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ALK Protein Expression Patterns in Squamous Cell Carcinoma of the Oral Cavity

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Abstract. *Background: Oral squamous cell carcinoma (OSCC) is characterized by a broad spectrum of genomic imbalances, including gross chromosomal (polysomy/aneuploidy) ones as well as specific gene alterations. Aberrant expression of anaplastic lymphoma kinase (ALK) seems to be a useful molecular marker for discriminating patients based on genetic signatures in a variety of solid malignancies, such as lung carcinoma. Our aim was to analyze ALK protein expression patterns in a series of OSCCs. Materials and Methods: Fifty (n=50) OSCC tissue sections were analyzed by implementing an ALK-based immunohistochemistry protocol. Digital image analysis was performed for measuring the corresponding protein expression levels. Results: ALK overexpression was observed in 14/50 (28%) OSCC tissue sections, whereas the rest 36/50 (72%) demonstrated low expression levels. ALK expression was negatively associated with grade (p=0.027) and stage (p=0.0028) of the examined cases. Conclusion: Abnormal ALK expression in subsets of patients with OSCC seems to be related to an aggressive phenotype (advanced stage/progressive dedifferentiation). ALK protein overexpression may be used as a significant marker for applying targeted therapeutic regimens.*

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Identifying specific genetic signatures in solid malignancies is a significant aspect of modern oncology (1). The cancerous cell microenvironment is characterized by gross chromosomal and specific gene numerical and structural aberrations (2-4). Based on molecular criteria, some subgroups of patients achieve extended survival due to high response rates to specific targeted therapeutic agents (5, 6). Epidermal growth factor receptor (*EGFR*, gene locus: 7p12) and anaplastic lymphoma kinase *(ALK*, gene locus: 2p23*)* represent crucial genes frequently deregulated in malignancies, including head and neck squamous cell carcinomas, especially laryngeal and oral squamous cell carcinoma (OSCC) (7-9). Novel targeted therapeutic agents include tyrosine-kinase inhibitors (TKIs) and monoclonal antibodies for subsets of patients that are eligible for them, *i.e.*, those demonstrating genetic profiles, such as mutations and amplification in *EGFR* or translocation in *ALK* genes, respectively (10-12). The *ALK* gene is located on chromosome

Table I. *Clinicopathological parameters of patients with oral squamous cell carcinoma according to total ALK receptor tyrosine kinase (ALK) protein expression.*

			ALK expression, n (%)		
Parameter	Subgroup	Frequency, n (%)	Overexpression	Low	*p*-Value
Whole cohort	(n=50)		14/50 (28%)	36/50 (72%)	**0.001**
Sex	Male	44 (88%)	12/50 (24%)	32/50 (64%)	0.213
	Female	6 (12%)	2/50 (4%)	4/50 (8%)	
HPV history	Positive	18 (36%)	8/50 (16%)	10/50 (20%)	0.245
	Negative	32 (64%)	6/50 (12%)	26/50 (52%)	
Grade	1	18 (18%)	1/50 (2%)	17/50 (34%)	**0.027**
	2	21 (58%)	8/50 (16%)	13/50 (26%)	
	3	11 (24%)	5/50 (10%)	6/50 (12%)	
Stage	I	9 (18%)	1/50 (2%)	8/50 (16%)	**0.0028**
	II	26 (52%)	1/50 (2%)	25/50 (50%)	
	III	15 (30%)	12/50 (24%)	3/50 (6%)	
Smoking status	Positive	38 (76%)	12/50 (24%)	26/50 (52%)	0.346
	Negative	12 (24%)	2/50 (4%)	10/50 (20%)	

Overexpression (moderate to high expression): RGB values ≤139 for stained cells; low expression: RGB values >151 for stained cells. Statistically significant *p*-values are shown in bold.

2 (2p23 band) and encodes a protein that acts as transmembrane tyrosine kinase receptor (6). *ALK* fusion with nucleophosmin (*NPM*) (gene location 5q35) is the result of t(2;5) chromosomal translocation. Specific histopathological entities based on this genetic mechanism include neuroblastoma, anaplastic large-cell lymphoma (an aggressive non-Hodgkin's lymphoma type), and non-small-cell lung carcinoma (NSCLC) (13, 14). In the current experimental study, we analyzed ALK protein expression levels in a series of OSCC tissue sections in order to detect significant associations with their corresponding clinico-histological features.

Materials and Methods

Study group. In the current protein analysis-based study, we selected 50 archival, formalin-fixed and paraffin-embedded tissue blocks derived from surgically resected and histologically confirmed primary OSCCs. The specimens were obtained from 44 male and six female patients with the majority of them to be smokers (n=38) and a significant subset were exposed to persistent human papillomavirus infection. The hospital Ethics Committee consented (Reference ID Research Protocol: 2226/09.09.2018) to the use of these tissues for research purposes, according to World Medical Association Declaration of Helsinki guidelines (2008, revised 2014). The corresponding tissue samples were fixed in 10% neutral-buffered formalin. Hematoxylin and eosin-stained slides of the corresponding samples were reviewed for confirmation of histopathological diagnoses. All lesions were classified according to the histological typing criteria of World Health Organization (15). Clinicopathological data of the examined cases are demonstrated in Table I.

Antibodies and immunohistochemistry (IHC). The IHC protocol was based on the selection of a ready-to-use monoclonal mouse anti-ALK (CD246) (clone ALK-1, dilution at 1: 100, 30 min at 25°C; DAKO, Glostrup, Denmark). IHC was applied using 4-μm serial sections of the tissue blocks. The corresponding slides were deparaffinized and rehydrated. All of them were enzyme digested for 10 min at 37°C. The EnVision™+; DAKO) detection system was applied for proceeding to the next detection steps. Blocking solution was applied to the slides for 10 min, followed by incubation with ALK antibody for 1 h at room temperature. Following incubation with secondary antibody for 10 min, diaminobenzidine-tetrahydrochloride (0.03%) containing 0.1% hydrogen peroxide was used as a chromogen and incubated for 5 min. Then the tissues were counterstained, dehydrated and cover-slipped. In order to determine the negative immunostaining pattern in control slides, the primary antibody was omitted. The IHC protocol was implemented on an automated staining system (I 6000; Biogenex, San Ramon, CA, USA). Focal or diffused cytoplasmic/perinuclear staining patterns for ALK protein expression were considered acceptable, according to the manufacturer's instructions (Figure 1). Microscopically normal appearing oral epithelia tissue sections that expressed ALK protein were used as positive controls.

Digital image analysis (DIA). ALK protein expression rates were measured quantitatively by calculating the corresponding staining intensity levels (densitometric evaluation) in immunostained malignant cells. A DIA protocol was implemented by applying a semi-automated system (hardware: CX-31 microscope – Olympus, Melville, NY, USA; Sony DSC-W220 digital camera, Tokyo, Japan; Windows XP/NIS-Elements Software AR v3.0 – Nikon Corp., Tokyo, Japan). Identification of fields of interest per slide (five optical fields at ×400 magnification) was followed by a digital database construction including the corresponding snapshots. ALK protein levels were measured by implementing a specific macro evaluating mainly cytoplasmic and mixed perinuclear staining for tumor cells, according to the manufacturer's mouse monoclonal anti-ALK guide). Based on an algorithm, a broad spectrum of continuous grey scale values (0-255) at the RedGreenBlue analysis

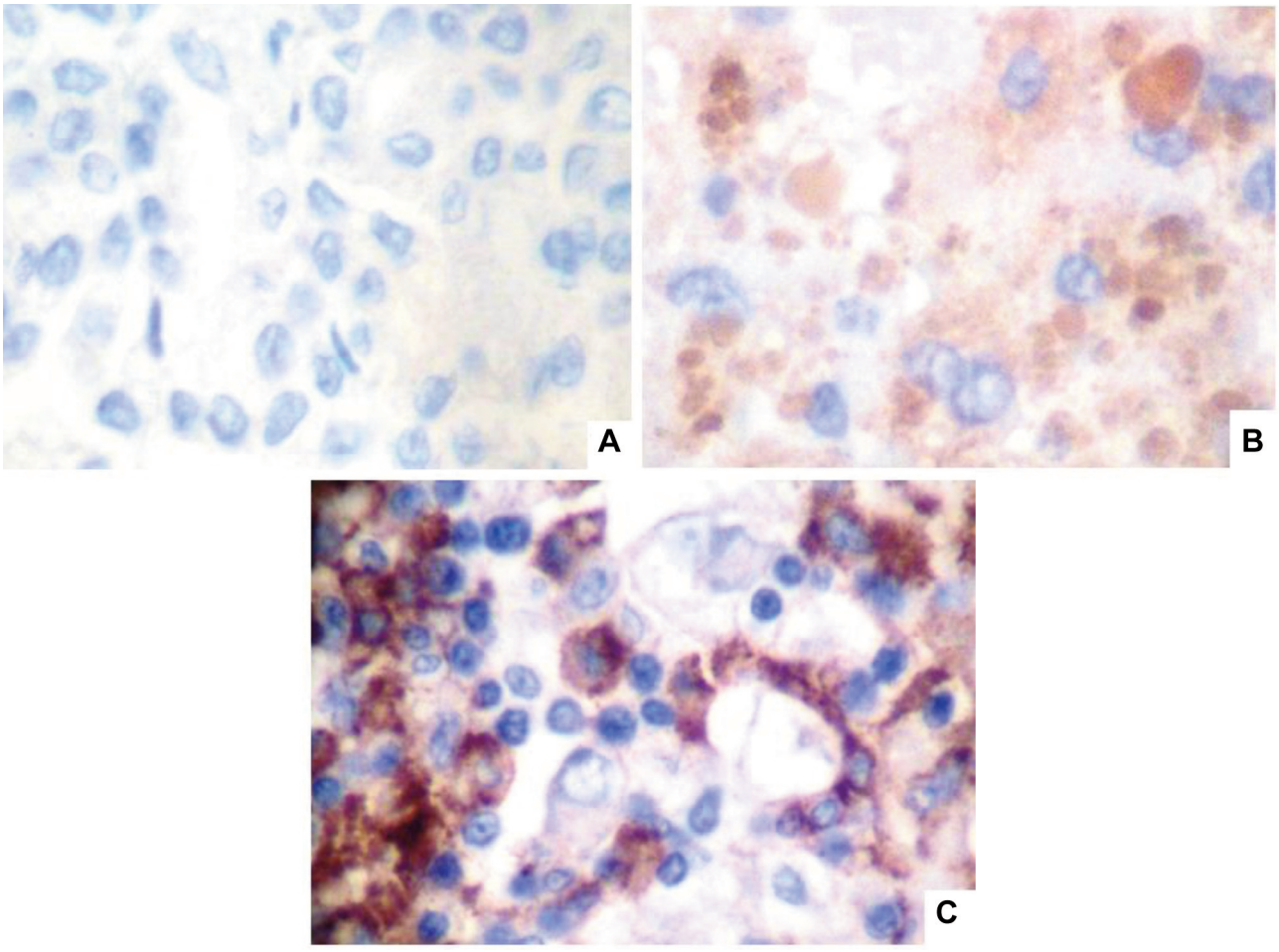

Figure 1. *Different ALK receptor tyrosine kinase (ALK) protein expression levels in oral squamous cell carcinoma tissues. A: Negative expression (absence). B. Low expression. C: High expression. Note differences in (focal or diffuse) negative, low and strong cytoplasmic/perinuclear staining patterns on diaminobenzidinetetra hydrochloride-based immunohistochemistry analysis (original magnification: ×400).*

was available for detecting and discriminating different protein expression levels. Immunostaining intensity levels decreasing to 0 demonstrate a progressive overexpression of the marker; in contrast, staining intensity values increasing to 255 represent a progressive loss of expression (Figure 2).

Statistical analysis. Statistical analysis was based on correlations between variables such as different protein expression rates, and clinicopathological features including sex, tumor grade and stage. Pearson chi-square test was implemented along with its 99% confidence interval. Spearman correlation coefficient was also utilized (SPSS v20; IBM, Armonk, NY, USA). Two-tailed values of $p \leq 0.05$ were considered statistically significant.

Results

Based on ALK IHC assay, the analyzed malignant tissue sections demonstrated a variety of protein expression levels. Results of the analysis are given in Table I. Concerning overall ALK expression, 14/50 (28%) OSCC tissue sections demonstrated high staining intensity levels, whereas the rest 36/50 (72%) demonstrated low expression. Aberrant ALK expression was negatively correlated with grade ($p=0.027$) and with stage ($p=0.0028$) of the examined cases. No statistical significance was observed associating ALK expression with sex, smoking status, or human papillomavirus history ($p=0.213$, $p=0.346$, and $p=0.245$, respectively). Interestingly, ALK expression was consistent in all of the examined cases, without internal bi-phasic patterns.

Discussion

Concerning solid malignancies, aberrant ALK expression as a result of gene rearrangements (fusion/mutation) is not a frequent event in head and neck squamous cell carcinoma (including OSCC) unlike NSCLC (16, 17). It is well established that

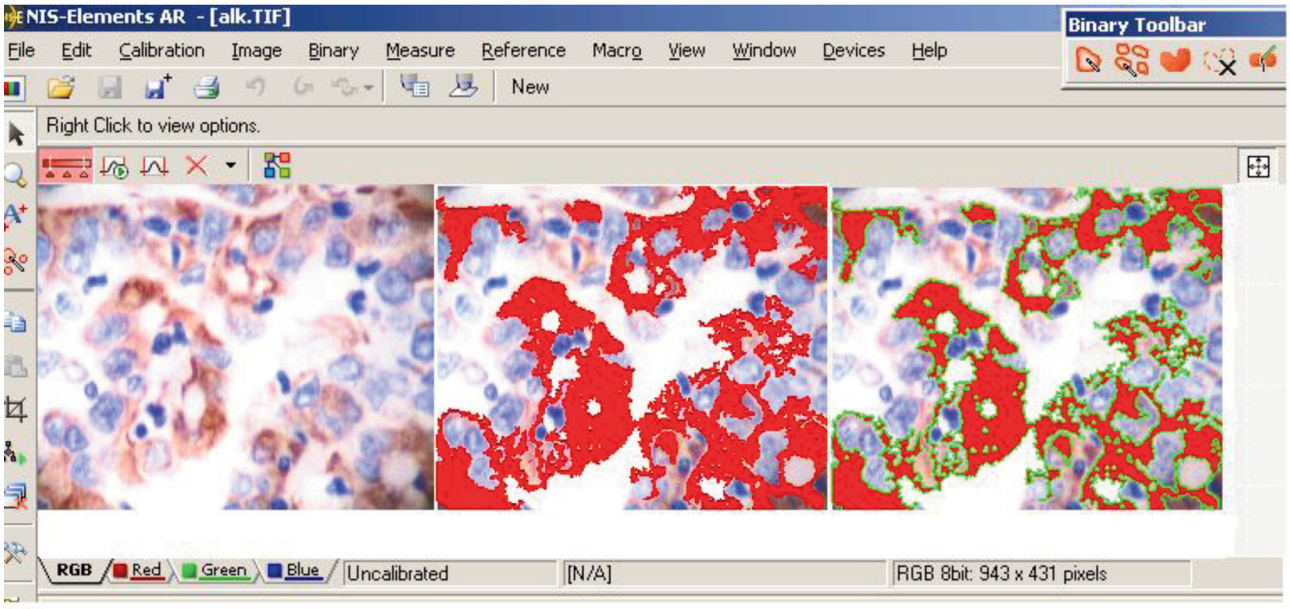

Figure 2. *Digital image analysis of ALK receptor tyrosine kinase (ALK) expression in an oral squamous cell carcinoma tissue section. Red areas represent different expression values of the marker. Green loops surrounding red areas represent the final stage of the digital analysis procedure providing objective numerical data as distinct staining intensity levels (diaminobenzidine tetrahydrochloride staining after immunolabeling; original magnification: ×400).*

translocation and fusion with *EML4* gene is the main mechanism of ALK deregulation in solid malignancies (carcinomas). Targeted regimens based on TKIs, such as crizotinib, are applied to subgroups of patients. Other frequently observed molecular signatures include epigenetic silencing of the *ALK* gene *via* DNA hypermethylation of promoter CpG islands in OSCCs. The role of this genetic mechanism in the biological behavior of OSCCs is currently under investigation. A molecular study reported increased levels of *ALK* promoter methylation in OSCCs without nodal metastasis, whereas relatively lower methylation levels were present in metastatic tumors (18). In our IHC-based protein analysis we detected a relatively significant subset of specimens that demonstrated overexpression of this marker. In these patients, ALK overexpression was associated with an aggressive phenotype (advanced stage/progressive dedifferentiation). Interestingly, co-analysis of EGFR and ALK protein expression levels seems to provide remarkable data for discriminating subgroups of patients with OSCC using specific genetic signatures. A combined western blot analysis of EGFR and ALK revealed progressively increasing ALK activity in OSCC tissues demonstrating advanced p-stage (19). Besides this, they observed that by inhibiting both ALK and EGFR molecules in cancerous cell lines, a significant reduction in EGFR phosphorylation was achieved. Similarly, in a previous study, we showed that EGFR overexpression is frequently detected combined with minimal ALK expression. Patients with OSCC demonstrating EGFR/ALK protein overexpression fulfill the genetic criteria for targeted therapeutic strategies (20).

Combined mutated EGFR- and fusion ALK-targeted therapeutic strategies are applied in patients with malignancies of different histological types. Initially anti-EGFR/ALK TKIs and monoclonal antibodies were used in patients with advanced NSCLC, including gefitinib, erlotinib, afatinib, apatinib, crizotinib, osimertinib, ceritinib, alectinib, brigatinib, lorlatinib, and alectinib (21-26). Interestingly, crizotinib was the first Food and Drug Administration-approved ALK inhibitor for utilization in locally advanced or metastatic ALK-positive NSCLC cases, whereas alectinib is a relatively novel, most-promising selective inhibitor (27, 28). Focused on anti-ALK targeted strategies, all of these agents are under investigation.

Furthermore, identification of specific micro-RNAs (miRs) that affect ALK expression and promote oncogenic activity is still ongoing Recently published molecular data show that *miR-1271* overexpression leads to progressive suppression of cell proliferation, increased colony deformation, and reduced migration and invasion of OSCC cells. Additionally, ALK was considered a target for *miR-1271* function by inversely associated to its expression in OSCCs (29). Applying ALK inhibitors combined with *miR-1271* enhancers seems to be a strong example for future targeted therapeutic approaches in subgroups of patients with specific characteristics. As well as miR micro-markers, additional gross chromosomal and specific genetic imbalances combined or not with EGFR/ALK deregulation are critical for prognosis and response to specific chemotherapy regimens in patients with OSCC (30-32).

In conclusion, our study revealed different ALK protein expression in subsets of patients with OSCC. Although ALK overexpression is detected in limited proportions of patients with OSCC, it is a critical molecule for partially explaining the aggressive phenotype of tumor in these patients. Furthermore, ALK overexpression in subsets of patients with OSCC seems to be related to an aggressive phenotype (advanced stage/progressive dedifferentiation). ALK protein overexpression should be used as a significant marker for discriminating patients with OSCC and implementing targeted therapeutic regimens for eligible ones with specific gene signatures.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

A. Chrysovergis, V. Papanikolaou, P. Pantos, E. Tsiambas, S. Mastronikolis: researcher, paper writing: P. Fotiades, P. Mamoulidis, P. Katsinis, A. Manoli, S. Papouliakos: statistics, clinical advisor, D. Spyropoulou, E. Kyrodimos, N. Mastronikolis, D. Peschos, V. Ragos: Academic advisor.

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