Biomarkers in wound drainage fluids of head and neck squamous cell carcinoma patients receiving neck dissection: A pilot study

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ABSTRACT

Aim: In a pilot prospective study, we aimed to test the feasibility and report on the preliminary results on the expression of molecular biomarkers in wound drainage fluids (WDFs) of operated head and neck squamous cell carcinoma (HNSCC) patients.

Material and methods: Nineteen patients undergoing primary tumor resection with en-block neck dissection were enrolled. In postoperative days 1–3, the expression of several biomarkers in WDFs was measured using enzyme-linked immunosorbent assay (ELISA) kits and correlated with clinical and histopathologic features.

Results: The expression of stromal cell-derived factor 1 (CXCL-12) was significantly increased in WDFs in presence of lymph node metastases, extranodal extension (ENE), and in case of close resection margins. In addition, Osteopontin expression was significantly increased in presence of ENE, whereas transforming growth factor beta (TGF-β) detection was significantly reduced. At multivariate analysis, CXCL-2 levels in both day 1 and 3 post-surgery were the only factor which retained significance in the prediction of close surgical margins (p = 0.028 and 0.025 for day 1 and day 3, respectively). Both CXCL-2 and Ostepontin assays were significantly correlated with ENE (p = 0.018 and 0.035 for day 1; 0.052 and 0.025 for day 3, respectively) whereas TGF- β expression was significant at day 1 only (p = 0.038)

Conclusions: Our pilot study showed that WDFs could qualify as a potential source of relevant postoperative information. Further studies are needed to confirm the prognostic impact of CXCL-12, Osteopontin and TGF-β expressed in WDFs on the personalized management of HNSCC.

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1. Introduction

The incidence of head and neck squamous cell carcinoma (HNSCC) is increasing: worldwide, it represents the sixth most common neoplasm, affecting more than 550.000 patients per year [1,2]. In over 60% of cases, HNSCC is diagnosed at a loco-regionally advanced stage, requiring a multimodal strategy in order to pursue a curative intent. In the post-operative setting the rationale of a combined therapeutic approach, when an estimated risk of residual microscopic disease is present, is to minimize the risk of a macroscopic recurrence. Indeed, HNSCC-related mortality is mainly driven by a predominant loco-regional pattern of failure [3]. It has long been known that the presence of lymph node metastases has the largest impact on prognosis, overall reducing cancer-specific survival (CSS) by about 50% [4], with extranodal extension (ENE) portending worse regional and distant metastatic failure rates [5]. In the setting of primary surgery, the potential causative role of manipulations during intervention in facilitating loco-regional seeding of viable tumor cells has never been fully elucidated, nor is the hypothetical impact of growth factors involved in wound healing in stimulating the proliferative capacity of HNSCC clonogens [6,7]. With the notable exception of Human Papilloma Virus (HPV) infection for oropharyngeal cancer, no molecular biomarkers are currently available in the clinic to tailor the choice of treatment at an individual level. In recent years, the biologic interplay of the tumor microenvironment in respect to hypoxia, inflammation and neo-angiogenesis was actively investigated [8]. Interest was drawn to assess the presence of growth factors in wound drainage fluids (WDFs) secreted by the host during

the wound healing process in response to surgery-related tissue damage that may boost residual tumor cells proliferation [9].

Among them, high levels of Epidermal Growth Factor Receptor (EGFR) protein and of its ligand were shown to be prognosticators of reduced disease-free survival (DFS) and CSS [10]. However, these findings were not corroborated by further evidence to support their post-operative assessment in clinical decision-making. Based on the hypothesis that WDFs obtained by neck dissection drain tubes could reliably provide information on the tumor microenviroment, we designed a pilot feasibility study aiming to determine the presence and potential prognostic relevance of the following soluble protein biomarkers: Epidermal Growth Factor (EGF), Vascular Endothelial Growth Factor (VEGF), Stromal cell-derived factor 1 (CXCL-12), Transforming Growth Factor-beta (TGF-β) and Osteopontin. Mainly, EGF, the EGFR ligand, was selected for its predominant contribution to the proliferation of epithelial head and neck tumors; VEGF as inducer of angiogenesis and lymphangiogenesis; CXCL-12 and Osteopontin because of their role in tumor invasion, metastasization and homing of cancer cells; TGF-β for its importance in promoting tumor cell growth, cell differentiation, and apoptosis [11–14]. The aim of our study was to explore the feasibility to detect the expression of these molecular biomarkers in WDFs following neck dissection. In addition, we sought to evaluate their correlation with standard clinico-pathological features of patients undergoing radical surgery for HNSCC.

2. Materials and methods

2.1. Patients' population

For this prospective pilot study, we enrolled patients affected by stage II-IV (according to TNM/AJCC 7th edition) HNSCC deemed amenable to undergo radical surgery at primary tumor site en-block with ipsilateral or bilateral elective or therapeutic neck dissection, with or without regional or free flap reconstruction. In our series 17 patients (84.2%) were naïve with no history of previous treatment, 2 patients (10,5%) had a previous history of chemoradiation therapy treatment in one case with curative intent for an oropharyngeal carcinoma and as adjuvant therapy for an oral cavity carcinoma (OCC) in the other; 1 patient (5,3%) was previously treated with transoral surgery for an early stage OCC. After surgery the collected specimens were sent to the Pathology Department of the University of Florence for a thorough histopathological analysis. Neck drain tubes were placed at the end of neck dissection procedure. WDFs of postoperative days 1 and 3 were collected from the ipsilateral neck and immediately processed. Fluids were centrifuged for 15 min at 3500 rpm, then divided in aliquots and stored at −70 °C until analysis. The expression of EGF, VEGF, CXCL-12, TGF-β and Osteopontin was measured using commercially available enzyme linked immunosorbent assay (ELISA) kits, with a mean minimum detectable dose of 0.089–0.740 pg/ml for EGF, 5.0 pg/ml for VEGF, 1.0–47 pg/ml for CXCL-12, and 0.006–0.024 ng/mL for Osteopontin, respectively. Each sample was analyzed in duplicates and then averaged for the mean value. Quality control pools of low, normal, and high concentrations for all parameters were included in each assay. The optical density of each well was evaluated by the use of a microplate reader (VICTOR Multilabel Plate Reader 1420 Perkin Elmer), set to a wavelength of 450 nm. In order to correct the error caused by optical surface imperfections, another measurement was performed with a wavelength of 570 nm. A subtraction between the two essays was performed. Results were expressed in pg/ml for EGF, VEGF, CXCL-12, TGF-β whereas the results of Osteopontin were expressed in ng/ml. Data on WDFs analysis at postoperative days 1 and 3 were ultimately available for 19 cases. This prospective pilot study

was reviewed and approved by the University of Florence Ethics Committee Review Board and written informed consent was obtained from all patients.

2.2. Statistical analysis

Statistical analysis was carried out using STATA 13 software (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP). The significance of the observed differences between molecular assays at day 1 and day 3 was verified with a T-Student test. The distribution of the data by dichotomous variables was analyzed with the Fisher's exact test. In particular one-sided tests were computed because by observing the two-ways tables the possibility of a one-direction relationship was more plausible. The Wilcoxon-Mann-Whitney test was used to test if molecular assays had a different distribution by some categorical variables (such as surgical margins, extranodal extension etc.). Only with regard to the tumor stage variable, the two-sided Fisher's exact test was performed and the ANOVA model was used to assess if the molecular levels (at day 1 and day 3) were differently distributed in each group. In order to investigate if there was some relationship between homolateral lymph node density and the five molecular assays the Sperman's correlation index was calculated. The basic requirements to perform a linear regression analysis were not satisfied but considering that this is a pilot study, we believed that observing more in depth if a given molecular level can vary in relation to the other molecular levels and the lymph node density was appropriate. So a linear regression through the backward elimination technique was performed for each of the five molecular levels (both at day 1 and 3). A p-value of 0.05 was chosen in order to establish if a variable had to remain in the model.

3. Results

From November 2014 to May 2016, 19 patients affected by stage II-IV HNSCC undergoing curatively-intended surgery were enrolled in our study. Patients' main clinical features are shown in Table 1. The mean age of the cohort was 67.7 years (SD ± 12.41; range, 51–90). According to the pathologic staging, 9 (47%) patients were classified as pT3, 6 (32%) patients as pT4a and 4 (21%) patients as pT2, respectively. The most common primary tumor site was oral cavity (12 patients; 63.1%), followed by oropharynx and larynx with 3 cases each (15.8% per site). Eleven (58%) patients presented a moderately differentiated carcinoma (G2), 4 (21%) a poorly differentiated carcinoma (G3) and 4 (21%) a well differentiated carcinoma (G1), respectively. In the analysed series, the most common surgical interventions were a partial glossectomy, glossectomy associated to a marginal mandibulectomy, total laryngectomy, glossectomy extended to the oropharynx, total laryngectomy, and a segmental mandibulectomy in 5 (26,3%), 3 (15,8%), 3 (15,8%), 2 (10,5%) and 2 (10,5%) patients, respectively. The remaining two patients underwent to an inferior maxillectomy in one case and a total auriculectomy in the other. In 16 patients (84.2%), the surgical resection margins were negative, whereas in the remaining 3 patients (16%) close margins were retrieved, defined as ≤ 3 mm. Pathologic lymph node metastases were detected in 9 cases (47%), 5 of which (56%) presented ENE. A mean of 67 ml of WDFs at day 1 and 42 ml at day 3 were collected per patient, respectively. The expression of EGF and TGF-β was significantly reduced from day 1 to day 3 (mean values of 162.20 ± 86.64 to 37.39 ± 46.19 pg/ml and 4254.94 ± 1437.97 to 1026.86 ± 571.68 pg/ml; p < 0.001 and p < 0.05, respectively). On the contrary, the expression of CXCL-12 and Osteopontin significantly increased from day 1 to day 3 (mean values of 15.82 ± 16.98 to 30.40 ± 11.24 ng/ml; and 479.08 ± 66.5 to 749.68 ± 93.71 pg/ml; p < 0.001

Table 1
patients characteristics.

	Tumor site	Grading	TNM*	Margins status	ENE†	Perineural invasion	Angio-vascular invasion	Lymph-vascular invasion
1	Oral Cavity	G2	T3N0	Neg	Neg	Neg	Neg	Neg
2	Oral Cavity	G1	T3N2c	Neg	Pos	Pos	Pos	Pos
3	Oral Cavity	G2	T3N1	Neg	Pos	Pos	Neg	Neg
4	Oropharynx	G2	T4aN2b	Neg	Neg	Neg	Neg	Neg
5	Oropharynx	G2	T3N0	Neg	Neg	Neg	Neg	Neg
6	Oropharynx	G2	T2N2a	Neg	Pos	Neg	Neg	Neg
7	External auricle	G3	T3N0	Neg	Neg	Neg	Neg	Neg
8	Oral Cavity	G3	T3N0	Neg	Neg	Neg	Neg	Neg
9	Oral Cavity	G2	T3N0	Neg	Neg	Neg	Neg	Neg
10	Larynx	G2	T4aN0	Neg	Neg	Neg	Neg	Neg
11	Oral Cavity	G2	T4bN0	Neg	Neg	Neg	Neg	Neg
12	Oral Cavity	G3	T3N1	Neg	Neg	Neg	Neg	Neg
13	Oral Cavity	G1	T2N0	Neg	Neg	Neg	Neg	Neg
14	Larynx	G1	T4aN1	Neg	Neg	Neg	Neg	Neg
15	Oral Cavity	G3	T2N0	Neg	Neg	Neg	Neg	Neg
16	Oral Cavity	G2	T4aN1	Close	Pos	Neg	Pos	Pos
17	Oral Cavity	G2	T2N1	Close	Pos	Pos	Neg	Neg
18	Oral Cavity	G3	T3N1	Close	Neg	Neg	Neg	Neg
19	Larynx	G1	T4aN0	Neg	Neg	Neg	Neg	Neg

*TNM: 7th edition of TNM staging system.
†ENE: Extranodal extension.

and p < 0.05, respectively) (Table 2). Linear regression (Table 3) showed that CXCL-2 levels significantly correlated with ENE and close surgical margins, both at day 1 and day 3 (p = 0.001 and 0.009 for ENE, 0.001 and 0.010 for close margins, respectively). At multivariate analysis, CXCL-2 levels in both timepoints were the only factor which retained significance in the prediction of close surgical margins (p = 0.028 and 0.025 for day 1 and day 3, respectively) (Table 4). Both CXCL-2 and Ostepontin assays were significantly correlated with ENE (p = 0.018 and 0.035 for day 1; 0.052 and 0.025 for day 3, respectively) whereas TGF- β expression was significant at day 1 only (p = 0.038) (Table 5).

4. Discussion

Currently, the lack of reliable biomarkers limits the possibility to tailor the best therapeutic approach on an individual basis in head and neck oncology. In this light, the identification of a panel of predictive biomarkers is one of the biggest challenges faced by the oncologic community. In the past few years, no conclusive evidence was generated on optimal prognostic stratification of HNSCC patients [15]. It has been reported that WDFs components can boost tumor cells proliferation in vitro [9]: thus, their exact determination could yield potential molecular targets for adjuvant therapies. In particular, Licitra et al. demonstrated that EGF-like molecules in WDFs obtained from surgically resected HNSCC can induce the proliferation of squamous cell carcinoma lines that appear to be triggered by EGFR expression and activation [11]. These preliminary evidences indicated that WDFs are enriched by several molecules (growth factors, cytokines) secreted by the host during the wound healing process in response to surgery-related

Table 2
wound drainage fluids-derived molecular assays.

	Day 1	Day 3
	Mean ± SD*	Mean ± SD*
CXCL-12	516.33 ± 422.51	815.49 ± 602.17
Osteopontin	15.82 ± 11.45	30.40 ± 11.24
VEGF	1666.54 ± 956.41	2210.47 ± 1190.53
EGF	162.20 ± 86.64	37.39 ± 46.19
TGF-β	4245.94 ± 1437.97	1026.86 ± 571.68

* SD: standard deviation.

tissue damage, ultimately enhancing residual tumor proliferation [9]. Similar studies were conducted on axillary drainage fluids of breast cancer: using reverse transcriptase (RT)-polymerase chain reaction (PCR), Greenberg et al. [16] demonstrated that a worse prognosis was correlated with the presence of Mucine 1 (MUC-1) while Zhang et al. [17] correlated carcinoembryonic antigen (CEA) and cytokeratin-19 (CK-19) with local relapse. In our study, we decided to analyse WDFs collected on postoperative days 1 and 3 in order to assess the changes between the early and rather bloody fluids versus late serous fluids. Based on the hypothesis that WDFs could be representative of a neoplastic microenvironment, in our explorative pilot feasibility study we tried to identify molecular biomarkers with a potential prognostic significance. Our study showed firstly the feasibility of testing molecular assay on postoperative WDFs, and secondarily highlighted how the collected data showed a potential significant association between an increased expression of CXCL-12 and Osteopontin with close surgical margins and ENE, whereas a decreased level of TGF- β was correlated with ENE, respectively. Both CXCL-12 and Osteopontin are known to be involved in tumor invasion process, metastatization and homing of cancer cells. Müller et al. demonstrated how CXCL-12 plays a critical role in determining the metastatic destination of tumor cells through the interaction with its tissue receptor CXCL-4 [18]. Furthermore, a study conducted by Standard et al. showed the contribution of Osteopontin in the process of cellular adhesion and metastatization [19,20]. Acting through its signaling pathway, In normal cells TGF-β is able to stop cellular proliferation by blocking cell cycle at G1 stage, to induce differentiation, and promote apoptosis. In many cancer cells, the TGF-β signaling pathway is mutated, resulting in impaired tumor proliferation. In addition, the derangement of TGF-β pathway influences surrounding stromal cells, immune cells, endothelial and smooth-muscle cells, resulting in immunosuppression and angiogenesis, conferring higher cancer invasiveness. Finally, TGF-β also turns off the anti-cancer immune response converting effector T-cells into regulatory (suppressor) T-cells [21].

Our results highlighted that increased levels of CXCL-12 and Osteopontin, together with decreased levels of TGF-β, were correlated with circumstances in which the presence of a minimal residual disease is more likely. In fact, the detection of close margins could suggest the persistence of residual cancer cells at the primary site, while the presence of ENE could reflect the persistence of cancer cells despite a neck dissection. Notably, the presence of

Table 3
results of linear regression with backward method for CXCL-12.

DAY 1	Coefficient	CI* 95%			p-value	R²†
Lymph node metastasis	395	133	–	656	**0.008**	0.91
Angio-vascular invasion	−848	−1,292	–	−405	**0.002**	
Homolateral lymph node density	12795	4,055	–	21,536	**0.010**	
ENE	779	450	–	1108	**0.001**	
Margins	1103	1604	–	601	**0.001**	
Intercept	218	22	–	414	**0.033**	
DAY 3	**Coefficient**	**CI* 95%**			**p-value**	**R²†**
ENE	757	227	–	1,287	**0.009**	0.59
Margins	816	1,402	–	230	**0.010**	
Intercept	742	449	–	1034	**0.000**	

*CI: Confidence Interval.
†R²: Coefficient of determination.
Bold numbers indicate significant values.

Table 4
correlation between surgical margins and molecular assays.

Margins		Negative N*=16			Close N*=3			
		Mean		SD†	Mean		SD†	p-value
CXCL-12	Day 1	121,47	±	27,02	595,30	±	420.21	**0.028**
	Day 3	177,24	±	27,44	935,16	±	581,62	**0.025**
Osteopontin	Day 1	17,38	±	11,81	7,99	±	5,29	0.173
	Day 3	29,26	±	11,95	36,45	±	1,19	0.695
VEGF	Day 1	1578,24	±	905,63	2108,06	±	1297,37	0.678
	Day 3	2210.19	±	1237,43	2211,99	±	1127,80	0.911
EGF	Day 1	167,65	±	95,17	136,74	±	1,12	0.450
	Day 3	34,49	±	41,69	51,91	±	74,78	0.953
TGF-β	Day 1	4209,92	±	1540.51	4606,13	±	0.00	0.752
	Day 3	1069,20	±	584,14	603,56	±	0.00	0.527

* N: number of patients.
† SD: standard deviation
Bold numbers indicate significant values.

Table 5
correlation between extranodal extension and molecular assays.

		No N*=14			Yes N*=5			
		Mean		SD†	Mean		SD†	p-value
CXCL-12	Day 1	404,25	±	276,03	807,72	±	619,00	**0.018**
	Day 3	626,62	±	447,14	1344,32	±	712,41	**0.052**
Osteopontin	Day 1	13,03	±	10.17	23,05	±	12,52	**0.035**
	Day 3	28,23	±	12,46	36,46	±	1,07	**0.025**
VEGF	Day 1	1724,31	±	1038,31	1516,34	±	783,77	0.961
	Day 3	2363,43	±	1255,33	1782,19	±	972,35	0.405
EGF	Day 1	163,16	±	73,76	159,90	±	122,69	0.673
	Day 3	43,26	±	50.79	22,15	±	30.44	0.153
TGF-β	Day 1	5047,52	±	524,81	2843,19	±	1489,57	**0.038**
	Day 3	1082,97	±	353,71	928,67	±	904,96	0.186

* N: number of patients.
† SD: standard deviation.
Bold numbers indicate significant values.

ENE and the status of surgical resection margins are the most important prognostic factors for the development of loco-regional and distant relapses. When compared with lymph node involvement without extracapsular spread, the identification of ENE leads to increased rates of loco-regional failure (28.9% vs 19.2% respectively) and of distant failure (24.4% vs 8.1%, respectively) [5]. Therefore, the high-risk feature of ENE translates into worse 5-year CSS and overall survival (OS) (48% 5-year CSS and 29% 5-year OS vs 66% and 51% rates without ENE, respectively) [22]. We hypothesize that the presence of a minimal residual disease might influence the post-surgical microenvironment, stimu-lating overexpression of CXCL-12 and Osteopontin, and decreasing the expression of TGF-β. These microenvironmental features could increase the aggressiveness of residual cancer cells, facilitating metastasis and invasion processes.

In a very preliminary way, we were able to describe a correlation between molecular WDFs assays and clinico-pathological characteristics of minimal residual disease. In the future, the implementation of WDFs analysis on top of histopathologic examination could be a useful tool to obtain a better prognostic stratification based on peri-operative biomarkers, allowing potentially for a more tailored adjuvant approach. Furthermore, in line with

previous experiences, we confirmed the feasibility of testing molecular assays from WDFs, potentially paving the way for further reseach in this field. Clearly, caution is advised when interpreting our study results, in view of its inherent limitations. The small sample size, the relative heterogeneity of primary tumors and disease stage and the low number of adverse pathologic features limit the strength of our findings. Taking into account the aforementioned considerations, no correlation was intended to be drawn between the expression of molecular biomarkers in WDFs and clinical outcome, therefore no inferences can be suggested on the potential long-term prognostic impact of the study results.

The biomolecular analysis of WDFs could represent a new source of information on tumor microenvironment. The feasibility of testing different assays directly from the neck surgical field in the immediate post-operative setting was demonstrated. A potential correlation between molecular expression in drainage fluids and the presence of histologic features associated with the persistence of minimal residual disease could be also hypothesized. Further prospective studies are warranted to clarify the usefulness of WDFs for optimal prognostication of operated HNSCC patients.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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