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Review article

Immunohistochemical markers of cancerogenesis in the lung

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Abstract: Lung cancer is the leading cause of cancer deaths for people of both sexes worldwide. Early diagnosis of pre-cancer lesions may be of crucial significance to lowering lung cancer mortality. The World Health Organization has defined three preneoplastic lesions of the bronchial epithelium: squamous dysplasia and carcinoma in situ, atypical adenomatous hyperplasia and diffuse idiopathic pulmonary neuroendocrine cell hyperplasia. These lesions are believed to progress to squamous cell carcinoma, adenocarcinoma and carcinoid tumors, respectively. Apart from WHO classification, two other lesions such as bronchiolization and bronchiolar columnar cell dysplasia (BCCD) can be observed and thought to be preneoplastic lesions leading to adenocarcinoma. In this review we summarize the data of morphological and cell cycle related proteins changes in both central and peripheral compartments of lung. Many molecular changes, which accompany the multistep process of the development of invasive types of cancer, may be observed thanks to the application of immunohistochemical markers. A deeper knowledge of molecular and genetic changes accompanying pre-cancer states may show new directions of early diagnostics of cancer development.

Key words: Preneoplastic lesions - Lung cancer - Squamous dysplasia - CIS - Bronchiolization - AAH - BCCD - Cell cycle related proteins - Immunohistochemistry

Introduction

Lung cancer is the leading cause of cancer deaths for people of both sexes worldwide [8,24,61]. This high mortality rate is first of all due to the fact that most cases of lung cancer are diagnosed at an advanced stage of development. An expanded diagnostics of preinvasive conditions, which are referred to as pre-cancer lesions, would certainly contribute to lowering the mortality rate. It is, however, a big challenge, considering that the whole lung is a potential area of canceration, and neoplasia has many different potential ways of development [41].

Lung cancer develops in two separate compartments - the central (transporting air) and the peripheral (respiratory) part of the lung. There are four histological types of cancer, including small cell cancer and a heterogeneous group of non-small cell cancer, which includes squamous cell carcinoma, adenocarcinoma (including a non-invasive type of bronchoaveolar carcinoma), large cell cancer and many other, much less frequent subtypes.

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 [14,47]. Thus, lung cancer may develop both in the primary bronchus, small bronchioli and alveoli. Squamous cell carcinoma is most often placed centrally whereas adenocarcinoma and large cell cancer are typically encountered in the peripheral part of the lung (Fig. 1 and 2).

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Morphology of pre-cancer lesions

The recently published WHO classification of lung tumours identifies three main pre-cancer states leading to the development of invasive types of cancer. These are: squamous dysplasia and carcinoma in situ (CIS), atypical adenomatous hyperplasia (AAH) and diffuse

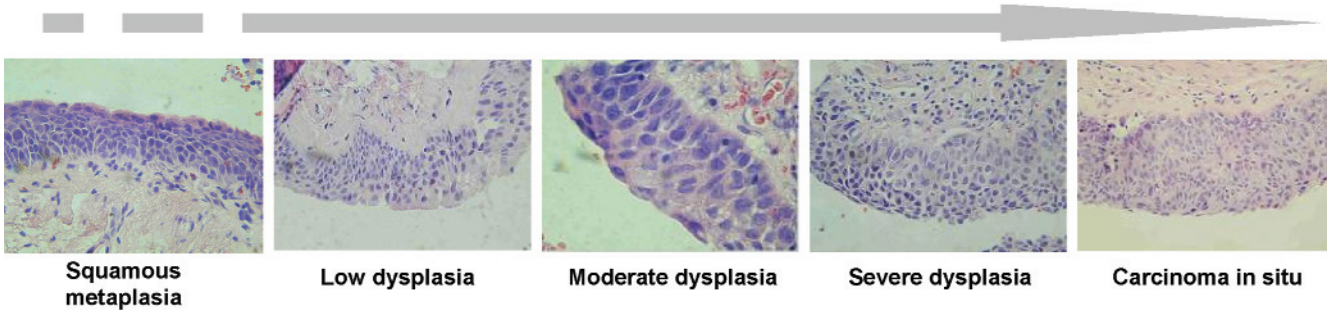

Fig. 1. Multistep progression to invasive squamous carcinoma.

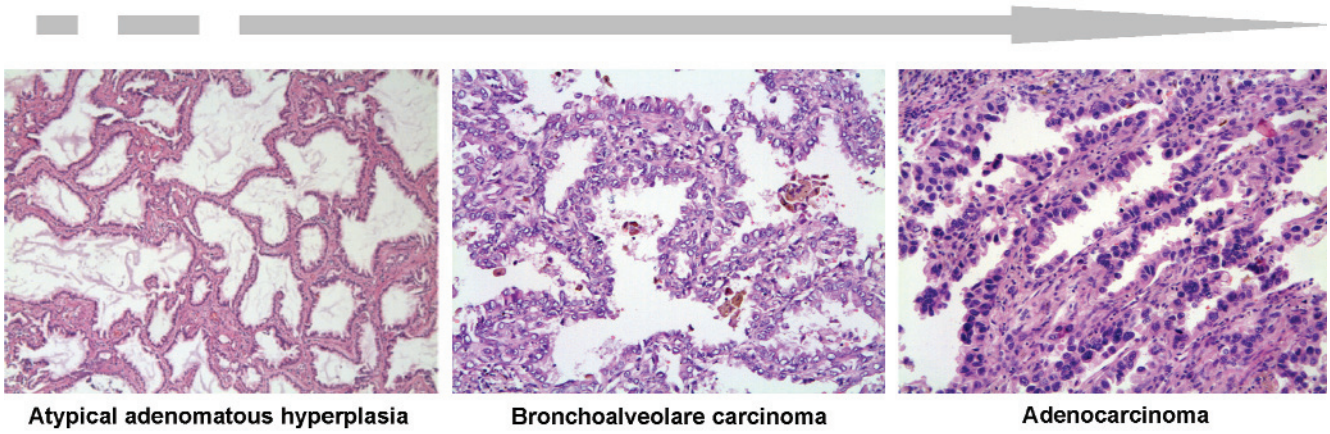

Fig. 2. Multistep progression to invasive adenocarcinoma.

idiopathic pulmonary neuroendocrine cell hyperplasia (DIPNECH) [9].

Progressive morphological changes of the epithelium, which in most cases lead to the development of an invasive type of squamous carcinoma, have been examined and described quite well [10,65]. These changes progress from epithelial cell hyperplasia, through squamous metaplasia and the accompanying dysplasia, to carcinoma in situ (Fig. 1). Hyperplasia may affect two types of cells: goblet and basal. The lesion is referred to as basal cell proliferation when the base layer of epithelium is thickened and it is more than 3 cell thick [14]. Metaplastic lesions occur mainly in the place where there is already a proliferation of basal cells. These cells, as a result of the differentiation process, create layers characteristic for stratified squamous epithelium. Squamous dysplasia, which is considered to be a real pre-cancer lesion, may differ in the stage of development (low, mild and high grade). Low-grade dysplasia is a lesion characterised by minimal aberration inside the cell. Only single atypical cells appear in the epithelium. The stratified structure of the epithelium is retained. A bigger irregularity is observed in the case of mild and high-grade dysplasia. In the latter there is a significant polymorphism affecting all the cells. Still, the stratified structure of the epithelium can be seen. In some cases of metaplasia or squamous dysplasia, we can observe significant thickening of the basal layer, while under the epithelium the vascularization process starts, which results in the formation of papillary processes. This lesion has been described as angiogenic metaplasia/squamous dysplasia [28]. Carcinoma *in situ* may be accompanied by thickening of the epithelium although it is not always the case. Epithelium is not stratified and the cells are not fully mature. We can observe a severe derangement of the cytology of the cells, such as the change of nucleus shape and size, a deranged relation between the nucleus and the cytoplasm and nuclear hiperchromasia. Cell division figures are observed on all levels [14,30].

The lesions affecting the respiratory part of the lung, which most often lead to the development of adenocarcinoma, are connected with 2 morphologically separate states.

Atypical adenomatous hyperplasia (AAH) is observed as solitary or multifocal lesions within a normal lung as well as lesions creating a continuity with a tumour consisting of adenomatous tissue. This lesion was first described in 1939 as proliferating, non-inflammatory lesion of the alveolar epithelium [19]. AAH is a small, proliferating lesion, whose size varies from 1mm to

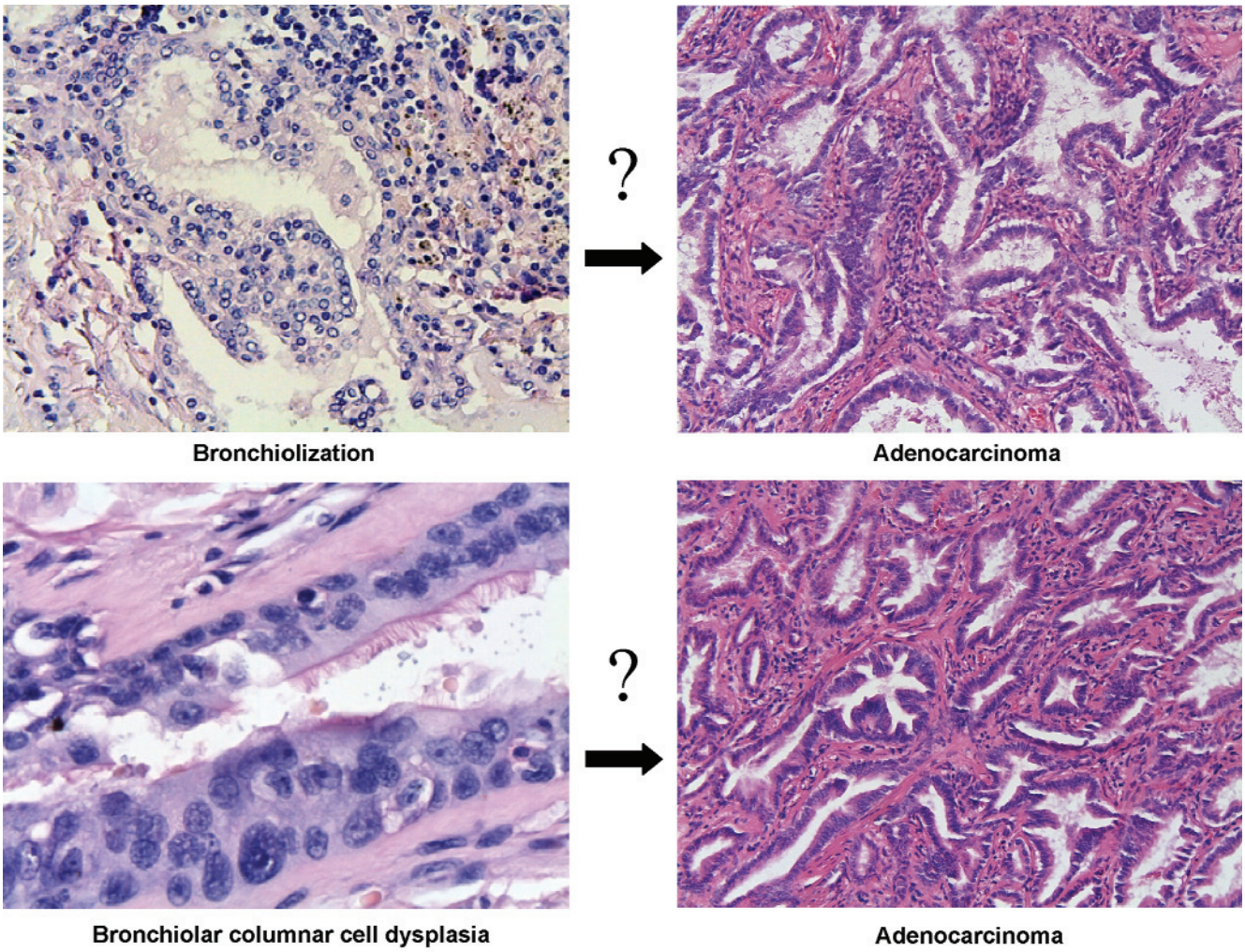

Fig. 3. Probable multistep progression to invasive adenocarcinoma.

about 7 mm, usually it is less than 3 mm. It most often occurs in the peripheral part of the lung and takes the form of a single layer of atypical cells similar to Clara cells or type II pneumocysts, covering alveoli and respiratory bronchioli. AAH cells are cuboidal or columnar. They show a different degree of cell atypia. Mitotic division figures are rarely visible in AAH. Intercellular connections are in most cases retained, with few occasional empty spaces between the cells. Contrary to the reactive proliferation of type II pneumocysts (bronchiolisation), in the proximity of atypical cells there are no, or they are very weakly manifested, features of interstitial inflammation and fibrosis. Some authors notice that AAH cells are at least twice as big as the neighbouring normotypical alveolar epithelium. These cells form a single layer consisting of round and/or cuboidal cells of low concentration. The nuclei of these cells show minimal features of atypia (changes in the size and shape as well as hiperchromasia). As AAH progresses, we can observe a slight thickening of alveolar septa, an increase in the concentration of cells and their overlaying with the formation of papillae. There is also the stratification of epithelial cells and a strongly manifested cell atypia. Despite a clear morphological diversification of these states, in practice AAH is not classified into low, mild and high grade [43,46,52,60].

Many authors put forward hypotheses that AAH leads both to the development of a non-invasive type of bronchioloalveolar carcinoma (BAC) and the invasive adenocarcinoma (AC)(Fig. 2). The frequency of AAH in the population is unknown. Nakahara *et al.* [44], examined 508 patients operated for primary lung cancer and found 118 (23.2%) patients with diagnosed 311 AAH lesions in total. On the other hand, the research conducted by Chapman and Kerr [11] covering 582 patients operated for primary lung cancer, points to 67 (12%) patients with diagnosed AAH.

Atypical adenomatous hyperplasia is more often observed in patients operated for adenocarcinoma (even up to 40% of cases) than in patients suffering from squamous carcinoma (about 9%) [30,31,33,46,

55,57,64]. Weng *et al.* [64] claim that the occurrence of AAH is irrelevant to sex whereas Chapman and Kerr [11] point to a more frequent AAH incidence in women operated for adenocarcinoma (30.2%) than in men (18.8%) operated for the same reason. Further research involving post-mortem examinations indicates incidental (2-3%) occurrence of AAH in patients who had not been diagnosed with primary or secondary carcinoma [56,68].

In 2003 Ullman and Bongiovanni [62] described a new, so far unobserved lesion, which they called "Bronchiolar Columnar Cell Dysplasia" (BCCD). Unlike AAH, which can be observed by means of macroscopic methods, BCCD requires the application of microscopic methods.

BCCD is characterised by the derangement in the organization of the epithelial layer of bronchioli. Normotypical columnar cells, as well as reserve cells are replaced with a uniform continuum of atypical columnar, cubic or polygonal cells, which may form multiple layers. From the cytological point of view, the atypia of these cells is manifested by the lack of diagonal orientation of the nucleus, its enlargement and the appearance of the nucleolus, hiperchromasia. We also observe derangements in nucleus shape and chromatin condensation. Multinuclear cells are also frequent. The authors of the quoted study believe that the hypothesis that BCCD lesions may precede the development of adenocarcinoma, is confirmed by the occurrence of lesions in the peripheral part of the lung, a similarity between BCCD and the lesions preceding the development of adenocarcinoma, which occur in other organs (prostate and pancreas) and have already been described well. An important argument are the results of LOH (the loss of heterozygosity) tests, which are performed on the BCCD epithelium [62]. They may indicate the way of development from BCCD to adenocarcinoma (Fig. 3).

Another lesion observed in the respiratory part of the lung, which may have a potential connection with cancerogenesis, is a reactive proliferation of type II pneumocysts, known as bronchiolisation (Fig. 3).

Bronchiolisation is most often observed as a focal lesion occurring within a normal respiratory epithelium. This lesion can be described as a metaplasia of this epithelium in the direction of type II respiratory epithelium cells or columnar cells of bronchioli. As the lesion develops, we can observe a diversity of sizes and shapes of cells. In our material (the lungs resected due to primary cancer) we came across the cases of bronchiolisation with the presence of atypical cells. The process of bronchiolisation is usually accompanied by focal lesions of interstitial fibrosis. In bronchiolisation foci the epithelium often forms micro-papillae. Lesions of this type have been observed both in connection with squamous carcinoma, adenocarcinoma and the mixed type, as well as in other pathological processes involving lung fibrosis [25].

Immunohistochemical markers in pre-cancer lesions

The current hypothesis referring to cancerogenesis suggests that the observed phenotype of the cells suspected to be responsible for the malignancy process is the consequence of lesions occurring in the genes taking part in the regulation of the cell cycle, cell proliferation and division, DNA repair, the transmission of growth signals and apoptosis [54]. Changes in the expression of genes as well as in the chromosome structure, which lead to cancerous transformation, are observed in the case of squamous metaplasia, dysplasia, CIS, as well as AAH or bronchiolisation. These changes occur in sequences and their number and frequency increases together with the development of atypia along both pathways of development; metaplasia - dysplasia - CIS and AAH - adenocarcinoma [20].

Bcl-2

Bcl-2 protein has anti-apoptotic qualities. Its over-expression provides the differentiating cancer cells with an unlimited freedom of development. It is observed in 25% of squamous carcinoma cases and about 10% of mature adenocarcinoma [7,29,50,63]. In the states preceding the development of an invasive type of squamous carcinoma, the over-expression of bcl-2 is slightly connected with the growing atypia. It occurs with the same frequency and intensity in the lesions such as low and high-grade dysplasia and CIS, with the exception of squamous metaplasia, where the over-expression of this protein is lower. Bcl-2 over-expression in metaplastic cells and those showing the features of low-grade dysplasia, is closely related to tumour proximity. Mild and high-grade dysplasia and CIS do not show such a tendency [5]. Bcl-2 over-expression in atypical adenomatous hyperplasia cells is significant and reaches about 70% [27]. Bcl-2 over-expression is related to the inactivation of pro-apoptotic bax protein. Bax/bcl-2 relation (BBR), which in normotypical epithelial cells is >1, is significantly opposite in pre-cancer lesions, starting from mild dysplasia, i.e. <1 [36].

Survivin

Survivin, which is another anti-apoptotic protein, does not occur in normotypical epithelial cells and low-grade AAH. Its expression is observed in 25% of squamous metaplasia cases, 40% of low-grade dysplasia cases and 61% of high-grade dysplasia cases, which

indicates an upward tendency in every stage of squamous carcinoma. The expression of survivin is stronger manifested in pre-cancer lesions situated in the immediate proximity of the tumour. It also applies to the cases of AAH which adhere to bronchioloalveolar carcinoma (BAC) [2].

p53

The expression of p53 protein is connected with the cell DNA damage. This protein stops the cell cycle by initiating the expression of p21 protein (the immediate inhibitor of cdk2), and at the same time triggers cell reconstruction systems. In the case of a bigger damage, it puts the cell on the pathway to apoptosis [18]. Point mutations, affecting most often the 5-8 exon of p53 gene, result in the formation of abnormal protein, which is not able to regulate transcription correctly. The formed protein, even though it is functionally abnormal, is very stable and accumulates in cells [18,37]. Derangements of p53 protein expression are the most often observed lesions in the invasive types of lung cancer [21] and seem to be playing a vital role in the multi-degree pathway of cancerous lesion development [14]. The expression (accumulation) of mutated p53 protein occurs already in early stages of invasive cancer development (Fig. 5). Different studies point to an upward tendency in p53 protein accumulation in the states preceding the development of squamous carcinoma. This accumulation ranges from 5% in squamous metaplasia cases to about 60% in an advanced high-grade dysplasia [3,6,13,22]. Brambilla *et al.* [5] reports a positive reaction to p53 in all examined cases of a pre-cancer nature (dysplasias, CIS). Just like in the case of bcl-2, the distance between the lesion and the tumour was an important factor, which influenced the frequency of positive reactions. In the material obtained from 'non-cancer' patients there was no p53 accumulation in the lesions considered as pre-cancer. The conclusion that can be drawn from that is that p53 over-expression in pre-cancer states has a significant influence on the development of a non-invasive lesion. Kerr *et al.* report 28% accumulation of p53 protein in atypical adenomatous hyperplasia [31]. In the same study, a positive expression in the tumour was observed in 53% cases. The research conducted by Kitamura *et al.* [32] indicates p53 expression in 5-8% of AAH cases and 8-62% of bronchioloalveolar carcinoma (BAC) cases. Martin *et al.* [38] reports that abnormal expression of p53 protein in single cells happens in 28% cases of normotypical epithelium of smokers. It has an upward tendency increasing together with the development of the lesion and affects 71% of cases already on the level of low-grade dysplasia. What is interesting is that in carcinoma in situ and invasive carcinoma, the positive reaction to p53 is encountered in 64% of cases (positive colour reaction in more than 10% of cells). Only in 2 out of 32 cases of bronchiolisation there was a strong, but focal, p53 expression [25]. 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 [4].

Telomerase

Telomerase is another factor which contributes to cell immortality. It may lead to blocking the normal shortening of telomeres, which protects the cells against the regular aging process. The expression of hTERT (human telomerase reverse transcriptase) has a clear upward tendency, starting from hyperplasia, where the expression is weakly manifested and occurs in about 70% of cases and squamous metaplasia (88%) and finishing with different grades of dysplasia (96-100%) and CIS (100%), where there is a strong manifestation of expression. The level of the expression of this enzyme depends significantly on lesion advancement, which has also been proved by the researchers by means of in situ hybridisation for hTERT mRNA. Authors also point to a correlation between hTERT expression and p53 accumulation as well as a reverse BBR. On the other hand, no significant relations between telomerase activation, D1 cyclin over-expression and the loss of p16 expression, have been noticed [36]. Similar studies of atypical adenomatous hyperplasia have shown telomerase expression in 27% of AAH cases with low-grade dysplasia and as many as 75% of AAH with high-grade dysplasia. The expression of hTERT in BAC was observed in as many as 98% of cases, which indicates a clear role of telomerase in the development pathway from AAH to BAC. Earlier studies had not shown the presence of telomerase in AAH [67].

p16

The loss of expression of protein p16, which is a specific inhibitor of cdk4/6, and, indirectly, also the inhibitor of pRb phosphorylation, is often observed in all types of carcinoma as well as in the states that precede them [1,6,12,23,40,58]. Jeanmart *et al.* [23] report the loss of expression of p16 in 12% of squamous metaplasia cases, 22% of low-grade dysplasia cases, 14% of high-grade dysplasia cases and 47% of the observed cases of CIS. The research conducted by the same team later showed the loss of expression of

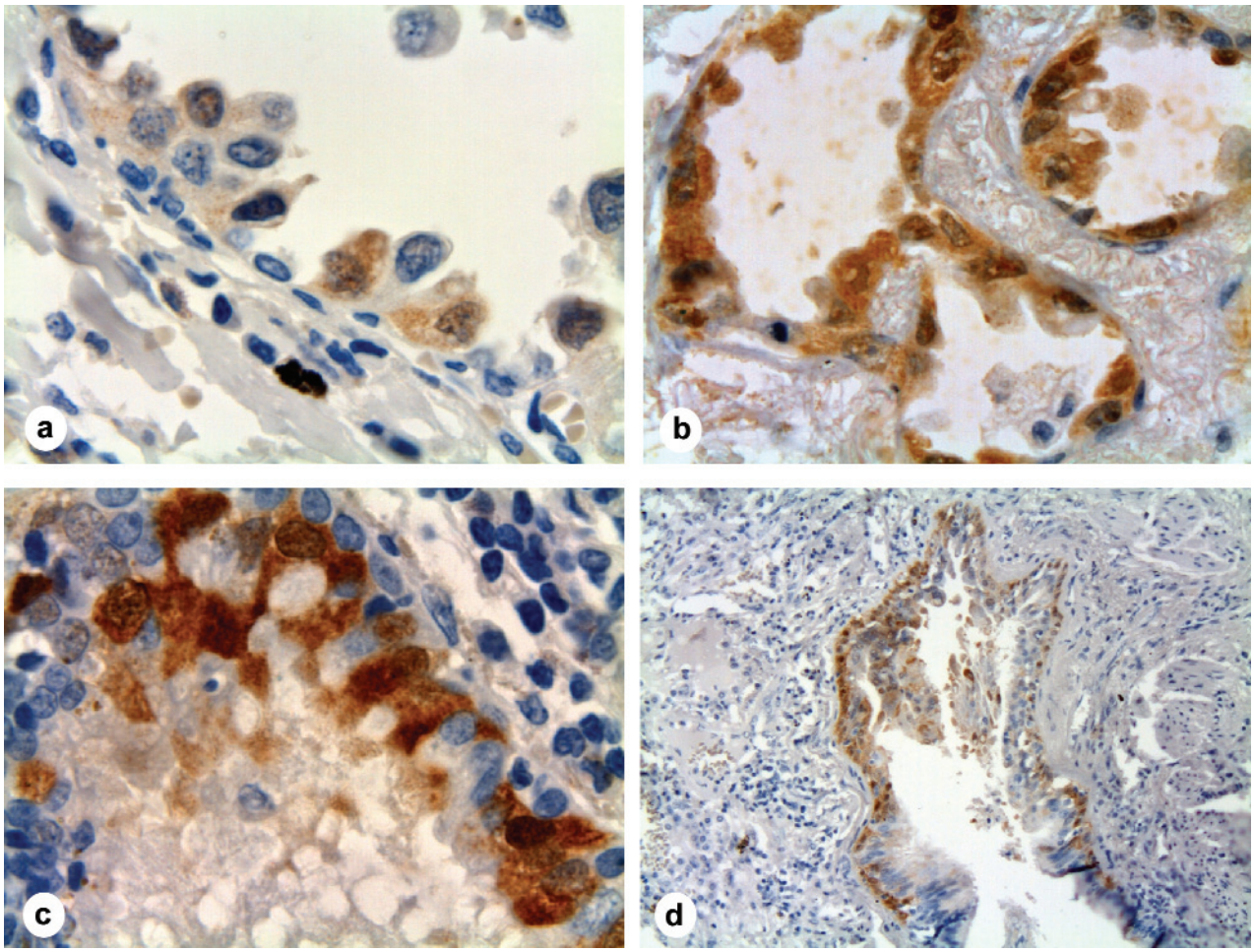

Fig. 4. Immunohistochemical expression of P16; **a**. in cells representing AAH, **b**. adenocarcinoma, **c**. cytoplasmic staining in cells representing BCCD, **d**. bronchiolus with BCCD.

p16 in 21% of hyperplastic epithelium, about 50% of lesions ranging from squamous metaplasia to CIS and increasing up to 63% in the cases of invasive cancer [36] state that the loss of expression of p16 does not differ much as regards the frequency of occurrence in AAH (20% of cases) and adenocarcinoma (14.7% of cases), thus, it does not play a crucial role in its development. The loss of expression might result from the silencing of *p16INK4a* gene by means of metilation, which has been described by many authors [35,49,66]. Numerous authors describing the expression of p16 in cytoplasm believe that it is a non-specific reaction. As a result, such cases are not taken into account. Recent studies of p16 protein on the sub-cell level indicate that such an interpretation may be wrong. It has been proved that cytoplasmic activity of p16 may be the consequence of the inactivation or mutation of protein, which results its moving to cytoplasm (Fig. 4) [48]. The relationship between the cytoplasmic localisation of p16 and the highly malignant breast cancer phenotype seems to confirm the above [16].

Cyclin D1

We often observe the over-expression of cyclin D1, which while creating complexes with cyclin-dependent kinases 4/6 (cdk 4/6) leads to the pRb phosphorylation and the activation of the E2F transcription factor. The over-expression has an upward tendency and is detectable in hyperplastic cells (9% of cases on average), metaplasia (6-11%), dysplasia of different grade (17-67%) and CIS (33-53%) [23,36]. Also in AAH the over-expression of cyclin D1 is high and ranges from 47 to 89% of cases depending on the degree of lesion advancement (AAH with low-grade dysplasia - AAH with high-grade dysplasia). However, a 28-60% decrease in expression has been observed at an early stage of adenocarcinoma development. Over-expression has been observed in 35% - 71% of the cases of invasive adenocarcinoma [34,59]. It has not been shown that the development of an invasive type of both squamous carcinoma and adenocarcinoma is related to the over-expression of cyclin D1, deranged expression of proteins p53, p16, pRb, hTERT and proliferation activity [23,34,36,47].

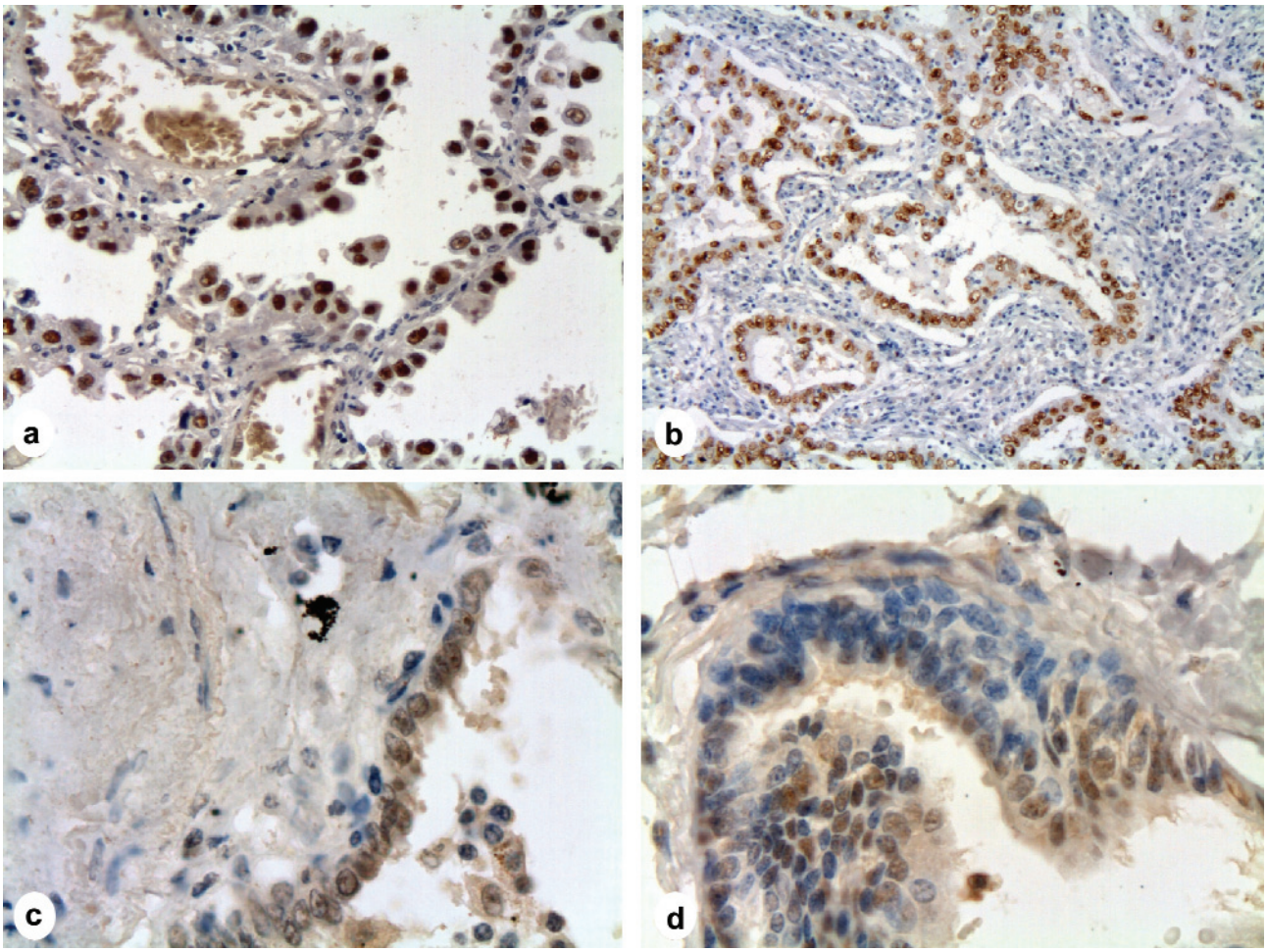

Fig. 5. Immunohistochemical expression of P53; **a**. in cells representing AAH, **b**. adenocarcinoma, **c**. bronchiolization, **d**. BCCD.

Ki-67

Proliferation index, as determined by a positive reaction to Ki-67, is an important factor differentiating the degrees of lesion development. Ki-67 is a nuclear and nucleolar protein, whose specific expression is observed throughout the whole cell cycle of proliferating cells, with the exception of G0 phase [17]. The increase in the proliferation activity correlated to the increasing degree of dysplasia and atypia, is a well-known phenomenon described in the studies of the epithelium of many organs [15,42,51,53]. Meert *et al.* [39] showed that Ki-67 expression depends on the degree of pre-cancer lesion development and increases significantly from low-grade dysplasia to CIS. A comparison of the topography, the intensity of colour reaction and the percentage of Ki-67 positive cells, indicates a significant difference between the stages of low-grade - mild dysplasia (47-67%) and high-grade dysplasia - CIS (91-100%). It clearly shows that proliferation activity during the development of squamous carcinoma is directly related to the increase of cell atypia. Similarly, during the development of adenocarcinoma, proliferation index increases together with the lesion development from AAH with low-grade and high-grade dysplasia to bronchioloalveolar carcinoma and invasive cancer [34].

Conclusion

Early diagnosis of pre-cancer lesions may be of crucial significance to lowering lung cancer mortality. Many molecular changes, which accompany the multi-step process of the development of invasive types of cancer, may be observed thanks to the application of immunohistochemical markers (Table 1). It must be noticed that the research concerning the above mentioned proteins has not been conducted in two lesions which are important for the development of invasive types of cancer, i.e. Bronchiolar Columnar Cell Dysplasia (BCCD) and bronchiolisation, which could be a valuable source of information about the potential pathways of development of invasive lung cancer. A deeper knowledge of molecular

Table 1. Immunohistochemical reactivity of markers involved in development of lung cancer

Marker IHC	Hyperplasia/ squamous metaplasia	Dysplasia mild/moderate/severe	CIS	AAH	Bronchiolization	BCCD
bcl-2	●	●●	●	●●●	?	?
Survivin	●	●●	●●●		?	?
hTERT	●	●●	●●●	●●	?	?
p53	●	●●	●●	●	●	?
P16	●●	●●	●●	●	?	?
Cyclin D1	●	●●	●●●	●●	?	?
Ki-67		●●	●●●	●●	?	?

● rarely observed; ●● moderately observed; ●●● often observed

and genetic changes accompanying pre-cancer states may show new directions of early diagnostics of cancer development.

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