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Immuno- and Hybridocytochemical Analysis of the Expression of Interleukin 2 and Its Receptor in Lung Cancers*

Immuno- i hybrydocytochemiczna analiza ekspresji interleukiny 2 i jej receptora w nowotworach płuc

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Abstract

Background. Interleukin 2 (IL-2) plays a significant role in the activation, differentiation and proliferation of hemopoietic cells, acting through a specific receptor complex (IL-2R). It is mainly produced by activated T helper cells (CD4+), but also by T cytotoxic lymphocytes (CD8+). Studies on IL-2/IL-2R in lung tumors focus mainly on detecting markers on immunocompetent cells isolated from patients' blood. Only a few studies describe tissue expression of IL-2 and its receptor in lung cancers.
Objectives. This study aimed at examining the expression of IL-2 and its receptor (IL-2Rα and IL-2Rβ) in lung tumor cells and in T lymphocytes that infiltrate the tumors (TILs).
Material and Methods. The material for studies included archival paraffin blocks with the following types of lung tumors: typical and atypical carcinoids (TC, AC), small-cell lung carcinoma (SCLC), non-small-cell lung carcinoma, squamous carcinoma (NSCLC-SQ) and adenocarcinoma (ADENO). Expression of the markers was demonstrated using immunocytochemical and in situ hybridization techniques, and the obtained data were subjected to statistical analysis.
Results. Expression of IL-2, IL-2Rα and IL-2Rβ was noted in all the studied types of pulmonary tumors. A positive immunocytochemical reaction was observed both in the tumor cells and in the tumor-infiltrating lymphocytes. The presence of IL-2 and IL-2Rα was confirmed by the detection of mRNA for both markers. IL-2Rβ expression was detected mainly in cell cytoplasm, and IL-2Rα on cell membranes of both neoplastic cells and TILs. The most pronounced total expression of IL-2 and IL-2Rβ was demonstrated in lung carcinoids, and the most pronounced expression of IL-2Rα was noted in squamous cell carcinomas. The most pronounced "neoplastic" expression of IL-2 was observed in TC, and of IL-2Rα in atypical carcinoids. The most pronounced "neoplastic" expression of the IL-2Rβ subunit was detected in atypical pulmonary carcinoids, while the least pronounced expression of this subunit was detected in small-cell lung cancer.
Conclusions. The prevalent expression of IL-2 and IL-2Rβ in tumor cells and of IL-2Rα in tumor-infiltrating lymphocytes, regardless of the lung tumor involved, may suggest their auto- and/or paracrine activity in the microenvironment of lung tumors. A direct relationship between the expression of the β subunit of IL-2R in tumor cells and tumor-infiltrating lymphocytes is observed in tumors of high proliferative activity (atypical carcinoids, small-cell and squamous pulmonary carcinomas) (**Adv Clin Exp Med 2011, 20, 2, 137–148**).

Key words: interleukin 2, receptor of interleukin 2, lung cancers.

Streszczenie

Wprowadzenie. Interleukina 2 (IL-2) odgrywa istotną rolę w aktywacji, różnicowaniu i proliferacji komórek hematopoetycznych, działając za pośrednictwem specyficznego kompleksu receptorowego (IL-2R). Jest wydzielana głównie przez aktywowane limfocyty T pomocnicze (CD4+), ale również przez limfocyty T cytotoksyczne (CD8+). Badania dotyczące IL-2/IL-2R w nowotworach płuc są głównie skupione na wykrywaniu tych markerów

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w komórkach immunokompetentnych izolowanych z krwi pacjentów. Nieliczne prace opisują tkankową ekspresję IL-2 i jej receptora w nowotworach płuc.

Cel pracy. Zbadano ekspresję IL-2 i jej receptora (IL-2Rα i IL-2Rβ) w komórkach zmienionych neoplastycznie (komórki guza płuc) oraz w limfocytach T naciekających guzy płuca (TILs).

Materiał i metody. Materiał do badań stanowiły archiwalne bloczki parafinowe z następującymi typami guzów płuc: rakowiaki typowe i atypowe (TC, AC), rak drobnokomórkowy (SCLC), rak płaskonabłonkowy (NSCLC-SQ) i rak gruczołowy (ADENO). Ekspresję markerów wykrywano z wykorzystaniem technik immunocytochemicznych i hybrydyzacji *in situ*. Wszystkie uzyskane dane poddano analizie statystycznej.

Wyniki. Ekspresję IL-2, IL-2Rα oraz IL-2Rβ wykrywano we wszystkich typach badanych nowotworów płuc. Pozytywną reakcję immunocytochemiczną obserwowano zarówno w komórkach nowotworowych, jak również w limfocytach infiltrujących guzy. Obecność IL-2 i IL-2Rα potwierdzono wykrywaniem mRNA dla obu markerów. Ekspresję IL-2Rβ umiejscowiano głównie w cytoplazmie komórek, a IL-2Rα na błonach komórkowych i to zarówno komórek neoplastycznych, jak i TILs. Największą całkowitą ekspresję IL-2 i IL-2Rβ wykazywały rakowiaki płuc, a IL-2Rα raki płaskonabłonkowe. Największą „guzową" ekspresję IL-2 obserwowano w TC, a IL-2Rα w atypowym rakowiaku płuc. Ekspresja „guzowa" podjednostki β IL-2R również była największa w atypowym rakowiaku płuc, a najmniejsza dotyczyła drobnokomórkowego raka płuc.

Wnioski. Przeważająca ekspresja IL-2 i IL-2Rβ w komórkach guzowych, a IL-2Rα w limfocytach naciekających guz niezależnie od typu nowotworu płuc, może sugerować ich auto- i/lub parakrynowe działanie w mikrośrodowisku guza płuc. Wprost proporcjonalna zależność między ekspresją podjednostki β IL-2R w komórkach guza i w TILs dotyczy nowotworów płuc o dużej aktywności proliferacyjnej (atypowe rakowiaki, drobnokomórkowe i płaskonabłonkowe raki płuc) (**Adv Clin Exp Med 2011, 20, 2, 137–148**).

Słowa kluczowe: interleukina 2, receptor interleukiny 2, raki płuc.

Interleukin 2 (IL-2) is one of the most important cytokines which control the proliferation and differentiation of cells in the immune system [1]. The cytokine is produced mainly by activated T helper lymphocytes (CD4⁺). Its expression can also be detected in T CD8⁺ lymphocytes, dendritic cells and thymocytes in the thymus [2].

The biological activity of IL-2 resulting in signal transduction and specific effects in the cell takes place due to specific interaction of IL-2 with a specific receptor. The receptor of IL-2 (IL-2R) consists of three subunits: the α chain (IL-2Rα, CD25, Tac, p55), β chain (IL-2Rβ, CD122, p70) and γ chain (IL-2Rγ, CD132). Depending on the links between individual subunits, three forms of IL-2R receptor with varying affinity of interaction with IL-2 can be distinguished [2–6].

Studies of the expression of IL-2 and its receptor have been conducted mainly on T and B cell leukemias, lymphomas and solid tumors such as cancers of the larynx, lungs, mammary gland, large intestine, liver, ovary, prostate and melanomas [4, 7–12]. The authors of these studies have focused their attention on the role of the IL-2/IL-2R complex in controlling cell growth and on the evaluation of the cytokine as a potential marker of tumors. It is intriguing that exogenous IL-2 inhibits the growth of certain human neoplastic cells via IL-2R, while the proliferation of other cells remains unchanged in the presence of a similar expression of IL-2Rβ on cell membranes [4, 13]. Some authors have presented evidence that endogenous IL-2 is in fact pre-required for the proliferation of certain tumor cells (such as squamous cell cancer of head and neck), while growth inhibition could have resulted from the use of anti-sense IL-2 [14]. Evidence is also available indicating that endogenous IL-2 may be involved as a growth factor for human neoplastic cells [4, 15].

Primary lung cancer is the most frequent malignant tumor in men and the second most frequent malignant tumor in women (following breast cancer), and is the most frequent cause of cancer deaths in both genders [16].

Studies on the role of IL-2 and its receptor in pulmonary tumors have dealt mainly with non-small cell lung cancers (NSCLC) [4, 9, 12, 17, 18]. The production of cytokines in tumors has mainly been examined in patients' immunocompetent blood cells (lymphocytes, monocytes) using various types of ELISA (Enzyme-Linked Immunosorbent Assay) test. Significantly reduced IL-2 production by those cells has been noted [17]. Lower production of IL-2 was documented in patients with metastasized forms of cancer than in patients without metastases [9, 11]. Moreover, patients with lowered ratios of CD4/CD8 lymphocytes manifested lower levels of IL-2 as compared to patients with normal CD4/CD8 ratios.

Also, in cases of small cell lung cancers (SCLC), the synthesis of certain cytokines (including interleukin-2) by immunocompetent blood cells was found to be significantly lower than in controls following stimulation with a mitogen. In addition, the secretion of IL-2 depended on the tumor load; lowered production of this cytokine was demonstrated in more advanced clinical stages of lung cancers. Lowered production of IL-2 in blood cells at the moment of SCLC diagnosis was found to be an important indicator of a poor prognosis for the patient's survival, independent of other indicators such as staging, NSE (neuron specific enolase) concentration, LDH (lactate dehydrogenase) concentration and the patient's age and gender. According to Fischer et al., the lowered production of IL-2 in small cell lung cancer may indicate immunosuppression in this type of cancer [19].

Studies on IL-2/IL-2R in lung tumors focus mainly on detecting markers on immunocompetent cells isolated from patients' blood. Few investigations describe tissue expression of IL-2 and its receptor in lung tumors.

Reports of detecting and localizing IL-2 in lung cancer tumor cells themselves are very rare. Studies on non-small cell lung cancer cell line cultures have found increased production of type 2 cytokines (mainly IL-5 and IL-10), but not of type 1 cytokines, including IL-2 [20, 21]. As in microenvironmental lymphocytes, in NSCLC tumor cells themselves, higher expression of IL-4, IL-10, TGF-α and TGF-β was demonstrated as compared to the expression of IL-2, IL-12, IL-18 or IFN-γ [22].

Studies on the role of the IL-2 receptor (IL-2R) in lung tumors have shown that activated T lymphocytes can produce the soluble α subunit of the receptor (sIL-2Rα), which is capable of effectively binding IL-2.

Recent studies related to the role of the IL-2 complex and its receptor have focused on the production of proteins in T regulatory lymphocytes (Treg), particularly those which are CD8-positive, in the contexts of immunomodulatory therapies and transplantological problems [23].

Lung cancer is the most frequently manifested tumor in men and the second most frequent in women (following breast cancer) [16]. The treatment of lung cancer still suffers from low efficacy. Numerous immunotherapy trials involving the signalling pathways of interleukin-2 and its receptor are being undertaken; some of these trials include lung carcinomas [3, 10]. The data related to cytokine expression in tumor cells are relatively incoherent, and the role of tissue expression of IL-2 and its receptor (particularly of its β subunit) as markers of clinical significance remains poorly defined. Research into the specific cellular source of IL-2 and its receptor in various types of lung tumors seems potentially significant, due to numerous findings of IL-2Rα overexpression, which leads to increased cell proliferation, drug resistance and augmented transforming activity in lung tumors [3, 10]. Therefore, in this study we examined the immunocytochemical and hybridocytochemical expression of IL-2 and its receptor (IL-2Rα and IL-2Rβ) in neoplastically transformed cells (lung tumor cells) and in the tumor-infiltrating lymphocytes (TILs).

Material and Methods

The experimental material for the study included archival paraffin blocks with the following types of lung cancers: typical and atypical carcinoids (TC, n = 14; AC, n = 11), small cell lung cancer (SCLC, n = 23), non-small cell lung cancer – squamous cell lung cancer (NSCLC-SQ, n = 23) and adenocarcinoma (ADENO, n = 15). The available clinical data on the patients included the histopathological diagnosis, the degree of histologic malignancy or grading (G) (NSCLC group) and the age and gender of the patient. The histological type of lung tumor was determined in routine diagnostic tests conducted by two independent pathologists.

The control material consisted of archival paraffin blocks with fragments of lymphatic organs (lymph nodes, appendixes) with inflammatory lesions and free of neoplasia (n = 10).

All the tissues were fixed of a buffered solution of 10% formalin, then embedded in paraffin, cut into 5 μm thick sections and deposited on SuperFrost/Plus glass microscope slides.

Paraffin sections were stained in the usual manner, using hematoxylin and eosin (H + E). The expression of markers (IL-2 – 1:100, R&D Systems; IL-2Rα – 1:100, Novocastra Labs; IL-2Rβ – 1:500, Abcam; CD3 – 1:500, Novocastra Labs; CD4 – 1:80, Dako; CD8 – 1:1, Dako) was detected using immunocytochemistry (the ABC technique, in accordance with Hsu et al. [24]) and *in situ* hybridization. In the cases of IL-2Rα, IL-2Rβ, CD3, CD4 and CD8 antibodies, an additional high temperature antigen unmasking technique was required. All the reactions entailed overnight incubation of the applied primary antibodies. In order to compare the expression of proteins (IL-2, IL-2R subunits and molecules of CD3, CD4 and CD8) in tumor cells and in lung tumor-infiltrating lymphocytes (TILs), identical reactions were performed in tissue material of reactive lymphatic organs (n = 10, positive control). The tests in the positive control group were aimed only at verifying the quality of the applied antibodies and were not subjected to semi-quantitative analysis or statistical analysis.

The immunocytochemical reactions were evaluated using a semi-quantitative, 12-point IRS (Immunoreactive Score) scale in accordance with Remmele and Stegner [25]. All the obtained data were subjected to statistical analysis.

Results

Comparison of IL-2, IL-2Rα and IL-2Rβ Expression Among the Examined Groups of Patients

Interleukin-2

Statistical analysis of the results of immunocytochemical detection of the total IL-2 expression (in tumors and TILs) demonstrated the highest mean IL-2 expression in typical and atypical carcinoids (Fig. 1). Expression of IL-2 was significantly higher in cases of TC and AC than in squamous cell lung cancer or pulmonary adenocarcinoma (Fig. 1, Table 1).

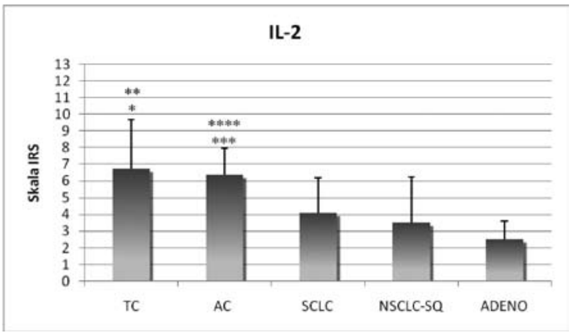

Fig. 1. Comparison of mean values of total IL-2 expression in particular groups of patients

Ryc. 1. Porównanie średnich wartości ekspresji IL-2 całkowitej w poszczególnych grupach chorych

* TC vs NSCLC-SQ, $p < 0.05$.
** TC vs ADENO, $p < 0.001$.
*** AC vs NSCLC-SQ, $p < 0.05$.
**** AC vs ADENO, $p < 0.001$.

Similar differences in expression were found when IL-2 expression only in tumor cells (g) was considered: IL-2 expression was significantly higher in both types of lung carcinoid cells than in squamous cell lung cancer and adenocarcinoma (Fig. 2A, Fig. 3A, Table 1).

Interestingly, IL-2 expression in tumor-infiltrating lymphocytes (n) was most pronounced in atypical lung carcinoids (Fig. 3B), and it was significantly higher than the analogous expression of the cytokine in TILs in typical carcinoids or pulmonary adenocarcinomas. No significant differences were noted in IL-2 expression in small-cell lung cancer, squamous cell lung cancer or adenocarcinomas (Fig. 2B, Table 1).

IL-2 Receptor α Subunit (IL-2Rα)

The most pronounced expression of total IL-2Rα was noted in patients with squamous cell lung cancer, but it was significantly higher only as compared to that in pulmonary adenocarcinoma ($p < 0.01$) (Fig. 4, Table 1).

Considering tumor cells (g) only, the highest expression of IL-2Rα was demonstrated in patients with atypical lung carcinoid (Fig. 6A), but it was significantly higher only as compared to the analogous expression in pulmonary adenocarcinoma (Fig. 5A). Relatively high mean IL-2Rα expression was also observed in cases of squamous cell lung cancer and SCLC; in both cases it was higher than

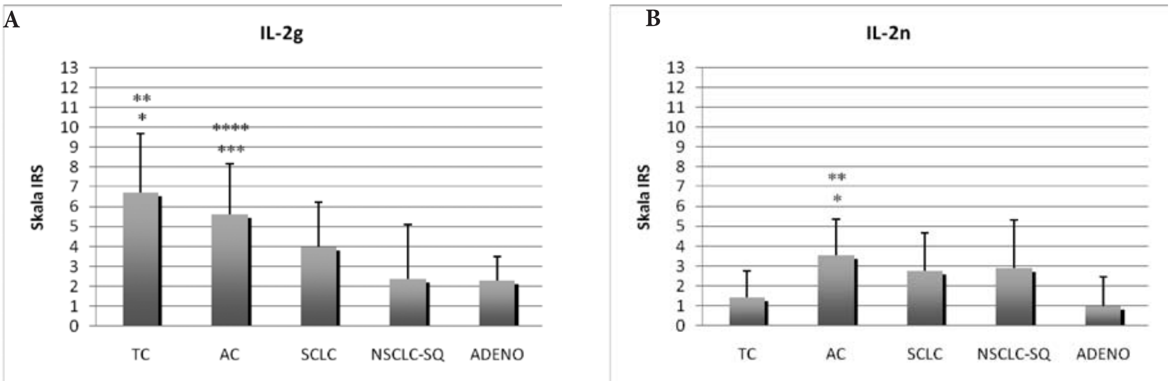

Fig. 2. Comparison of mean values of IL-2 expression in A) tumor cells (g) and B) tumor infiltrating lymphocytes (n) in particular groups of patients

Ryc. 2. Porównanie średnich wartości ekspresji IL-2 w komórkach nowotworowych guza (g) (A) i w limfocytach naciekających guzy (n) (B) między poszczególnymi grupami chorych

A) * TC vs NSCLC-SQ, $p < 0.001$.
** TC vs ADENO, $p < 0.001$.
*** AC vs NSCLC-SQ, $p < 0.05$.
**** AC vs ADENO, $p < 0.05$.
B) * TC vs AC, $p < 0.05$.
** AC vs ADENO, $p < 0.05$.

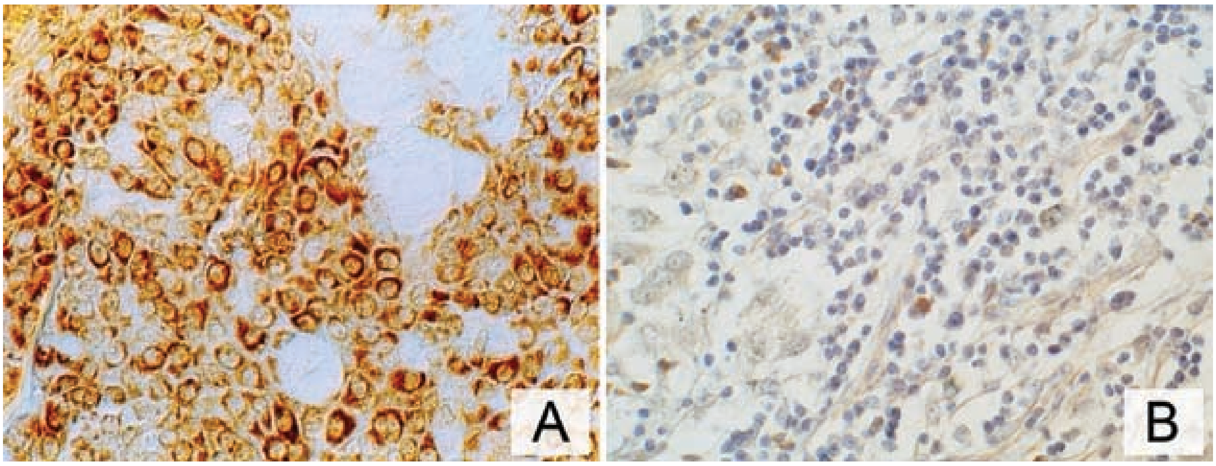

Fig. 3. Immunocytochemical localization of Il-2 in A) tumor cells in a typical carcinoid patient and B) individual cells of connective tissue stroma in an atypical carcinoid patient. ABC technique. Objective ×40; nuclei counterstained with hematoxylin (B)

Ryc. 3. Immunocytochemiczna lokalizacja Il-2 w komórkach nowotworowych u chorego z typowym rakowiakiem płuc (A) i w pojedynczych komórkach zrębu łącznotkankowego guza u chorego z atypowym rakowiakiem płuc (B). Technika ABC. Obiektyw 40×; jądra komórkowe podbarwione hematoksyliną (B)

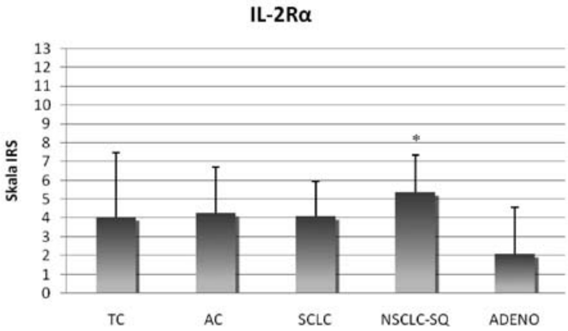

Fig. 4. Comparison of mean values of total IL-2α expression in particular groups of patients

Ryc. 4. Porównanie średnich wartości ekspresji IL-2Rα całkowitej w poszczególnych grupach chorych

* NSCLC-SQ vs ADENO, $p < 0.01$.

the analogous expression in adenocarcinoma (Fig. 5A, Table 1).

In tumor-infiltrating lymphocytes (n), the most pronounced IL-2Rα expression was observed in NSCLC-SQ (Fig. 6B), which was significantly higher than the analogous expression in either AC or lung adenocarcinoma (Fig. 5B).

IL-2 Receptor β Subunit of (IL-2Rβ)

The most intense mean expression of the IL-2 receptor β subunit was noted in the group of patients with atypical lung carcinoid (Figure 7A); it was significantly higher than in small-cell lung cancer (SCLC) (Fig. 7B). The remaining groups of lung cancers did not differ significantly in total expression of the marker (Fig. 8, Table 1).

Analysis of IL-2Rβ expression in tumor cells only and in tumor-infiltrating lymphocytes demonstrated that in both cases the highest expression was in atypical lung carcinoids. No significant differences among individual groups were detected in terms of IL-2Rβ expression within the tumor (Fig. 9A) or in the tumor microenvironment (n) (Fig. 9B, Table 1).

Analysis of the Expression of Markers Typical for T-Lymphocyte Subpopulations (CD3, CD4 and CD8) in the Microenvironment of Lung Cancers

The expression of markers which differentiate pulmonary tumor-infiltrating lymphocytes pertained to subpopulations of T lymphocytes. Most frequently, the cells were present in greater numbers in the connective tissue sublayer of lung cancers. Occasional individual immunopositive T lymphocytes were also noted between tumor cells or scattered over the entire area of the histological preparation.

The expression of CD3 (a marker molecule of T lymphocytes), CD4 (a subpopulation of T lymphocytes: the so-called helper lymphocytes Th1 and Th2) and CD8 (a subpopulation of T lymphocytes: cytotoxic T lymphocytes Tc) was analyzed on a 12-point IRS scale, scoring only cells with the entire circumference of cell membrane labelled.

The presence of a positive reaction for T-lymphocyte marker molecules was detected almost exclusively on cell membranes of tumor-infiltrating lymphocytes (Fig. 10A, B, C), which was comparable to the immunocytochemical reaction noted in reactive lymph nodes (the positive control) (Fig. 10D). As a rule, the immunocytochemical reaction was

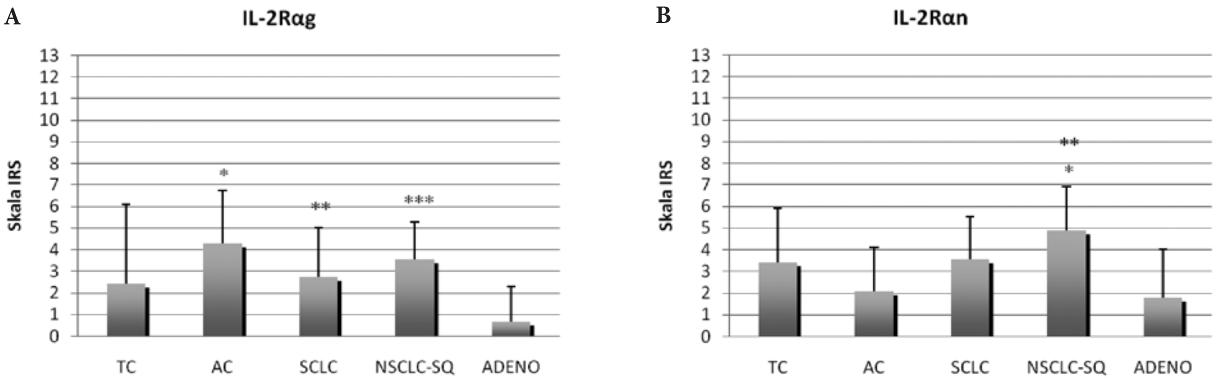

Fig. 5. Comparison of mean values of IL-2α expression in A) tumor cells (g) and B) tumor infiltrating lymphocytes (n) in particular groups of patients

Ryc. 5. Porównanie średnich wartości ekspresji IL-2α w komórkach guza (g) (A) i w limfocytach naciekających guzy (n) (B) między poszczególnymi grupami chorych

A) * AC vs ADENO, $p < 0.01$.
** SCLC vs ADENO, $p < 0.05$ NSCLC-SQ vs ADENO, $p < 0.001$.
*** NSCLC-SQ vs ADENO, $p < 0.001$.
B) * NSCLC-SQ vs AC, $p < 0.05$.
** NSCLC-SQ vs ADENO, $p < 0.01$.

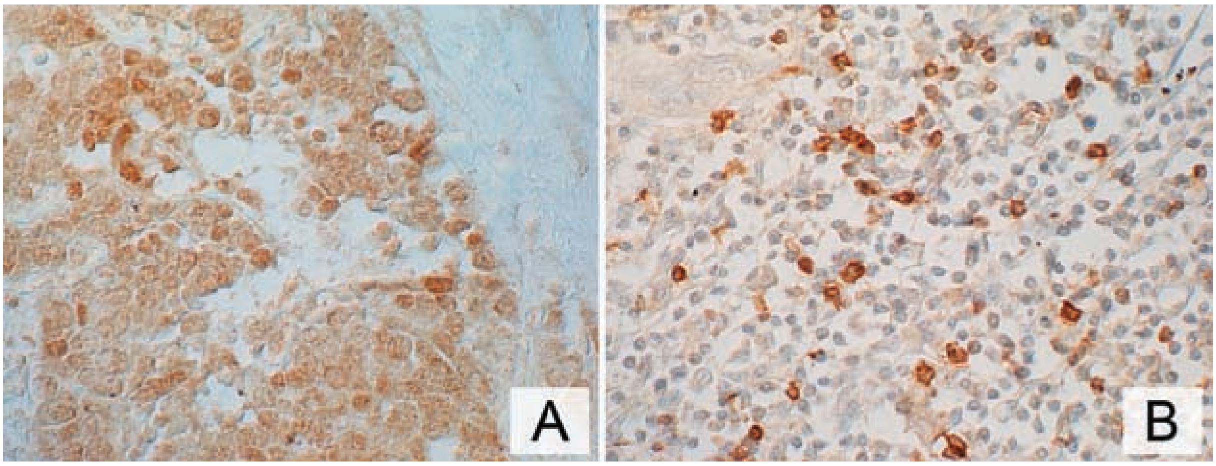

Fig. 6. Immunocytochemical localization of IL-2α in A) tumor cells in an atypical carcinoid patient and B) tumor-associated cells in a squamous cell carconoma patient. ABC technique. Objective ×40; nuclei counterstained with hematoxylin (B)

Ryc. 6. Immunocytochemiczna lokalizacja IL-2Rα w komórkach nowotworowych u chorego z atypowym rakowiakiem płuc (A) i komórkach towarzyszących guzowi u chorego z płaskonabłonkowym rakiem płuc (B). Technika ABC. Obiektyw 40×; jądra komórkowe podbarwione hematoksyliną (B)

very intense, covering the entire membrane of T lymphocytes.

In patients with typical lung carcinoid, significant correlations were demonstrated between the expression of the CD4 molecule and the total IL-2 expression ($r = 0.9380$), and between CD8 expression and the total IL-2Rβ expression ($r = 0.8660$). Similarly, in the SCLC group, a direct relationship was detected between CD3 expression and the total tissue expression of IL-2Rβ ($r = 0.6211$).

Hybridocytochemical Studies

Studies using *in situ* hybridization in lung cancers were conducted following the immunocytochemical examination of a given pulmonary tumor fragment. IL-2 and IL-2Rα mRNAs were detected using digoxygenin-labelled probes. Hybridocytochemical analysis of lung cancers was conducted on a total of 22 randomly selected representatives from the different patient groups; the data were not subjected to quantitative analysis. For all 22 of these patients, positive results were obtained in the hybridocytochemical reaction for both IL-2 mRNA

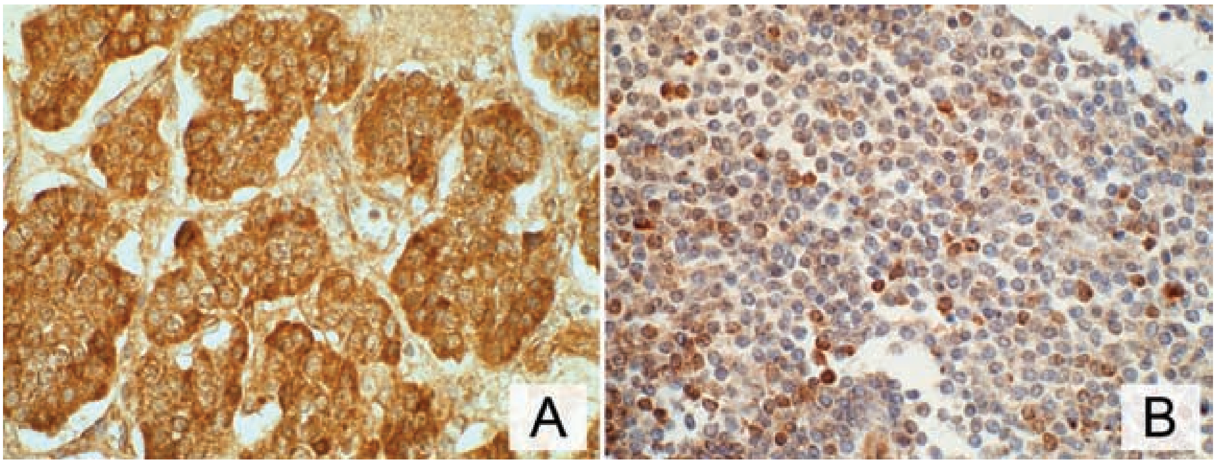

Fig. 7. Immunocytochemical localization of IL-2β in A) tumor cells in a typical carcinoid patient and B) tumor-infiltrating cells in a small cell lung cancer patient. ABC technique. Objective ×40; nuclei counterstained with hematoxylin

Ryc. 7. Immunocytochemiczna lokalizacja IL-2Rβ w komórkach nowotworowych u chorego z typowym rakowiakiem płuc (A) i komórkach infiltrujących guz u chorego z drobnokomórkowym rakiem płuc (B). Technika ABC. Obiektyw 40×; jądra komórkowe podbarwione hematoksyliną

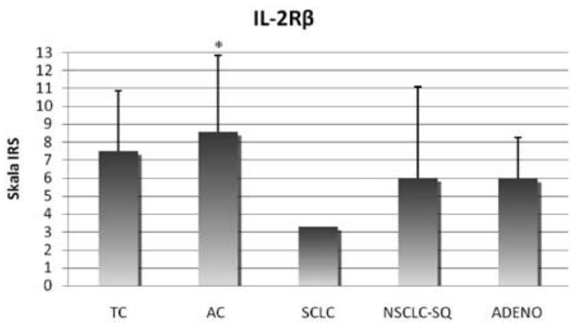

Fig. 8. Comparison of mean values of total IL-2β expression in particular groups of patients

Ryc. 8. Porównanie średnich wartości ekspresji IL-2Rβ całkowitej w poszczególnych grupach chorych

* AC vs SCLC, $p < 0.05$

and IL-2Rα mRNA. In the cases where IL-2 mRNA was examined, the positive hybridocytochemical reaction was in all cases (except one – a single patient in the SCLC group) consistent with a positive result in the immunocytochemical reaction and with the presence of the IL-2 protein both in the tumor cells and in individual mononuclear cells in the tumor microenvironment. In cases of IL-2 mRNA, nuclear localization prevailed, although the reaction product was also detected in the cell cytoplasm (Fig. 11A). In cases where IL-2Rα mRNA was examined, a positive hybridocytochemical reaction was noted even when presence of the receptor could not be shown using the immunocytochemical approach. In cases of IL-2Rα, the product of the hybridocytochemical reaction was in most cases noted mainly in cell nuclei, similar to the mRNA for IL-2 itself (Fig. 11B).

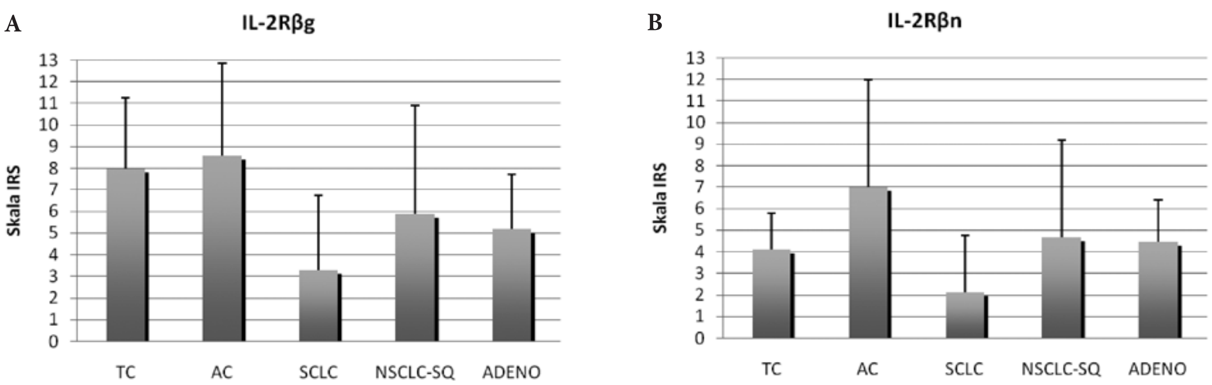

Fig. 9. Comparison of mean values of IL-2β expression in A) tumor cells (g) and B) tumor infiltrating lymphocytes (n) in particular groups of patients

Ryc. 9. Porównanie średnich wartości ekspresji IL-2β w komórkach nowotworowych guza (g) (A) i w TILs (n) (B) między poszczególnymi grupami pacjentów

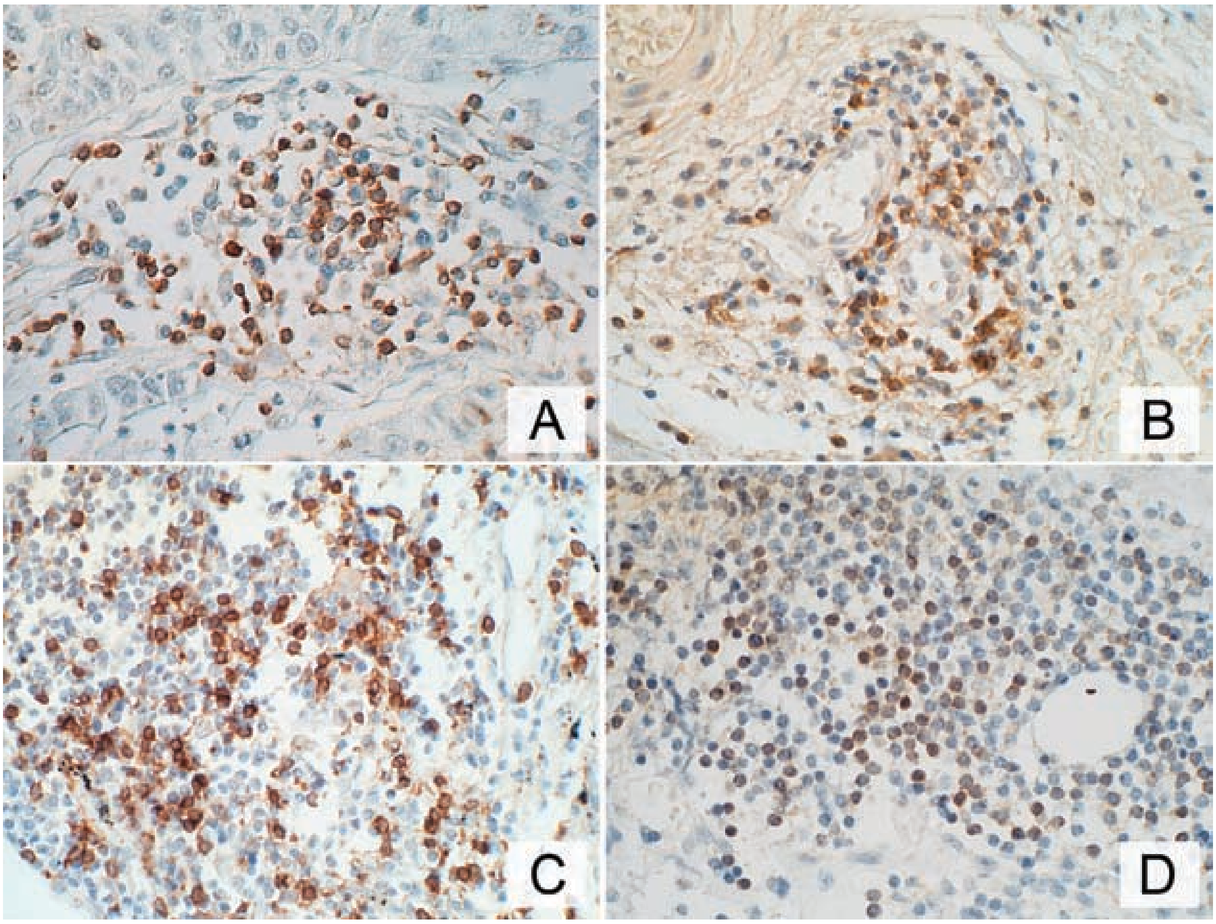

Fig. 10. Immunocytochemical localization of A) CD3 in tumor-infiltrating cells in an adenocarcinoma patient; B) CD4 in tumor-infiltrating cells in a typical carcinoid patient; C) CD8 in tumor-infiltrating cells in a squamous cell carcinoma patient; and D), CD4 molecule in reactive lymph node cells (positive control). ABC technique. Objective ×40; nuclei counterstained with hematoxylin

Ryc. 10. Immunocytochemiczna lokalizacja: CD3 w komórkach infiltrujących guz u chorego z gruczołowym rakiem płuc (A), CD4 w komórkach naciekających guz u chorego z typowym rakowiakiem płuc (B), CD8 w komórkach naciekających guz u chorego z płaskonabłonkowym rakiem płuc (C) oraz lokalizacja cząsteczek CD4 w komórkach reaktywnego węzła chłonnego (kontrola pozytywna) (D). Technika ABC. Obiektyw 40×; jądra komórkowe podbarwione hematoksyliną

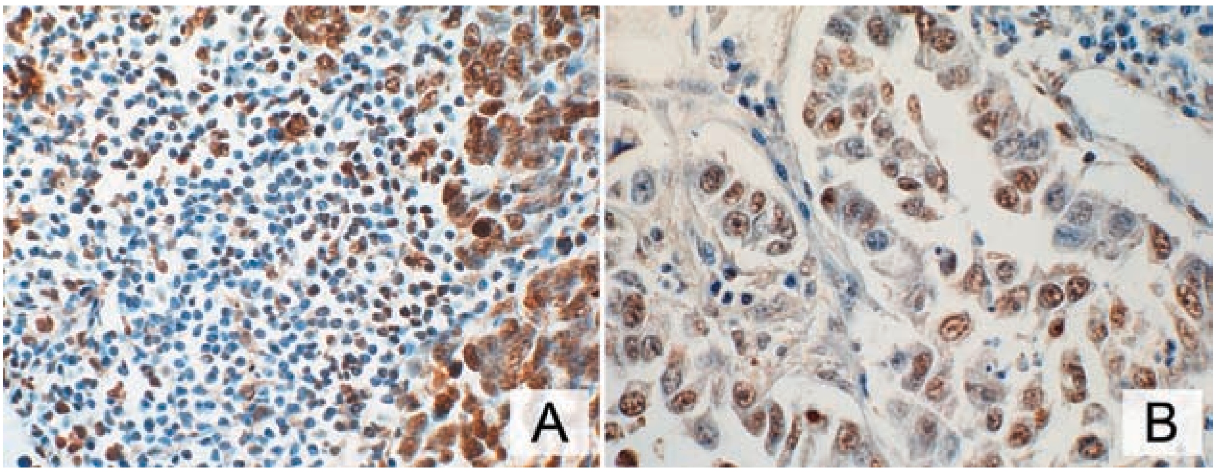

Fig. 11. Hybridocytochemical localization of A) IL-2 in tumor cells and tumor-infiltrating cells in a non-small cell lung cancer patient; and B) IL-2Rα in tumor cells in an adenocarcinoma patient. Objective ×40; nuclei counterstained with hematoxylin

Ryc. 11. Hybrydocytochemiczna lokalizacja: IL-2 w komórkach nowotworowych i naciekających guz u chorego z niedrobnokomórkowym rakiem płuc (A); oraz IL-2Rα w komórkach nowotworowych u chorego z gruczołowym rakiem płuc (B). Obiektyw 40×; jądra komórkowe podbarwione hematoksyliną

Table 1. Comparative analysis of total IL-2, IL-2Rα and IL-2Rß expression, their expression in tumors and in the infiltrating cells in the analyzed groups of patients (Dunn's test)

Tabela 1. Analiza porównawcza ekspresji IL-2, IL-2Rα, IL-2Rß całkowitych oraz w guzie i w nacieku między badanymi grupami chorych (test Dunna)

	$IL\text{-}2^g$	$IL\text{-}2^n$	$IL\text{-}2^c$	$IL\text{-}2R\alpha^g$	$IL\text{-}2R\alpha^n$	$IL\text{-}2R\alpha^c$	$IL\text{-}2Rß^g$	$IL\text{-}2Rß^n$	$IL\text{-}2Rß^c$
TC vs AC	ns	$p < 0.05$	ns	ns	ns	ns	ns	ns	ns
TC vs SCLC	ns	ns	ns	ns	ns	ns	ns	ns	ns
TC vs SQ	$p < 0.001$	ns	$p < 0.05$	ns	ns	ns	ns	ns	ns
TC vs ADENO	$p < 0.001$	ns	$p < 0.001$	ns	ns	ns	ns	ns	ns
AC vs SCLC	ns	ns	ns	ns	ns	ns	ns	ns	$p < 0.05$
AC vs SQ	$p < 0.05$	ns	$p < 0.05$	ns	$p < 0.05$	ns	ns	ns	ns
AC vs ADENO	$p < 0.05$	$p < 0.05$	$p < 0.001$	$p < 0.01$	ns	ns	ns	ns	ns
SCLC vs SQ	ns	ns	ns	ns	ns	ns	ns	ns	ns
SCLC vs ADENO	ns	ns	ns	$p < 0.05$	ns	ns	ns	ns	ns
SQ vs ADENO	ns	ns	ns	$p < 0.001$	$p < 0.01$	$p < 0.01$	ns	ns	ns

p – significance level; ns – statistically non-significant; g – tumor; n – infiltrating cells; c – total.

p – poziom istotności; ns – zmiany statystycznie nieistotne; g – guz; n – naciek; c – całkowita.

Discussion

The immunocytochemical and hybridocytochemical studies were conducted in the usual manner, on formalin-fixed and paraffin-embedded tissue material of typical and atypical lung carcinoids and two principal lung cancers: small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). In the latter histological subtype the tissue material included squamous cell lung cancer and adenocarcinoma. The credibility of the obtained results was supported by statistical analysis.

The conducted analysis of cellular IL-2 expression was focused first of all on establishing whether it was present in lung cancer cells (at the levels of protein and mRNA) and on comparing that to its analogous expression in lymphocytes infiltrating the tumors (TILs).

Studies *in vitro* and *in vivo* have demonstrated that, like lymphocytes infiltrating pulmonary tumors, neoplastic cells themselves are also capable of producing cytokines, growth factors, chemotactic molecules and proteases, which influence tumor growth, its immunogenicity and the immune response of the host [4, 15].

In lung cancers, IL-2 and its receptor have mainly been studied in patients' immunocompetent blood cells (lymphocytes, monocytes), and the studies have been focused primarily on non-small cell lung cancers [4, 9, 12, 17, 18].

The current study has demonstrated that the cellular expression of IL-2 varies depending on the histologic subtype of lung cancer. In the majority of the tumors studied, cytoplasmic expression of the IL-2 protein was detected in tumor cells and in TILs. It was only in some individual typical lung carcinoids that the product of immunocytochemical reaction was also detected in cell nuclei; this might have corresponded to growth control in the tumor cells [4]. IL-2 mRNA expression was confirmed in all the studied patients' tissue material. The transcript for IL-2 and for the α chain of its receptor was localized mainly in the cell nuclei of both tumor cells and TILs. This confirmed the results of Li et al., who used in situ hybridization to detect the presence of IL-2 mRNA in lung cancers (NSCLC), albeit in lower quantities than the mRNAs for IL-4, IL-10 and TGF [22].

Comparing the „quantitative" intensity of IL-2 expression in the various types of tumors examined in this study, significantly more IL-2 was detected in typical lung carcinoids (TC), followed by atypical lung carcinoids (AC), than in squamous cell lung cancers and adenocarcinomas. TCs manifested an intense immunocytochemical reaction located mainly in the tumor cells themselves (and in individual cell nuclei). ACs mostly presented a moderately intense immunocytochemical reaction in the neoplastic cells and TILs; once again the predominant expression was in the neoplastic cells. However, the statistical analysis showed that TILs associated with ACs manifest the most pronounced IL-2 expression, as compared to other types of lung tumors. Atypical lung carcinoids are among the neuroendocrine lung cancers with worse survival prognoses than typical lung carcinoids [26].

Among the lung cancers studied, the least pronounced expression of IL-2 in TILs was detected in adenocarcinoma – significantly lower than in TCs and ACs. This might result from suppressed production of the cytokine by TILs in this type of cancer and may indirectly indicate a poor prognosis. Reduced production of IL-2, indicating immunosuppression, has been demonstrated in the case of SCLC [19]. In the current study, SCLC and NSCLC-SQ did not differ significantly from each other either in total IL-2 expression or in its expression in tumor/TIL compartments. Other authors have described a selective suppression of cytokine secretion (IL-2, IFN-α and γ) in pulmonary carcinomas, dependent on the size of the tumor and differing among patients with SCLC or NSCLC [17]. In the present study no significant differences between SCLC and NSC LC were detected in tissue expression of the cytokine.

It is worth emphasizing that in the adenocarcinomas examined in this study, a positive correlation was documented between IL-2 expression in TILs and the expression of the α subunit of its receptor (IL-2Rα, CD25) in the same cells (indicative of autocrine action?) and in tumor cells of the same patients. This may indirectly indicate a potential for paracrine action of IL-2 on adenocarcinoma tumor cells.

IL-2 expression in TILs was associated with the T lymphocytes CD3+. On the other hand, the total expression of IL-2 itself in the patients studied manifested a positive correlation with the expression of the marker molecule of CD4+ helper T-lymphocytes, but only in typical lung carcinoids. Such relationships were not detected in any other type of the cancers.

Subunit α of the IL-2 receptor participates in binding IL-2, but it is not involved in the processes of internalization and signal transduction. Nevertheless, the presence of the subunit in the IL-2R complex is a pre-condition for high-affinity binding of IL-2 and the cytokine's full biological activity [27].

Cellular expression of the IL-2R α subunit (CD25) was more pronounced in squamous cell lung cancers than in the other tumors analyzed in this study, but a significant difference was documented only in relation to adenocarcinomas, which manifested the lowest expression.

Some recent studies have been aimed at establishing a correlation between the expression of IL-2Rα (CD25) in tumor cells and their growth *in vivo* [3]. The results of those studies prove that overexpression of IL-2Rα is responsible for intensifying the proliferation of neoplastic cells. It is assumed that neoplastic IL-2Rα-positive cells proliferate more rapidly and demonstrate chromosomal instability, which may promote increased tumor aggressiveness, more pronounced resistance to drugs and a less favorable prognosis for the patient [3, 10, 28]. Elevated expression of IL-2Rα mRNA has also been demonstrated in non-small cell lung cancers (squamous cell lung cancer and adenocarcinoma) as compared to such expression in a normal tissue [3].

The current study documented that the presence of the IL-2R α chain (IL-2Rα, CD25) in all subtypes of lung cancers varies in terms of detectability and intensity of expression. Production of the IL-2R subunit α and IL-2R (mRNA and protein) by all cells in all the representatives of lung cancers studied was confirmed, with the most pronounced expression in cells of squamous cell lung cancer and the least intense production in pulmonary adenocarcinoma cells (with a significant difference between them).

Using *in situ* hybridization, production of IL-2Rα mRNA was confirmed in all the lung cancers studied, although the product was not evaluated in a quantitative manner. Nuclear localization of mRNA for the α chain of IL-2R was most often noted.

The immunohistochemical approach also showed that the presence of CD25 was prevalent in TILs accompanying squamous cell lung cancer, and that its expression was significantly higher than in TILs accompanying adenocarcinoma and/or atypical lung carcinoids. A strong positive correlation was documented between the expression of IL-2Rα in tumors and TILs in TCs and adenocarcinoma ($r = 0.7032$). Naturally, CD25 expression was also documented in neoplastic cells in NSCLC-SQ, but the highest quantities were produced by atypical lung carcinoid tumor cells. CD25 molecules in TILs showed an exclusive membrane localization. In immunopositive neoplastic cells in pulmonary cancers, the product of immunocytochemical reaction was usually detected in the cytoplasm of the cells. In the tumor cells themselves the most pronounced tissue expression of the IL-2R subunit was observed in the group of atypical lung carcinoids, followed by NSCLC-SQ and SCLC, and these expressions were significantly higher than the expression in lung adenocarcinoma.

The available literature related to IL-2 receptor expression in pulmonary TILs (including regulatory T lymphocytes) emphasizes that the lymphocytes carry both active chains of IL-2R (α and β) [29], and that they proliferate when cultured in the presence of IL-2 [30]. The role of IL-2 produced by lung cancers and of its receptor expression in TILs and/or neoplastic cells remain difficult to evaluate unequivocally. Further research, including functional studies, is required for an accurate assessment of the essence of tumor cell/TIL interactions in relation to the role of IL-2/IL-2R complex components. The prevalent immunocytochemical and hybridocytochemical expression of IL-2 and of IL-2Rβ (CD122) in tumor cells and of IL-2Rα in TILs regardless of the type of lung cancer involved may suggest their auto- and/or paracrine activity in the microenvironment of pulmonary tumors.

Due to its intra- and extracellular domains, IL-2Rβ plays the most important role in the transduction of a cellular signal following interaction with IL-2 [6].

The most pronounced expression of IL-2Rβ was demonstrated by atypical lung carcinoids (comparable to that in typical lung carcinoids); it was the least pronounced in small-cell lung cancers (SCLC). The difference was significant.

The role of IL-2Rβ (CD122) expression in human neoplastic cells has not been widely documented in the available literature, and the few existing papers on the subject pertained not to lung cancers but to other solid tumors [4]. IL-2Rβ seems to play a significant role in growth control in neoplastic cells, although the mechanisms involved have not been clearly identified. Some authors have noted distinct exogenous IL-2 activity, mediated by IL-2Rβ and γ subunits (growth inhibition), and the distinct effect of endogenous IL-2 effect on processes of cellular growth (growth control or stimulation of growth?) [4]. The authors of those studies have also suggested that IL-2Rβ can also be used by other cytokines than IL-2 and/or by growth factors.

In this study, a cytoplasmic localization of IL-2Rβ was most frequently documented in neoplastic cells, and a membraneous/cytoplasmic localization in tumor-infiltrating lymphocytes; the authors have not been able to confirm other studies' documentation of a nuclear localization of the marker. According to Lin et al. (1995), constitutively produced IL-2 is linked to its receptor on the cell surface. Subsequently, it is internalized and translocated to the cell nucleus along with the β and γ chains. However, using flow cytometry Lin et al. also showed a cytoplasmic localization of IL-2Rβ [4].

In the current study, immunocytochemical investigations made it possible to demonstrate the variable expression of this type of IL-2 receptor in tumor cells as well as in tumor-infiltrating cells. No significant differences were found between tumors and TILS in terms of the localization of the expression in the lung cancers studied.

In none of the studied cases were there significant positive correlations between the expression of IL-2 itself and the expression of IL-2Rβ in lung cancers. On the other hand, almost ideal positive correlations were found between the "tumorous" and "infiltrative" expression of the IL-2Rβ subunit in AC, SCLC and squamous cell lung cancer. In typical lung carcinoids a very high positive correlation was detected between the expression of IL-2Rβ (CD122) and the expression of the cytotoxic T-lymphocyte (CD8) marker molecule. In the case of SCLC, expression of this form of IL-2 receptor correlated with the expression of T-lymphocyte marker molecule (CD3). This may indirectly indicate the presence of IL-2Rβ epitopes on T lymphocytes, including the subpopulation of suppressor/cytotoxic cells (CD8+), in accordance with data in the literature [2].

Cellular expression of IL-2 and both subunits of its receptor in neoplastic cells and TILs in the principal groups of lung cancers (carcinoids, SCLC, NSCLC), may indicate the markers' involvement in growth control of both tumor cells and lymphocytes of the tumor microenvironment. To evaluate the prognostic significance of the obtained data further studies are required, and complete clinical data on patients with various types of lung cancers.

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