New immunohistochemical markers in the differential diagnosis of nonsmall cell lung carcinoma

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Background/aim: The aim of this study was to investigate Napsin-A, NTRK-1, NTRK-2, Desmoglein-3, and Desmocollin-3 in the differential diagnosis and prognosis of nonsmall cell lung cancer.

Materials and methods: The expression of Napsin-A, NTRK-1, NTRK-2, and Desmoglein-3 was examined by immunohistochemistry in 50 squamous cell carcinomas and 50 adenocarcinomas. Desmocollin-3 was investigated in 29 squamous cell carcinoma and 29 adenocarcinoma cases. Associations between expression profiles of Napsin-A, NTRK-1, NTRK-2, Desmoglein-3, and Desmocollin-3 in lung cancers and clinicopathological variables were analyzed.

Results: Napsin-A staining was statistically significant in detecting adenocarcinomas versus squamous cell carcinomas. The sensitivity of Napsin-A for adenocarcinomas was 96% and the specificity was 100%. NTRK-2 and Desmocollin-3 staining were statistically significant in detecting squamous cell carcinomas versus adenocarcinomas. Desmoglein-3, Napsin-A, and NTRK-2 had no effect on survival. Disease-free survival time was significantly shorter in cases that were moderately positive with NTRK-1.

Conclusion: Our data suggest that Napsin-A, NTRK-2, and Desmocollin-3 are useful markers in the differentiation of nonsmall cell lung cancer.

Key words: Napsin-A, NTRK-1, NTRK-2, Desmoglein-3, Desmocollin-3, lung cancer

1. Introduction

Lung cancer is a widely seen disease making up 13% of all cancers, being responsible for 18% (1.4 million) of cancer-related deaths (1). It is the major cause of cancer-related mortalities in both sexes across the world. The most important reason for high mortality is the diagnosis being determined at a late stage in 70% of cases, when no effective treatment is available (2,3). Moreover, resection in patients who present with an advanced stage of lung cancer is not appropriate and such patients can only benefit from chemotherapy. The primary pathological diagnosis in advanced stage patients, who represent the majority of the cases, is established through a limited number of specimens from small biopsies and cytology samples (2).

The four major types of lung cancer are small cell carcinoma (SCC) and nonsmall cell carcinomas (NSCLC) including squamous cell carcinoma (SqCC), adenocarcinoma (AC), and large cell carcinoma (LCC). Until recently, it was sufficient to distinguish SqCC from NSCLC, as management was similar for all subtypes of NSCLC (4,5). With the development of new biological agents, accurate distinction between AC and SqCC is now critical as histological subtyping is important in clinical decision making (6). In particular, if the tumor represents a less differentiated or heterogeneous area in small biopsy and cytological samples, the contribution of available immunohistochemical markers to a differential diagnosis becomes even more limited. There is now a greater obligation on pathologists to accurately subtype NSCLC in small biopsy specimens with the help of immunohistochemical and molecular markers (7).

Napsin-A is an aspartic proteinase enclosed in the cytoplasm of type II pneumocytes and alveolar macrophages in the lungs and plays a role in the maturation of surfactant protein-B. Besides the lungs, it is also found in the kidneys' proximal tubule and convoluted tubule epithelia (8). It regulates the absorption and catabolism of the proteins in the proximal tubules of the kidneys (9).

It is a specific marker for pulmonary adenocarcinomas and for a small portion of papillary-type renal cell and papillary-type thyroid carcinomas. It is thought to be a very useful marker when used in combination with TTF-1 in distinguishing between primary adenocarcinomas and metastatic adenocarcinomas of the lungs (8).

The neurotrophic tyrosine kinase receptors NTRK-1 and NTRK-2 are from the family of neuron growth factor receptors responsible for neuronal development. They play a major role in neuronal proliferation and cell survival. Activation of the NTRK gene in the alveolar cells of the lungs regulates the survival and proliferation of alveolar cells. On the other hand, inhibition of NTRK induces apoptosis and reduces clonogenicity. Strong expressions of NTRK-1 have been reported in normal epidermis, oral mucosa, and epidermal carcinomas (basal cell carcinoma and squamous cell carcinoma). NTRK-2 is found in normal lung, liver, and heart tissues. Unlike NTRK-1, it has not been detected in normal epidermis, normal oral mucosa, basal cell carcinoma, or squamous cell carcinomas of the skin. NTRK-1 and NTRK-2 have been found to be expressed more strongly in immunohistochemical terms in squamous differentiation than in glandular differentiation. It has been reported that they can be useful in distinguishing SqCC from AC, especially in small samples such as needle biopsies that cannot be diagnosed in histological terms (6,10).

Desmoglein-3 is an adhesion protein of the cadherin family that is expressed in the stratified squamous epithelium (3). Desmocollin-3 shows stronger expression in the basal layers of the stratified squamous epithelium and is localized in the desmosomal junction of epithelial cells. It shows weaker expression in the basal layers of columnar epithelia of trachea and bronchi in the lungs (11). Desmocollin-3 and Desmoglein-3 are suggested to be valuable new immunohistochemical markers in squamous differentiation (3,11).

The aim of the present study was to determine the role of TTF-1, p63, Napsin-A, NTRK-1, NTRK-2, and Desmoglein-3 and Desmocollin-3 in the diagnosis of SqCC and AC of the lung and to investigate the possible use of these markers in the differential diagnosis and prognosis of SqCC and subtypes of AC. The sensitivity and the specificity values of each marker were compared.

2. Materials and methods

We retrospectively investigated 100 cases of pulmonary carcinoma, 50 being SqCC and 50 AC, which had been diagnosed at the Pathology Department of Pamukkale University Faculty of Medicine between 2007 and 2013. The cancer stages ranged between I and IV and no chemotherapy was administered to the patients but 58% underwent lobectomy, 28% pneumonectomy, and 14%

wedge resection. Records from the electronic hospital data were searched for demographic information, the tumor's preoperative phase, types of treatment, durations of follow-up, and disease outcomes (disease-free survival, overall survival, relapse, and cancer-related mortality data). The diagnosis of pulmonary carcinoma in the histological sections of the resection materials of each patient, the tumor's differentiation, and the presence of metastatic lymph node dissection materials were reviewed and assessed.

In the histopathological examinations, the SqCC patients were assessed based on the 2004 classification of the WHO. The AC cases were reclassified in view of the guidelines published by IASLC/ATS/ERS collectively in 2011 (12), which are recommended for resection specimens. Tumor staging was carried out according to the criteria in the American Joint Committee for Cancer Staging and End Results Reporting (AJCSS) for 2009 (13,14).

A specimen reflecting the tumor tissue best was chosen from each case. Five pieces of 3-μm sections were taken for each case from the chosen paraffin blocks and placed on positive loaded slides to work on Napsin-A, NTRK-1, NTRK-2, Desmoglein-3, and Desmocollin-3 antibodies. Then, with the help of a VENTANA BenchMark LT device, they were stained automatically using anti-Napsin-A antibody (clone EPR6252, Abcam, 1/250 dilution), NTRK-1 antibody (clone 0193R, Bioss, 1/100 dilution), NTRK-2 antibody (clone 0175R, Bioss, 1/100 dilution), anti-Desmoglein-3 antibody (clone 6585R, Bioss, 1/100 dilution), anti-Desmocollin-3 antibody (clone EPR7486, Abcam, 1/50 dilution), p63 (clone 7 JUL, Novocastra, dilution 1/25), and TTF-1 (clone SP141, Novocastra, ready-to-use).

Cases were assessed assuming that granular cytoplasmic staining displays positivity for Napsin-A. The positive controls of Napsin-A were type II pneumocytes and alveolar macrophages. Cytoplasmic staining with or without circumferential membranous involvement was considered positive for NTRK-1 and NTRK-2. Sporadic nuclear positivity was also assessed. The skin was used as the positive control of NTRK-1 and the alveolar macrophages were used as the positive control for NTRK-2. Skin tissue was used as positive control for Desmoglein-3 and Desmocollin-3. Cytoplasmic and nuclear staining with Desmoglein-3 in varying amounts was considered as positive. Cytoplasmic staining and marked membranous staining were considered as positive for Desmocollin-3.

The semiquantitative and subjective grading system was used for Napsin-A, NTRK-1, NTRK-2, Desmoglein-3, and Desmocollin-3 to examine the intensity of staining and the staining rate of tumor cells in 4 categories: 0: no staining, 1: mild staining, 2: moderate staining, 3: marked staining.

The statistical analyses were carried out using SPSS 17.0 for Windows. The Kruskall–Wallis H test was used to compare the variables between the SqCC and AC groups, logistic multivariate regression analysis was used to compare the immunohistochemical staining between the two groups, and the Kaplan–Meier test was used for the analysis of survival.

3. Results
The clinical follow-up information was obtained for all the subjects included in the study. The clinicopathologic data are summarized in Table 1. Histologically, 50 of the subjects (50%) showed SqCC and 50 of them (50%) AC morphology. From those who had AC, 16 (32%) had papillary, 13 (26%) acinar, 8 (16%) solid, 7 (14%) lepidic, 5 (10%) micropapillary, and 1 (2%) minimal invasive subtypes of adenocarcinoma. From the SqCC cases, 16 (32%) showed well differentiated, 21 (42%) moderately differentiated, and 13 (26%) poorly differentiated SqCC morphology. From the AC cases, 8 (16%) showed well differentiated, 29 (58%) moderately differentiated, and 13

(26%) poorly differentiated AC morphology. The smallest tumor diameter was 1 cm and the largest 10 cm, with a mean diameter of 4.01 ± 2.09 cm. Angiolymphatic invasion was found in 49 of the tumors (49.0%). Tumors were found at the bronchial surgery border in 2 (2.3%) of the 84 patients who underwent lobectomy and pneumonectomy. One of these patients was in the SqCC group and the other in the AC group. Pleural involvement evaluation revealed that the tumor infiltrated the visceral pleura in 15 (15%) patients.

According to the new AJCSS lung cancer staging system for 2009, the distribution of the cases by their stages was as follows: in the SqCC group, 8 patients were at Stage IA (8%), 12 patients at Stage IB (12%), 8 patients at Stage IIA (8%), 12 patients at Stage IIB (12%), 4 patients at Stage IIIA (4%), and 6 patients at Stage IV (6%). In the AC group, 15 patients were at Stage IA (15%), 4 patients at Stage IB (4%), 12 patients at Stage IIA (12%), 5 patients at Stage IIB (5%), 4 patients at Stage IIIA (4%), and 10 patients at Stage IV (10%). There was no statistically significant correlation between the distribution of cases by stages or tumor types ($P > 0.05$).

Table 1. Characteristics and clinicopathologic findings of lung cancer cases.

	SqCC Number of cases (n)	AC Number of cases (n)	Percent (%)
Age			
40–65	33	32	65
>65	17	18	35
Sex			
Male	50	39	89
Female	0	11	11
Localization			
Right lung	28	31	59
Left lung	22	19	41
Metastasis status			
Yes	6	10	16
No	44	40	84
Recurrence status			
Yes	3	9	12
No	47	41	88
Survival status			
Alive	30	34	64
Exitus	20	16	36

SqCC: squamous cell carcinoma, AC: adenocarcinoma

All patients in the SqCC group were male; 39 (78%) of the patients in the AC group were male and 11 (22%) were female. The difference between tumor type and sex was statistically significant (P = 0.01). No statistically significant difference was seen between tumor type and age groups, the localization of the tumor, bronchial surgery border, pleural involvement, angiolymphatic invasion, stage, relapse, distant organ metastasis, or exitus (P > 0.05).

The most sensitive marker was p63 (100%) for SqCC in all SqCC cases, followed by Desmocollin-3 (96.6%), Desmoglein-3, and NTRK-2 (both 94%). The most specific marker for SqCCs was p63 (98%), and then came NTRK-2 (36%) and Desmocollin-3 (34.5%). TTF-1 (100%) was the most sensitive marker for AC in all AC and SqCC cases; it was followed by Napsin-A (96%). The most specific marker for ACs was Napsin-A (100%) and then came TTF-1 (98%) (Table 2).

We found a statistically significant difference in favor of adenocarcinoma versus SqCC (P < 0.05) in the analysis of Napsin-A staining and tumor type. Excluding the solid type, all other AC subtypes showed marked staining with Napsin-A. Napsin-A staining in one of the AC cases is shown in Figure 1a. Napsin-A staining was also positive in tumor-free areas, type-II pneumocytes, foamy histiocytes, and some necrotic areas.

In all of the 100 cases, varying degrees of cytoplasmic staining with NTRK-1 were observed in many areas although nuclear staining was less frequent. No relationship between NTRK-1 staining and tumor type was observed (P > 0.05). Comparison of the degrees of staining revealed that 14 of SqCCs (28%) showed mild, 15 (30%) moderate, and 21 (42%) marked staining, whereas 6 of the adenocarcinomas (12%) displayed mild, 11 (22%) moderate, and 33 (66%) marked staining. Comparison of mildly stained materials with moderately and markedly stained cases exposed no statistically significant difference between these two groups in terms of the degree of staining with NTRK-1 (P > 0.05).

Analysis of NTRK-2 staining and tumor type revealed a statistically significant difference in detecting SqCC (P < 0.05). NTRK-2 staining in one of the SqCC cases is shown in Figure 1b. Analysis of Desmoglein-3 staining and tumor type showed no statistically significant difference (P > 0.05). Apparent staining in tumor giant cells was remarkable in addition to positive staining with Desmoglein-3 in nontumor pulmonary parenchyma, type II pneumocytes, inflammatory cells, secretions, endothelial cells, and chondrocytes.

Analysis of Desmocollin-3 staining and tumor type showed a statistically significant difference in detecting SqCC (P < 0.05). Desmocollin-3 staining in one of the SqCC cases is shown in Figure 1c. With Desmocollin-3, positive staining was seen in some type-II pneumocytes, alveolar macrophages, bronchial epithelium, endothelial cells, and in seromucous gland cells in nontumor pulmonary parenchyma.

Fifty patients in the SqCC group were followed up for between 2 and 74 months and 20 of them (40%) had died by the end of the follow-up period. The disease-free survival time was 46.8 ± 4.5 months for SqCC patients. Patients in the AC group were followed up for between 1 and 76 months and 16 of them (32%) had died by the end of the

Table 2. Tumor type and expression of markers in lung cancer cases.

	SqCC n (%)	AC n (%)	P
TTF-1 (+) TTF-1 (−)	1 (2) 49 (98)	50 (50) 0 (0)	<0.05
p63 (+) p63 (−)	50 (50) 0 (0)	1 (2) 49 (98)	<0.05
Napsin-A (+) Napsin-A (−)	0 (0) 50 (100)	48 (96) 2 (4)	<0.05
NTRK-1 (+) NTRK-1 (−)	36 (72) 14 (28)	44 (88) 6 (12)	>0.05
NTRK-2 (+) NTRK-2 (−)	47 (94) 3 (6)	32 (64) 18 (36)	<0.05
Desmoglein (+) Desmoglein (−)	47 (94) 3 (6)	46 (92) 4 (8)	>0.05
Desmocollin (+) Desmocollin (−)	28 (96.6) 1 (3.4)	19 (65.5) 10 (34.5)	<0.05

SqCC: squamous cell carcinoma, AC: adenocarcinoma

follow-up period. The disease-free survival time was 50.1 ± 5.2 months for AC patients. The relationship between tumor type and disease-free survival was statistically insignificant (P > 0.05).

Analysis of stage and tumor type revealed a mean survival time of 64.7 ± 3.8 months at stage I, 44.4 ± 5.1 months at stage II, 13.11 ± 3.5 months at stage III, and 27.0 ± 4.3 months at stage IV. Being at stage I had a protective effect independent of other factors (odds ratio = stage I/ stage IV: 0.15 95% confidence interval: 0.04–0.52) and prolonged survival time. The relationship between tumor stage and disease-free survival was also statistically significant (P < 0.05).

Analysis of immunohistochemical markers and survival time showed that the relationship between survival and Napsin-A (P > 0.05), NTRK-2 (P > 0.05), Desmoglein-3 (P > 0.05), and Desmocollin-3 (P > 0.05) was statistically insignificant. However, disease-free survival time was shorter in NTRK-1 moderately stained cases than in mild stained cases (P > 0.05) (odds ratio for NTRK-1 moderately positive = 3.7; 95% confidence interval: 1.3–10.6) (Figure 2).

Apart from this, no significant relationship was found between survival and tumor type, tumor subtype, tumor diameter, patient age, sex, tumor localization, lymph node metastasis, or angiolymphatic invasion.
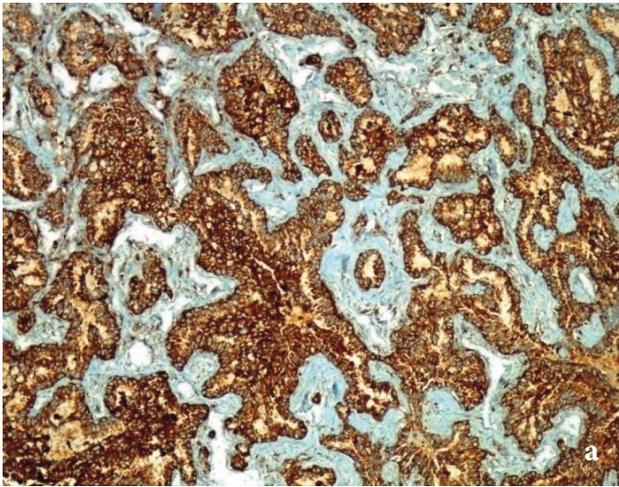

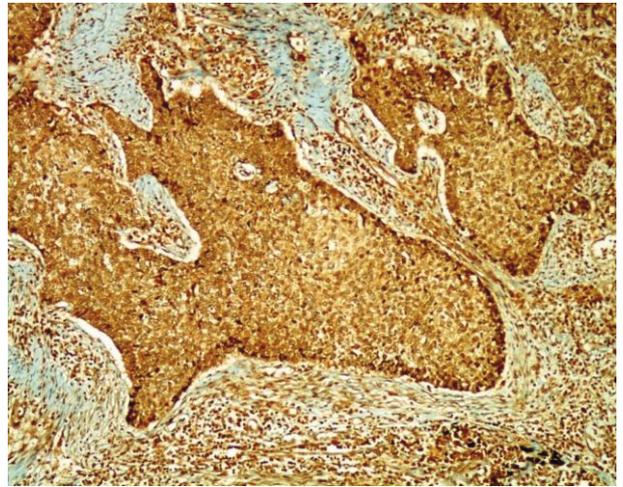**Figure 1a.** Marked Napsin-A staining in one of the AC cases (Napsin-A × 100 ihc).

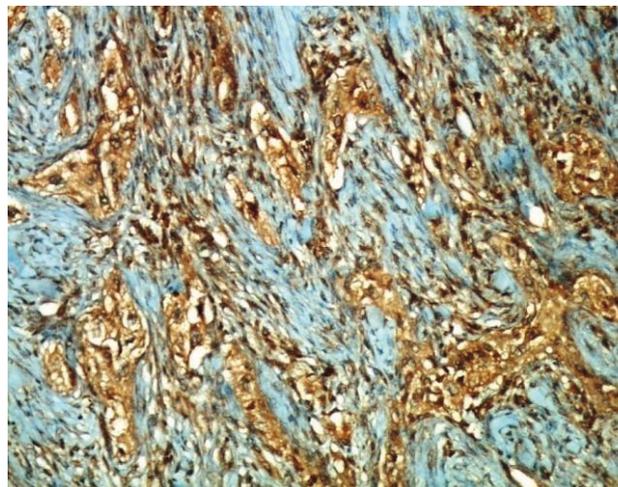**Figure 1b.** NTRK-2 staining in SqCC (NTRK-2 × 100 ihc).

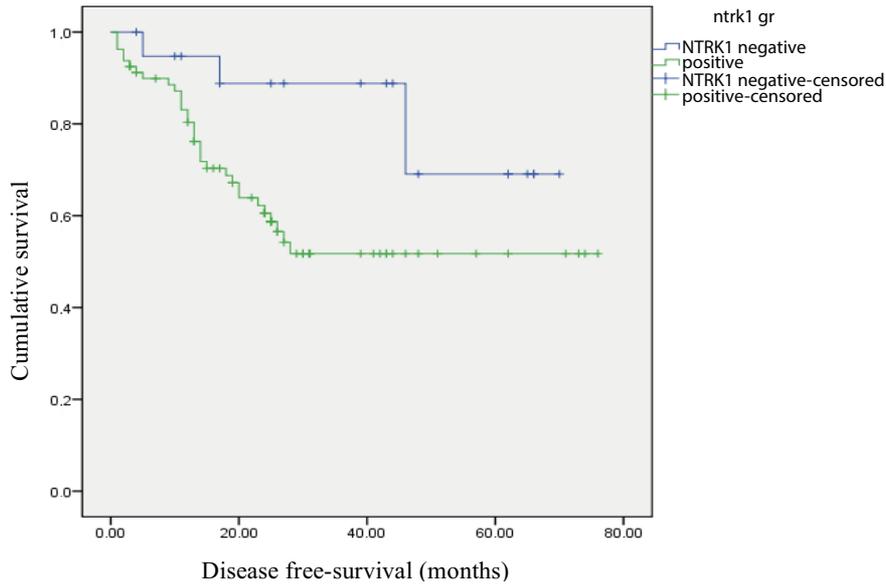**Figure 1c.** Marked Desmoglein-3 positivity in SqCC (Desmoglein-3 × 100 ihc).

Figure 2. Relation between NTRK-1 staining and disease-free survival rate.

4. Discussion

We found that TTF-1 was the most sensitive marker and Napsin-A the most specific marker in pulmonary adenocarcinomas, while p63 showed the highest specificity and sensitivity in SqCC. With respect to sensitivity, p63 was followed by Desmocollin-3 (96.6%) and Desmoglein-3 (94%).

Many studies have shown that Napsin-A positivity ranges between 58% and 91% in pulmonary adenocarcinomas (8,15–19). In our study, Napsin-A was the marker with the highest specificity (100%) and the second highest sensitivity (96%) after TTF-1. As reported in many studies in the literature, we also found manifest Napsin-A negativity for SqCC (20–23).

A study that investigated Napsin-A staining in bronchioloalveolar carcinomas reported that most of the cases were Napsin-A positive. In their series of mucinous and nonmucinous bronchioloalveolar carcinomas, Napsin-A was more sensitive than TTF-1 (20). In our study, 8 of the 50 AC cases were lepidic predominant type (nonmucinous bronchioloalveolar carcinoma as per 2004 WHO) and all of them showed marked staining with Napsin-A as was the case in the above study. Napsin-A was reported to show significantly higher positivity than surfactant protein A in acinar, papillary, and mixed subtypes of adenocarcinomas (19). We also found higher degrees of staining with Napsin-A in the acinar and papillary subtypes, in concordance with this result.

In another study investigating 686 primary pulmonary cancers, NTRK-1 showed high specificity (92.8%) but relatively low sensitivity (71.6%) in SqCC (10). In the same study, NTRK-2 was also found to be specific (96.4%) for

SqCC but with quite low sensitivity (51.3%). The sensitivity of NTRK-1 for SqCC was 72% and the specificity was 28% in our study. Our study presents comparable results with a sensitivity of 94% and a specificity of 6% for NTRK-2 in SqCC cases. Our findings suggest that NTRK-2 can be a useful marker in showing squamous differentiation. Zhang et al. studied 60 patients with nonsmall cell lung cancer with NTRK-2 and reported that NTRK-2 expression was positive in 40 (66.7%) of the patients. A statistically significant correlation was found in these cases only between the NTRK-2 expression and the TNM stage and lymph node metastasis (24). In our study, 94% of the SqCC cases and 64% of the AC cases were found NTRK-2 positive and, in contrast with this study, we observed a significant difference in favor of SqCC between the NTRK-2 expression and the tumor's histological type.

In a study by Tsuta et al., Desmocollin-3 was found to be the most sensitive marker (with 100% specificity) in SqCC (25). We found the specificity of Desmocollin-3 to be quite lower (3.4%) in SqCC, but its sensitivity was higher (96.6%).

Another study revealed Desmocollin-3 positivity in nearly a half of the 62 undifferentiated LCC cases and TTF-1 negativity in all of these cases (11). The authors concluded that Desmocollin-3 was expressed in a portion of pulmonary LCC and that these cases were not stained with TTF-1. They stated that an immunohistochemical panel including Desmocollin-3 and TTF-1 would assist in determining morphology in such cases. In accordance with this, we also found that Desmocollin-3 is a useful marker in determining squamous differentiation and we also suggest that Desmocollin-3 should be present in the

panel when dealing with undifferentiated tumors whose differential diagnoses cannot be established in small biopsy and cytology materials.

Fukuoka et al. showed that Desmoglein-3 had 88% specificity and 98% sensitivity for SqCC compared with AC. They also found that Desmoglein-3 positivity was associated with a poor prognosis (26). Cui et al. reported that Desmocollin-3 was a potential diagnostic marker for SqCC in primary pulmonary tumors and that Desmocollin-3 DNA hypermethylation was correlated with poor prognosis and the Desmocollin-3 gene acted like a new tumor suppressor gene that inhibits EGFR (27). We could not report any significant effect on prognosis with Desmoglein-3.

A study exploring the effect of Napsin-A on prognosis in pulmonary AC reported that Napsin-A negativity was an independent prognostic factor reducing patient survival (28). In our study, 47 of the pulmonary AC cases were Napsin-A positive. Only 3 cases were not stained with Napsin-A and since the number of patients was small in this group, the prognostic significance of Napsin-A in AC was not studied statistically. When the entire 100 cases were assessed, Napsin-A was found not to have a significant effect on survival.

A study suggested that NTRK-1 staining does not have a significant effect on the prognosis of SqCC and AC but is a positive prognostic factor for SqCC in patient survival irrespective of sex, stage, age, and smoking (10). They observed improvements in the disease-related survivals of NTRK-2 positive SqCC cases but the effect of NTRK-2 on survival in AC cases was statistically insignificant (10). Contrasting this, we found that disease-free survival time was significantly shorter in moderately NTRK-1 positive stained cases compared with mildly positive cases, irrespective of the histological subtype and independent of other variables. However, we observed that NTRK-2 did not have any significant effect on the prognosis of SqCC and we did not see any significant relationship between NTRK-2 and TNM or lymph node metastasis.

In conclusion, we showed that Napsin-A, NTRK-2, and Desmocollin-3 staining have statistically significant meaning in detecting squamous cell carcinomas versus adenocarcinomas. Desmoglein-3, Napsin-A, and NTRK-2 had no effect on prognosis but disease-free survival time was significantly shorter in cases that were moderately positively stained with NTRK-1.

We think that including Napsin-A, NTRK-2, and Desmocollin-3 in the panel together with p63, TTF-1, and mucin will improve differential diagnosis in cases of "NSCC, not otherwise specified" that cannot be diagnosed with available stains. However, larger studies are needed to test this observation.

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