The most recent classification of the lung cancer expanded the diagnostic criteria of its histological subtypes and included its immunophenotypic profile. We performed the study to compare the reliability of selected markers in high-grade non-small cell lung carcinoma (NSCLC) in the oligobiopsies with the matched postoperative samples. We evaluated expression of p40, p63, TTF1, cytokeratin 5/6, cytokeratin 7, napsin A, desmoglein 3, desmocollin 3 and mucin secretion as detected by mucicarmine staining.

The study cohort included 123 cases of poorly-differentiated NSCLC. The tissue oligobiopsy material was available in 38 cases. Tissue microarrays (TMAs) from all postoperative cases were constructed.

Comparing the immunophenotype between postsurgical samples and oligobiopsies we found an almost perfect agreement for most of performed IHC reactions. The highest concordance of results was found for desmoglein 3, CK7, and p40, whereas the lowest – for desmocollin 3.

Immunoprofile of the oligobiopsies corresponded well to that in the resection specimens. The most useful markers in poorly differentiated ADs are: TTF1 and napsin A, and for non-keratinizing SCCs: p40, p63, CK5/6 and desmoglein 3.

Key words: high-grade non-small cell lung carcinoma, phenotype, immunohistochemistry, oligobiopsy.
is contraindicated in patients with SCC as it may cause fatal pulmonary haemorrhages [3]. Therefore, the correct pathological diagnosis is crucial for the optimal treatment.

The histological diagnosis of the lung tumors is often based on the small tissue biopsy, as approximately 70% lung cancer patients are not candidates for surgery [8]. In most cases, the diagnosis of histological subtype is straightforward based on standard morphologic criteria. However, in poorly differentiated tumors, it may be a diagnostic challenge, especially if only a small surgical biopsy is available. Additionally, scant cellularity, crush artifacts, tumor heterogeneity or only focal morphological differentiation may also cause the diagnostic problems. Thus, histochemical detection of mucin and immunohistochemistry are often essential tests expanding the routine morphological examination. The specific phenotypic panel characteristic for most cases of SCC (p40, p63, cytokeratin 5/6) and AD (TTF1, napsin A and cytokeratin 7) is helpful in such cases.

We compared the immunophenotype of high-grade NSCLC in the biopsy and in the matched surgical resection samples. We evaluated the following markers: p40, p63, TTF1, cytokeratin 5/6 (CK 5/6), cytokeratin 7 (CK 7), napsin A, desmoglein 3, desmocollin 3. The histochemical mucicarmine staining was also assessed.

Material and methods

Study design and specimen characteristics

One hundred twenty three cases of formalin-fixed paraffin-embedded (FFPE) surgical resection tumor specimens of high-grade NSCLC were obtained from archival files of the Department of Pathology, Medical University of Gdańsk in the period from 2003 to 2015. Oligobiopsy material was available in 38 cases (bronchoscopy or core biopsy). In the remaining cases the diagnosis of NSCLC was established by cytological smears from fine needle aspiration biopsy or intraoperative examination. No neoadjuvant radiotherapy or chemotherapy was administered before surgery. Tissue microarrays (TMAs) from all cases were constructed using Manual Tissue Microarrayer 1 (Beecher Instr. Inc, Sun Prairie, WI) and FFPE blocks from surgical resections. In brief, morphologically representative areas of tumors were determined from the blocks and four cores (each 2 mm in diameter) were obtained from each tumor: two cores from the peripheral portion of the neoplasm and two cores from its central part, omitting the necrotic areas. Punches of normal tonsil samples were added to the array as a built-in internal control. To analyze the heterogeneity of tumors, the TMA recipient blocks were constructed from the different tumor samples than those used for immunohistochemical studies.

The diagnosis in all tumors was verified according to 2015 WHO classification [7]. The total cohort of tumors included: 41 AD with solid and/or micropapillary dominating pattern, 61 non-keratinizing SCC – (including 10 cases of basaloid SCC), 4 large cell carcinomas (LCO), 5 adenosquamous carcinomas (ADSCC), 6 large cell neuroendocrine carcinomas (LCNC), 3 pleomorphic carcinomas, 2 combined large cell neuroendocrine carcinomas (1 combined LCNC and SCC , 1 LCNC and AD) and 1 spindle cell carcinoma.

Application of the modified WHO classification criteria for large cell carcinoma (LCC), yielded in change of diagnosis. From the original group of 17 LCCs, only four cases were retained. 10 out of 13 tumors were reclassified as solid variant of AD, and the remaining three into non-keratinizing poorly differentiated SCC.

Immunohistochemical study

The tissue blocks and TMA were cut into 4 µm thick sections, mounted on silane-covered slides and subjected to standard procedures. The slides were stained using antibodies against p63 [ab111449, Abcam], napsin A [ab 75021, Abcam], desmocollin 3 [ab 14416, Abcam], desmocollin 3 [ab 150554, Abcam], cytokeratin 7 [OV-TL, Dako], cytokeratin 5/6 [D5/16, Dako], p40 [CE IVD, Zytomed], TTF1 [SPT24, Novocastra], mucicarmine staining kit [Roche], according to standard manufacturer’s protocols by Autostainer Link 48, Dako; BenchMark GX, Ventana Roche and NexES Special Stainer, Ventana. Incubation of the primary antibodies against – desmocollin 3 and desmoglein 3, was performed overnight at 4°C. The material was analyzed using a transmission light microscope (Olympus BX 41) with 400x magnification by 2 pathologists. Pneumocytes served as internal controls for TTF1, napsin A and CK 7 reactivity; bronchial basal cells – for p63, p40, CK 5/6, desmoglein 3 reactivity and mucus in the bronchial glands for mucicarmine. The samples of the normal skin were used as control for desmocollin 3. Negative controls (by substituting Phosphate Buffer Saline (PBS) for the primary antibody) were included in each staining bath.

In all sections, immunoreactivity was scored semi-quantitatively by recording percentage of reactive tumor cells. The average percentage of positive tumor cells within the four tumor cores was used as the immunoreactivity distribution for each patient. Diffuse reactivity was defined as labeling of ≥50% tumor cells and focal reactivity if labeling index was from 1–49%. Intensity of reactivity was graded as weak...
(1+, less than normal cells); moderate (2+, same as normal cells); and strong (3+, stronger than normal cells). H (‘histologic’) scores were derived by multiplying percentage of immunoreactive cells (0 to 100) by intensity score (0, 1+, 2+, 3+), yielding a number between 0 and 300.

**Data analysis**

A comparison of the phenotype of NSCLC in the whole sections and in oligobiopsies or TMAs was performed by two tests – Spearman rank correlation and Cohen’s κ coefficient. Spearman rank correlation (r) was used to assess whether expression of markers in whole sections and oligobiopsies or TMAs was independent or coordinated, with r value of 1.0 indicating a perfect direct relationship, r value of 0 indicating the absence of a relationship, and r value of –1.0 indicating a perfect inverse relationship. In addition, the data was measured by estimating Cohen’s kappa coefficient (κ) with the usage Statistica12,0 software. The level of agreement based on κ values was assessed using the Landis and Koch criteria: 0.00-0.20, slight agreement; 0.21-0.40, fair agreement; 0.41-0.60, moderate agreement; 0.61-0.80, substantial agreement; and 0.81-1.00, almost perfect agreement. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for each marker against the gold standard represented by the morphologic and IHC diagnosis. Performance of markers on a continuous scale was analyzed by receiver operator characteristic curves (ROC), in which area under the curve ranges (AUC) between 0.5 – indicating no predictive value and 1 – indicating perfect predictive accuracy (Medcalc software, version 12.2.1.0).

**Results**

**Expression profiles of high-grade NSCLC in whole section versus in oligobiopsies or TMAs**

The detailed results of expression of the analyzed markers are shown in Table I.

Consistency of the immunophenotype between postsurgical samples and oligobiopsies was assessed by calculated H score. We found an almost perfect agreement for most of performed IHC reactions (Table II). The highest concordance of results was found for desmoglein 3, CK 7, and p40, whereas the lowest – for desmocollin 3.

Analysis of H score results achieved in the tumor resections in the comparison with results in TMAs revealed almost perfect agreement for p40, CK 5/6, CK 7, TTF1 (Table II), and the lowest for desmocollin 3. Moreover, the analysis performed for the microarrays indicated very high concordance for most antibodies (Spearman rank correlation ranged between 0.903 a 0.976). Only for mucicarmine and desmocollin 3 was it lower: 0.770 and 0.745, respectively.

In the group of markers characteristic for poorly differentiated SCC the highest concordance of results between surgical resection samples and oligobiopsies/TMAs was achieved for p40 and desmoglein 3, and slightly lower for CK 5/6 and p63.

Among markers typical for AD, TTF1 protein showed very high concordance between whole tissue sections and TMAs, whereas napsin A showed high consistency. Comparing resections and oligobiopsies, both antibodies reached the moderate concordance.

**Table I.** The immunohistochemical expression and histochemical reaction of markers in whole sections versus oligobiopsies versus TMA in SCC and AD

| Marker       | Whole sections | Oligobiopsies | TMA       |
|--------------|----------------|---------------|-----------|
| p40          | 57/61 (93.4%)  | 22/26 (84.6%) | 55/61 (90.2%) |
| p63          | 58/61 (95.1%)  | 22/26 (84.6%) | 47/61 (77.0%) |
| CK 5/6       | 50/61 (82.0%)  | 21/26 (80.8%) | 55/61 (90.2%) |
| Desmoglein 3 | 56/61 (91.8%)  | 22/25 (88.0%) | 51/61 (83.6%) |
| Desmocollin 3| 36/61 (59.0%)  | 20/26 (76.9%) | 27/61 (44.3%) |

**SCC**

|         | Whole sections | Oligobiopsies | TMA       |
|---------|----------------|---------------|-----------|
| TTF1    | 37/41 (90.2%)  | 5/7 (71.4%)   | 37/41 (90.2%) |
| CK 7    | 41/41 (100.0%) | 7/7 (100.0%)  | 41/41 (100.0%) |
| Napsin A| 31/41 (75.6%)  | 4/7 (57.1%)   | 28/41 (68.3%) |
| Mucicarmine | 14/41 (34.1%) | 1/7 (14.3%)   | 12/41 (29.3%) |

**AD**
Immunohistochemical profile of high-grade NSCLC

TTF1 antibody showed both the highest sensitivity (92.5%) and specificity (93.85%) for poorly differentiated ADs. Subsequently, CK7 was characterized by the highest sensitivity – 100%, but very low specificity 72.31% for this histological type. Napsin A showed the second highest specificity – 98.46%, but lower sensitivity – 75.0%. The highest specifici-
ity (100%), but low sensitivity (35%) was demonstrated by mucicarmine. Tables III and IV show the results of these analyses.

For SCC, p40 indicated the highest sensitivity (91.67%) and specificity (91.11%), while desmoglein 3 showed sensitivity of 90.0% and specificity of 88.89%, p63 sensitivity of 93.33% and specificity of 66.67%. CK 5/6 showed specificity of 93.33% and sensitivity of 83.33%.

All studied cases of LCNC showed the expression of neuroendocrine markers (chromogranin, synaptophysin) and diffuse nuclear staining for TTF1. CK7 positivity was observed in 5 out of 6 cases. Moreover, in 2 out of 6 tumors

Fig. 2. High grade squamous cell carcinoma – representative immunohistochemical staining (magnification 10×): A) HE staining; B) diffuse staining with p40; C) CK5/6; D) negative reaction with desmocollin 3 and E) TTF1; F) weak-positive staining with desmoglein 3
strong and diffuse expression of napsin A was detected.

Two of three cases of pleomorphic carcinomas showed phenotype characteristic for AD (diffuse reaction with TTF1, strong diffuse staining for napsin A in one case). In one case, immunoprofile typical for SCC was identified: diffuse reaction with p63, p40 and CK 5/6. Spindle cell carcinoma showed focal expression of CK 7 and very weak and focal staining for napsin A.

Identification of the mucin within the cells in solid carcinomas is a very helpful feature for the diagnosis of AD. It was detected in 34.2% of poorly differentiated ADs in our study. Occasional cases of SCCs with the presence of up to 2-3 vacuoles of mucus in neoplastic cells were noted in our material. However, this feature does not fulfill the criteria of AD diagnosis, as intracellular mucin should be present in ≥ 5 tumor cells in each of two high-power fields [8, 10, 11]. Thus, if tiny fragments of tissue are available, a pathologist may detect single droplets of mucus in the cells of NSCLC, but this does not necessarily favor the diagnosis of AD (due to sample error).

Cytokeratin 7 is highly expressed in all ADs, but at the same time it is present in many SCCs (23%) as well as in majority of LCNCs and LCCs.

Discussion

Currently, the precise determination of the carcinoma subtype is decisive for the treatment of NSCLCs as it may alter the therapeutic approach to the patient. However, an important challenge in the pathological diagnosis stems from the fact that most of patients with this cancer are not operable. Therefore, the small biopsy material may be the only

Table III. Immunohistochemical and histochemical profiles of poorly differentiated adenocarcinomas – whole sections

|                | AD (n = 41) |
|----------------|-------------|
|                | No of positive cases | Sensitivity (%) | Specificity (%) | AUC | PPV (%) | NPV (%) |
| p40            | 3            | 7.32          | 12.31          | 0.10 | 5.00    | 17.39   |
| p63            | 13           | 31.71         | 9.23           | 0.20 | 18.06   | 17.65   |
| CK 5/6         | 3            | 7.32          | 21.54          | 0.14 | 5.56    | 26.92   |
| CK 7           | 41           | 100           | 73.85          | 0.87 | 70.69   | 100.00  |
| TTF1           | 37           | 90.24         | 93.85          | 0.92 | 90.24   | 93.85   |
| Napsin A       | 31           | 75.61         | 100.00         | 0.88 | 100.00  | 86.67   |
| Mucicarmine    | 14           | 34.15         | 100            | 0.67 | 100.00  | 70.65   |
| Desmoglein 3   | 3            | 7.32          | 13.31          | 0.10 | 5.00    | 17.39   |
| Desmocollin 3  | 10           | 24.39         | 44.62          | 0.35 | 21.74   | 48.33   |

AUC = the area under the curve; PPV = positive predictive value; NPV = negative predictive value

Table IV. Immunohistochemical and histochemical profiles of poorly differentiated squamous cell carcinoma – whole sections

|                | SCC (n = 61) |
|----------------|-------------|
|                | No of positive cases | Sensitivity (%) | Specificity (%) | AUC | PPV (%) | NPV (%) |
| p40            | 57           | 93.44         | 93.33          | 0.93 | 95.00   | 91.30   |
| p63            | 58           | 95.08         | 68.89          | 0.82 | 80.56   | 91.18   |
| CK 5/6         | 50           | 81.97         | 91.11          | 0.87 | 92.59   | 78.85   |
| CK 7           | 14           | 22.95         | 2.22           | 0.13 | 24.14   | 2.08    |
| TTF1           | 3            | 4.92          | 15.56          | 0.10 | 7.32    | 10.77   |
| Napsin A       | 0            | 0             | 31.11          | 0.16 | 0       | 18.67   |
| Mucicarmine    | 0            | 0             | 68.89          | 0.34 | 0       | 33.70   |
| Desmoglein 3   | 56           | 90.32         | 91.11          | 0.91 | 93.33   | 87.23   |
| Desmocollin 3  | 36           | 59.02         | 77.78          | 0.68 | 78.26   | 58.33   |

AUC = the area under the curve; PPV = positive predictive value; NPV = negative predictive value
one a pathologist may deal with. An important issue correlated with this problem is morphological heterogeneity of the pulmonary tumors and the significance of supplementary techniques in optimization of the tumor diagnosis.

We focused on high-grade NSCLC, which is a major problem in daily practice as routine morphology is usually insufficient for the unequivocal diagnosis. It usually requires the phenotyping of the tumor cells. Most of the former studies included carcinomas with all degrees of differentiation, which in vast majority do not require immunohistochemistry for their classification. Contrary to that, the precise diagnostics of high-grade NSCLC is necessary both when post-surgical tissue samples or tiny oligobiopsies are available [12, 13].

In our paper we correlated the phenotype of lung carcinomas achieved in the surgical tumor tissue and respective preoperative biopsies. Moreover, we analyzed the results of immunohistochemical staining of multiple cores set in the TMAs. We tried to correlate the tumor phenotype present in the oligobiopsies and oligobiopsy-simulating TMAs with that shown in the whole tumor resections. Many NSCLC are heterogeneous in terms of morphology: the poorly differentiated areas may be intermingled with the areas showing focal features of squamous and glandular differentiation. Likewise, the expression of the respective markers does not always show a diffuse pattern, and in many cases immunoreactivity is only focal. The oligobiopsy may contain only poorly differentiated component of the tumor and in such a case difficulties in the interpretation may arise what is especially evident in high-grade NSCLC [13, 14].

The divergences in results of IHC between biopsies and resection may be caused by heterogeneity of lung carcinomas. Biopsies or TMA cores are only tiny fragments of the entire tumors. To verify the tumor heterogeneity, we have used two tissue blocks from different areas of each tumor: one for the construction of TMA and the second for the whole sections for IHC study.

The small biopsy may not be representative for the whole tumor, thus the IHC results from the oligobiopsies may differ from those obtained in the postoperative material. This could in part explain the reported lack of consistency and accuracy in subtyping of NSCLCs in the literature [11, 13, 14, 15, 16]. The discrepancies may also depend on technical factors. Larger tissue samples may require extended fixation and may cause slower penetration of the fixative that result in altered protein structure than in small biopsy specimens.

We found oligobiopsies as a reliable material for high-grade NSCLC phenotyping enabling the recognition of the histological subtype. We confirmed the previous reports, which concluded that immunohistochemical discrepancies between biopsies and resection specimens are uncommon [15, 16].

High-grade NSCLC are difficult for unequivocal diagnosis based on routine histologic assessment. For example, a portion of solid component of ADs can mimic squamous morphology. Therefore, IHC is often required in poorly differentiated tumors. There are only a few reports dealing with the immunohistochemical phenotype of high-grade NSCLC cohorts based on whole section samples and using multiple markers [12, 13, 15, 17].

TTF1 and napsin A appeared as the most useful markers in the diagnosis of poorly differentiated ADs. TTF1 had a very high sensitivity and specificity (92.5% and 93.85%, respectively) in high-grade NSCLC. In majority of cases, the intensity of IHC reaction was strong or moderate. The low intensity usually correlated with small proportion of stained nuclei. In the literature, the sensitivity for TTF1 in ADs ranged from 54% to 86%, and specificity from 89% to 97% [14, 15, 18, 19, 20]. In multiple studies which included ADs of various histological grades, the sensitivity and specificity for TTF1 widely ranged from 62-98% and 89-97%, respectively [12, 21, 22]. We found TTF1 expression in all LCNCs and in 5% of SCCs. These data are also consistent with the previous studies, in which focal reactivity was noted in 2-5% of SCCs [12, 19, 22, 23].

Among the established markers for AD, napsin A expression has been reported in 59–87% of pulmonary ADs, which is in line with our results. It seems to be a moderately sensitive (64-79% to 87%) and highly specific (94-100%) marker for lung ADs [20, 24, 25, 26], but its expression is associated with higher differentiation of the tumor [22, 27].

P40 and p63 are generally accepted diagnostic markers for SCC. We have determined high sensitivity for both of them. The specificity for p63 is much lower in the group of high-grade NSCLC than for keratinizing SCCs. According to the other authors the sensitivity of p63 ranges from 75% to 100%, whereas specificity between 52% and 88% [12, 13, 14, 15, 19, 20, 21, 22, 28, 29, 30].

Similarly to other studies, we found that p40 has the highest specificity for SCC (93.3%). Therefore, p40 together with TTF1 appear as potentially most reliable markers distinguishing pulmonary SCC from AD. The data in the literature also report high specificity (96.9-100%) and sensitivity of p40: 96.8-100% [20, 28, 29].

We found high CK 5/6 expression in 81.9% of poorly differentiated SCCs, with the specificity of 91.1%. The results in our study showed considerably higher specificity of CK 5/6 than p63 for SCCs. Its high specificity remains in line with earlier studies [14, 20]. The mean reactivity for CK 5/6 is lower
in poorly-differentiated compared with well- and moderately-differentiated SCCs [12, 18, 19, 29]. Among less commonly used markers of squamous differentiation, desmoglein 3 showed high specificity and sensitivity in high-grade NSCLC (respectively 91.1% and 90.3%). We found the positive reaction in 3/41 ADs - in two of them the reaction being focal and in the remaining one was diffuse. The observations of desmoglein 3 reactivity in AD differ: Fukukoka et al. found it in 54.8% tumors [31], Savci-Heijink et al. in 2% of moderately differentiated AD [32], Gomez-Morales et al. and Saaber et al. in none of analyzed cases [33, 34].

The lowest concordance of results between surgical resection samples, oligobiopsies and TMAs was found for desmocollin 3. The expression was moderate (59.02% in whole sections). This antibody often showed the focal reaction, while the staining had usually intermediate intensity. Other authors noted similarly expression of desmocollin-3 in poorly differentiated SCC: 60.9% [29] and 52.2% [19], however, they also found considerably higher specificity of 99-100% [19, 22, 29]. The discrepancies may depend on various clones of antibodies applied or technical factors.

In our study we implemented many immunohistochemical markers to assess their expression in NSCLC samples of various sizes, however, in routine practice the limited number of IHC stainings is used in order to spare the valuable material for other (e.g. genetic) studies. In a way, we found by reevaluation of our cases that LCC is a heterogeneous group of tumors. They lack specific morphologic signs of differentiation but show unambiguous markers if tested by IHC and molecular analysis [35, 36].

In conclusion, we demonstrated that immunoprofiling of the oligobiopsies corresponded well to that in the resection specimens. The most useful markers for poorly differentiated ADs are TTF1 and napsin A, and for non-keratinizing SCCs p40, p63, CK5/6 and desmoglein 3.

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