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Cancer stem cell and its niche in malignant progression of oral potentially malignant disorders

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ABSTRACT

Objective: The purpose of this study was to determine association between cancer stem cells (CSCs) and their niche with progression of oral potentially malignant disorders.

Materials and methods: Patients with histologically confirmed oral potentially malignant disorders, stratified into high/low risk lesions based on the degree of dysplasia and oral cancer were included in this study. Immunohistochemical profiling of markers of CSCs (CD44), endothelial cells (CD31) and CSC-vascular niche cross-talk (CXCR4 and SDF1) were carried out. Statistical analysis was performed to correlate the relationship of markers with histopathology grade (ANOVA, and χ^2 test, unpaired *t* test) using GraphPad InStat v3.06.

Results: The study included 550 samples (349 patients) and analysis showed progressive increase in expression levels of CSC and its niche markers with increase in grade of dysplasia as compared to the normal cohort ($p < 0.05$). Co-expression analysis revealed that, in comparison to the normal cohort, a larger percentage of patients showed increased expression of CD31 and CD44 ($CD31^{high}/CD44^{high}$; $p < 0.05$) and of CXCR4 and SDF1 ($CXCR4^{high}/SDF1^{high}$; $p = 0.04$), suggesting an association of the CSCs and the vascular niche. Further, distribution of patients with $CD44^{high}/CXCR4^{high}$ ($p < 0.05$) and $CD31^{high}/SDF1^{high}$ ($p = 0.01$) was significantly increased in the high-risk group (18%), suggesting a correlation between $CD44^{+}/CXCR4^{+}$ cells, the vascular niche and progression of oral dysplastic lesions.

Conclusion: The increased expression of CSCs, the vascular niche and their cross talk markers are associated with increase in severity of dysplasia suggesting their role in the progression of oral potentially malignant disorders and may hence be used in identifying high-risk OPMD.

Introduction

Oral cancer, which is the sixth most common cancer, accounts for 300,000 cases worldwide [1]. A large proportion of oral cancer is preceded by the development of oral leukoplakia [2,3], an oral potentially malignant disorder (OPMD). The histologic progression of OPMD from hyperplasia, different grades of dysplasia to carcinoma-in-situ and invasive carcinoma and the associated genomic changes are well studied [4]. Cancer stem cells (CSCs) have been increasingly implicated in oral carcinogenesis and field cancerization [5], and are

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known to be regulated by stroma and endothelial cells constituting the CSC-niche [6,7]. We hypothesized that increase in expression of markers specific to the CSCs, its vascular niche, and their cross talk correlates with progression of OPMD. These molecules, if validated in OPMD, could serve as potential markers for malignant transformation and targets for chemoprevention.

Among the multiple pathways implicated in the epithelial cell-niche cross talk (Notch1, TGFβ, and SDF-1/CXCR4) [8–11], the SDF-1/CXCR4 axis has been implicated in CSC-niche cross talk in many cancers including head and neck (HNSCC) [12]. *In vitro* studies investigating their role in HNSCC have shown that CXCR4 promotes migration and invasion specifically in CD44+ cells [13], while SDF-1 released from the Cancer Associated Fibroblast (CAFs) and endothelial cells induces increased migratory and invasive properties in the cancer cells [14]. Additionally, SDF-1/CXCR4 has been associated with poor prognosis in patients diagnosed with head and neck cancer [15,16]. A definitive correlation between the CSCs, their vascular niche and the molecular signals involved in this cross-talk, should provide evidence towards their role in oral carcinogenesis

Materials and methods

Patient details

This is a retrospective study of patients with histologically confirmed OPMD and oral cancer presented to the Department of Head and Neck Surgery, Roswell Park Cancer Institute, New York, USA (2006–2013) and Department of Head and Neck Oncology at Mazumdar Shaw Medical Centre, Bangalore, India (2011–2013). The clinical and demographic details of these patients were obtained from the hospital medical records. Patients who have been previously treated for malignancy, diagnosed with infectious/inflammatory lesions and non-squamous malignancies were excluded from the study. Patients with clinically and histologically normal mucosa were used as controls for the study. The study was approved by the Institutional Ethical Committee of the respective hospitals (RPCI IEC No: I66805 and MSMC IRB approval dated 10.08.2010).

The oral mucosa of the patients enrolled in the study were categorized into normal, oral potentially malignant disorders (OPMD) and oral squamous cell carcinoma (OSCC) based on the histopathology reports. The OPMD patients were categorized as high and low risk based on established binary classification criteria [17]. Hyperplasia, parakeratosis and mild dysplasia were categorized as low-risk lesions, while moderate and severe dysplasia and carcinoma in situ as high-risk lesions. The carcinoma patients included micro-invasive carcinoma, and cancers of all stages (I-IV) and differentiation (well, moderate and poor).

Immunohistochemistry

The Formalin Fixed Paraffin Embedded (FFPE) sections were deparaffinized, re-hydrated and antigen retrieval was carried out according to standard protocols. The sections were then incubated with the primary antibodies overnight at 4 °C in a humidified chamber. The antibodies used were for CD44 (AM310-5M; Biogenex Life Sciences Pvt. Ltd., Hyderabad, India; Ready-to-use and MA4400; Thermo Fischer Scientific, Rockford, IL, USA; dilution: 1:50), CD31 (endothelial cell clone JC70A (M0823, DAKO, California, USA; dilution: 1:50), CXCR4 (ab2074; Abcam, Cambridge, MA, USA; 1:50) and SDF-1 (97958; Cell signaling technology, Danvers, MA, USA; 1:200). The two CD44 antibodies were evaluated for their comparative staining patterns (Supplementary Fig. 1). Sections were then washed twice with 1X-TBST buffer, stained with a secondary detection kit (Real TM EnVision™ Detection system DAKO, Denmark) and counterstained using Mayers-Hematoxylin and mounted using DPX mountant. All the slides were scanned (Aperio ScanScope XT 1509, AT2, Leica biosystems, IL, USA) at 20× magnification, reviewed independently by two observers and images analyzed using the Aperio Imagescope v.11.2.0.780 (Leica biosystems, IL, USA). The reviewers were blinded to the histopathological diagnosis during the entirety of scoring.

Immunohistochemical scoring and analysis

The expression of CD31 was scored to denote the presence or absence of microvessels. Slides were initially viewed under minimal magnification to identify areas of maximal staining, or “hot spots”, a minimum of three CD31 positively stained hotspots were identified. The micro vessel density (MVD) was evaluated by counting the number of CD31 positive vessel lumen under a high power field (hpf) using a magnification of 400× field [18–20]. Four non-overlapping fields were counted per hot spot, the total number of vessel lumens/hpf recorded, and an average number of lumens per hot spot calculated.

Analysis of CD44 and SDF-1 was carried out by evaluating the staining in terms of the distribution (percentage of positive epithelial cells) and level of expression (intensity of staining) [21,22]. Sections were considered positive when > 10% of the tumor cells were stained with strong intensity. The percentage of positive cells (0–100%) was multiplied with the intensity of staining (weak/1+, moderate/2+, strong/3+) to obtain the H−score of CD44 and SDF1 expression with a maximum score being 300 (100% x 3+). Cytoplasmic/membrane and nuclear low and high expression of CXCR4 evaluated by counting percentage of positive cells (1 = < 10%, 2 = 10–50%, 3 = > 50%) for cytoplasmic and membrane staining (1 −3). Positive nuclear staining was defined as a nuclear score of 6 or more [i.e. any slide with > 50% of the cells expressing nuclear expression (3) with intermediate or strong]. Cells were counted in at least three fields (at ×400) within the lesion [23]. The scoring for all the antibodies was done by a panel of three experienced observers.

Statistical analysis

All statistical analyses were performed using the R 3.3.1 statistical computing language. Statistical testing included Pearson correlation, ANOVA, and $\chi 2$ test of independence. Unpaired *t* test was carried out using GraphPad InStat version 3.06 (Graph Pad Software, San Diego California USA). Statistical significance was set at $p < 0.05$ for all tests. The data is presented as Mean ± SEM for all relevant analyses. Univariate and multivariate analysis of frequency distributions of the patient cohorts (normal, low risk, high risk and carcinoma) within the marker expression patterns ($marker^{high}$ and/or $marker^{low}$) was carried out and the statistical significance evaluated by Chi square or Fisher's test.

Results

Clinical characteristics of the patients

Five hundred and fifty ($n = 550$) samples from 349 patients were categorized into normal, different grades of dysplasia and squamous cell carcinoma based on their histopathological diagnosis. Among the study cohort ($n = 349$), majority of the patients were males ($n = 211$, 60.6%) with a median age of 57 years (range 21–91). While the most common histologic diagnosis was non-dysplastic lesions ($n = 227$, 41.27%), the remaining patients were distributed into mild ($n = 148$, 26.9%), moderate ($n = 47$, 8.45%) and severe dysplasia ($n = 34$, 6.2%) and carcinoma ($n = 94$, 17.09%) (Table 1). The clinically and histologically normal samples ($n = 6$) from healthy subjects, undergoing dental procedures, served as the control.

Correlation of microvascular density (MVD) with grades of dysplasia

The expression of CD31 (Fig. 1A), as an indicator of MVD, was