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Immunoexpression of P16^{INK4a}, Rb and TP53 proteins in bronchiolar columnar cell dysplasia (BCCD) in lungs resected due to primary non-small cell lung cancer

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Abstract: Lung cancer is the leading cause of death worldwide. High mortality comes out mainly of the fact that majority of the cases are diagnosed in advanced stadium. An expanded diagnostics of precancerous conditions would certainly contribute to lowering the mortality rate. Many of the molecular changes accompanying the multistep cancer development could be observed using the immunohistochemistry method. In this paper we describe the morphology and cell cycle proteins immunoexpression of the novel probable preinvasive lesion - bronchiolar columnar cell dysplasia (BCCD). Thirty cases of BCCD selected out of 193 patients population, treated for primary non-small cell lung cancer were investigated. Loss of P16^{INK4a} protein was observed in 70% of all cases and was statistically significant in patients with adenocarcinoma. Two cases show abnormal cytoplasmic localization of this protein. TP53 protein accumulates in 26.7% of all BCCD. Rb protein was active in 48.3% of the BCCD cases. In two cases we observed differentiation of the cells composing BCCD into multilayer epithelium of the squamous type, which occurs with formation of desmosomes. We suppose that BCCD may be preneoplastic lesion leading to adenocarcinoma as well as to peripheral squamous cell lung cancer.

Key words: BCCD - Bronchiolar columnar cell dysplasia - Preneoplastic lesion - Lung cancer - Adenocarcinoma - Peripheral squamous cell carcinoma

Introduction

Lung cancer is the leading cause of cancer deaths worldwide [1-3]. High mortality rate is first of all due to the fact that most cases of lung cancer are diagnosed in an advanced stage of development. Better diagnostics of preinvasive conditions, which are thought to be precancerous lesions, would certainly contribute to lowering the mortality rate. However, it is a big challenge, considering that the whole lung is a potential field of the cancerogenesis, and that the neoplasia has many different potential ways of development [5].

A long term exposure of the epithelium lining the airways to different carcinogens, including most of all cigarette smoke, causes a number of mutations of the cells placed in different compartments. These multiphase changes with a diversified morphology result in the development of a fully invasive type of cancer [6,7]. Thus, lung cancer may develop both in the primary bronchus, small bronchioli and alveoli.

The multistep carcinogenesis of the peripheral non-small cell lung cancer of the lung still seems to be unclear. Adenocarcinoma (AC) of the lung, which arises mainly peripherally, is the common histological type of lung cancer among patients around the world. The frequency of the appearance of its precancerous lesion - atypical adenomatous hyperplasia (AAH) is rather unknown [8-12]. Its occurrence is still unproportionally infrequent compared to the number of diagnosed AC. Thus, it is necessary to search for new preneoplastic conditions that may complete the classification of the precancerous lesions leading to development of peripheral lung cancer. In 2003 Ullman [4]

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characterized new eventual lesion - bronchial columnar cell dysplasia (BCCD) arising in small bronchioles that may lead to adenocarcinoma. The thesis that adenocarcinoma may arise from BCCD was supported with LOH studies showing loses of 6q23-ter, 12q23-ter and 13q14-21 and gains 7q11-q21, 13q32-ter and 19q13.1 chromosomes both in BCCD and AC specimens. In this paper we describe the morphological and immunohistochemical analysis of P16^{INK4a}, TP53, Rb and Ki-67 proteins expression in this preneoplastic lesion concerning small bronchioles that may lead both to adenocarcinoma as well as peripheral-squamous carcinoma development.

Materials and methods

Material samples. We examined specimens taken form a population of 193 patients (148 males, 45 females) surgically treated for primary non-small cell lung cancer (lobectomy, bilobectomy or pulmonectomy) in Department of Thoracic Surgery, Medical University of Bialystok in 2003-2006. The age of the patients ranged from 40 years to 77 years (mean 60.4 years). Four to six tissue samples of tumour and 10 to 15 samples of not invaded lung were taken from each specimen of all patients belonging to the studied population. Samples were routinely formalin-fixed and paraffin-embedded. For histological evaluation each 5 µm section was H+E stained. Slides of each sample were investigated separately by two pathologists experienced in lung cancer pathology using criteria for preneoplastic lesions according to the WHO 1999 classification. All the cases were divided into three groups corresponding to histological types of primary lung cancer.

Immunohistochemistry. Selected samples representing BCCD (according to morphological criteria by Ullman *et al.*) obtained from macroscopically normotype tissue were immunohistochemicaly examined. Paraffin blocks were cut for 5 µm thick slices, placed on a microscopic slide and left in 56°C for 24 hours. Then deparaffinization with subsequent xylens was made. Deparaffinized specimens were then placed in following alcohol solutions (absolute alcohol, 96%, 70%) in room temperature. Next, rinsed with water specimens placed in citric buffer (pH=6.0; 55-60°C). Series of 4 slides were then microwaved (630W/20 min). Immunohistochemical analysis was performed using monoclonal mouse anti-human antibodies directed against P16^{INK4a} (E64H, 1:25, DakoCytomation), TP53 (DO-7, 1:50, DakoCytomation), Rb (1F8, 1:20, Novocastra Laboratories) and Ki67 (MIB-1, 1:75, DakoCytomation) proteins. As a detection kit for P16^{INK4a}, TP53 and Ki-67 biotin-strepatvidin-acidic phosphatase (DakoCytomation) was used. For Rb detection we used Avidin/Biotin Blocking System (Novocastra Laboratories). As a chromogen for all proteins Substrate - chromogen solution (DAB) (DakoCytomation) was used. Nuclei were then stained with Mayer hematoxylin. Positive controls were made using tissue samples proposed by antibody manufacturer which showed high expression of proteins. Negative control were made with the same tissue without antibody. Expression of the proteins was recognized as positive when at least 10% of the cells of observed lesion were stained.

Statistical analysis. The association between immunohistochemical and histological parameters have been measured using Spearman rank correlation. Statistical analysis was performed with Statistica 6.0. Differences were recognized as significant when p was <0.05.

Results

The distribution of the preneoplastic lesions

In studied population of 193 patients we described as much as 137 preneoplastic lesions in 101 patients. In some of the cases we observed more than one lesion. In most cases bronchiolization states as an additional change. The studied population was divided into three groups corresponding with histological type of the primary tumour. In squamous carcinoma group (Group SqC, n=54) leading lesion was squamous metaplasia without or with dysplasia (63.5%). Carcinoma in situ was present in 8.6% of the cases. Interestingly, BCCD was observed in 17.6% while AAH and bronchiolization were present respectively in 2.7% and 8.1% of the cases. The difference in number of the metaplasia in this group comparying to others was statistically significant (p<0,001). In adenocarcinoma group (Group AC, n=34) percentage of squamous metaplasia and BCCD was aproximate (respectively 34.9% and 30.2%). In this group AAH was observed in 11.2% and bronchiolization in 23.3% of the cases. The occurrence of BCCD in this group in comparison to others was statistically significant (p<0,05). Large cell lung cancer group (LCLC) was to small to provide any statistical analysis.

Morphology of BCCD

We observed that BCCD changes are mainly focal, usually concern a short section of the bronchiolar mucosa. That creates cardinal difficulties for far-reaching studies of these lesions. The abnormalities relate to the structure, number of the cells, as well as cytological state of the epithelium. Columnar epithelium, typical for bronchioles with vertically oriented nucleus is most often replaced by cells showing horizontal orientation of the nucleus. Sometimes the cells take polygonal or lengthened form. Often the epithelium arrange into two, three or more layers. The cells showed cytological atypia. Mostly we observed enlarged nuclei, showing horizontal orientation to the basal layer. Some of the polinuclear cells were also described. Nuclei usually is hiperchromatic, often with prominent nucleoli. Chromatin seems to be condensed and decomposed in the cell. Shapes of the nuclei are often irregular. Incidentally cells can differentiate into multilayer squamous type epithelium. It occurs with creation of the desomsomes. Very often foci of BCCD are found in bronchioles, which shows focal disruption or disappearance. Fig. 1 to 4 show all described characteristics.

Immunohistochemistry of BCCD

Thirty cases of BCCD found during histopathological investigation were studied for immunoexpression of

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the key cell cycle proteins $P16^{INK4a}$, TP53 and additionally proliferative index (Ki-67) was measured.

$P16^{INK4a}$ protein. Twenty one (70%) of the cases we observed loss of expression of the P16INK4a protein. Two cases (6.7%) represent abnormal expression of the protein in cytoplasm. Mixed nuclear - cytoplasmic expression was observed in seven cases (23.3%). The Spearman correlation rank test showed statistical significance between lack of expression of these protein and histological type of the tumor which was adenocarcinoma ($p<0.001$).

TP53 protein. In eight cases of BCCD (26.7%) we observed overexpression of TP53. Seven cases showed nuclear expression, one case mixed nuclear - cytoplasmic expression. In remaining 22 cases we didn't observe positive staining for TP53.

Rb protein. The presence of Rb protein in nuclei of the cells was found in 14/29 cases of BCCD (48.3%). In all remaining cases expression of this protein was not observed. In one case there was no possibility to establish status of the Rb protein expression, because the lesion was cut out while preparation of the slides.

Mitotic activity (Ki-67). Diversified expression of Ki-67 (from 10 to 80% of stained cells) was observed in 10/28 cases of BCCD (35.7%). In all remaining cases we didn't observed the expression of the protein or it considered only single cells of the case. In two cases there was no possibility to establish status of the mitotic activity, because the lesion was cut out while preparation of the slides.

Figures 5-10 presents immunostaining of the described proteins.

Discussion

High mortality because of the lung cancer comes out mainly of the fact that majority of the cases are diagnosed in advanced stadium. An expanded diagnostics of preinvasive conditions, which are referred to as precancerous lesions, would certainly contribute to lowering the mortality rate. It is, however, a big challenge, considering that the whole lung is a potential field of cancerogenesis, and neoplasia has many different potential ways of development [5].

Many studies show that the development of non-small cell lung cancer, in particular squamous cell lung cancer, is preceded by long-term period in which the genetic - molecular changes in cells of the respiratory epithelium take place. These multistep changes of different morphology provides to growth of fully invasive cancer [7,13-15].

Table 1. Expression of the proteins in SqCLC, AC and LCLC groups of patients.

Type of expression/Protein		Group		
		SqCLC-group	AC-group	LCLC-group
Nuclear expression	$P16^{INK4a}$	-	-	-
	TP53	2	4	1
	Rb	7	5	2
	Ki-67	5	4	1
No expression	$P16^{INK4a}$	5	12 ($p<0.001$)	4
	TP53	11	8	3
	Rb	6	7	2
	Ki-67	8	8	2
Abnormal expression*	$P16^{INK4a}$	6	1	-
	TP53	-	1	-
	Rb	-	-	-
	Ki-67	-	-	-

*cytoplasmic or nuclear cytoplasmic localization of the protein

The multistep changes leading to development of the invasive squamous cell lung cancer are well recognized and described by many authors [6,16-19].

The transitions leading to the development of adenocarcinoma, which states in Poland for 30% of all diagnosed cases of non-small cell lung cancer is still unclear. Many authors contribute that AAH leads to non invasive form of bronchiolo-alveolar cancer as well as to invasive adenocarcinoma. Unfortunately the occurrence of this lesion in the population is not known. In this study AAH states for 6.7% of all diagnosed preneoplastic lesions and although it was more often present in AC-group (11.2%) than in SqCLC-group (2.7%) we can tell that the occurrence of this change was occasional. However studies of Nakahara [9] showed that AAH states for 23.2% of 508 examined cases, whereas Chapman and Kerr [8] indicated only 12% cases of AAH in studied population of 582 patients. The frequence of the AAH in our studies is quite low comparing to other literature data. This disproportion is apparent and could result of two facts. First of all, in countries of Western Europe and USA the frequence of adenocarcinoma is higher. It has been estimated that for the last 20 years in European countries the number of diagnosed adenocarcinoma and lesions preceding its development among the young men and women at all age raised up for about 10% [1]. In Poland the leading type of NSCLC is still squamous cell lung cancer. Secondly in 2006 pathologists were

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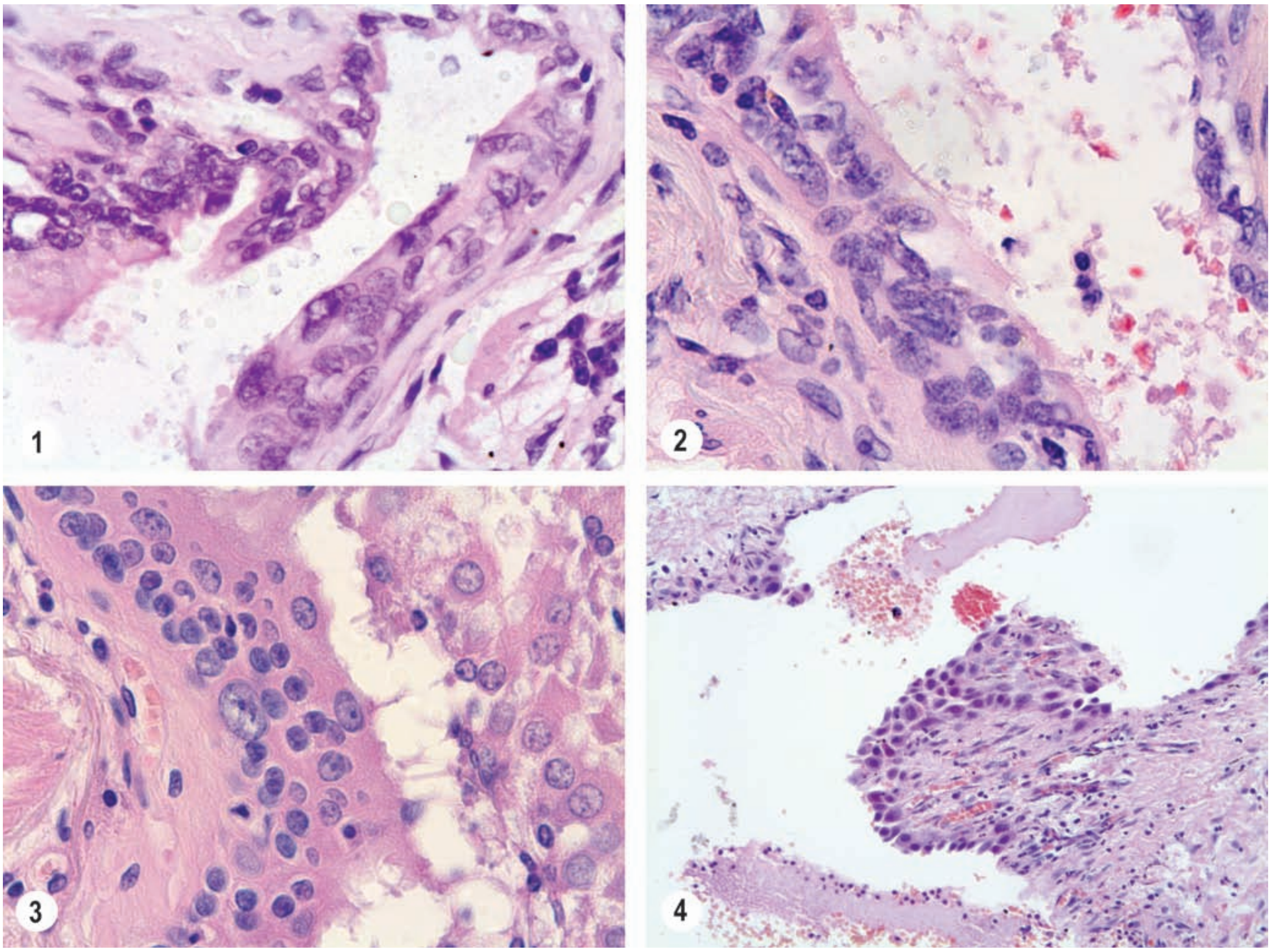

Fig. 1. Bronchiolus with focal BCCD lesion - one layer of columnar or cuboidal epithelium was lined by multilayer epithelium formed by elongated or polygonal cells with marks of atypia (M, primary cancer SqCLC, H+E, magnification × 400). **Fig. 2.** Fragment of the bronchiolus wall with focal BCCD. Nuclei of the cells are concentrated, of different shapes (round, polygonal or elongated). Nuclear chromatin in granular aggregation (F, primary cancer AC, H+E, magnification × 400). **Fig. 3.** Fragment of the bronchiolar mucosa with chaotically lined epithelium with morphological marks of atypia. Nuclei of different size with prominent nucleoli and unevenly arranged chromatin (M, primary cancer SqCLC, H+E, magnification × 400). **Fig. 4.** Fragment of bronchiolus. Preserved an island of the epithelium with marks of squamous metaplasia with dysplasia (special and rare subtype of BCCD). Cells of the epithelium connected with each other through desomsomes (M, primary cancer AC, H+E, magnification × 100).

presented to more precise criteria of differentiating AAH than in previous years [20].

Described results of others and our studies clearly show the disproportion of the frequence of AAH and development of adenocarcinoma in population. Thus, it seems that the WHO classification of the precancerous lesions leading to the peripheral tumors of the lung is still incomplete. It is necessary to search for the peripheral situated changes which could fill the theory of invasive adenocarcinoma development. It is also worthy to thing of the point the development of the rare peripheral squamous cell lung cancer which states for 5-6% of all surgically treated NSCLC [21,22]. In our study in two cases we observed the differentiation of the cells of bronchiolar epithelium into the squamous multilayer epithelium, which reveal with desomsomes formation. Thus, there occurs the hypothesis that bronchioles might be a place of carcinogenesis for both adenocarcinoma and squamous cell lung cancer development.

Noted cases of the BCCD (most of all in group with primary adenocarcinoma - 30.2%, and squamous cell lung cacer - 17.6%) seems to support this thesis. The additional argument that BCCD may be a real preneoplastic lesion are studies of the immunoexpression of the cell cycle crucial proteins.

Described BCCD cases are extremely difficult for diagnosis because concern small bronchioles (0.3 - 1 mm). The changes are often very small, focal transformation of the epithelial cells, which could be revealed only after accurate diagnosis of all histological material.

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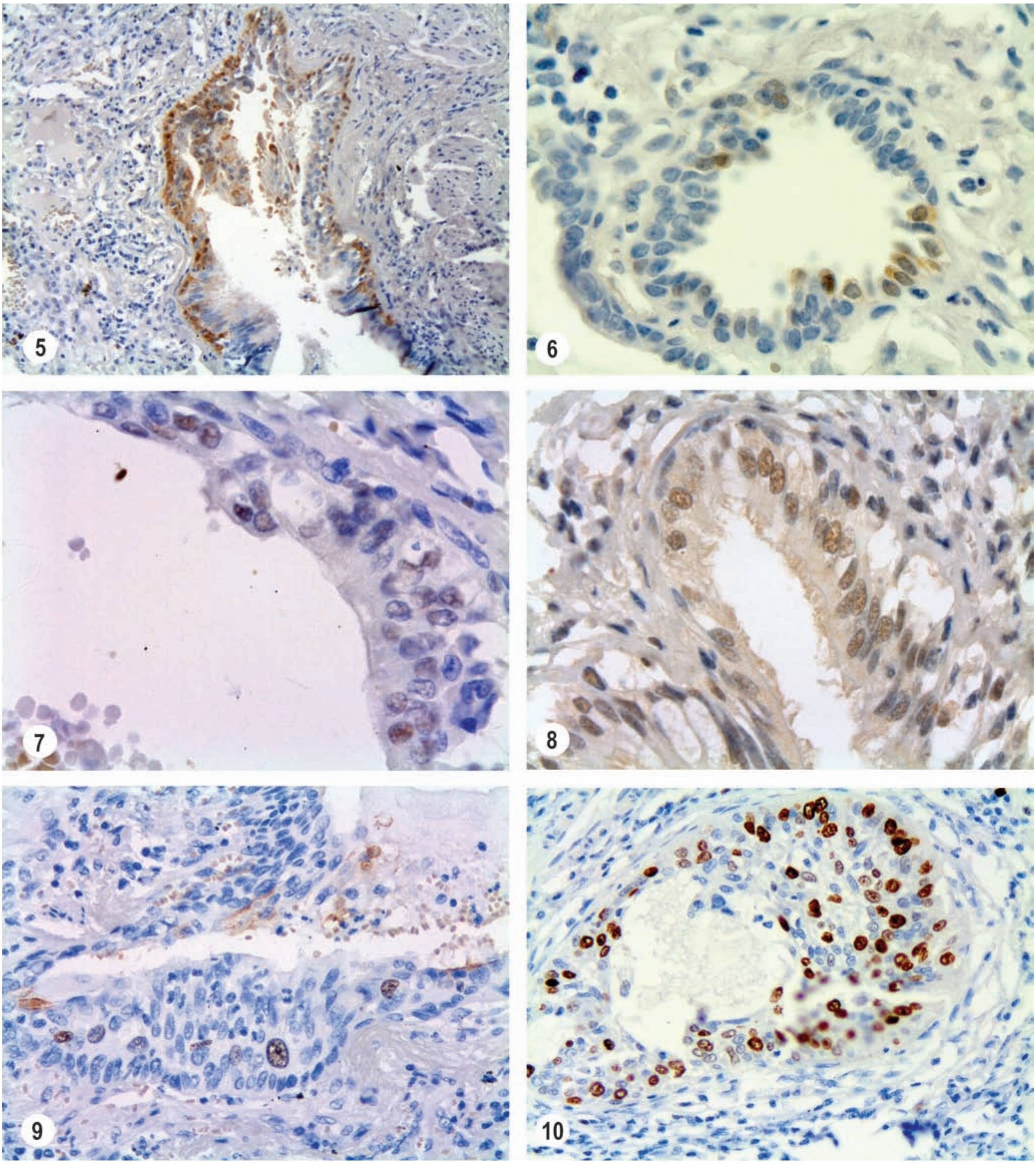

Fig. 5. High cytoplasmic - nuclear expression of P16^{INK4a} in bronchiolus with BCCD (M, primary cancer SqCLC, magnification × 40). **Fig. 6.** Medium cytoplasmic - nuclear expression of P16^{INK4a} in bronchiolus with BCCD (F, primary cancer AC, magnification × 200). **Fig. 7.** Nuclear expression of TP53 in bronchiolus with BCCD (M, primary cancer SqCLC, magnification × 400). **Fig. 8.** Nuclear expression of TP53 in bronchiolus with BCCD (M, primary cancer LCLC, magnification × 200). **Fig. 9.** Low mitotic activity (Ki-67 protein) in bronchiolus with BCCD (M, primary tumour SqCLC, magnification × 200). **Fig. 10.** High mitotic activity (Ki-67 protein) in bronchiolus with BCCD (M, primary cancer SqCLC, magnification × 200).

It is thought that observed phenotype of the cells suspected for neoplastic transformation is result of the changes in genes and proteins that participates in regulation of the cell cycle and apoptosis as well as the repairing of the DNA [23]. The changes in gene expression and the structure of chromosomes that lead

Folia Histochem Cytobiol. 2008:46(1): 93 (89-96)
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to cancer development are often observed in cases of squamous metaplasia, dysplasia and CIS and AAH. Changes occur sequentially so their number and frequency raises with progress of atypia from metaplasia - dysplasia - CIS and AAH - adenocarcinoma [13].

Loss of expression of the P16^{INK4a} protein observed in our study is common phenomenon in majority of cancers and its preceding lesions [18,24-30]. In our study 92.3% of the BCCD cases showing loss of expression of P16^{INKa} were from AC-group. In seven of them it was probably linked with absence of the active Rb protein in the cells. In cases from SqCLC-group only one of five cases of loss of the protein might be due to lack of Rb protein. Cytoplasmic expression observed in this study is often refused by other authors as non-specific reaction. Recent study of the protein at sub-cellular level shows that this interpretation could be wrong. It's been shown that cytoplasmic activity of this protein could be the result of its inactivation or the mutation of the P16^{INK4a} gene [31]. This thesis is supported by study showing cytoplasmic localization of P16^{INK4a} protein and highly malignant phenotype of breast cancer [32].

Aberrances of TP53 protein are mainly observed changes in invasive cancers. It play a leading role in multistep cancer development [6,34]. Accumulation of the TP53 is observed at an early stages of cancer growth. The accumulation of this genetic and epigenetic changes concerning TP53 states from 5% in squamous metaplasia to even 60% in severe dysplasia cases [35-38]. Brambilla [37] found no accumulation of the TP53 in preneoplastic changes from non cancer cases, so it may show that overexpression of TP53 results in invasive cancer development. Accumulation of the protein in atypical adenomatous hyperplasia was observed in 5 to 28% of the cases, where tumor tissue showed overexpression of TP53 in 53 to 64% of the cases [33], [39]. In our study accumulation of TP53 was observed in 26.7% of the cases. Most of the cases were from the AC-group (38.5%). In SqCLC-group the number of cases with overexpression of TP53 was nearly twice lower (15.5%). It must be taken into account that the immunohistochemical method is able to define over-expression only in the case of protein p53. Only the methods of molecular biology are able to define mutation in exons, which do not lead to the accumulation of protein in the cell and, therefore, are not detectable by means of the immunohistochemical method [40].

The *Rb* gene mutations are often observed in series of cancers [43-46]. Lack of expression of the Rb protein is the highest in small cell lung cancer (about 90%). In non-small cell lung cancers the frequency of loss of expression is much lower (about 30%) [34,47,48]. In precancerous lesions of the lung lack of expression of the Rb protein is low and concerns up to 18% of the cases [25,49,50]. In our study the Rb loss was quite high in BCCD cases from SqCLC-, AC- and LCLC-groups (46.1%, 53.8% and 50% respectively). This high percentage could be accidental event that results from low amount of examined cases. Although, we could suggest the new way of transformation from BCCD to small cell lung cancer development, which not exclude according to new articles concerning development from single stem cells mediating in inflammation [41,42].

Other important marker differentiating stages of development of the preneoplastic lesion is proliferative index (Ki-67). Raise of the mitotic activity that correlates with growing level of dysplasia and atypia is well known phenomenon described in epithelium of many organs [51-53]. Meert [54] showed that the expression of Ki-67 depends on level of development of the preneoplastic lesion and grows significantly from low dysplasia to CIS. Comparison of the topography and intensity of the staining shows significant difference between low dysplasia - moderate dysplasia (47-67% of stained cells) and severe dysplasia -CIS levels (91-100% of stained cells). Similarly mitotic activity raises up with increasing level of atypia in AAH to bronchiolo-alveolar cancer and invasive adenocarcinoma [49].

In our study increased mitotic activity was observed in 10 cases of BCCD (35.7%). Five cases (38.5%) from SqCLC-group showed different levels of mitotic activity from 10 to 80% of stained cells. The raised proliferative index was nearly similar in cases from AC-group (30.8%). Seven cases represents low staining for Ki-67 (below 10% of the cells).

Conducted experiments did not show any dependence between expression status of each protein and presence of BCCD. Considering the fact that neoplastic transformation is a multistep process the result is not surprising. Concluding the shown above results we may suppose that BCCD may be preneoplastic lesion leading to adenocarcinoma as well as to peripheral squamous cell lung cancer. The hypothesis should be supported with further evidence, especially with results of genetic - molecular studies.

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