ACTA OTORHINOLARYNGOLOGICA ITALICA 2020;40:90-98; doi: 10.14639/0392-100X-2419

Head and neck

Stem cell markers in oral and oropharyngeal squamous cell carcinomas in relation to the site of origin and HPV infection: clinical implications

Marker di staminalità nei carcinomi squamocellulari del cavo orale e orofaringe in relazione al sito di origine e a infezione da HPV: implicazioni cliniche

Davide Rizzo[1*], Cristina Graziani[2*], Roberto Gallus[3], Gian Franco Zannoni[4], Donatella Lucchetti[2], Claudio Parrilla[3], Alma Boninsegna[2], Jacopo Galli[3], Gaetano Paludetti[3], Francesco Bussu[1,3**], Alessandro Sgambato[2]

[1] Otolaryngology Division, Azienda Ospedaliera Universitaria, Sassari, Italy; [2] Department of General Pathology, Università Cattolica del S. Cuore, Rome, Italy; [3] Department of Otolaryngology, Università Cattolica del S. Cuore, Rome, Italy; [4] Department of Histopathology, Università Cattolica del S. Cuore, Rome, Italy

[*] D. Rizzo and C. Graziani contributed equally to this work.

[**] Present address: University of Sassari, Dipartimento di Scienze Mediche, Chirurgiche e Sperimentali, Sassari, Italy

SUMMARY

The expression of potential stem cell markers in HNSCCs was investigated to assess their potential clinical role. 69 primary, previously untreated oral (OSCC) and oropharyngeal squamous cell carcinomas (OPSCC) were enrolled; personal, clinical and follow-up data were collected. HPV infection and expression of 5 potential stem cell markers (CD44, CD133, Oct-4, Nanog, and Sox-2) were evaluated. HPV+ OPSCC showed lower expression of Nanog. The cytoplasmic expression of Nanog was associated with significantly worse prognosis in OPSCC, but not in OSCC. Sox-2 staining was more intense among OPSCCs. Sox-2 nuclear staining was associated with worse prognosis. Nanog expression was associated with HPV- OPSCC and may have a role as a surrogate diagnostic marker. In general, the expression profile of some stem cell markers in HNSCC seems to vary according to the site of origin and HPV infection. Nanog and Sox-2 may also have prognostic value.

KEY WORDS: molecular markers, prognosis, HPV diagnosis, Sox-2, Nanog

RIASSUNTO

In questo studio è stata esaminata l'espressione di potenziali marcatori di staminalità nei carcinomi della testa e collo (HNSCC) per valutarne il loro possibile ruolo clinico. Sono stati arruolati 69 carcinomi squamocellulari del cavo orale (OSCC) e dell'orofaringe (OPSCC) primitivi e non precedentemente sottoposti a trattamento, raccogliendo i dati anagrafici, clinici e sul follow up. Abbiamo valutato l'eventuale infezione da HPV e l'espressione di 5 potenziali marker di staminalità (CD44, CD133, Oct-4, Nanog, and Sox-2). Gli OPSCC positivi per HPV hanno mostrato minor espressione di Nanog, mentre la sua espressione citoplasmatica è stata associata con una prognosi significativamente peggiore negli OPSCC ma non in OSCC. La colorazione di Sox-2 si è rivelata più intensa tra gli OPSCC, e la sua espressione nucleare è associata con una peggiore prognosi. L'espressione di Nanog è associata a OPSCC HPV-negativi e può avere un ruolo come marker diagnostico surrogato. In conclusione il profilo di espressione di alcuni marker di cellule staminali nei HNSCC sembra essere differente a seconda del sito di origine del tumore e dell'infezione da HPV. Inoltre Nanog e Sox-2 potrebbero presentare un significato prognostico.

PAROLE CHIAVE: *markers molecolari, prognosi, diagosi di HPV, Sox-2, Nanog*

Received: November 13, 2018
Accepted: August 26, 2019

Correspondence
Davide Rizzo
Otolaryngology Division, Azienda Ospedaliera Universitaria, viale San Pietro, 07100 Sassari, Italy
Fax +39 079208067
E-mail: davide.rizzo@aousassari.it

Funding
This work was supported in part by Grant D.1.-2015 from the Università Cattolica del S. Cuore, as part of its program for the promotion and diffusion of scientific research.

Conflict of interest
The Authors declare no conflict of interest.

How to cite this article: Rizzo D, Graziani C, Gallus R, et al. Stem cell markers in oral and oropharyngeal squamous cell carcinomas in relation to the site of origin and HPV infection: clinical implications. Acta Otorhinolaryngol Ital 2020;40:90-98. https://doi.org/10.14639/0392-100X-2419

Introduction

Various malignant tumours are considered to originate from a typical cell of origin. Nevertheless, within the same tumour, cancer cells often exhibit functional heterogeneity, exhibiting distinct proliferative and differentiation potentials (referred to as tumour heterogeneity) [1,2]. The cancer stem cell (CSC) model is a carcinogenic theory, demonstrated primarily for haematologic malignancies - although evidence is also accumulating in solid neoplasms - that coherently accounts for such heterogeneity within the tumour cell population [3,4]. The CSC model proposes a hierarchical organizsation of cells within the tumour, in which a subpopulation of tumour cells displays some characteristics that are similar to normal stem cells. These so-called cancer stem cells (CSCs) have the ability to give rise to all cell types in a particular neoplasm. Thus, these cells are responsible for sustaining tumour growth as well as for local relapse and metastasis. CSCs share important properties with normal tissue stem cells, including self-renewal (by symmetric and asymmetric division) and differentiation capacity, albeit aberrant, but this does not imply that the cell of origin of a given tumour was necessarily a stem cell.

From a clinical perspective, the CSC concept has significant implications as these cells, which are thought to be more resistant to chemotherapy and targeted therapy, should be the primary target of every non-surgical therapeutic approach in order to provide long-term disease-free survival.

The isolation of CSCs from different malignancies has been aimed, on a speculative level, at confirming that the CSC model is valid for a certain neoplastic disease. Moreover, identification of a population of cells, on which the effectiveness of different therapeutic approaches could be tested, would also be highly relevant from a clinical perspective. A number of cell surface markers have been demonstrated to be useful for identification of CSCs, while it is not yet known whether these merely represent surrogate markers or have a meaningful role in regulating CSC function. In head and neck oncology, the CD44 protein (CD44) has been proven to be the most reliable surface marker [5,6], even if measurement of the activity level of some enzymes has been demonstrated as a potentially reliable approach, as in the case of aldehyde dehydrogenase (ALDH) [7,8].

Other cellular markers, such as octamer-binding transcription factor 4 (OCT-4), homeobox protein NANOG (Nanog) and SRY (sex determining region Y)-box 2 (SOX-2), are not suitable for easy isolation of the CSCs as they are either not expressed on the membrane surface or lack detectable enzymatic activity. Nevertheless, such markers have been reported to be associated with stem cells and to have a possible clinically predictive role in head and neck cancers [9-12].

Head and neck squamous cell carcinomas (HNSCCs) represent most of the malignancies arising from the mucosal lining of the upper aero-digestive tract. They are an extremely heterogeneous group of tumours from both molecular [13,14] and clinical points of view. The main clinical heterogeneity factor is the site of origin, which substantially defines different diseases, each with their own typical risk factors, presentation at diagnosis, tendency to local and distant metastasis, chemo- and radiosensitivity as well as prognosis. In this context, high risk HPV infection, whose role in oropharyngeal carcinogenesis is well established [15], defines a group of oropharyngeal squamous cell carcinomas with peculiar clinical [16-18] and molecular [19] features.

The aims of the present work were to study the expression of different potential stem cell markers in HNSCCs arising from the oral cavity and oropharynx in relation with the above-cited heterogeneity factors, namely, site of origin and HPV infection as well as to assess their potential clinical utility as prognostic markers.

Materials and methods

Patient characteristics

We retrospectively collected data from 69 patients affected by primary, previously untreated oral (OSCC) and oropharyngeal squamous cell carcinomas (OPSCC) and treated between March 2008 and December 2011, at Policlinico Agostino Gemelli - Università Cattolica del Sacro Cuore, Rome, Italy. All patients had been examined at the same institution by a multidisciplinary head and neck tumour board, which provided therapeutic recommendations following histological diagnosis and staging according to TNM classification, VII edition [20]. FFPE tumour samples adequate for immunohistochemistry (IHC) and DNA extraction were available. All 39 patients with OSCC underwent primary surgery ± radiotherapy ± chemotherapy, while all 30 patients with OPSCC underwent primary radiochemotherapy, reserving surgery for the salvage setting.

Authorisation for this retrospective study was obtained by the local ethics committee.

HPV detection

For HPV detection in FFPE samples, we used previously described and validated methods [17,18]. FFPE samples were sectioned for DNA extraction and collected in 1.5 ml micro-tubes. One ml of xylene was then added to each micro-tube and incubated for 30 min at room temperature. The samples were then centrifuged at 14,000 rpm for 3 min, and the supernatant was discarded; this procedure

was repeated twice. The pellet was then washed twice with absolute ethanol (5 min at room temperature). The samples were then incubated overnight with 1 ml of Lysis Buffer (BioMérieux, Rome, Italy) at 37°C.
Nucleic acid extraction was performed using the NucliSens easyMAG platform (BioMérieux, Rome, Italy), according to the manufacturer's protocols. Detection of HPV DNA was performed using the Digene Hybrid Capture 2 (HC2) assay (Qiagen Inc., Valencia, CA, USA), which allows for detection of 18 HPV genotypes and differentiation between high risk (HR) (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 58, 59, and 68) and low-risk (types 6, 11, 42, 43, and 44) (LR) HPV.

Immunohistochemistry (IHC) for stem cell markers

FFPE tumour specimens were evaluated by IHC for the expression of 5 potential stem cell markers: CD44, CD133 protein (CD133), Oct-4, Nanog and Sox-2. Tissue sections were cut at lengths of 2 to 4 mm and deparaffinised. After antigen unmasking for 10 ± 1 minutes at 95 to 99°C in Tris buffer, pH 9.0, slides were allowed to cool to room temperature in the solution for 20 ± 1 min. Endogenous peroxidases were blocked with 3% hydrogen peroxide for 5 ± 1 minutes. The IHC Vectastain® Abc Kit (Vector Laboratories, Inc., Burlingame, CA) was used according to the manufacturer's protocol. The slides were stained with corresponding primary antibodies, namely, Anti-CD44 (Monoclonal Mouse, Phagocytic Glycoprotein-1, Clone DF1485. Code n. M7082) at a 1:50 dilution, Anti-CD133 (CD133/1 (AC133) pure human, monoclonal Myltenyi Biotec) at a 1:10 dilution, anti-OCT4 (C52G3, rabbit, cod. 2890 Cell Signaling Technology), anti-NANOG (C52G3, rabbit; cod. 4903 Cell Signaling Technology) and anti-SOX2 (D6D9 XP, rabbit; cod. 3579 Cell Signaling Technology), and incubated overnight at 4°C. Biotinylated secondary antibodies and VECTASTAIN® ABC Reagent were applied for 45 and 30 min, respectively. After development using a substrate-chromogen solution (AEC, Dako, Copenhagen, Denmark) for 2 min, the immunostained slides were counterstained using haematoxylin (Dako). Four "blinded" histopathologists evaluated the immunohistochemistry in independent readings. The cases that varied among the readers were re-evaluated to obtain a consensus.
The rate of cells with immunoreactivity (from 0 to 100%) was evaluated from 5 different fields and a total of at least 100 cancer cells.
Staining intensity was scored from 0 (no staining) to 3 (strong staining). For CD44, membrane and cytoplasmic staining were evaluated. For OCT-4, NANOG and SOX2, which are considered to be transcription factors with prominent nuclear expression, both cytoplasmic and nuclear expression patterns were specifically evaluated.

Statistical analysis

Statistical analysis was performed using JMP in software, release 7.0.1, from the SAS Institute (Cary, NC, USA). Confidence intervals for hazard ratios were determined by Cox multivariate analysis using STATA version 10, by StataCorp LP.
Correlations between categorical and numerical variables were evaluated by a Wilcoxon test, as most of the numerical variables in the present work did not display a normal distribution.
The oncological endpoint in prognostic evaluation was disease-specific survival (DSS). Univariate survival analysis according to nominal variables was performed by drawing Kaplan-Meyer curves and by evaluating statistical significance using a Wilcoxon test. Multivariate analysis was performed using Cox regression.

Results

Characterisation of the tumours and presence of HPV

Patient and tumour characteristics are shown in Table I. All patients were available for follow-up; the median length of follow-up was 40 months.
The most frequent subsite from which the SCCs originated was the mobile tongue (33%), followed by the tonsil (29%). We observed a marked prevalence of advanced cases (stage III and IV) (approximately 80%). More than 65% of patients in our study cohort presented with clinically positive lymph nodes at diagnosis.
Within the subgroup of OPSCC, the frequency of HR HPV infection was 33% (10/30), and all but one HPV-positive case originated from the tonsil. No HR HPV infection was detected in OSCCs. As expected and as previously described [21], HR HPV infection was associated with a markedly better survival among OPSCCs ($p = 0.045$ for Wilcoxon test).
Clinical TNM staging displayed a prognostic value in the entire series ($p = 0.016$ for Wilcoxon test) as well.

Description of the distribution of markers among HPV+ OPSCC, HPV-OPSCC and OSCC

In Table II, the IHC results for the different stem cell markers in the entire series, OSCC and OPSCC patients, are shown.
In most tumours, a distinct population of CD44+, usually representing approximately 10% of cancer cells, was identifiable. Most of these cells displayed membrane staining (Tab. II, Fig. 1A) in both OPSCCs and OSCCs. Nevertheless, the intensity of membrane staining for CD44 was significantly higher among OSCCs ($p = 0.0035$ for Wilcoxon test). More interestingly, such significance was

Table I. Descriptive statistics of the main variables concerning patients and tumour parameters.

Characteristic		69 patients
Age at diagnosis		
Median		62
Range		45-79
Follow-up period in months		
Median		40
Range		8-87
Smoking habits		
Non-smoker		20 (29%)
Current smoker		38 (55%)
Former smoker		11 (16%)
Alcohol consumption		
More than 4 glasses/day		23 (33.3%)
Less than 4 glasses/day		46 (66.7%)
Sex, no. (%)		
Male		53 (76.8%)
Female		16 (23.2%)
Site of origin, no. (%)	**Subsite of origin, no. (%)**	
Oral cavity 39 (56.5%)	Mobile tongue	23 (33.3%)
	Hard palate	2 (2.9%)
	Floor of mouth	10 (14.5%)
	Retromolar trigone	4 (5.8%)
Oropharynx 30 (43.5%)	Tonsil	20 (29%)
	Base of tongue	8 (11.6%)
	Soft palate	2 (2.9%)
AJCC stage, no. (%)		
I		3 (4.3%)
II		11 (16%)
III		14 (20.3%)
IVa		37 (53.6%)
IVb		4 (5.8%)
cT classification, no. (%)		
T1		7 (10.1%)
T2		22 (31.9%)
T3		10 (14.5%)
T4a		26 (37.7%)
T4b		4 (5.8%)
cN classification, no. (%)		
N0		24 (34.8%)
N1		16 (23.2%)
N2a		2 (2.9%)
N2b		12 (17.4%)
N2c		15 (21.7%)
Grading, no. (%)		
G1		20 (29%)
G2		26 (37.7%)
G3		23 (33.3%)
HPV DNA in FFPE samples, no. (%)		
Negative		59 (85.5%)
High risk HPV		10 (14.5%) (All in the oropharynx)

lost when excluding HPV positive OPSCCs, even if the comparisons of the expression of stem cell markers between the HPV positive (n = 10) and HPV negative (n = 20) OPSCCs did not demonstrate significant differences.

CD44 staining did not show any correlation with prognosis in our series.

As for CD133 staining, its expression was detected in only one sample, and with a low staining intensity.

Among the other markers evaluated, Oct-4 and Nanog were found to be expressed in less than 50% of HNSCCs, with prominent cytoplasmic expression (Tab. II; Figs. 1B, C, D, E, F, G, H, I, L). They did not display different expression profiles according to the site of origin of the tumour. Nevertheless, HPV positive cancers, and especially HPV+ OPSCC, showed significantly (in the Wilcoxon test) lower expression of Nanog in the cytoplasm ($p = 0.0041$ for intensity of staining, $p = 0.0054$ for the percentage of stained cells). Interestingly, the cytoplasmic expression of Nanog was associated with significantly worse prognosis in OPSCC ($p = 0.0012$ for Wilcoxon test, Fig. 2), but not in the OSCC subgroup when analysed separately.

Sox-2 staining was prevalently localised in the nucleus (Fig. 1M, N) and was significantly more intense and frequent among OPSCCs ($p = 0.0006$ for intensity of staining, $p = 0.0001$ for rate of stained cells), while it did not show any significant correlation with HPV infection.

Survival analysis

Sox-2 nuclear staining was associated with worse prognosis when evaluated within the entire series (Fig. 3).

Cox multivariate analysis for DSS took into account age, gender, tumour site, clinical stage, CD44 membrane staining, Oct-4 staining, Nanog cytoplasmic staining and Sox-2 nuclear staining (but not HPV infection, due to its strong correlation with cytoplasmic Nanog staining). To improve the readability and potential clinical applicability of the results, we transformed all of the numerical variables (namely age, CD44 membrane staining, Oct-4 staining, Nanog cytoplasmic staining, and Sox-2 nuclear staining) into nominal variables using the medians as cut-off values. The only parameter retaining prognostic significance at multivariate analysis was Nanog cytoplasmic staining ($p = 0.043$), while age at diagnosis, clinical stage and Sox-2 nuclear staining showed significant trends (Tab. III).

Discussion

Research on stem cell markers, in oncology in general and in HNSCCs in particular, may be interesting for at least two aims [2]: definition of the subpopulation of cancer stem cells, which should be specifically targeted by treatments, and the molecular characterisation of tumours for outcome prediction and treatment selection.

Table II. IHC for stem cell markers.

Marker	Entire series (n = 69)	OPSCC (n = 30)	HPV+ OPSCC (n = 10)	HPV- OPSCC (n = 20)	OSCC (n = 39)
CD44					
Membrane staining intensity					
0	5 (7.3%)	4 (13.3%)	2 (20%)	2 (10%)	1 (2.6%)
1	9 (13%)	5 (16.7%)	2 (20%)	3 (15%)	4 (10.3%)
2	18 (26.1%)	11 (36.7%)	4 (40%)	7 (35%)	7 (17.9%)
3	37 (53.6%)	10 (33.3%)	2 (20%)	8 (40%)	27 (69.2%)
Cytoplasmic staining intensity					
0	20 (29%)	11 (36.7%)	3 (30%)	8 (40%)	9 (23.1%)
1	35 (50.7%)	12 (40%)	5 (50%)	7 (35%)	23(59%)
2	13 (18.8%)	7 (23.3%)	2 (20%)	5 (25%)	6 (15.4%)
3	1 (1.5%)	0	0	0	1 (2.5%)
CD133					
Staining intensity	1 case w weak staining	No staining	No staining	No staining	1 case w weak staining
Oct-4					
Site of staining					
Nuclear	2 (2.9%)	2 (6.7%)	1 (10%)	1 (5%)	0
Cytoplasmic	27 (39.1%)	13 (43.3%)	5 (50%)	8 (40%)	14 (35.9%)
None	40 (58%)	15 (50%)	4 (40%)	11 (55%)	25 (64.1%)
Staining intensity					
0	40 (58%)	15 (50%)	4 (40%)	11 (55%)	25 (64.1%)
1	9 (13%)	6 (20%)	2 (20%)	4 (20%)	3 (7.7%)
2	14 (20.3%)	6 (20%)	3 (30%)	3 (15%)	8 (20.5%)
3	6 (8.7%)	3 (10%)	1 (10%)	2 (10%)	3 (7.7%)
Rate (%) of stained cells					
Mean	15.3	19.3	23.7	17.7	12.3
SD	25	27.9	29.7	27.76	22.5
Nanog					
Site of staining					
Nuclear	5 (7.2%)	3 (10%)	3 (30%)	0	2 (5.1%)
Cytoplasmic	28 (40.6%)	13 (43.3%)	0	13 (65%)	15 (38.5%)
Nuclear and cytoplasmic	1 (1.5%)	1 (3.4%)	0	1 (5%)	0
None	35 (50.7%)	13 (43.3%)	7 (70%)	6 (30%)	22 (56.4%)
Staining intensity					
0	35 (50.7%)	13 (43.3%)	7 (70%)	6 (30%)	22 (56.4%)
1	7 (10.2%)	3 (10%)	2 (20%)	1 (5%)	4 (10.3%)
2	12 (17.4%)	5 (16.7%)	1 (10%)	4 (20%)	7 (17.9%)
3	15 (21.7%)	9 (30%)	0	9 (45%)	6 (15.4%)
Rate (%) of stained cells					
Mean	22.6	30	12.5	36.6	17
SD	29.3	33.7	28.1	33.8	24.4
Sox-2					
Site of staining					
Nuclear	35 (53.7%)	23 (76.7%)	7 (70%)	16 (80%)	14 (36.8%)
Cytoplasmic	12 (17.9%)	4 (13.3%)	3 (30%)	1 (5%)	8 (21.1%)
Nuclear and cytoplasmic	4 (6%)	2 (6.7%)	0	2 (10%)	2 (5.3%)
None	15 (22.4%)	1 (3.3%)	0	1 (5%)	14 (36.8%)
Staining intensity					
0	15 (22.1%)	1 (3.3%)	0	1 (5%)	14 (36.8%)
1	10 (14.7%)	5 (16.7%)	2 (20%)	3 (15%)	5 (13.2%)
2	16 (23.5%)	6 (20%)	2 (20%)	4 (20%)	10 (26.3%)
3	27 (39.7%)	18 (60%)	6 (60%)	12 (60%)	9 (23.7%)
Rate (%) of stained cells					
Mean	42.4	61.72	65	60.5	27.6
SD	32.5	26.1	25	26.9	29.4

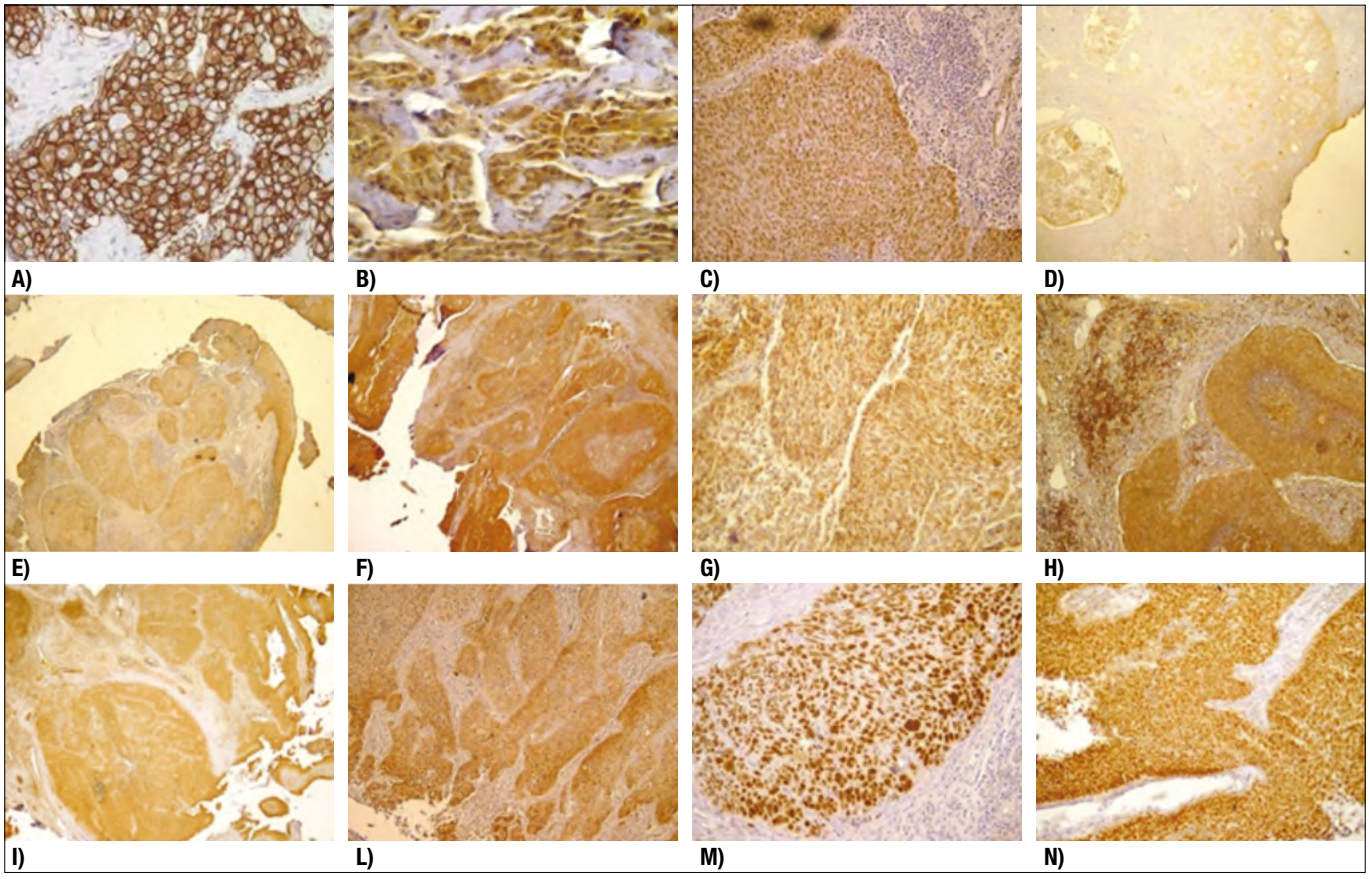

Figure 1. Immunostaining for CD44, Oct-4, NANOG and Sox-2 is shown. (**A**) membrane (3+) and cytoplasmic (2+) immunostaining for CD44 in a case of squamous cell carcinoma of the oropharynx, G2, T4bN2cM0, stage IV; (**B**) nuclear Oct-4 immunostaining, in a case of squamous cell carcinoma of the oral cavity (mobile tongue), T4bN0M0, stage IVb, G2, staining intensity of 3, 90% diffusion of staining; (**C**) nuclear Oct-4 immunostaining from a patient with squamous cell carcinoma of the oropharynx, tongue base, G2, T4N2cM0, stage IV; (**D**) cytoplasmic Oct-4 immunostaining from a case of squamous cell carcinoma of the oral cavity, T3N2bM0, stage IVa, G3, staining intensity of 2, 10% diffusion of staining; (**E**) cytoplasmic Oct-4 immunostaining from a case of squamous cell carcinoma of the oropharynx, T4N2cM0, stage IVa, G3, staining intensity of 2, 70% spread; (**F**) cytoplasmic Oct-4 immunostaining from a case of squamous cell carcinoma of the oral cavity, T4bN1M0, stage IVa, G3, staining intensity of 2, 70% spread; (**G**) nuclear Nanog immunostaining from a case of squamous cell carcinoma of the oropharynx, T4aN2cM0, stage IVa, G3, staining intensity of 3, 80% spread; (**H**) nuclear Nanog immunostaining from a case of squamous cell carcinoma of the oral cavity, T4aN1M0, stage IVa, G3, staining intensity of 2, 70% spread; (**I**) cytoplasmic Nanog immunostaining from a case of squamous cell carcinoma of the oral cavity, T3N1M0, stage III, G2, staining intensity of 3, 80% spread; (**L**) cytoplasmic Nanog immunostaining from a case of squamous cell carcinoma of the oropharynx, T3N2cM0, stage IVa, G3, staining intensity of 3, 70% spread; (**M**) nuclear Sox-2 immunostaining from a case of squamous cell carcinoma of the oropharynx (tonsil), G3, T4aN2cMx, stage IV; (**N**) nuclear Sox-2 immunostaining from a case of squamous cell carcinoma of the oral cavity, T3N2cM0, stage IVa, G3, staining intensity of 3, 100% diffusion of staining.

From the first perspective, the present work confirms the potential utility of CD44 localised on the cell membrane, almost constantly expressed in approximately 10% of cancer cells, consistent with observations in previous reports [5,6,21]. CD44 membrane or cytoplasmic expression did not influence DSS in the present series. CD44 was differentially expressed on the cell membranes of OSCCs and OPSCCs, suggesting, as plausible, that molecular differences associated with the different sites of origin in head and neck [22] also involve the subpopulations of CSC. Excluding HPV-related OPSCC from the analysis eliminated the statistical significance of such differences, confirming that HR-HPV has a role in determining the phenotype of OPSCCs stem cells.

CD133 was substantially undetectable and therefore does not appear to be a valuable stem cell marker in HNSCC. However, we cannot definitively rule out its role as a stem cell marker in HNSCC since the inability of the antibody utilised to detect CD133 molecule in FFPE samples might also be responsible for the results obtained.

The impact of HPV infection on the phenotype of HNSCC cells is even more evident when analysing Nanog cytoplasmic expression, which was always absent in HPV-related OPSCC, while it was frequent in the others.

To our knowledge, such negative correlation between HR-HPV infection and Nanog expression in HNSCC has not been previously described. Nanog is a transcriptional factor that plays a critical role in regulating the cell fate of the pluripotent inner cell mass during embryonic development [23]. Nanog cytoplasmic expression was demonstrated to be a strong prognostic predictor in OPSCC and was the only prognostic marker retaining its significance at Cox multivariate analysis in the entire series. A previous study on OSCC showed correlation of Nanog expression with stage at diagnosis, and, when associated with other markers, with prognosis [24]. In the present study, we show a prognostic role of Nanog expression, but apparently limited to the oropharynx, and probably correlated with HPV infection. No prognostic significance was detected either for the expression of Oct-4, which is a member of the family of POU domain transcription factors, expressed in pluripotent embryonic stem and germ cells [25-27] and functionally related to Nanog [28]. Furthermore, differently from previous hypotheses [28] and descriptions in OSCC [24], both proteins in the present series were prevalently localised in the cytoplasm.

Conversely, Sox-2 displayed the expected nuclear localisation and was shown to have prognostic value at univariate analysis in the entire series, as previously described [11], even if such significance was not retained at multivariate analysis in the present work. Nuclear expression of Sox-2 was significantly higher among OPSCCs, reconfirming the phenotypic differences among CSCs from different sites in the head and neck.

In conclusion, in the present study, CD44 appears to be a reliable marker for identification of the CSC subpopulation in HNSCC. Nevertheless, when evaluating the expression of membrane CD44 itself, and also nuclear

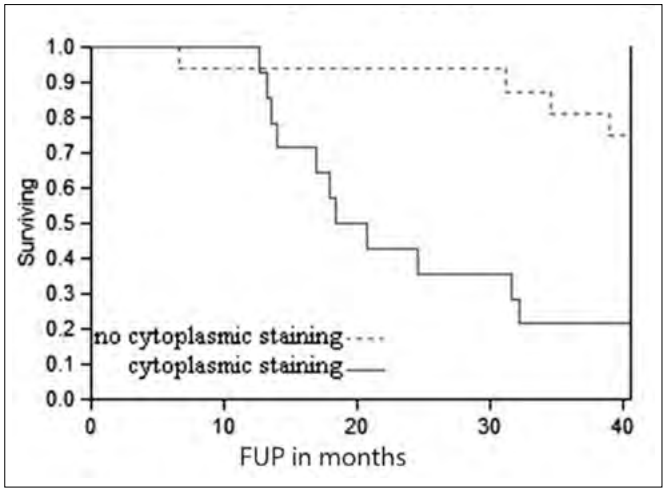

Figure 2. In the OPSCC group, the absence of Nanog cytoplasmic staining was associated with significantly better prognosis (p = 0.0012 for Wilcoxon test).

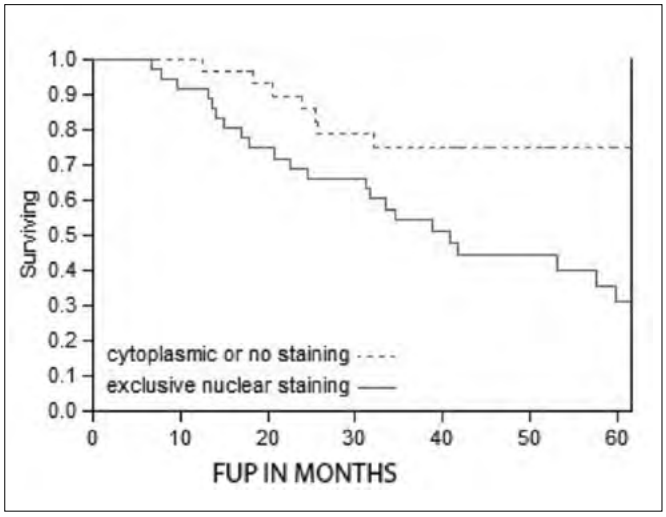

Figure 3. In the entire series of OSCCs and OPSCCs, nuclear staining for Sox-2 was associated with worse prognosis (p = 0.012 for Wilcoxon test).

Table III. Univariate and multivariate analysis of prognostic covariates for disease-specific survival.

Characteristic	Multivariate analysis		
	HR[a]	CI (95%)[b]	p
Age at diagnosis			
Over 65	1		
Under 65	0.48	0.22-1.03	0.06
Sex			
Female	1		
Male	1.52	0.61-3.8	0.365
Clinical stage			
I-II-III	1		
IVa-IVb	2.31	0.88-6.1	0.089
Primary site			
Oral cavity	1		
Oropharynx	1.67	0.65-4.26	0.283
CD 44 membrane staining			
Staining intensity 0, 1, 2	1		
Strong staining (score 3)	0.78	0.32-1.9	0.584
OCT-4 staining			
No staining	1		
Presence of stained cells	1.15	0.52-2.52	0.733
Nanog cytoplasmic staining			
No staining	1		
Presence of stained cells	2.45	1.02-5.84	**0.043**
Sox-2 staining			
No nuclear staining	1		
Presence of nuclear staining	2.24	0.9-5.56	0.083

[a]: hazard ratio; [b]: 95% confidence intervals.

Sox-2, clear differences emerged between different sites in the head and neck. Previous approaches in the study of CSCs have sometimes grouped HNSCCs together, but our results suggest that different markers could be used in the future for isolation as well as for targeting of CSCs in SCCs arising from different head and neck sites.

Other markers, such as Nanog, are influenced by HR-HPV infection. HPV infection is currently considered the most promising molecular marker in head and neck oncology, and has also been included by NCCN in the diagnostic work up for oropharyngeal SCC [29]. Debate about the standard detection method for HPV in FFPE samples is still ongoing, and the reliability of p16 expression as surrogate marker is questioned [18,30]. The absence of Nanog may be useful in this situation, being another effective indicator of HPV infection, which deserves to be evaluated in combination with other parameters (p16 and pRb, for example) to define the HPV related phenotype in OPSCCs, with potentially relevant clinical implications. In fact, Nanog might become an alternative, or more probably, an integration to p16 IHC, for diagnosis of HPV driven carcinogenesis in the oropharynx. At a cellular level, such differences in Nanog expression, still awaiting a consistent explanation, may turn out to be a useful clue to explain the clear phenotypic differences between HPV+ and HPV- SCCs.

As prognostic stratification, currently relying on clinical parameters only, is considered unsatisfactory, the definition of molecular predictive factors aimed to delineate homogeneous groups of patients for prognostic stratification and treatment selection (molecular characterisation) is potentially one of the most relevant areas of translational research in the head and neck. From this perspective, both Sox-2 and Nanog look promising as prognostic markers, although larger studies, also evaluating additional head and neck sites, are required before confirmation of this hypothesis and introduction into daily clinical practice.

References

1 Heppner GH, Miller BE. Tumor heterogeneity: biological implications and therapeutic consequences. Cancer Metastasis Rev 1983;2:5-23. https://doi.org/10.1007/bf00046903

2 Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer 2008;8:755-68. https://doi.org/10.1038/nrc2499

3 Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 1997;3:730-7. https://doi.org/10.1038/nm0797-730

4 Reya T, Morrison SJ, Clarke MF, et al. Stem cells, cancer, and cancer stem cells. Nature 2001;414:105-11. https://doi.org/10.1038/35102167

5 Prince ME, Sivanandan R, Kaczorowski A, et al. Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. Proc Natl Acad Sci USA 2007;104:973-8. https://doi.org/10.1073/pnas.0610117104

6 Okamoto A, Chikamatsu K, Sakakura K, et al. Expansion and characterization of cancer stem-like cells in squamous cell carcinoma of the head and neck. Oral Oncol 2009;45:633-9. https://doi.org/10.1016/j.oraloncology.2008.10.003

7 Chen YC, Chen YW, Hsu HS, et al. Aldehyde dehydrogenase 1 is a putative marker for cancer stem cells in head and neck squamous cancer. Biochem Biophys Res Commun 2009;385:307-13. https://doi.org/10.1016/j.bbrc.2009.05.048

8 Clay MR, Tabor M, Owen JH, et al. Single-marker identification of head and neck squamous cell carcinoma cancer stem cells with aldehyde dehydrogenase. Head Neck 2010;32:1195-201. https://doi.org/10.1002/hed.21315

9 Atlasi Y, Mowla SJ, Ziaee SA, et al. OCT-4, an embryonic stem cell marker, is highly expressed in bladder cancer. Int J Cancer 2007;120:1598-602. https://doi.org/10.1002/ijc.22508

10 Bourguignon LY, Peyrollier K, Xia W, et al. Hyaluronan-CD44 interaction activates stem cell marker Nanog, Stat-3-mediated MDR1 gene expression, and ankyrin-regulated multidrug efflux in breast and ovarian tumor cells. J Biol Chem 2008;283:17635-51. https://doi.org/10.1074/jbc.M800109200

11 Freier K, Knoepfle K, Flechtenmacher C, et al. Recurrent copy number gain of transcription factor SOX2 and corresponding high protein expression in oral squamous cell carcinoma. Genes Chromosomes Cancer 2010;49:9-16. https://doi.org/10.1002/gcc.20714

12 Sholl LM, Long KB, Hornick JL. Sox2 expression in pulmonary non-small cell and neuroendocrine carcinomas. Appl Immunohistochem Mol Morphol 2010;18:55-61. https://doi.org/10.1097/PAI.0b013e3181b16b88

13 Huang Q, Yu GP, McCormick SA, et al. Genetic differences detected by comparative genomic hybridization in head and neck squamous cell carcinomas from different tumor sites: construction of oncogenetic trees for tumor progression. Genes Chromosomes Cancer 2002;34:224-33. https://doi.org/10.1002/gcc.10062

14 Bosch FX, Ritter D, Enders C, et al. Head and neck tumor sites differ in prevalence and spectrum of p53 alterations but these have limited prognostic value. Int J Cancer 2004;111:530-8. https://doi.org/10.1002/ijc.11698

15 Gillison ML, Koch WM, Capone RB, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst 2000;92:709-20. https://doi.org/10.1093/jnci/92.9.709

16 Ang KK, Harris J, Wheeler R, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med 2010;363:24-35. https://doi.org/10.1056/NEJMoa0912217

17 Bussu F, Sali M, Gallus R, et al. HPV infection in squamous cell carcinomas arising from different mucosal sites of the head and neck region. Is p16 immunohistochemistry a reliable surrogate marker? Br J Cancer 2013;108:1157-62. https://doi.org/10.1038/bjc.2013.55

18 Bussu F, Sali M, Gallus R, et al. Human papillomavirus (HPV) infection in squamous cell carcinomas arising from the oropharynx: detection of HPV DNA and p16 immunohistochemistry as diagnostic and prognostic indicators - a pilot study. Int J Radiat Oncol Biol Phys 2014;89:1115-20. https://doi.org/10.1016/j.ijrobp.2014.04.044

19 Stransky N, Egloff AM, Tward AD, et al. The mutational landscape of head and neck squamous cell carcinoma. Science 2011;333:1157-60. https://doi.org/10.1126/science.1208130

20 Edge SB, Byrd DR, Compton CC, et al. AJCC Cancer Staging Manual. Seventh edition. New York: Springer; 2010.

[21] Al-Hajj M, Wicha MS, Benito-Hernandez A, et al. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 2003;100:3983-8. https://doi.org/10.1073/pnas.0530291100

[22] Freier K, Joos S, Flechtenmacher C, et al. Tissue microarray analysis reveals site-specific prevalence of oncogene amplifications in head and neck squamous cell carcinoma. Cancer Res 2003;63:1179-82.

[23] Chambers I, Colby D, Robertson M, et al. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 2003;113:643-55. https://doi.org/10.1016/s0092-8674(03)00392-1

[24] Chiou SH, Yu CC, Huang CY, et al. Positive correlations of Oct-4 and Nanog in oral cancer stem-like cells and high-grade oral squamous cell carcinoma. Clin Cancer Res 2008;14:4085-95. https://doi.org/10.1158/1078-0432.CCR-07-4404

[25] Burdon T, Smith A, Savatier P. Signalling, cell cycle and pluripotency in embryonic stem cells. Trends Cell Biol 2002;12:432-8. https://doi.org/10.1016/s0962-8924(02)02352-8

[26] Okamoto K, Okazawa H, Okuda A, et al. A novel octamer binding transcription factor is differentially expressed in mouse embryonic cells. Cell 1990;60:461-72. https://doi.org/10.1016/0092-8674(90)90597-8

[27] Rosner MH, Vigano MA, Ozato K, et al. A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. Nature 1990;345:686-92. https://doi.org/10.1038/345686a0

[28] Wang J, Rao S, Chu J, et al. A protein interaction network for pluripotency of embryonic stem cells. Nature 2006;444:364-8. https://doi.org/10.1038/nature05284

[29] Pfister DG, Spencer S, Brizel DM, et al. Head and neck cancers. vers.1.2015. Clinical Practice Guidelines in Oncology. National Comprehensive Cancer Network.

[30] Agrawal N, Frederick MJ, Pickering CR, et al. Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. Science 2011;333:1154-7. https://doi.org/10.1126/science.1206923