Expression of p53, bcl-2 and nm23 proteins in squamous cell lung cancer

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Abstract

Introduction: The evaluation of lung cancer molecular profiles is an essential element of the therapeutic process in that type of neoplasm. The analysis of apoptotic and metastasis-linked proteins is an important goal because of the key role of those processes in carcinogenesis. The aim of this study was to evaluate the expression of apoptosis regulatory proteins p53 and bcl-2 as well as antimetastatic marker nm23 in squamous cell lung cancer, taking into account the clinical and pathological data.

Material and methods: Thirty tissue specimens from patients undergoing therapeutic or diagnostic thoracic surgery were included in the study. All markers were assessed with immunohistochemistry method on paraffin-embedded tissue.

Results: Nm23 expression was observed less frequently in specimens with cancer cell emboli in blood vessels or lymph node metastasis. In cancers with lymph node metastasis, the coexpression of p53 and bcl-2 was found statistically more often than in lymph node negative cases. There was no correlation between p53, bcl-2 and nm23 expression and 2-years survival time.

Conclusions: Our study indicates a marked heterogeneity of p53, bcl-2 and nm23 expression in squamous cell lung cancer and the potentially unfavorable influence of p53 and bcl-2 coexpression. Less frequent nm23 expression seems to be connected with morphological signs of metastatic process.

Key words: p53, bcl-2, nm23, immunohistochemistry, lung cancer
death that is essential for oncogenic function of this protein [3]. Decreased cell ability for apoptosis in response to injury factors results in the appearance of cell clones with DNA alteration. That is widely accepted as a crucial step in carcinogenesis. The nm23 gene, encoded nm23 protein, is known as metastasis suppressor. The mechanism of antimetastatic nm23 action is not finally explained. Results of previously published studies indicate the possibility of nm23 interactions with centrosomal kinases, regulation of GTP-ases and direct influence on DNA structure. The above mentioned processes can raise and enhance the ability of programmed cell death and diminished cell motility [4].

The apoptotic regulatory protein’s prevalence in lung cancer has been widely described, but data concerning immunoreactivity of p53 and bcl-2 proteins in relation to clinicopathological parameters separately analysed for squamous cell type is rare and incomplete [5]. The precise role of nm23 protein in lung cancer remains controversial, because the nm23 protein function can be distinct in cancers of different primary sites or the same anatomic site but different histology [4].

The aim of our study was the immunohistochemical evaluation of p53, bcl-2 and nm23 expression in squamous cell lung cancers in relation to basic clinical and pathological parameters such as: clinical advance, tumor size, presence of lymph node metastasis and tumor cell emboli in blood vessel. The initial analysis of p53, bcl-2 and nm23 expression impact on prognosis was also concerned. The 2-years survival time was compared with the immunoreactivity of three studied proteins. The assessment of survival time was also presented in a group of patients with concomitant p53 and bcl-2 presence as compared to cases without that type of coexpression.

### Material and methods

Thirty lung cancer tissue specimens were included in this study. All were obtained from patients who underwent radical resection (lobectomy or pneumonectomy with neoadjuvant or adjuvant chemotherapy as needed) or diagnostic thoracotomy in advanced cases with post-operative chemotherapy and radiotherapy in IIIIB stage and palliative chemotherapy in IV stage cases. The pathological diagnosis was based on WHO criteria [6] and advance stage was assessed according to TNM classification [7]. The detailed characteristic of the study group is shown in Table 1.

The expression of studied molecular markers in different TNM stages was done separately for I, II and IIIA stages but IIIB and IV stages were analysed as one group because of the small number of cases. The assessment of p53, bcl-2 and nm23 was done separately for the N status and different tumor size — T1 versus T2 (T3 and T4 cases were excluded because of the small number of cases). The analysis of studied protein was done separately for patients with or without cancer cells blood vessel emboli according to recently published data [8].

The immunohistochemical staining was performed on paraffin-embedded tissue specimens using the avidin/biotin method (ABC Kit Novocastra) with monoclonal antibodies against p53 and bcl-2 (clone DO7 and Bcl-2/100/D5, respectively Novocastra Lab. Ltd, UK) and streptavidin/biotin kit (LSAB, Dako Denmark) with polyclonal antibody against nm-23 (Polyclonal Rabbit Anti Human nm-23 protein, DAKO A0096). Sections of 5-µm thickness were deparaffinised in xylene (3 × 5 min), rehydrated through a graded alcohol and washed in distilled water. The next step was heat-induced epitope retrieval — for p53 and bcl-2 in microwave oven (800 W, 2 × 15 min) and for nm23 in bath water (800 W, 2 × 15 min). Non-specific endogenous peroxidase reactivity was blocked using periodic acid (2.28%, 30 s) and then sodium borohydride (0.02%, 2 min) for p53 and bcl-2 or hydrogen peroxide (3%, 10 min) for nm23. Tissue specimens were treated with primary antibodies at room tempe-

| Table 1. Characteristics of 30 patients with squamous cell lung cancer |
|---------------------------------|-----|----------------|
| Feature                        | Number of cases | Percentage |
| Gender                         |           |             |
| Men                            | 24         | 80          |
| Women                          | 6          | 20          |
| Age                            |             |             |
| £ 60 year                      | 12         | 40          |
| > 60 year                      | 18         | 60          |
| Tumor                          |             |             |
| T1                             | 6          | 20          |
| T2                             | 18         | 60          |
| T3                             | 2          | 7           |
| T4                             | 4          | 13          |
| Lymph nodes                    |             |             |
| N0                             | 13         | 43          |
| N1                             | 7          | 23          |
| N2                             | 10         | 34          |
| Metastases                     |             |             |
| M0                             | 28         | 93          |
| M1                             | 2          | 7           |
| Stage                          |             |             |
| I (A and B)                    | 9          | 30          |
| II (A and B)                   | 9          | 30          |
| III A                          | 6          | 20          |
| IIIIB and IV                   | 6          | 20          |
| Carcinomatous emboli           |             |             |
| Absent                         | 16         | 53          |
| Present                        | 14         | 47          |
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When incubated with 3,3′-diaminobenzidine (DAB) as a chromogen. The preparations were counterstained with hematoxylin. Negative controls were performed by omitting the primary antibodies. The results of the immunostaining were evaluated in light microscope. The case was scored as positive if more than 10% of cancer cells had a nuclear accumulation of p53 protein. The staining for bcl-2 and nm23 proteins was considered positive in cases where more than 20% of cancer cells showed cytoplasmic staining.

The statistical results were evaluated by CSS Statistica for Windows (version 5.0, Sta-Soft Inc. Tulsa OK, USA). Correlation between studied parameters was analysed by the chi squared test with Yates correction as needed. P value <0.05 was considered as statistically significant. The survival curves were done by Kaplan-Meier method.

**Results**

**P53, bcl-2 and nm23 expression in lung cancer**

The nuclear staining of p53 protein was revealed in 20 (66%) squamous cell lung cancers. No cytoplasmic reaction with anti-p53 antibody was visible in all analysed cases. Although marked heterogeneity in the immunostaining was noticed according to the percentage of positive cells and the intensity of staining, the cases with high (more than 50%) percentage of p53 immunoreactive cancer cells were predominant.

Expression of bcl-2 protein was detected in 17 (57%) cases. Bcl-2 protein immunostaining was located in cytoplasm and the percentage of bcl-2 positive cancer cells varied among specimens from 20% to 100%. In most analysed cases the bcl-2 positive lymphocytes were present and qualified as positive intrinsic control.

The nm23 immunostaining was located in cytoplasm of the cells in 22 (73%) of the cancer specimens. Similarly for apoptosis regulatory proteins, considerable disparity of colourful reaction was visible. The frequency of unfavourable coexpression of p53 and bcl-2 was analysed distinctly and that characterised phenotype was present in 12 (38%) cases.

The comparison of p53, bcl-2 and nm23 expression with clinical and pathological parameters

In cases with blood vessel emboli the expression of nm23 was present statistically less frequently than in cases without blood vessel involvement (54% vs. 88%, p = 0.034). Such type of relationship was not found for p53 and bcl-2.

Figure 1 represents the expression of studied markers in relation to N status. The coexpression of p53 and bcl-2 was detected significantly more often in the group of cancer with lymph node metastasis (N+ group) as compared to the group with negative nodes (N0 group) (53% and 15% respectively, p = 0.042). Analysis of separate markers presence revealed double the percentage of bcl-2 positive cases in N+ group than N0 ones. The difference was close to the border of statistical significance (65% and 38% respectively, p = 0.078). The nm23 expression in N+ group was detected in a markedly, but not statistically significant, lower percentage of cases than in N0 group. (65% vs. 84%, p > 0.05). There were no marked differences in p53 expression between N0 and N+ group of cases (61% and 71%).

P53, bcl-2, nm23 protein expression and p53+/bcl-2+ phenotype failed to correlate with T1 or T2 status. There were no marked differences in studied markers expression according to TNM stage except bcl-2 protein, the presence of which was observed more often in higher stage cases (III and IV) than in less advanced ones (I–II) (33% in I, 44% in II, 100% in IIIA stages and 60% in IIIB with IV group) but the difference was below statistical significance level. Sixteen patients in our group survived more than two years (seven in stage I, five in II, two in IIIA, two in IIIB and IV group). That represents 53%. In none of the analysed cases the correlation of p53, bcl-2 and nm23 expression and 2-years survival was revealed (Fig. 2).

**Discussion**

The pathological p53 protein has an elongated half-life time which results in accumulation in
the cell nucleus and can be detected via the immunohistochemical method. The nuclear immu-
noreactivity with commercially available anti-p53 antibodies demonstrates a high level of correspon-
dence with the mutation of the p53 gene, especially missence type [9]. The results of our study indi-
cating a high (66%) percentage of p53 positive ca-
se of squamous cell lung cancers are in accordance
with the previously published results [10, 11].

Moreover, in squamous cell lung cancer the p53
expression can be detected more often than in whole groups of non-small cell lung cancer (NSCLC) and adenocarcinomas [11–13].

The meaning of this fact remains unclear. It is probable that pathological p53 protein plays an especially important role in squamous cell lung cancer development, serving as a molecular sign of carcinogenic influence of tobacco smoke. It was proven that benzopyrene from cigarette smoke could interact with p53 gene forming its selective adducts
which resulted in arising and preservation of some type of p53 gene alterations like point T→G mutation [14]. More discrepancies were found according to bcl-2 protein. Most authors have indicated a lower percentage of bcl-2 positive cases among NSCLC cancer as compared to our results, but only a few publications separately analysed squamous cell lung cancer. Yaren et al. [15] de-
scribed a similar to ours percentage of bcl-2 posi-
tive cases in that type of cancer. The high percen-
tage of bcl-2 positive squamous cell lung cancers
could reflect the important role of bcl-2 in that type of cancer.

Emboli as a result of cell penetration into blo-
od vessel are considered to be one of most impor-
tant features of malignancy that present tumor
ability to form distance metastases. In concordan-
ce with recently published results, the detection
of blood vessel invasion can stratify patients among
TNM stages and serve as a new potential progno-

Figure 2. Cumulative Kaplan-Meier proportion of survival according to nm-23 (A), bcl-2 (B), p53 (C) proteins expression and coexpression of p53 and bcl-2 (D)
Negative correlation between nm23 expression and cancer cell blood vessel emboli revealed in our study could confirm the nm23 suppressing function on metastases formation. The nm23 negative role in metastasis process was underlined by other authors. Especially clinically important was the observation by Ohta et al. [16] indicating that nm23 absence could be a predictive factor for micrometastasis creation.

There were many published results on p53, bcl-2 and nm23 correlations with TNM stage of lung cancer, but only a few studies concerned that relation for squamous cell lung cancer. We did not find the correlation of p53 expression and N status of the tumour, which agrees with other authors’ results according to whole NSCLC group [13, 17] as well as squamous cell subtype [5]. The relation of bcl-2 immunoreactivity with lymph node metastasis in lung cancer is unclear. Most authors hypothesise that bcl-2 had negative or no correlation with lymph node metastasis [13, 15] but there are a few studies suggesting the opposite [18]. Unfortunately, in the above mentioned studies the results were done for the whole group of NSCLC without detailed analysis of histopathological subtypes.

The significantly higher percentage of p53+/bcl-2+ phenotype among N+ tumours detected in our study suggests that the enhanced expression of bcl-2 protein connected with the simultaneous inhibition of normal p53 function may represent more aggressive tumour cases. Decreased ability of cells to programmed death enhance possibility of cell surviving in extremely unfavourable conditions during metastatic process.

The lack of significant correlation of p53 and bcl-2 with T status in NSCLC was reported by others. Piyathilake et al. [5] also published data on squamous cell lung cancer. In their study, in accordance with ours, there was a majority of less advanced cases with T1 or T2 feature, and the influence of apoptosis alteration for T status remains unexplained.

The relationship with nm23 expression and clinical or pathological advance stage is not clear for NSCLC and its histopathological subtypes. Bosnar et al. [19] did not reveal the correlation between nm23 presence and tumor size or clinical advance stage in squamous cell lung cancer patients. Lai et al. [20] found higher nm23 expression among squamous cell lung cancer cases without mediastinal lymph node metastases. There were some reports confirming the hypothesis that two or more molecular markers evaluation could be useful for identifying patients with a high risk of metastasis. Chen et al. [21] proved that lower nm23 protein and E-cathderin expression correlated with higher advanced stage and lymph node metastasis [21]. The same conclusion was presented by Kogan et al. a year before [22].

The assessment of prognosis in squamous cell lung cancer is based on TNM classification; the role of molecular markers is still controversial. The key role of tumour resistance to apoptosis in cancer progression implicates the potentially negative influence of p53 and bcl-2 markers on prognosis. We did not confirm the negative prognostic value of p53 and bcl-2 in squamous cell lung cancer. Mitsudami et al. [9] in their detailed meta-analysis reported that bcl-2 had negative or no correlation with lymph node metastasis [13, 15] but there are a few studies suggesting the opposite [18]. Unfortunately, in the above mentioned studies the results were done for the whole group of NSCLC without detailed analysis of histopathological subtypes. The prognostic value of bcl-2 protein is unclear. Despite the bcl-2 negative influence on apoptosis, the results of the previously published studies indicate positive or no prognostic value of bcl-2 in lung cancer [23] and this accorded our results.

There is no agreement on nm23 relationship with prognosis for lung cancer patients. In most published results concerning NSCLC there were no detailed analysis of squamous or adenocarcinoma subtypes. Some authors confirmed the opposite correlation between nm23 protein expression and prognosis [24], but others did not prove such a relationship [25]. Immunohistochemical evaluation of at least two markers including nm23 seems to be promising. In the Hsu et al. [26] study longer survival time was observed in a group with nm23 positive and FAK (Focal Adhesion Kinase) negative phenotype of lung cancer cells. Relatively rare studies on squamous cell lung cancer indicated lack of nm23 influence on prognosis in lung cancer as in our group of cases [19].

The relationship of p53, bcl-2 and nm23 expression with prognosis and TNM status in lung cancer is controversial. The bcl-2 expression was confirmed in bronchial epithelium with preneoplastic changes and even in normal bronchial epithelium where the basal cells expressed bcl-2 [27, 28]. That indicated that bcl-2 expression could appear in the early stages of the evolution of squamous cell lung cancer, providing the growth advantage. Ohmura et al. [13] had the interesting hypothesis that bcl-2 protein appeared in bronchial epithelial cells in response to cell stress done by cancerogenous factors from cigarette smoke resulting in genetic instability and simultaneous presence of me-
chanism retrieving stability like telomerase activation. The expression of altered p53 protein was also described in preneoplastic bronchial changes preceding squamous cell lung cancer development [5]. It seems that early bcl-2 expression with p53 protein alteration has a great impact on the promotion of neoplastic changes rather than progression. That hypothetic sequence of apoptosis markers presence in bronchial epithelium can partially explain the difficulty in assessing the correlation of p53 and bcl-2 immunoreactivity in cancer tissue with survival. Many other factors can affect the difficulty of estimating the clinical value of molecular markers expression in lung cancer tissue: these include the subjectivity of immunohistochemical method or the incomplete representation of different case stages in studied material, especially lack of advanced ones.

Conclusions

On the basis of achieved results, the correlation of p53 and bcl-2 coexpression in lung cancer tissue with the presence of lymph node metastases was revealed. Our results indicate also that absence of nm23 protein expression in primary lung cancer is connected with lymph node metastases and cancer emboli in blood, which could confirm the suppression function of that protein on metastases creation.

The promising results of our analysis suggest that further studies on the discrimination of new molecular markers, especially connected with basic cell life processes, are necessary for determining modern and more effective regimens of lung cancer treatment.

References