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

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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 un peggiore prognosi. L’espressione di Nanog è associata a OPSCC HPV-negativi e può avere un ruolo come marker diagnostico surrogate. 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, diagnosi di HPV, Sox-2, Nanog

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Conflict of interest
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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 organization 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 Mylytenyi 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, 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
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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: 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 (28.1%) | 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 | Cytoplasmic | None | 0 | 14 (35.9%) |
| 0      | 2 (2.9%) | 27 (39.1%) | 15 (50%) | 11 (55%) | 25 (64.1%) |
| 1      | 9 (13%)  | 12 (40%) | 5 (50%) | 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 | 22.6 | 22.6 | 12.3 |
| SD     | 25 | 27.9 | 30 | 29.3 | 22.5 |
| Nanog  |                        |                |                     |                     |              |
| Site of staining | Nuclear | Cytoplasmic | Nuclear and cytoplasmic | None | 15 (43.8%) |
| 0      | 35 (50.7%) | 13 (43.3%) | 7 (70%) | 6 (30%) | 22 (56.4%) |
| 1      | 1 (0.2%) | 3 (10%) | 1 (10%) | 4 (20%) | 7 (17.9%) |
| 2      | 12 (17.4%) | 5 (16.7%) | 1 (10%) | 14 (36.8%) | 1 (5%) |
| 3      | 15 (21.7%) | 9 (30%) | 0 | 9 (45%) | 6 (15.4%) |
| Rate (%) of stained cells | Mean | 22.6 | 30 | 12.5 | 36.6 |
| SD     | 29.3 | 33.7 | 28.1 | 33.8 | 24.4 |
| Sox-2  |                        |                |                     |                     |              |
| Site of staining | Nuclear | Cytoplasmic | Nuclear and cytoplasmic | None | 15 (43.8%) |
| 0      | 35 (53.7%) | 23 (76.7%) | 7 (70%) | 16 (80%) | 14 (36.8%) |
| 1      | 12 (17.9%) | 4 (13.3%) | 3 (30%) | 1 (5%) | 8 (21.1%) |
| 2      | 4 (6%) | 2 (6.7%) | 0 | 2 (10%) | 2 (5.3%) |
| 3      | 15 (22.4%) | 1 (3.3%) | 0 | 1 (5%) | 14 (36.8%) |
| Staining intensity | Mean | 42.4 | 61.72 | 65 | 60.5 |
| SD     | 32.5 | 26.1 | 25 | 26.9 | 29.4 |
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. 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 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 OPSCC 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. 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. 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 and functionally related to Nanog. Furthermore, differently from previous hypotheses and descriptions in OSCC, 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, 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 expression...
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. 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. 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.

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