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ORIGINAL ARTICLE

Introducing Cytology-Based Theranostics in Oral Squamous Cell Carcinoma: A Pilot Program

Anna Patrikidou[1] · **Rosalia Maria Valeri**[2] · **Kyriaki Kitikidou**[4] · **Charikleia Destouni**[2] · **Konstantinos Vahtsevanos**[3]

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Abstract We aimed to evaluate the feasibility and reliability of brush cytology in the biomarker expression profiling of oral squamous cell carcinomas within the concept of theranostics, and to correlate this biomarker profile with patient measurable outcomes. Markers representative of prognostic gene expression changes in oral squamous cell carcinoma was selected. These markers were also selected to involve pathways for which commercially available or investigational agents exist for clinical application. A set of 7 markers were analysed by immunocytochemistry on the archival primary tumour material of 99 oral squamous cell carcinoma patients. We confirmed the feasibility of the technique for the expression profiling of oral squamous cell carcinomas. Furthermore, our results affirm the prognostic significance of the epidermal growth factor receptor (EGFR) family and the angiogenic pathway in oral squamous cell carcinoma, confirming their interest for targeted therapy. Brush cytology appears feasible and applicable for the expression profiling of oral squamous cell carcinoma within the concept of theranostics, according to sample availability.

Keywords Cytology · Biomarker · Oral squamous cell carcinoma · Angiogenesis · Theranostics

✉ Anna Patrikidou
anna.patrikidou@gustaveroussy.fr

[1] Department of Cancer Medicine, Gustave Roussy, Cancer Campus, Grand Paris, 114 rue Edouard Vaillant, 94805 Villejuif, France

[2] Department of Cytopathology, "Theagenio" Cancer Hospital, Thessaloniki, Greece

[3] Department of Oral and Maxillofacial Surgery, "Theagenio" Cancer Hospital, Thessaloniki, Greece

[4] Department of Forestry and Management of the Environment and Natural Resources, School of Agricultural Sciences and Forestry, Democritus University, Alexandroupoli, Greece

Introduction

OSCC Epidemiology

Head and neck squamous cell carcinoma (HNSCC) is the 6th commonest cancer, with an estimated worldwide incidence of 0.65 million new cases and 350,000 deaths annually [1]. Within HNSCC, oral squamous cell carcinoma (OSCC) accounts for 90 % of oral cancer, and represents an anatomical region of moderate prognosis in the cartography of the head and neck tumors. The overall 5-year cancer-specific survival is 62 %, ranging from 82 % for the localised disease to 57 % for the node-positive tumors and 35 % for the metastatic disease [2].

Theranostics and Targeted Therapy

The concept of blocking key pathways of tumour survival and growth is supported by abundant evidence, has found celebrated applications in targeted therapy of solid tumours, and is currently undergoing daring expansion in all fields of oncology. The concept of theranostics is an emerging treatment strategy that combines the modalities of therapy and diagnostic imaging [3]. In this context, it aims to develop material or apply existing technologies with the capacity of monitoring the treated tissue and efficacy in the long-term period. It can also be used for identifying patients most likely to benefit from tailored cancer targeted therapy, therefore personalising treatment from the early diagnostic stages. In order to achieve this, the selection and validation of appropriate biomarkers is essential.

Prognostic Biomarkers in OSCC

The network-based meta-analysis of the HNSCC transcriptome data has identified enriched signaling pathways and hotspots of transcriptional profiles implicated in the regulation of early, advanced and metastatic HNSCC. Amongst these vascular endothelial growth factor (VEGF) and EGFR signaling, integrin signaling, p53 signaling, antigen presentation genes and epithelial-matrix interaction pathways are significantly overexpressed hubs [4]. Unsurprisingly, these pathways have also been identified as prognostic biomarkers, with a relationship between survival parameters of OSCC patients and their expression levels, mainly using immunohistochemistry [5].

Angiogenesis Biomarkers

The VEGF family and their receptors play a pivotal role in OSCC, and is most frequently used by a tumor to switch on its angiogenic phenotype. VEGF represents an independent negative prognostic factor for OSCC [6–9]. Anti-VEGF therapies are successfully integrated in the management of major solid tumors such as colorectal, lung and ovarian cancer, and are currently investigated in HNSCC, in combination with chemotherapy or other targeted therapy [10–13].

CD34 is a highly sensitive marker for endothelial cell differentiation and has also been studied as a marker for angiogenesis in vascular tumors. When overexpressed around tumor microvessels in cancer cell nests and marginal areas of cancer infiltration it is associated with early lymph node metastasis and poor survival in OSCCs [14, 15]. This pattern of stromal CD34 expression seems to be dynamic and specific: the border of invasive squamous cell carcinomas is characterized by a loss of CD34-positive fibroblasts paralleled by a gain of α-SMA-positive myofibroblasts [16]. Furthermore, the existence of CD34-positive penetrating vessels within tumor nests was significantly associated with risk of cervical node metastasis [17, 18].

Platelet-derived Endothelial Cell Growth Factor (PDGF) is overexpressed [15, 19] and is an independent prognostic factor for poor survival in OSCCs [15]. Furthermore, as PDGF is identical to thymidine phosphorylase, an essential enzyme for the activation of prodrugs of 5-fluorouracil (5FU), it is predictive of response to 5FU-based chemotherapy [15].

Cell Growth and Proliferation Biomarkers

The EGFR family members including EGFR, ErbB2 (Neu, HER2), ErbB3, and ErbB4 play a critical role in cancer development. Aberrant expression of EGFR and its dimerisation partner HER2 are indicators of poor prognosis in OSCC [20, 21]. Frequent co-expression of ErbB receptors may enhance oncogenicity due to receptor heterodimerization and predict worse disease outcome in patients with OSCC [21]. EGFR, overexpressed in 80–90 % of HNSCC, is an early event associated with more aggressive disease, regional lymph node metastasis, resistance to chemotherapy and poorer survival [22, 23]. The frequency of HER2 overexpression varies between 6 % and over 80 % depending on tumor type and has been associated with shorter disease-free (DFS) and overall survival (OS) in OSCC in some studies [20, 21, 24–26], although not all studies [27, 28]. EGFR and Her2 have been shown to be more frequently overexpressed in oral cavity tumors compared to other HNSCC localizations [24–29]. Agents targeting EGFR are now approved for HNSCC treatment. Intriguingly, the activation status of HER2 but not EGFR predicts resistance to the EGFR inhibitor gefitinib in HNSCC [30], suggesting that interactions between family members are important.

c-KIT (CD117) is a transmembrane tyrosine kinase receptor, structurally related to PDGFR. Its natural ligand is stem cell factor (SCF). The SCF/c-KIT signaling pathway is closely related to the regulation of tumor cell proliferation, differentiation, adhesion, and apoptosis. c-kit was found to be overexpressed in 86 % of oral/oropharyngeal tumors, however not correlated with DFS [31]. Like CD34, c-kit seems to have a selective pattern of expression in tumor-associated myofibroblasts in OSCCs [16]. In another study, reactivity to c-kit was confined to stromal cells, many of which were arranged as a barrier near the front of invasion in OSCC tumors. Most of these CD117+ cells were of mesenchymal origin, enhancing the formation of tumor stroma [32].

Cyclooxygenase (COX) is the rate-limiting enzyme in the formation of prostaglandins. COX-2 is overexpressed in OSCC, and is a predictor of poor survival [33] and poor DFS [34], although not in all studies [35, 36]. Prostaglandins can enhance tumor growth and metastasis by stimulating angiogenesis [34] and invasiveness, in addition to inhibiting apoptosis and immune surveillance. Short-term administration of a COX-2 inhibitor restored anti-tumoral immunity and increased infiltration into the tumor of monocytes and Th1 and CD25+ activated lymphocytes [37]. Thus, in vivo inhibition of the COX-2 pathway may potentiate cancer immunotherapy. Selective inhibitors of COX-2, such as celecoxib, have chemopreventive action in advanced oral premalignant lesions [38]. Combined COX-2 and EGFR inhibition in the neoadjuvant setting decreased tumoral proliferation in HNSCC patients [39]. Studies in combination with EGFR inhibitors in the adjuvant (NCT01515137) or metastatic setting (NCT00392665), as well as for radiosensitisation (NCT00581971) are currently ongoing.

Cytology-Based Theranostics in OSCC

The immunohistochemical (IHC) expression of the above-mentioned markers has been investigated to a variable degree in OSCC. However, the feasibility and reliability of their immunocytochemistry detection (ICC) has not to date been investigated for the majority of the above markers.

The purpose of our study was to investigate the expression profile of selected markers in cytological brushes of OSCCs using ICC and to verify their prognostic significance. This does not only involve use of archived tissue to establish the medical utility of a marker, but also assesses the applicability and prognostic validity of a non-invasive, easily repetitive technique.

Materials and Methods

Patient Cohort

Patients with histology-proven OSCC for whom both clinocopathological data and archived cytological material were available were selected. All patients were treated at the Department of Oral & Maxillofacial Surgery of the "Theagenio" Cancer Hospital of Thessaloniki, Greece in the period 2003–2011. The study was approved by the Institutional Review Board of "Theagenio" Cancer Hospital.

Data Collection

Data were extracted from the patient notes and collected in a dedicated electronic database, and were subsequently coded for statistical analysis. Data were collected on patient demographics, disease characteristics (localization, staging, histology, treatment modalities), disease outcome (relapse-free survival [RFS], OS and last known status) and biomarker ICC scoring.

Marker Selection

A set of 7 markers (EGFR, c-erb-B2, COX-2, PDGF, VEGF, CD117/c-KIT, CD34) was selected from the spectrum of prognostic gene expression changes in OSCC. They were selected to represent pathways for which commercially available or investigational agents exist for clinical application in other types of tumors, including epithelial tumors. EGFR was selected to serve as an internal control for the sensitivity/specificity of the cytology-based diagnosis, since it extensively studied in HNSCC [Ang 2002, Kong 2006]. Finally, we aimed to have a molecular tumor profile readily available for future personalized medicine treatment planning.

Marker analysis was subject to specimen availability, so not all markers could be investigated in each patient. Analysis was performed on primary tumor specimens exclusively.

Specimen Collection

All brush biopsy specimens were collected by two trained cytopathologists of the Department of Cytopathology, "Theagenio" Cancer Hospital of Thessaloniki, Greece. Specimens were collected, processed, and archived using departmental standard operational procedures.

Immunocytochemical Techinique

Profiling was performed using conventional cytology and Liquid Based Cytology (ThinPrep®, Cytyc Co, USA), subject to specimen availability. There was no specific intentional order for marker analysis, and patient samples were analyzed according to specimen availability. Assay-specific procedures were determined individually for each marker. Monoclonal antibodies against VEGF (clone EP1176Y, 1:50, Biocare Medical), PDGF (clone P-GF.44C, 1:50, Novocastra), c-Kit (CD117) (Clone T595, 1:25, Novocastra), and CD34 (clone QBEnd/10, 1:50, Biocare Medical) were used. Cytoplasmic and cell membrane staining for VEGF-A and CD34, nuclear and cytoplasmic for PDGF and membranous staining for c-Kit (CD117) was evaluated. A single examiner (RMV) evaluated the results to ensure consistency and eliminate inter-examiner variability bias. The staining positivity was scored as strong (3+ when >50 % of cells were stained), intermediate (2+ when 15–50 % of cells were stained, weak (1+ when 10–15 % of cells were stained) and 0 (when less than 10 % of the cells were stained).

Statistical Analysis

The correlation of immunopositivity to several factors like smoking history, histological grade, pathological and clinical staging, treatment modality, local control of the disease, RFS and OS was investigated.

The Wilcoxon rank sum and χ^2 tests were used to compare variables between groups. OS was defined as the time from surgery to death from any cause. RFS was defined as the time from surgery to disease relapse (local, regional or distant). Censoring was at the date of last contact for surviving or non-relapsing patients respectively. The Kaplan-Meier method and Cox proportional hazards models were used to assess the association between predictor variables and time-to-event outcomes. All P values are two-sided. $P < 0.05$ was considered statistically significant. Analyses were conducted the SPSS software version 10.

Data mining was done with the use of the CHAID (Chi-squared Automatic Interaction Detection) tree-growing algorithm. Significance level for splitting and merging was set at 5 %, adjusted using the Bonferroni method. The feature selection node was also used for data mining, using the following set of criteria: maximum percentage of missing values: 70 %; maximum percentage of records in a single category: 90 %; maximum number of categories as a percentage of records: 95 %; minimum coefficient of variation: 0.1; minimum standard deviation: 0.0. Artificial neural network forecasting was done with use of the Multilayer Perceptions (MLP) method.

Results

Patient Cohort

The cohort included 99 patients (67 males [67.7 %], 32 females [32.3 %]) with OSCC. All tumors were localized in the oral cavity and lip. Baseline characteristics are presented in Table 1. Details on treatment modalities and outcome measurements are given in Table 2.

Marker Analysis

The immunocytochemical expression analysis of the different markers is outlined in Table 3.

Survival Analysis-RFS and OS

Four clinicopathological factors were identified in the univariate analysis as significant for OS: M stage ($p = 0.016$), surgery ($p = 0.000$), local control at the end of definite treatment ($p = 0.000$) and relapse ($p = 0.007$) (Table 4). Four markers were significant in the univariate analysis: EGFR ($p = 0.003$) (3+ expression associated with shorter OS and increased risk of death), PDGF ($p = 0.002$) (lack of expression associated with longer OS and decreased risk of death), VEGF ($p = 0.035$) (lack of expression associated with longer OS and decreased risk of death), and CD34 ($p = 0.010$) (3+ expression associated with shorter OS and increased risk of death) (Table 5, Fig. 1).

In regard with RFS, two markers were significant in the univariate analysis: EGRF ($p = 0.046$) (3+ expression associated with shorter RFS and increased relapse risk), and PDGF ($p = 0.005$) (lack of expression associated with longer RFS and decreased relapse risk) (Table 5, Fig. 2). Six clinicopathological factors were also significant for RFS: localization ($p = 0.016$; lip primaries had longer RFS), N stage ($p = 0.004$), M stage ($p = 0.032$), surgery ($p = 0.002$), radiotherapy ($p = 0.001$), and local control at the end of definite treatment ($p = 0.000$) (Table 4).

No factor was identified as significant in the multivariate analysis for either RFS or OS.

MLP prediction did not identify any clinicopathological factor as significant for OS (the biomarkers were not included in the analysis). Analysis of the relative importance of the factors indicated the smoking history as the most important factors for OS prediction (importance: 0.1425, non-significant) and the relapse as the least significant (importance: 0.0251).

Survival Analysis-Last Known Status

Data mining using the classification model CHAID indicated that the last known status depended on relapse. For patients with no relapse, last known status depended on c-erbB2; e.g. all 4 patients with c-erbB2 0 or 1+ did not relapse and died of another cause.

For patients who relapsed, last known status depended on T stage; e.g. 15 patients with T stage >1 relapsed and died of their disease. For patients with T stage 1 who relapsed, last known status depends on local control at the end of definitive treatment. For

Table 1 Patient baseline characteristics

	Males (N = 68)	Females (N = 31)	Overall (N = 99)
Age at diagnosis			
median (95 % CI)	69.5 (65–79)	75 (53–92)	71 (65–92)
Smoking history (n, %)			
yes	60 (88.2)	7 (22.6)	67 (67.7)
no	1 (1.5)	1 (3.2)	2 (2.0)
unknown	7 (10.3)	23 (74.2)	30 (30.3)
Localisation (n, %)			
mobile tongue	8 (11.8)	7 (22.6)	15 (15.1)
lip	50 (73.5)	16 (51.6)	66 (66.6)
gingivae/alveolar ridge	3 (4.4)	3 (9.7)	6 (6.1)
oral cavity (other)	7 (10.3)	5 (16.1)	12 (12.2)
Differentiation (n, %)			
grade 1	14 (20.6)	12 (38.7)	33 (33.3)
grade 2	18 (26.5)	2 (38.7)	30 (30.3)
grade 3	27 (39.7)	10 (32.3)	8 (8.1)
unknown	6 (8.8)	1 (3.2)	28 (28.3)
T stage (n, %)			
T1	30 (44.1)	11 (35.5)	41 (41.4)
T2	17 (25)	10 (32.3)	27 (27.3)
T3	6 (8.8)	3 (9.7)	9 (9.1)
T4	2 (3)	2 (6.4)	4 (4.1)
unknown	13 (19.1)	5 (16.1)	18 (18.1)
N stage (n, %)			
N0	46 (67.6)	20 (64.5)	66 (66.7)
N1	4 (5.9)	1 (3.2)	5 (5)
N2	4 (5.9)	4 (12.9)	8 (8.1)
N3	1 (1.5)	0 (0)	1 (1)
unknown	13 (19.1)	6 (19.4)	19 (19.2)
M stage (n, %)			
M0	53 (77.9)	26 (83.9)	79 (79.8)
M1	1 (1.5)	0 (0)	1 (1)
unknown	14 (20.6)	5 (16.1)	19 (19.2)
Overall stage (n, %)			
I	30 (44.1)	10 (32.3)	40 (40.4)
II	18 (26.5)	8 (25.8)	26 (12.3)
III	7 (10.3)	6 (19.4)	13 (13.1)
IV	2 (3)	2 (6.4)	4 (4)
unknown	11 (16.1)	5 (16.1)	16 (16.2)

Table 2 Patient treatment and outcome data

	Males (N = 68)	Females (N = 31)	Overall (N = 99)
Surgery (n, %)			
yes	55 (80.9)	24 (77.4)	79 (79.8)
no	13 (19.1)	7 (22.6)	20 (20.2)
unknown	0 (0)	0 (0)	0 (0)
Radiotherapy (n, %)			
yes	28 (41.2)	15 (48.4)	43 (43.4)
no	29 (42.6)	9 (29.0)	38 (38.4)
unknown	11 (16.2)	7 (22.6)	18 (18.2)
Chemotherapy (n, %)			
yes	6 (8.8)	2 (6.4)	8 (8.1)
no	51 (75)	23 (74.2)	74 (74.7)
unknown	11 (16.2)	6 (19.4)	17 (17.2)
Local control with definite treatment (n, %)			
yes	50 (73.5)	14 (45.2)	64 (64.7)
no	6 (8.8)	9 (29)	15 (15.1)
unknown	12 (17.6)	8 (25.8)	20 (20.2)
Relapse (n, %)			
yes	18 (26.5)	11 (35.5)	29 (29.3)
no	38 (55.9)	14 (45.1)	52 (52.5)
unknown	12 (17.6)	6 (19.4)	18 (18.2)
RFS			
median (95 % CI)	78 (75.7–80.3)	48 (46.2–49.8)	71 (66.4–73.5)
OS			
median (95 % CI)	101 (98.5–103.5)	70 (64.3–75.6)	90 (87.3–92.6)
Last known status (n, %)			
DoD	8 (11.8)	8 (25.8)	16 (16.2)
AWD	0 (0)	0 (0)	0 (0)
ADF	32 (47)	10 (32.2)	42 (42.4)
DOC	14 (20.6)	7 (22.7)	21 (21.2)
DUC	3 (4.4)	0 (0.0)	3 (3)
LFU	11 (16.2)	6 (19.3)	17 (17.2)

RFS: relapse-free survival; DoD: died of disease; AWD: alive with disease; ADF: alive, disease-free; DOC: died of another cause; DUC: died of unknown cause; LFU: lost to follow-up

patients with T stage > 1 who relapsed, last known status depends on VEGF (if VEGF was overexpressed they were more likely to relapse and die of their disease). Finally, for patients with VEGF = 0, T stage > 1, and relapse, last known status depends on whether surgery was used as treatment modality.

The above data was confirmed using feature selection node. The most important inputs relative to the last known status, in highest to lowest ranking, were the following: relapse (0.999999), local control with definite treatment (0.999997), c-erb-B2 (0.999643), localization (0.997135), T stage (0.983131) and surgery (0.9967229).

Discussion

Despite diagnostic and therapeutic improvements, OSCC prognosis especially for the advanced stages remains poor [2]. Therefore, the importance of markers prognostic of tumor aggressiveness and predictive of response to treatment is paramount [5, 18].

Brush cytology is a valuable and reliable diagnostic tool, used for early diagnosis and clinical follow-up of oral cancer. Cytological preparations are readily, painlessly and directly obtained without need of an endoscope, without significant damage of the tissues, so it can be repeatable in clinical follow-up [40].

Liquid-based cytology offers an automated or semi-automated processing and distribution of cells in a thin, evenly dispersed layer, to enhance specificity (95 %-100 %) and sensitivity (80 %) [41]. It increases sample quality and diagnostic accuracy, mainly because of better cytomorphologic picture and cleaner background. It is easier and less time-consuming to screen and interpret the slides, as the cells are limited to a smaller area. Its great advantage is that it allows creation of archival material and application of new techniques on the same sample [42]. The ability to stain for cellular proteins and more recently genes and gene products in cells, or obtain tumoral DNA or RNA [43] represents a major advancement.

Our results indicate that, in addition to the known significance of the EGFR overexpression, the angiogenetic pathway is of prognostic significance in OSCC. An angiogenic switch involving increased expression of PDGF, VEGF and CD34 in the tumor and its microenvironment seems to characterize a more aggressive phenotype with less favorable survival outcomes in our cohort, in concordance with previous reports [6, 44]. However, more insight on the potentially dynamic temporo-spatial role of these markers ought to be further investigated. Insight from other cancers reveals sometimes opposing roles; for example, CD34 was found to play a biphasic role in tumor progression in melanoma, maintaining early-stage vascular integrity but accelerating late-stage growth via altering immune cell infiltration [45].

Table 3 Marker expression profiling results

	0	*1+*	*2+*	*3+*
EGFR	23 (37 %)	16 (26 %)	13 (21 %)	10 (16 %)
c-erb-B2	38 (70 %)	8 (15 %)	7 (13 %)	1 (2 %)
COX2	18 (32 %)	15 (27 %)	18 (32 %)	5 (9 %)
PDGF	30 (79 %)	2 (5 %)	6 (16 %)	0 (0 %)
VEGF	38 (81 %)	2 (4 %)	5 (11 %)	2 (4 %)
CD117 (c-KIT)	35 (72 %)	8 (16 %)	5 (10 %)	1 (2 %)
CD34	32 (78 %)	3 (7 %)	4 (10 %)	2 (5 %)

Percentages refer to overall number of analysed samples

It ought to be noted that the relatively small patient number and the limited quantity of available material compromised our analysis. As a result of the latter not all markers could be analyzed for every patient, and this may entail non-intentional bias. The reason for this was that the study did not involve a prospective collection of specimens, but rather consisted of archival material primarily intended for initial diagnostic purposes. Conversely, a prospective database construction would have been compromised by the lack of long survival/follow-up data, and therefore a "prospective-retrospective" design using archival material was used [46]. To our knowledge this is the only cytological biomarker expression and outcome analysis of this extent in OSCC. Nevertheless, the retrospective nature of our study entails weaknesses such as

Table 4 Survival functions

		Age at diagnosis (yrs)			RFS (mo)			OS (mo)		
		Median	Percentile 05	Percentile 95	Median	Percentile 05	Percentile 95	Median	Percentile 05	Percentile 95
Smoking	no	56	56	75	95	93	95	195	93	195
	yes	62	49	78	64	14	144	79	25	216
Localisation	tongue	72	35	81	51	0	103	51	12	103
	lip	65	56	80	77	16	204	90	21	305
	gingivae/alveolar ridge	78	75	92	5	0	50	70	10	73
	oral cavity (other)	60	58	69	107	48	124	124	48	144
Grade	gr 1	67	49	92	61	0	183	64	21	196
	gr 2	60	56	79	95	13	204	107	25	305
	gr 3	62	57	80	60	16	144	119	16	216
T stage	T1	65	56	79	63	16	204	101	21	305
	T2	69	49	89	83	11	124	83	25	144
	T3	72	62	79	60	0	103	73	12	103
	T4	92	70	92	0	0	5	70	7	70
N stage	N0	67	51	80	67	14	204	79	25	305
	N1	72	68	79	16	5	103	103	12	110
	N2	66	57	92	48	0	84	70	12	154
	N3	52	52	52	2	2	2	2	2	2
M stage	M0	67	56	81	77	11	204	90	25	305
	M1	70	70	70	0	0	0	7	7	7
Overall stage	stage I	65	56	79	63	14	204	90	21	305
	stage II	68	49	89	78	16	124	90	25	144
	stage III	69	57	78	60	0	103	73	12	154
	stage IVa	92	75	92	0	0	5	70	10	70
	stage IVb	52	52	52	2	2	2	2	2	2
	stage IVc	70	70	70	0	0	0	7	7	7
Surgery	no	69	64	92	35	0	90	46	7	90
	yes	64	51	80	78	16	204	101	25	305
Radiotherapy	no	61	49	81	77	25	204	77	25	305
	yes	69	56	79	63	8	124	90	17	195
Chemotherapy	no	67	51	80	71	13	204	79	20	305
	yes	59	57	69	67	16	124	119	67	154
Local control with definitive treatment	no	67	60	92	11	0	107	70	7	144
	yes	66	51	79	77	16	204	90	31	305
Relapse	no	69	49	80	61	16	107	63	21	110
	yes	58	56	81	95	0	204	154	19	305

RFS: relapse-free survival; OS: overall survival

Table 5 Correlation of marker expression profile to survival outcomes

	Median RFS (mo)			Median OS (mo)		
		95 % Confidence Interval			95 % Confidence Interval	
		Lower Bound	Upper Bound		Lower Bound	Upper Bound
PDGF						
0	121	71	170	144	0	303.6
1+	0	0	0	9.5	7	12
2+	50	25	77	21	0	52.
Overall	111	69	153	71	0	149
VEGF						
0	36	28	40	36	27.5	60.4
1+	49	57,214	68,786	49	37.2	68.7
2+	10.5	0	21	7	7	7
3+	17	17	17	17	17	17
Overall	37	29	41	39	29.6	42
CD34						
0	31	56	62	35	29	69
1+	60.5	52	67	60.5	52	67
2+	28.5	24	45	32	27	45
3+	0	0	0	0	0	0
Overall	37	58	60	39	57	61
EGFR						
0	25	0	36	36	17	131
1+	34	20	48	66	37	135
2+	50	8	107	76	21	151
3+	8	5	11	10	8	137
Overall	25	3	107	39	0	151
c-erb-B2						
0	66	11	144	90	26	216
1+	50	19	84	73	19	154
2+	55	5	57	57	10	79
3+	90	90	90	90	90	90
Overall						
COX2						
0	100	0	206	131	131	131
1+	107	6.5	207.5	144	0	321
2+	95	58	131	73	43	103
3+	5	0	13	10	0	26
Overall	100	86	113	131	50	211
CD117						
0	67	13	204	101	17	305
1+	59	4	83	71	17	110
2+	124	20	124	124	20	124
3+	95	95	95	195	195	195
Overall						

important number of missing data in terms of clinical characteristics, such as smoking history, alcohol consumption and metabolic disorders (insulin resistance).

Further to the established role of cetuximab, there could possibly be a place for therapeutic targeting for tumors over-expressing these markers (15 %-30 % of tumours in our

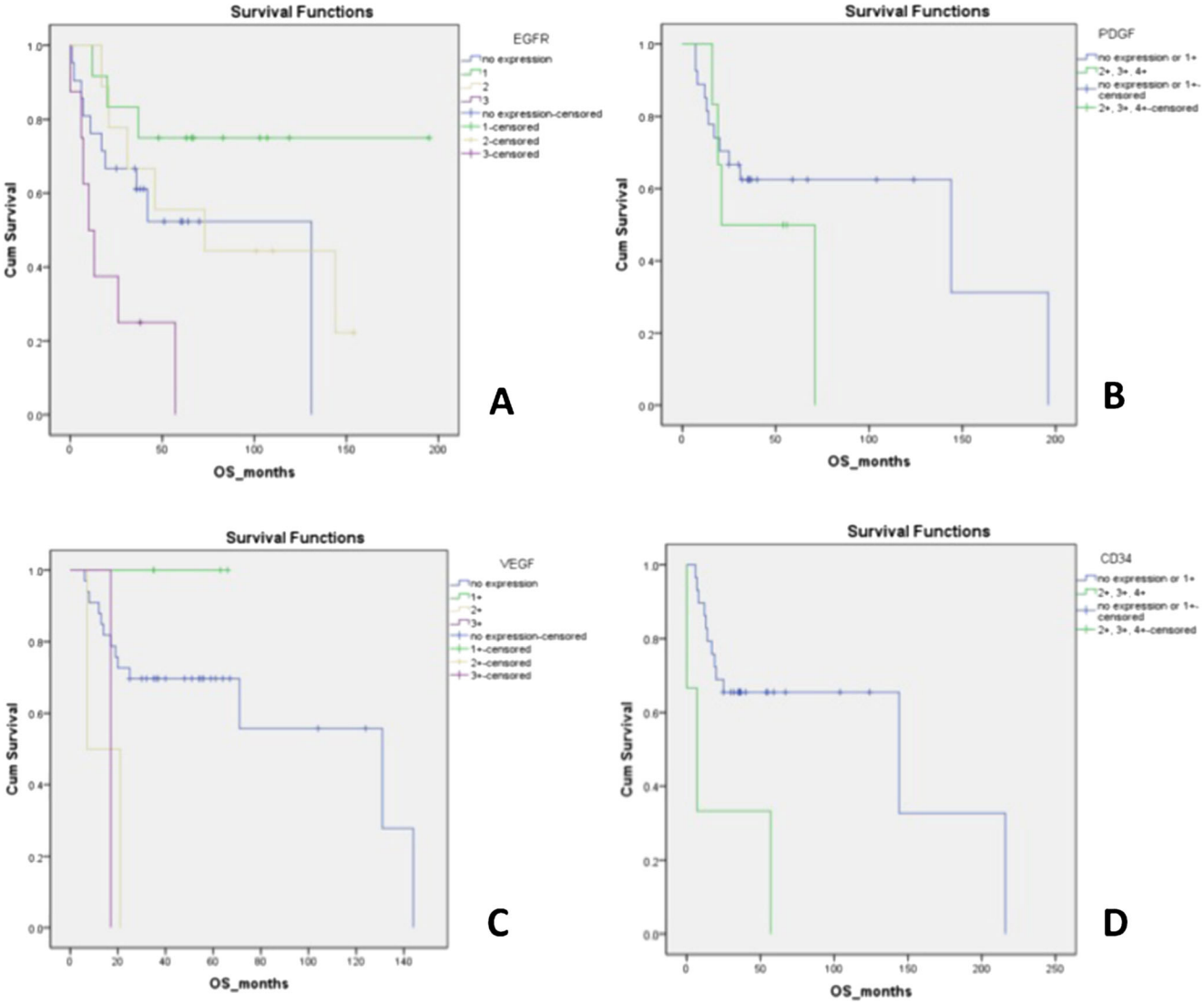

Fig. 1 Overall survival according to the status of informative markers in univariate analysis. A: EGFR; B: PDGF; C: VEGF; D: CD34

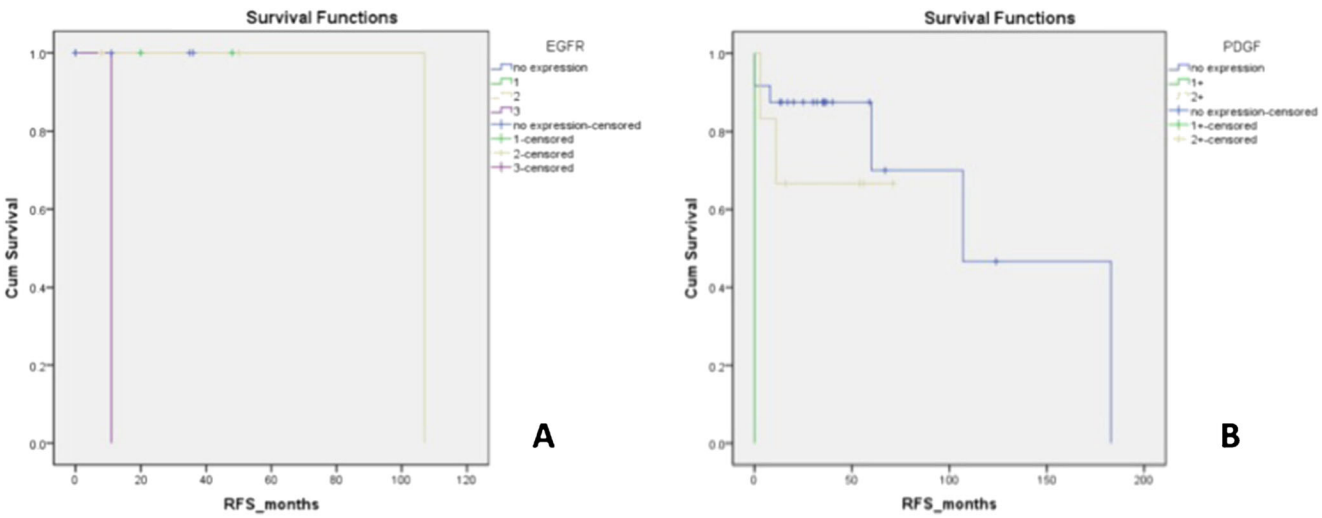

Fig. 2 Relapse-free survival according to the status of informative markers in univariate analysis. A: EGFR; B: PDGF

cohort), utilizing drugs such as bevacizumab (anti-VEGF monoclonal antibody), cediranib (oral selective inhibitor of VEGFR-1, −2 and −3) [47], afatinib (oral inhibitor of the broader erb-B family) [48] or even combined pathway targeting, e.g. cetuximab & bevacizumab [10, 11], erlotinib & bevacizumab [12], bevacizumab & pemetrexed [13]. It ought to be noted, however, that data on the use of anti-angiogenic agents in HNSCC is still immature, and that an enhanced risk of bleeding is reported in trials with VEGF inhibitors [13]. Moreover, there have been disappointing results on targeted therapies either as monotherapy or as combination therapies [49, 50].

Cytology can be used for repeated tumor profiling throughout the disease course, to evaluate the influence of treatment (chemotherapy, radiotherapy) on the tumoral molecular profile, and direct treatment according to the marker expression profile at diagnosis, between treatment lines, upon progression and/or relapse. However, further validation and standardization is required for the ICC scoring of these markers, especially in the aim of optimizing their use towards targeted therapy. The most celebrated example of targeted treatment in head and neck is EGFR targeting. If we borrow the knowledge obtained from EGFR targeting in lung cancer, an optimized IHC EGFR scoring may serve as a predictive factor for the efficacy of cetuximab and help select patients more appropriately for cetuximab treatment [51, 52].

Examples of theranostics application are the combination of molecular-targeted cancer imaging and therapy to improve cancer diagnosis and minimize the side effects of conventional treatments, such as ErbB2 targeting for bioluminescence imaging and therapy [53], or nanotheranostics applications [54]. To our knowledge, there is no published theranostics application in OSCC. Our results aim to serve as an introduction towards such a proof-of-concept, utilizing a well-established and broadly used existing technology, such as brush cytology.

This project was introduced as a forward step for oral cancer management in our Unit, within the wider concept of applied tumor targeted therapy in HNSCC. The identification of driver genetic alterations, the elucidation of cross-talk between oncogenic pathways and the detection of susceptibility to synthetic lethality facilitate the move towards biomarker-directed therapy and precision medicine. Our research is ongoing with the analysis of further markers (integrins, cadherins, NFκB etc) and a larger future analysis is planned.

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Compliance with Ethical Standards

Conflict of Interest The authors have no conflicts of interest to declare.

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