotic pathway are known (5). P53 is one of the important regulators involved in these processes. The p53 gene regulates the apoptotic process as well as the cell cycle (19). Mutated p53 may lead to uncontrolled proliferation and suppression of p53-dependent apoptosis. There are some studies which describe that p53 expression was associated with proliferation but not with apoptosis (4). This

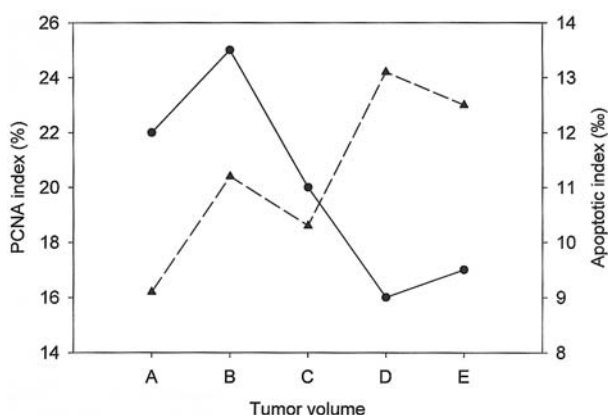


Figure 1. PCNA index and apoptotic index as a function of tumor volume of human lung carcinomas. Abscissa (tumor volumes): A,  $<50 \text{ cm}^3$ ; B,  $51-100 \text{ cm}^3$ ; C,  $101-200 \text{ cm}^3$ ; D,  $201-250 \text{ cm}^3$ ; E,  $>250 \text{ cm}^3$ .

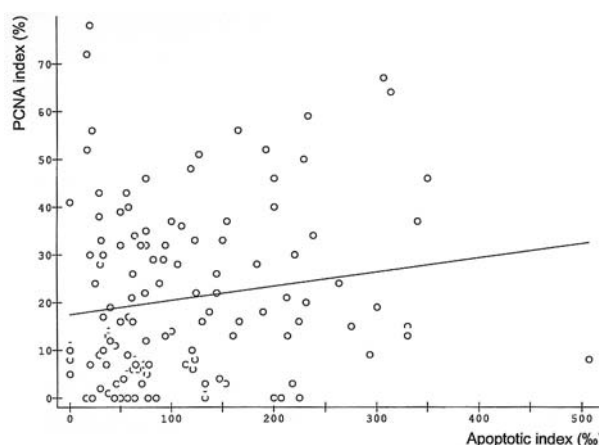


Figure 2. Relationship between PCNA index and apoptotic index of human lung carcinomas ( $n=135$ ). Linear regression analysis:  $r=0.15$ ,  $p<0.05$ .

could indicate that mutant p53 may be more closely related to the modulation of cell proliferation than of apoptosis. However, besides or together with p53, many other proteins are also involved in the control of cell proliferation and cell death (5). Thus, depending on the activity of these proteins, the rate of cell death may exceed the proliferation rate and *vice versa* (20).

In conclusion, the net increase of the number of cells during progression of lung carcinomas is accompanied by an increase in apoptosis rather than an increase in cell proliferation, suggesting that in large lesions apoptotic-related mechanisms are most important and that this imbalance between apoptosis and proliferation may be responsible for retardation of tumor growth.

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