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Proteoglycan-based diversification of disease outcome in head and neck cancer patients identifies NG2/CSPG4 and syndecan-2 as unique relapse and overall survival predicting factors

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Abstract

Background: Tumour relapse is recognized to be the prime fatal burden in patients affected by head and neck squamous cell carcinoma (HNSCC), but no discrete molecular trait has yet been identified to make reliable early predictions of tumour recurrence. Expression of cell surface proteoglycans (PGs) is frequently altered in carcinomas and several of them are gradually emerging as key prognostic factors.

Methods: A PG expression analysis at both mRNA and protein level, was pursued on primary lesions derived from 173 HNSCC patients from whom full clinical history and 2 years post-surgical follow-up was accessible. Gene and protein expression data were correlated with clinical traits and previously proposed tumour relapse markers to stratify high-risk patient subgroups.

Results: HNSCC lesions were indeed found to exhibit a widely aberrant PG expression pattern characterized by a variable expression of all PGs and a characteristic *de novo* transcription/translation of GPC2, GPC5 and NG2/CSPG4 respectively in 36%, 72% and 71% on 119 cases. Importantly, expression of NG2/CSPG4, on neoplastic cells and in the intralesional stroma (Hazard Ratio [HR], 6.76, $p = 0.017$) was strongly associated with loco-regional relapse, whereas stromal enrichment of SDC2 (HR, 7.652, $p = 0.007$) was independently tied to lymphnodal infiltration and disease-related death. Conversely, down-regulated SDC1 transcript (HR, 0.232, $p = 0.013$) uniquely correlated with formation of distant metastases. Altered expression of PGs significantly correlated with the above disease outcomes when either considered alone or in association with well-established predictors of poor prognosis (i.e. T classification, previous occurrence of precancerous lesions and lymphnodal metastasis). Combined alteration of all three PGs was found to be a reliable predictor of shorter survival.

Conclusions: An unprecedented PG-based prognostic portrait is unveiled that incisively diversifies disease course in HNSCC patients beyond the currently known clinical and molecular biomarkers.

Keywords: Proteoglycans, Squamous cell carcinoma, Biomarker, NG2/CSPG4, Tumour relapse

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Background

Head and neck squamous cell carcinomas (HNSCC) have an estimated frequency of 38,160 new cases in the US (updated to August, 2014) [1] and an estimated occurrence of more than 442,000 new cases worldwide according to GLOBOCAN 2012 [2,3], thereby representing the primary lethal cancer entity in patients with head and neck tumours. Loco-regional relapsing is the most severe clinical problem encountered in these tumours, while the pre-operative presence of lymphnodal infiltration is a recognized prognostic factor [4,5]. Especially in patients presenting smaller primary lesions, occult secondary lesions in lymphnodes significantly complicate the clinical management of these individuals [6-13]. The currently adopted methods to predict disease recurrence, such as staging and grading, are too arbitrary and do not allow for a sufficiently accurate clinical stratification of the patients [14,15]. This deficit calls upon the need to identify distinct molecular markers that more reliably would predict disease progression, recurrence and metastasis formation, and many such have been proposed over the last decade (Table 1). Thus far, however, only three such markers have been considered as meaningful, i.e. HPV infection, TP53 mutation status and overexpression of EGFR [16-20], but their full independence from clinical parameters is still dubious.

One class of molecules with the potential of acting as clinically relevant factors in HNSCC, especially for oral cavity and oropharynx cancer, is that comprising cell surface-associated proteoglycans (PGs). In fact, changes in their relative expression are progressively being associated with neoplastic transformation, propagation of local tumour masses, and formation of distant metastases. This not only in HNSCC, but also in numerous epithelial and non-epithelial tumour types. Both PGs produced by the HNSCC cells themselves and PGs associated with the intra-lesional tumour stroma may play critical roles in the control of HNSCC growth, dissemination and therapeutic refraction, and may therefore be contemplated as putative biomarkers as well as therapeutic targets. There are currently 15 cell surface PGs known in the human genome with the most representative ones belonging to either the transmembrane syndecan group, i.e. syndecan-1-4 (SDC1-SDC4) [21-25], or the GPI-anchored glypican group, i.e. glypican-1-6 (GPC1-GPC6) [23,26-28]. The unique structural traits of cell surface PGs enable them to modulate directly and/or indirectly several facets of the tumour cell phenotype and behavior, including growth kinetics, invasiveness and metastatic ability.

Previously documented, representative examples of the implication of diverse PGs expressions for disease outcome are afforded by the recently consolidated tumour-suppressing effect of GPC5 in lung carcinomas arising in “never smokers” [29-31], as well as by the well-established

prognostic/predictive up-regulation of GPC1 in pancreatic cancer [32,33]. As a corollary, GPC3 is a recognized prognostic/predictive factor and therapeutic target in hepatocellular carcinoma [34-37]. SDC1, the only PG for which there is some documentation in oral squamous cell carcinoma, seems to be associated with the differentiation status of the tumour cells [38-40]. Clinical correlation of SDC1 expression with disease status specifically refers to its modulation in epithelial neoplastic cells [41-47] and tumour stroma [48], while the PG has been proposed to influence migration and invasion of oral squamous cell carcinoma cells *in vitro* by interacting with the β 1 integrin subunit and the laminin β 1 chain [48].

NG2/CSPG4 has been proposed to impact on tumour-igenesis and evidence has been accrued suggesting that NG2/CSPG4 alone is able to confer metastatic potential to cancer cells by serving as a multivalent mediator of the cancer cell-host microenvironment interactions and by enhancing drug resistance and protecting cells from stress-induced programmed cell death [49,50]. In an increasing number of tumours, prognostic implications of NG2/CSPG4 are being unveiled and these discoveries accentuate the potential of the PG as a therapeutic target. Recently, a direct link between methylation and CSPG4 expression in HNSCC HPV-negative/stage IVa subgroup were proved, where high protein expression and low promoter methylation were significantly associated with an adverse progression-free and overall survival [51].

Based upon previously accrued information about the role of PGs in cancer and the currently available experimental evidences along this line, we have addressed the possibility that the pattern of expression of individual PGs, or groups of PGs, may act as either pro- or anti-tumourigenic and thereby be predictive, or indicative, of a discrete disease course in oral cavity HNSCC disease course.

Methods

Patients

Patients from whom surgical specimens were evaluated were treated surgically at the S. Orsola-Malpighi Hospital, at the Bellaria University Hospital in Bologna and at the Maxillo-Facial Surgery Division, Department of Head and Neck Surgery of the University of Parma. A total of 173 surgical specimens of primary oral cavity HNSCC were collected after informed consent obtained from each enrolled patients, all of them in adulthood (Additional file 1: Table S1; Additional file 2: Figure S1). Patients were referred to adjuvant radiation therapeutic treatment according to the guidelines defined by the National Comprehensive Cancer Network (NCCN) Clinical Practice (Version 2.2014; www.nccn.org). Clinical data were collected within the 2 years-post surgical follow-up every 6 months (Additional file 3; Additional file

Table 1 Previously proposed prognostic biomarkers in HNSCC¹

Biomarker ²	Clinical outcome	Method of detection	N. of cases/%/type of modulation	Annotation
ADAM17	Lymph nodal metastasis/Loco-regional relapse	IHC/WB	50/46/Up	None
CD44	OS/DFS	IHC	138/59/Down	None
E-cadherin	Recurrence/OS	IHC	50/20/Up 112/59/Down	None
EGFR	OS	IHC	109/73/Up 59/58/Up	None
Estrogen-R2	OS	IHC/nPCR + sequencing	67/51/Up	Laryngeal/hypopharyngeal cancer
FHIT	OS/DFS	IHC	53/61/Down	None
GLUT1	OS	IHC	40/26/up	Poor radiation response
HIF1A	OS/DFS	IHC	85/63/Down	None
Keratin-18	OS	IHC	308/54/Up	None
Keratin-8	OS	IHC	308/54/Up	None
Laminin γ2	DSS	DNA Microarray	119/NS/Up	None
MCM5	OS	IHC	97/61/Up	None
MET	OS	IHC	69/82/Up	None
Moesin	OS	IHC	103/NS/Up	Cytoplasmic expression pattern
Mucin-1	OS/DFS/Lymphnodal metastasis	IHC	206/39/Up	Within 5-years follow-up
Mucin-4	OS/DFS/Lymphnodal metastasis/Loco-regional relapse	IHC	150/41/Up	Within 5-years follow-up
p21	OS	IHC	192/71/Down	None
p27	DFS	IHC	192/80/Down	Only in patients with lymphnodal infiltration
p57	OS	IHC	67/87/Down	None
p63	OS	IHC	62/NS/Up	None
P-cadherin	Disease recurrence/Loco-regional relapse/OS	IHC	50/20/Down 67/45/Down 108/16/Down	None
Podoplanin	DSS	IHC	35/56/Up	None
Rb	DFS	IHC	220/49/Down	Only in p53 ⁺ /pRb ⁻ patients
RUNX3	OS	IHC/WB	108/46/Down	None
S100A2	DFS/Cervical metastasis	RT-PCR + seq/IHC	135/26/Down 52/NS/Down	Nuclear expression pattern
SPARC	OS/DFI	DNA Microarray/IHC	62/NS/Up	None
STAT1	OS	IHC	89/NS/Up	None
Survivin 3α	OS	RT-PCR	97/NS/Up	Only in lymphnodes
TERT	OS	IHC	62/NS/Up	None
Ezrin	OS	IHC	47/85/Up	Cytoplasmic expression pattern

¹Specifically referred to oral and oropharyngeal squamous cell carcinoma;

²Alterations of TP53, CCND1 and FGFR4 genes are not included;

Abbreviations: **OS**, Overall Survival; **DFS**, Disease Free-Survival; **DSS**, Disease Specific-Survival; **DFI**, Disease Free-Interval; **IHC**, Immunohistochemistry; **WB**, Western Blotting; **nPCR**, nested Polymerase Chain Reaction; **NS**, Not Specified.

2: Figure S1). The present study has been approved by the local ethics committees (Comitato Etico Provinciale di Parma –Parma University Hospital e Comitato Etico Provinciale di Bologna-Bologna University Hospital) and was conducted in compliance with the Helsinki Declaration's Ethical Principles for Medical Research Involving Human Subjects.

RNA extraction and qPCR

Total RNA from healthy specimens and 119 neoplastic specimens were extracted using Trizol[®] according to the manufacturer's instructions and in combination with Qiagen RNAeasy Mini Kit (Qiagen). Total RNA (1 μ g) was reverse-transcribed with the QuantiTect[®] Reverse Transcription Kit (Qiagen). Each TaqMan Low Density

Array was designed for quantification of the human PGs. The assays were chosen among the TaqMan Gene Expression Assay library (Additional file 3) and the cards were run on ABI PRISM 7900 HT Fast Real-Time PCR System (Applied Biosystems Inc., Foster City, CA, USA). Changes in gene expression levels were calculated using the “relative quantification method”. Relative gene expression fold-change were expressed as $\text{Log}_2(2^{\Delta\Delta Ct})$ and to visualize the obtained expression profiles we used heatmap graphing by EPCLUST – Expression Profile data CLUSTERing and analysis software (www.bioinf.ebc.ee/EP/EP/EPCLUST/) [52]. The data presented herein have been deposited in NCBI’s Gene Expression Omnibus [53] and are accessible through GEO Series accession number GSE33788 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE33788>) (Additional file 3).

Tissue microarray (TMA) construction

Tissue specimens from a total of 163 patients, which were independently assured to contain representative areas of the neoplastic lesions, were selected for TMA construction according to a previously described procedure [54,55]. Cases were considered representative when at least 50% of the section was composed of neoplastic cells. For each case, the core portion of the section with the highest percentage of tumour cells was used for analysis (Additional file 3).

Immunohistochemistry

Details on the antibodies used, characteristics of control tissues and experimental procedures are reported in Additional file 3. Relative antigen expression was assessed semi-quantitatively according to the arbitrary scoring: “-” = no positive cells were detected, “+” <10% of cells were positive, “++” <50% of cells were positive, “+++” <90% of cells were positive, and “++++” ≥90% of cells were positive.

Statistical and bioinformatic analyses

Demographic data, presence of recognized risk factors for development of HNSCC, clinical diagnostic parameters, gene expression and protein distribution patterns for the PGs GPC1-6, SDC1-4 and NG2/CSPG4 were comparatively evaluated for their potential correlation with the following disease outcomes: loco-regional recurrence, lymphodal metastasis, distant metastasis, disease-related deaths and probability of incurring into one or more of these clinical outcomes. Estimation of influence of each variable considered for the above disease outcomes was analyzed independently with both the Log-rank and Wilcoxon’s rank test. Survival rate was estimated using the Kaplan-Meier method from the time of surgery to the end of the follow-up. Cox’s multivariate proportional hazards regression method was used to extract a parsimonious

set of independent variables. All analyses were performed using the Statgraphics Centurion XVI software (StatPoint Technologies, Inc, Virginia, USA). *P* values <0.05 were considered to be significant (Additional file 3).

Results

Transcriptional profiles of PGs in primary oral cavity

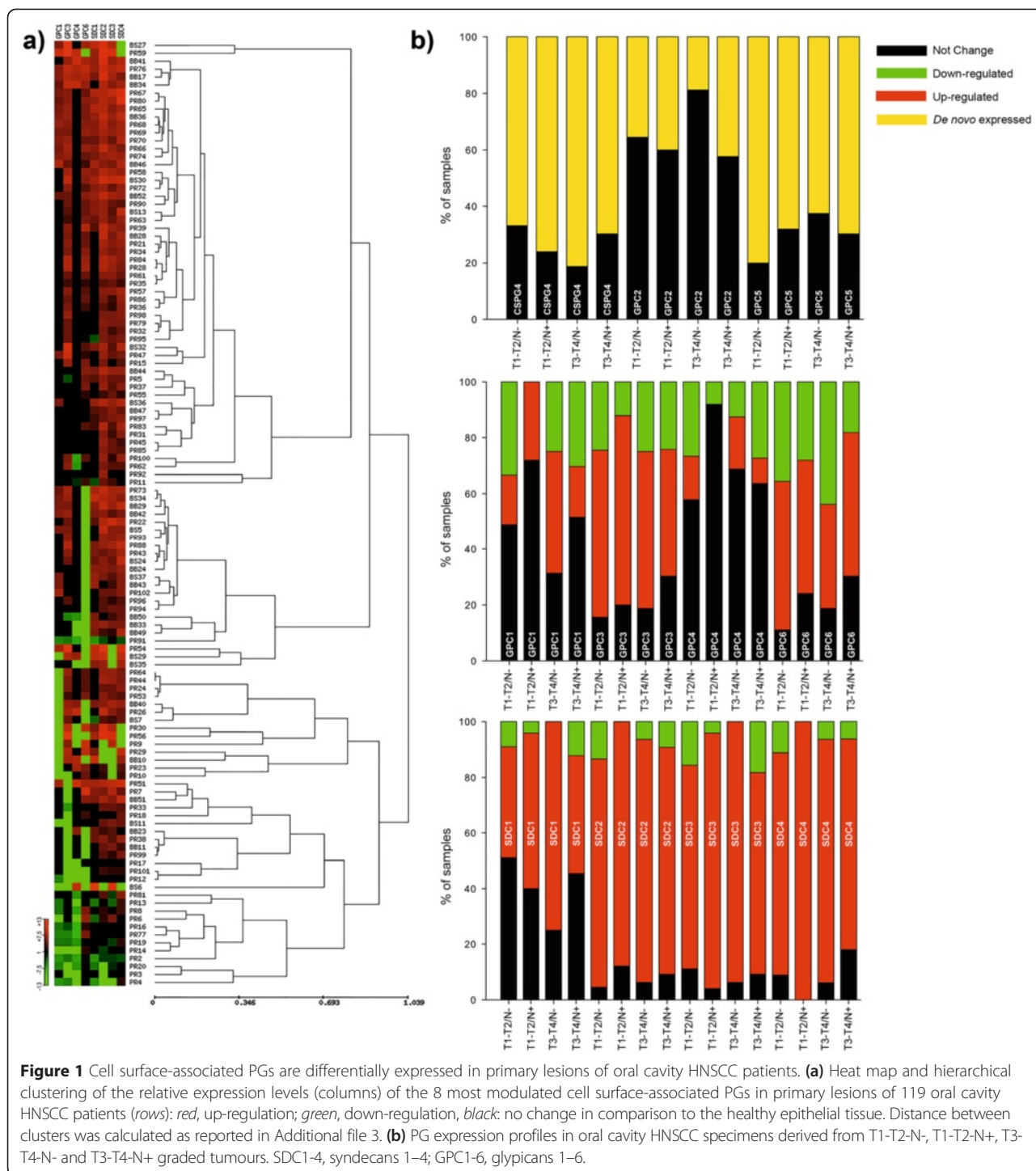
HNSCC lesions

Analyses of the relative mRNA expression levels of the eleven prevalent cell surface-associated PGs conducted on a total of 119 primary oral cavity HNSCC lesions revealed that 3 of the PGs, including NG2/CSPG4, GPC2 and GPC5, were *de novo* expressed in neoplastic cells, i.e. were not detectable in the healthy control tissues, but were detectable in cancer cells. These were transcribed in 71% (NG2/CSPG4), 36% (GPC2) and 72% (GPC5) of the lesions, respectively. The remaining 8 PGs, for which transcripts were expressed at a frequency of 84% (GPC1), 86% (GPC3), 88% (GPC4), 70% (GPC6), 100% (SDC1), 94% (SDC2), 93% (SDC3) and 95% (SDC4) of the tumour cases, respectively, were found to be differently modulated. Thus, SDC2, SDC3 and SDC4 were up-regulated in 79-84% of the patients, whereas GPC4 was enhanced in 11% and GPC3 in 57% of the specimens. However, GPCs were more frequently down-regulated (GPC3, 22%; GPC4, 21%; GPC1, 24%; and GPC6 30%) than SDCs (SDC1, 8%; SDC2, 8%; SDC3, 11%; and SDC4, 7%; Figure 1a; Additional file 4: Table S2).

We next compared the PG expression patterns exhibited by discrete groups of patients differentiated by tumour staging, i.e. T1-T2/N- versus T1-T2/N+ and T3-T4/N- versus T3-T4/N+. GPC2 was expressed in a mere 19% of the T3-T4/N- classified lesions, whereas it was a two-fold more frequently transcribed in patients belonging to the other three classes (T1-T2/N-, 36%, T1-T2/N+, 40% and T3-T4/N+, 42%). GPC5 and NG2/CSPG4 were detectable in samples of a large proportion of patients, ranging from 62% in T3-T4/N- to 80% in T1-T2/N-, but their relative expression levels did not discriminate between the above patient subsets (Figure 1b). GPC1 was similarly differently expressed in the distinct groups of patients with a 1.5-fold higher frequency in the T3-T4/N- patients compared to the other patient subsets. GPC4 was down-regulated in about 27% of the T1-T2/N- and T3-T4/N+ subgroups, and <13% in the T1-T2/N+ and T3-T4/N- patient subgroups (Figure 1b). Syndecans were generally up-regulated in most of the lesions (Figure 1b), with SDC4 showing enhanced expression in 100% of T1-T2/N+ patients.

Immunolocalization of PGs in oral cavity HNSCC lesions

Intralesional distribution of PGs was further examined in oral cavity HNSCC lesions and control healthy tissue using empirically validated, pre-selected antibodies against



each of the PGs (Figure 2; Figure 3; Additional file 5: Figure S2). The percentage of cases in which GPCs could be disclosed on the epithelial neoplastic cells varied from 18% (30 out of 163 cases) for GPC3 to 72% for GPC1 (118 out of 163 cases; Table 2). Relative frequency of expression was in the order: GPC1 > GPC4 (41%; 67 out of 163 cases) > GPC6 (37%; 61 out of 163

cases) > GPC3. GPCs were often detected within the cytoplasm as well as on the cell membrane, consistent with their thoroughly described internalization and recycling patterns. The hybrid cell membrane/cytoplasmic distribution of these PGs was characteristically observed for GPC1, GPC3 and GPC4, with GPC1 being most strongly associated with these two cellular compartments

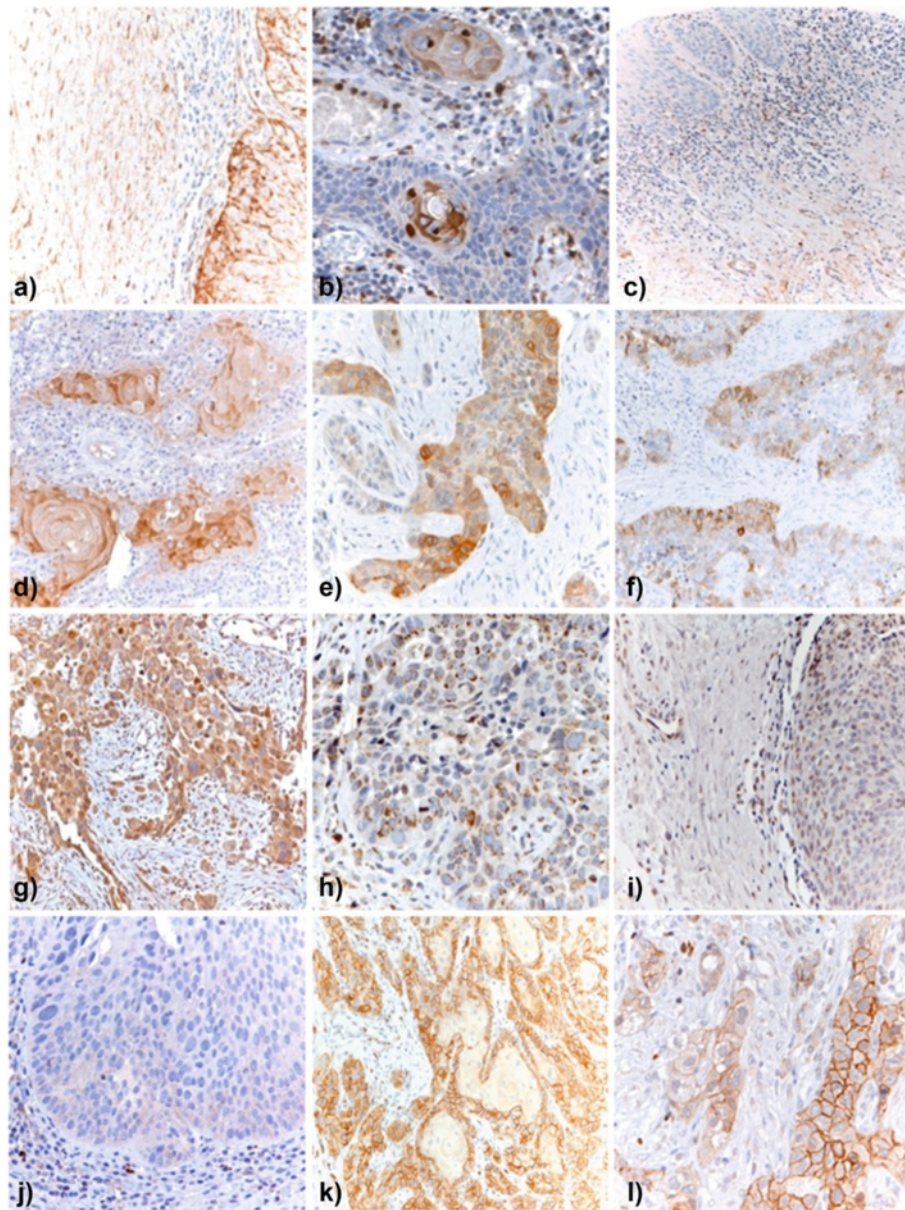


Figure 2 In situ immunolocalization of GPCs and NG2/CSPG4 in oral cavity HNSCC primary lesions. Representative patterns of GPC and NG2/CSPG4 distribution in oral cavity HNSCC lesions. **(a, b)** representative views of GPC1 expression in lesions with different degrees of keratinizing neoplastic cells. **(c, d)** representative images of GPC1 expression in stromal cells of pre-malignant lesions **(c)** and lack of expression in the stromal cells of HNSCC tissue **(d)**. GPC3 was detected in neoplastic cells **(e)**, but not stromal fibroblasts **(f)**, whereas GPC4 **(g)** and GPC6 **(h)** were primarily found to be associated with the neoplastic cells. **(i)** Shows the lack of expression of GPC6 in the intralesional stroma. NG2/CSPG4 was found to be abundantly expressed in both well- **(k)** and moderately-differentiated **(l)** oral cavity HNSCC lesions, whereas it was similarly absent from potentially pre-malignant lesions **(j)**.

in keratinizing neoplastic cells (Additional file 5: Figure S2). GPC3 was entirely absent in healthy tissue, while GPC4 showed a widespread distribution both on normal epithelial cells and in the intralesional stromal compartment (Additional file 5: Figure S2). GPC6 appeared to be preferentially retained within intracellular vesicles (Figure 2), as deduced by the appearance of GPC6-positive granules throughout the cytoplasm of neoplastic cells. This seemed

rather specific for tumour cells since it was not observed in healthy epithelial cells (Additional file 5: Figure S2). GPC1 and GPC6 were rarely seen in the intralesional stroma of oral cavity HNSCC lesions (13 out of 163 and 16 out of 163 of the cases, respectively), whereas GPC3 was consistently absent from this compartment and GPC4 showed a somewhat more frequent expression in stromal cells (19%; 31 out of 163 of the cases; Table 2; Figure 2).

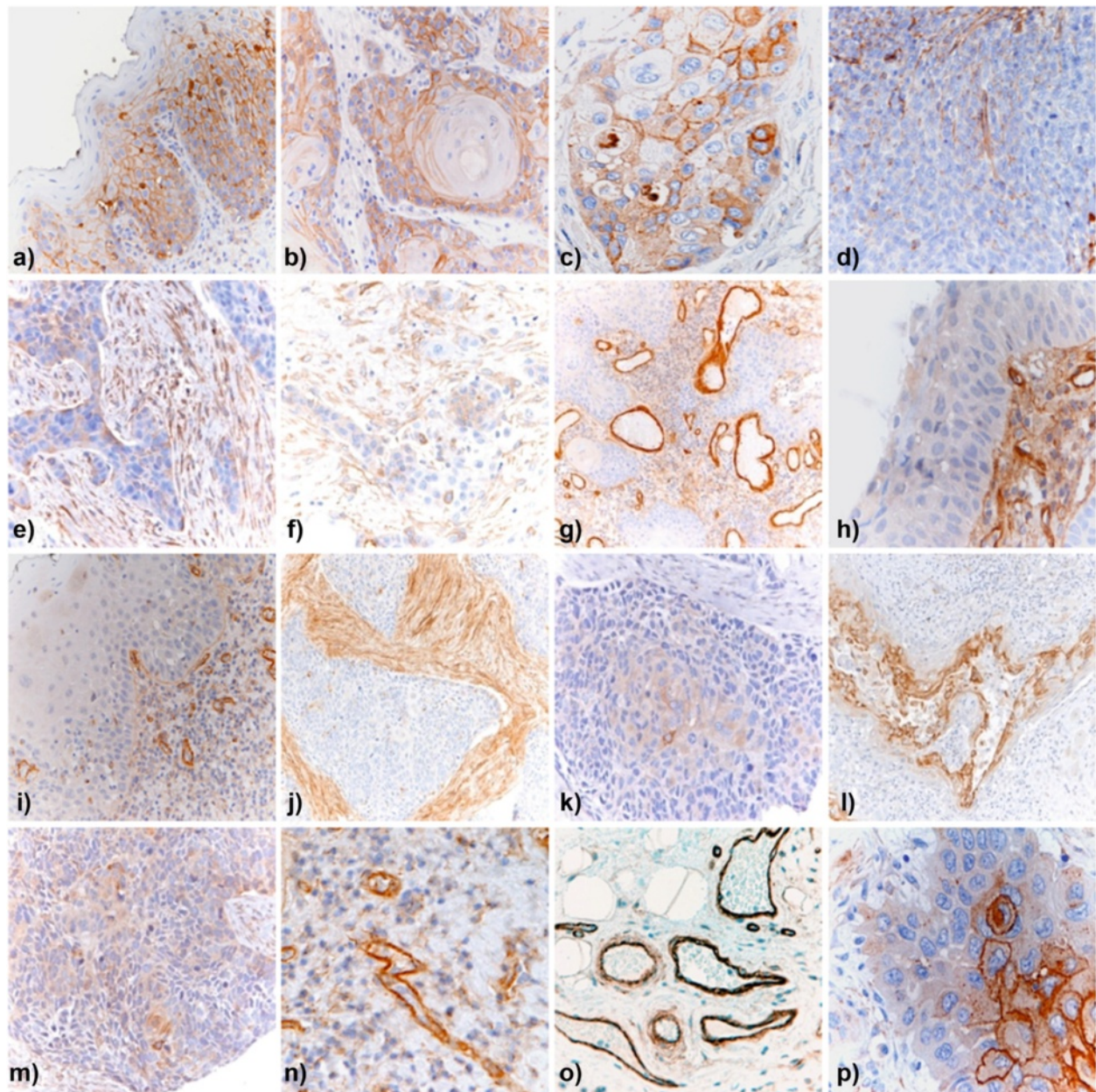


Figure 3 Immunodetection of SDCs in oral cavity HNSCC primary lesions. Representative view of the SDC1 expression pattern, inversely correlating with the overall differentiation status of the tumour (**a**, dysplastic tissue; **b, c**, well-differentiated; **d**, poorly differentiated), while being particularly abundant in the center of neoplastic nests (**p**) and in the stromal compartment (**e-f**). SDC2 was seen strongly associated with tumour vessels (**g, n, o**) and was the only PG to be widely expressed in the different degree of dysplastic tissue (**h-j**). SDC3 (**k**) and SDC4 (**m**) immunolocalized in the epithelial tumour cells, but not in the stromal compartment (**l**, SDC3; **m**, SDC4).

HNSCC lesions showed variable expression of SDCs with a relative frequency of positive cases decreasing in the order: SDC1 > SDC3 > SDC4 > SDC2 (Table 2). In fact, a total of 149 cases out of 163 lesions that were evaluated for the *in situ* expression of the SDC1/CD138 protein had epithelial neoplastic cells presenting the PG on the cell surface, or in intracellular locations (Figure 3). In contrast, a mere 57 (35%), 32 (19.6%), and 19 (12%)

out of 163 examined lesions had epithelial neoplastic cells staining positively for respectively SDC2, SDC3 and SDC4 (Table 2). The relative number of cancer cells that expressed these PGs in each lesion markedly differed and a similar divergence was seen in terms of subcellular localization of the molecules. Thus, neoplastic cells with plasma membrane-associated SDC1 were mainly keratinizing cells located at the center of the neoplastic nests

Table 2 Patterns of the in situ distribution of PGs in HNSCC lesions (% of cases)

PG	Tumor cell positivity ¹		Overall staining intensity ²		Subcellular localization ³		Stromal expression	
GPC1	-	(27.5)	-	(27.6)	Membrane	-	-	(92.0)
	+	(29.2)	+	(33.1)	Cytoplasmic	-	+	(6.7)
	++	(33.1)	++	(30.1)	Both	(72.4)	++	(0.6)
	+++	(7.4)	+++	(6.7)			+++	(0.6)
	++++	(2.5)	++++	(2.5)			++++	-
GPC3	-	(81.6)	-	(81.6)	Membrane	-	-	(100.0)
	+	(14.1)	+	(14.1)	Cytoplasmic	-	+	-
	++	(3.1)	++	(4.3)	Both	(18.4)	++	-
	+++	(1.2)	+++	-			+++	-
	++++	-	++++	-			++++	-
GPC4	-	(58.9)	-	(59.5)	Membrane	-	-	(81.0)
	+	(20.9)	+	(14.1)	Cytoplasmic	(40.5)	+	(5.5)
	++	(19.0)	++	(25.8)	Both	-	++	(12.9)
	+++	(1.2)	+++	(0.6)			+++	(0.6)
	++++	-	++++	-			++++	-
GPC6	-	(62.2)	-	(62.6)	Membrane	-	-	(90.2)
	+	(30.1)	+	(31.9)	Cytoplasmic	(37.4)	+	(9.8)
	++	(7.4)	++	(5.5)	Both	-	++	-
	+++	-	+++	-			+++	-
	++++	-	++++	-			++++	-
SDC1	-	(8.6)	-	(8.6)	Membrane	(42.9)	-	(79.1)
	+	(31.3)	+	(25.8)	Cytoplasmic	(37.4)	+	(9.2)
	++	(33.7)	++	(50.3)	Both	(10.4)	++	(10.4)
	+++	(22.1)	+++	(10.4)			+++	(1.2)
	++++	(4.3)	++++	(4.9)			++++	-
SDC2	-	(88.3)	-	(88.3)	Membrane	(1.8)	-	(26.4)
	+	(6.7)	+	(8.0)	Cytoplasmic	(8.6)	+	(20.2)
	++	(4.3)	++	(3.1)	Both	-	++	(25.8)
	+++	(0.6)	+++	(0.6)			+++	(21.5)
	++++	-	++++	-			++++	(6.1)
SDC3	-	(65.0)	-	(65.0)	Membrane	-	-	(100.0)
	+	(27.0)	+	(28.2)	Cytoplasmic	-	+	-
	++	(6.1)	++	(6.7)	Both	(34.4)	++	-
	+++	(1.8)	+++	-			+++	-
	++++	-	++++	-			++++	-
SDC4	-	(80.4)	-	(80.4)	Membrane	-	-	(100.0)
	+	(16.0)	+	(19.6)	Cytoplasmic	-	+	-
	++	(3.7)	++	-	Both	(17.8)	++	-
	+++	-	+++	-			+++	-
	++++	-	++++	-			++++	-
NG2/CSPG4	-	(34.2)	-	(34.2)	Membrane	(62.6)	-	(96.7)
	+	(38.8)	+	(36.8)	Cytoplasmic	-	+	(3.3)
	++	(21.7)	++	(19.7)	Both	-	++	-
	+++	(2.6)	+++	(7.2)			+++	-

Table 2 Patterns of the in situ distribution of PGs in HNSCC lesions (% of cases) (Continued)

++++	(2.6)	++++	(2.0)	++++	-
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¹PG expression was assessed semi-quantitatively according to the arbitrary scoring: “-”, no positively staining cells were detected; “+”, <10% of cells were positive; “++”, ≥10% and <50% of positive cells; “+++”, ≥50% and <90% of positive cells; “++++”, ≥90% of positive cells;

²Refers to the average staining intensity within the examined lesion, according to the arbitrary scoring: “-” = absent; “+”, faint; “++”, weak; “+++”, moderate; “++++”, strong;

³Immunostaining was prevalently cell membrane-associated (“Membrane”) or diffuse cytoplasmatic (“Cytoplasmic”).

(Figure 3). Fibroblasts of the tumour stroma that surrounded the neoplastic nests were positive for SDC1 in 34 out of 163 (21%) of the tumours. In 14 out of 163 of the lesions (8.6%), SDC2 was immunolocalized within the cytoplasm of neoplastic cells, while it appeared widespread in the stromal cells of the majority of the lesions (73.6%, 120 out of 163 of the cases; Figure 3) and was particularly enriched in lesions containing desmoplastic stroma. Intriguing was the fact that in 100% of the lesions, SDC2 could be observed in the wall of both normal and intra-lesional blood vessels, suggesting that it was associated with both endothelial and neovascular pericytes (Figure 3). In contrast to SDC1 and SDC2, both SDC3 and SDC4 were undetectable in the healthy epithelium, or the tumour stroma, but could be immunolocalized both subcellularly and on defined portions of the cell surface of neoplastic cells, with a particular concentration in focal plaque-like structures (Figure 3; Additional file 5: Figure S2). Finally, the diversity of SDCs expressions in oral cavity HNSCC lesions was even more remarkable when considering the relative distribution of these PGs in the stromal compartment. In this case, the frequency of occurrence of the PGs was largely reversed with respect to that seen in the cancer cells and decreased in the order: SDC2 > SDC1 > SDC3 = SDC4 (Table 2). Deviating from the pronounced intracellular distribution of SDC3 and SDC4 was that of NG2/CSPG4 which showed an exclusive cell membrane localization in all samples in which the PG could be disclosed (63%). NG2/CSPG4 was rarely detected in the stromal compartment (5 out of 163; Table 2; Figure 2), where, if occurring, was concentrated on the membrane of basal cells (Additional file 5: Figure S2).

Altered expression of discrete PGs correlates with disease outcome

All demographic and clinical-pathological traits of the patients were initially compared by univariate analysis of the cumulative PG expression data, except for patient categories comprised of less than 13 patients (independently defined as a cut-off level of “statistical” exclusion). In these correlation analyses we considered five primary disease outcomes, including loco-regional tumour recurrence, lymphnodal metastasis, distant metastases, disease-related death and a situation in which at least one of the former disease outcomes was manifested (Table 3).

In order to test whether there is a relationship between PGs transcript and protein expressions and clinicopathological parameters, a Chi-Square test was applied and just three such correlation resulted statistically significant: N classification and SDC2 stromal positivity, $p = 0.002$; alcohol consumption and SDC1 mRNA up-regulation, $p = 0.021$, and presence of precancerous lesion and SDC1 mRNA up-regulation, $p = 0.016$.

Although radiation therapy and excessive alcohol consumption independently correlated with one or more of the above clinical outcomes (Table 3), these parameters were not considered in the multivariate logistic regression analyses because of being potentially confounding indicators. The first because almost all patients presenting lymphnodal infiltrations had been routinely subjected to radiation therapy, the second because, despite of its well-recognized importance as a risk factor in HNSCC, the admission of this habit was measured by a self-provided questionnaire and no details were available on the accuracy of the information provided by the patients. A further consideration is that self-reported excessive alcohol intake is often denied, causing underestimation of the cohort of patient that may fall under this “risk category”.

Advanced T classification ($p = 0.007$), T3-T4 grouping ($p = 0.001$; Figure 4), positive NG2/CSPG4 transcript expression ($p = 0.029$; Figure 4), or GPC1 positivity in stromal cells ($p = 0.007$) were all conditions strongly associated with a high loco-regional tumour relapse rate (Table 3). Stromal GPC6 expression could, however, not be included as a parameter in the multivariate logistic regression model due to the low number of cases contained within this category and the borderline statistical significance in univariate analyses ($p = 0.058$). Application of the Cox proportional hazard model revealed that T3-T4 classification of the tumour (HR, 6.36, $p = 0.001$) and *de novo* expression of NG2/CSPG4 mRNA (HR, 6.76, $p = 0.017$) were independent, robust prognostic factors for local tumour recurrence (Table 4; Figure 5). If combining T-grouping and mRNA expression of NG2/CSPG4, the probability to develop a secondary loco-regional lesion was further increased ($p < 0.001$; Table 3; Figure 4).

Significant correlations with post-surgical lymphnodal metastases were further disclosed between gender ($p = 0.043$), the presence of precancerous lesions ($p = 0.003$; Figure 4), the occurrence of lymphnodal infiltration at time of surgery ($p = 0.001$), stromal expression of SDC2

Table 3 Univariate analysis of PG expression in relation to known prognostic indicators

Prognostic indicator/PG	N. of cases	Clinical outcomes										
		Loco-regional recurrence		Lymphnodal metastasis		Distant metastasis		Disease-related death		Any of the clinical outcomes ¹		
		% cases	p value	% cases	p value	% cases	p value	% cases	p value	% cases	p value	
<i>Prognostic indicator</i>												
Sex	Male	99	14.1	0.342	15.2	0.043	10.1	0.847	22.2	0.155	36.4	0.094
	Female	74	7.0		5.4		9.5		13.5		24.3	
Age	≤45 yrs	17	11.8	0.689	11.8	0.964	0.0	-	5.9	0.146	17.6	0.184
	>45 yrs	156	12.2		10.9		10.9		19.9		32.7	
Smoking	No	66	13.6	0.689	9.1	0.466	9.1	0.718	12.1	0.109	25.8	0.253
	Yes	105	11.4		12.4		10.5		21.9		34.3	
Alcohol ²	No	83	10.8	0.521	8.4	0.231	4.8	0.026	6.0	<0.001	21.7	0.012
	Yes	88	13.6		13.6		14.8		29.5		39.8	
Familial cancer history	No	144	12.5	0.732	10.4	0.615	9.7	0.880	18.8	0.876	31.3	0.994
	Yes	27	11.1		14.8		11.1		18.5		33.3	
Precancerous lesions	No	121	12.4	0.869	5.8	0.003	9.9	0.970	18.2	0.869	28.1	0.305
	Yes	51	11.8		21.6		9.8		19.6		37.3	
Tumor site	OC	156	12.2	0.530	11.5	0.204	10.3	0.776	17.9	0.858	31.4	0.399
	OP	14	7.1		0.0		7.1		21.4		21.4	
	OC+OP	3	33.3		33.3		0.0		33.3		66.7	
T classification	T1	51	3.9	0.007	7.8	0.326	2.0	0.073	5.9	0.038	17.6	0.018
	T2	60	8.3		8.3		16.7		21.7		30.0	
	T3	17	23.5		17.6		5.9		23.5		47.1	
	T4	45	22.2		15.6		11.1		26.7		42.2	
	T1-T2	111	6.3	0.001	8.1	0.065	9.9	0.957	14.4	0.043	24.3	0.005
	T3-T4	62	22.6		16.1		9.7		25.8		43.5	
N classification	Negative	97	9.3	0.079	5.2	0.001	5.2	0.016	9.3	<0.001	19.6	<0.001
	Positive	76	15.8		18.4		15.8		30.3		46.1	
Differentiation Degree	Well	20	15.0	0.441	0.0	0.223	5.0	0.392	15.0	0.952	20.0	0.44
	Moderate	50	16.0		12.0		14.0		18.0		36.0	
	Poor	78	7.7		12.8		7.7		16.7		30.8	
Radiotherapy	No	83	8.4	0.082	4.8	0.005	3.6	0.007	8.4	<0.001	20.5	0.002
	Yes	90	15.6		16.7		15.6		27.8		41.1	
<i>PG (mRNA)³</i>												
SDC1	↓ / =	61	11.5	0.553	6.6	0.396	4.9	0.016	11.5	0.036	21.3	0.013
	↑	58	13.8		10.3		19.0		25.9		41.4	
SDC2	↓ / =	19	5.3	0.253	5.3	0.519	5.3	0.329	10.5	0.314	15.8	0.104
	↑	100	14.0		9.0		13.0		20.0		34.0	
SDC3	↓ / =	24	8.3	0.405	8.3	0.896	4.2	0.193	20.8	0.855	25.0	0.352
	↑	95	13.7		8.4		13.7		17.9		32.6	
SDC4	↓ / =	19	5.3	0.253	5.3	0.519	5.3	0.329	15.8	0.780	26.3	0.601
	↑	100	14.0		9.0		13.0		19.0		32.0	
GPC1	=	62	12.9	0.803	6.5	0.502	12.9	0.668	17.7	0.940	32.3	0.625
	↓ / ↑	57	12.3		10.5		10.5		19.3		29.8	
GPC2	=	76	10.5	0.393	6.6	0.363	10.5	0.596	21.1	0.335	27.6	0.307

Table 3 Univariate analysis of PG expression in relation to known prognostic indicators (Continued)

	T3-T4/=	13	7.7									
	T3-T4/ <i>De novo</i>	36	30.6									
Precancerous lesions/ SDC2 stroma	-/-	29		0	0.001							
	-/+	84		8.3								
	+/-	14		7.1								
	+/+	35		28.6								
	N status ⁶ /SDC1 mRNA	Negative/ ↓/=	31		0	0.004						
	Negative/↑	30		10.0								
	Positive/↓/=	30		10.0								
	Positive/↑	28		28.6								
N status/SDC2 stroma	-/-	33		0	<0.001							
	-/+	57		14.0								
	+/-	10		10.0								
	+/+	63		33.3								
N status/SDC1 mRNA	Negative/ ↓/=	31									<0.001	3.2
	Negative/↑	57										33.3
	Positive/↓/=	10										40.0
	Positive/↑	63										50.0
PGs pattern												
SDC1 mRNA ↑ NG2/CSPG4 mRNA <i>de novo</i> + SDC2 stroma	Yes	36	16.7	0.271	11.1	0.405	25	<0.001	33.3	0.002	50	<0.001
	No	73	12.3		8.2		4.1		11		23.3	

¹This refers to the situation in which patients manifested at least one of the four adopted clinical outcomes within the follow-up period;

²Excessive alcohol consumption was based upon self-provided information;

³PG transcript expression was defined as "↓", down-regulated; "↑", up-regulated; "=", not changed; and "*De novo*", *de novo* expressed, when compared to a healthy mucosal tissues pool that was used as sample calibrator;

⁴Protein expression data are reported as detectable or non-detectable by indirect immunohistochemistry;

⁵Univariate analyses combining the prognostic indicators that were deemed to be independent poor predictors of each of the five clinical outcomes as accomplished through the Cox proportional hazard model;

⁶N status positive or negative is according to N classification AJCC staging system;

p values <0,05 were considered to be significant (in bold); *p* values within ≥ 0,05 and <0,06 were considered borderline and were included in the following multivariate regression model; *p* value was not calculated where a monotone likelihood was established.

Abbreviations: OC, oral cavity; OP, oropharynx.

(*p* = 0.015; Figure 4) and altered GPC6 protein expression in tumour cells (*p* = 0.01; Table 3). As predictable, "sentinel lymphnodes" at diagnosis significantly influenced the later appearance of more prominent lymphnodal lesions, but this factor was not considered in the multivariate logistic regression model because of its unuttered prognostic implication. Multivariate analysis corroborated that the occurrence of precancerous lesions (HR, 3.773, *p* = 0.005), and more incisively the presence of SDC2 in the stromal compartment (HR, 7.652, *p* = 0.007), but not GPC6 expression or gender of the patient, were independent prognostic markers for post-surgery secondary infiltrations of lymphnodes (Table 4; Figure 5). If we then accounted for both a history of precancerous lesions and SDC2 stromal expression, the probability to develop post-

surgical lymphnodal infiltration was significantly increased (*p* = 0.001; Table 3; Figure 4).

Contrary to the above associations, univariate logistic analyses revealed that the N classification, at time of surgical removal of the primary tumour mass, and up-regulated transcription of SDC1 (Figure 4) were significantly associated with the formation of distant metastases (*p* = 0.016 for both correlations; Table 3). The frequency of GPC6 expression in neoplastic cells did not satisfy the limit of significance (*p* = 0.052), but could be a potentially interesting indicator to take into account in future investigations on larger cohorts of patients. Conversely, multivariate analyses reinforced the impact of N classification (HR, 4.38, *p* = 0.012) and down-regulated or unaltered SDC1 expression (HR, 0.232, *p* = 0.013) as independent factors predicting the

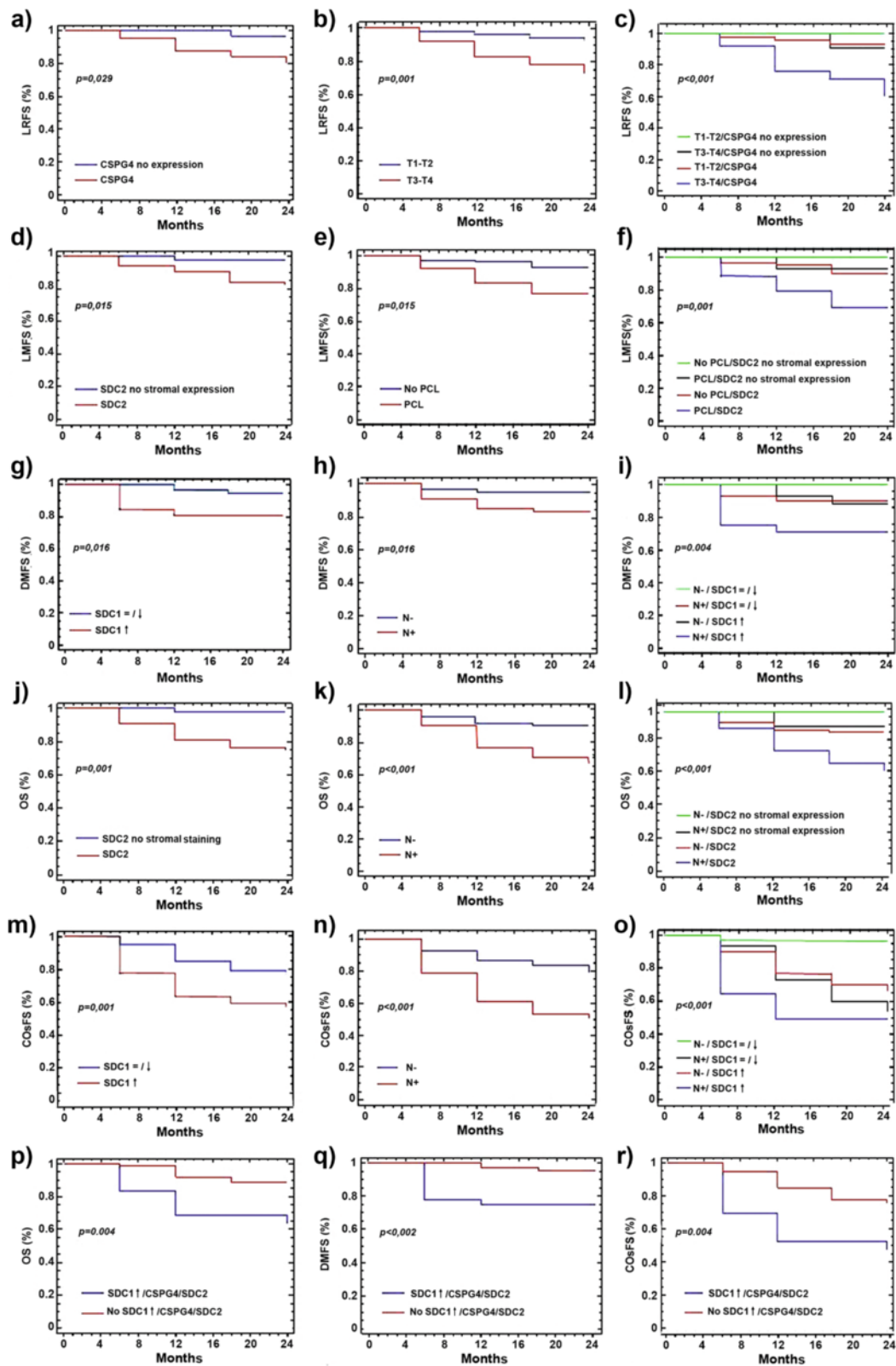


Figure 4 (See legend on next page.)

(See figure on previous page.)

Figure 4 Differential PG expression correlates with clinical outcome. Survival and probability curves for the following correlations: **(a)** loco-regional relapse vs *de novo* expression of NG2/CSPG4, **(b)** loco-regional relapse vs T group classification; **(c)** loco-regional relapse vs coincident NG2/CSPG4 expression and advanced T classification; **(d)** lymphnodal metastases vs enhanced SDC2 expression in stromal cells; **(e)** lymphnodal metastases vs manifestation of precancerous lesions; **(f)** lymphnodal metastases vs the combination of both previous prognostic indicators; **(g)** distant metastases vs up-regulated SDC1 expression; **(h)** distant metastases vs infiltration of cervical lymph nodes; **(i)** distant metastases vs the combination of both previous prognostic indicators; **(j)** overall survival vs enhanced SDC2 expression in the stromal compartment; **(k)** overall survival vs N classification; **(l)** overall survival vs the coincidence of both previously indicated events; **(m)** the occurrence of any of the clinical outcomes vs up-regulated SDC1 expression; **(n)** the occurrence of any of the clinical outcomes vs N classification; **(o)** the occurrence of any of the clinical outcomes vs the combination of both above listed events; **(p)** overall survival, **(q)** distant metastases and **(r)** the occurrence of any of the clinical outcomes vs the combination of SDC1 up-regulation, *de novo* expression of NG2/CSPG4 and stromal enhancement of SDC2 positivity. *Abbreviations:* LRFS, loco-regional relapse-free survival; LMFS, lymphnodal metastasis-free survival; DMFS, distant metastasis-free survival; OS, overall survival, COsFS, clinical outcomes-free survival.

formation of distant metastases, albeit with opposite trends, (Table 4; Figure 5). When we next considered the combination of the unfavourable conditions represented by lymphnodal infiltration and up-regulation of SDC1 transcription, we unfolded a significantly increased probability to develop distant metastases ($p = 0.004$; Table 3; Figure 4). Noteworthy was also the fact that 91% of patients with up-regulated SDC1 transcription that developed distant metastases within the follow-up period invariably succumbed the disease.

Advanced T classification ($p = 0.038$), T3-T4 grouping ($p = 0.043$), positive N classification ($p < 0.001$; Figure 4), up-regulation of SDC1 transcription ($p = 0.036$) and stromal expression of SDC2 or GPC1 ($p = 0.001$ and $p = 0.012$ respectively; Table 3, Figure 4) were also found to be strongly associated with disease-related death. Cervical lymphnodal involvement (HR, 2.971, $p = 0.005$) and, more markedly, synthesis of SDC2 in the stromal cells (HR, 8.671, $p = 0.003$), established two independent predictors of survival (Table 4; Figure 5). The combination of these two conditions further decreased the survival probability of the patients ($p < 0.001$, Table 3; Figure 4).

In the evaluation of situations in which patients presented at any of the mentioned clinical outcomes, we similarly found a tight correlation between advanced T classification ($p = 0.018$), T3-T4 grouping ($p = 0.005$), positive N classification ($p < 0.001$; Figure 4), up-regulation of SDC1 mRNA ($p = 0.013$; Figure 4) and SDC2 stromal reactivity ($p = 0.002$). Finally, in multivariate analyses, N involvement (HR, 3.203, $p < 0.001$) and down-regulation or unaltered SDC1 expression (HR, 0.429, $p = 0.012$), but not T classification or SDC2 detection in stromal cells, were independent factors with opposite trends for the prediction of poor prognosis (Table 4; Figure 5). Even in this case the combination of positive N classification and up-regulation of SDC1 mRNA expression significantly increased the probability of the patients to incur into a dismal disease course ($p < 0.001$; Table 3; Figure 4).

We finally evaluated the disease course in patients scoring positively for the 3 dismal prognostic indicators, i.e. *de novo* expression of NG2/CSPG4, stromal

abundance of SDC2 and up-regulation of SDC1 mRNA, which, in an independent manner, associated with one or more of the adverse clinical outcomes. This conditions was found in 36 of the 173 patients (21%) and within this patient subgroup 17% and 25%, respectively, developed loco-regional secondary lesions or distant metastases. Lymphnodal metastasis was observed in 11% of the patients, whereas 33% of them succumbed to the disease. Survival analyses revealed a strong association between the PG pattern analyzed and the presence of distant metastases ($p < 0.002$), disease-related deaths ($p = 0.004$) and a cumulative bad prognosis ($p < 0.004$). The Cox regression model ascertained that patients not expressing simultaneously the three bad independent disease course markers resulted to have a best prognosis in terms of putative development of distant metastases (HR, 0.146, $p = 0.002$), for survival (HR, 0.272, $p = 0.004$) and for incurring into any of the unfavourable clinical events under consideration (HR, 0.363, $p = 0.003$) (Table 4; Figure 5).

Discussion

Despite the relatively high incidence of oral cavity HNSCC, very few reliable prognostic and/or predictive molecular markers are currently available for the routine clinical management of the patients. In light of this deficiency, we have explored the possibility that variation in the expression of cell surface PGs, widely recognized to be key factors in the control of tumour progression [33,56-59], could afford more effective means of prognosticating patients affected by these tumours. Indeed, we find that, upon neoplastic transformation, epithelial cells of the oral cavity and oropharynx modify their transcriptional/translational rates of virtually *all* currently known cell surface PGs. This led us to conclude that transformation-dependent modulation of PG synthesis may be part of the globally altered pattern of gene expression in these cells, as well as contribute to the cancer cell's acquisition of a defined repertoire a cell surface molecules capable of dictating their malignant behavior.

Table 4 Multivariate analyses of the prognostic implication of altered PG expression for the different clinical outcomes¹Estimated regression coefficient and confidence interval;

Prognostic indicator/PG		Estimate ¹ (95% CI)	SE ²	p value ³	HR ⁴	Clinical outcome
T classification						
	T2 ⁵	0.359 (0.153/0.565)	0.105	0,012	1.432	Loco-regional relapse
	T3	1.857 (1.556/2.158)	0.153		6.404	
	T4	2.160 (1.956/2.364)	0.104		8.671	
	T3-T4 vs T1-T2	1.850 (1.691/2.009)	0.081	0.001	6.360	Loco-regional relapse
NG2/CSPG4 mRNA ⁶						
	<i>De novo</i> expression vs no expression	1.911 (1.735/2.087)	0.090	0.017	6.760	Loco-regional relapse
Precancerous lesions						
	Presence vs Absence	1.328 (1.184/1.471)	0.073	0.005	3.773	Lymphnodal metastases
SDC2 stroma						
	Positive vs Negative	2.035 (1.885/2.184)	0.076	0.007	7.652	Lymphnodal metastases
SDC2 stroma						
	Positive vs Negative	2.160 (2.022/2.298)	0.070	0.003	8.671	Disease-related deaths
N classification						
	Positive vs Negative	1.477 (1.326/1.628)	0.077	0.012	4.380	Distant metastasis
N classification						
	Positive vs Negative	1.089 (0.967/1.211)	0.062	0.005	2.971	Disease-related deaths
N classification						
	Positive vs Negative	1.164 (1.003/1.325)	0.082	<0.001	3.203	Any of the clinical outcomes ⁷
SDC1 mRNA						
	↓ / = vs ↑	-1.460 (-1.612/-1.309)	0.077	0.013	0.232	Distant metastasis
SDC1 mRNA						
	↓ / = vs ↑	-0.845 (-1.007/-0.684)	0.082	0.012	0.429	Any of the clinical outcomes
SDC1 mRNA up-regulation + NG2/CSPG4 mRNA <i>de novo</i> expression + SDC2 stroma						
	Single condition vs combination of all 3 conditions ⁸	-1.924 (-2.088/-1.760)	0.084	0.002	0.146	Distant metastasis
		-1.302 (-1.459/-1.146)	0.080	0.004	0.272	Disease-related deaths
		-1.014 (-1.194/-0.833)	0.092	0.003	0.363	Any of the clinical outcomes

¹Estimated regression coefficient and confidence interval;²Standard error of estimated regression coefficient;³p value <0,05 were considered to be significant;⁴Hazard Ratio estimated from Cox proportional hazard regression model;⁵Compared to T1 stage;⁶PG transcript expression (↓, down-regulated; ↑, up-regulated; =, not changed; *De novo* expression, *de novo* expressed in comparison to a healthy mucosal tissues pool that was used as sample calibrator) could be grouped according to the trend of each PG gene in relation to the clinical outcomes;⁷Is referred to a patient that had at least one of the other outcomes within the follow-up;⁸Refers to the comparison between a situation in which *all three* indicated conditions were manifested ("*combination of all 3 conditions*") versus either condition alone or the combination of *any two* conditions;

Abbreviations: CI, Confidence Interval; HR, Hazard Ratio; SE, standard error.

SDCs are widely recognized to undergo malignancy-associated changes in their expressions in several types of carcinomas, including those of thyroid, breast, colon, skin, stomach and urogenital tract, and SDC1 is recognized to be the best documented prognostic biomarker [39,41,43,40,46,60-64]. Its expression pattern frequently correlates with the differentiation status of the cells and thereby with their malignancy degree [47,54,65-68]. This

characterizing trait of the SDC1 tumour-associated expression was corroborated here, along with its widespread distribution in neoplastic HNSCC lesions.

Although much less studied, SDC2 has also been reported to be associated with malignant carcinoma lesions in various anatomical sites/organs including head and neck [63,69,70]. In this study, transcription/translation of SDC2 was found to be more prominent in the

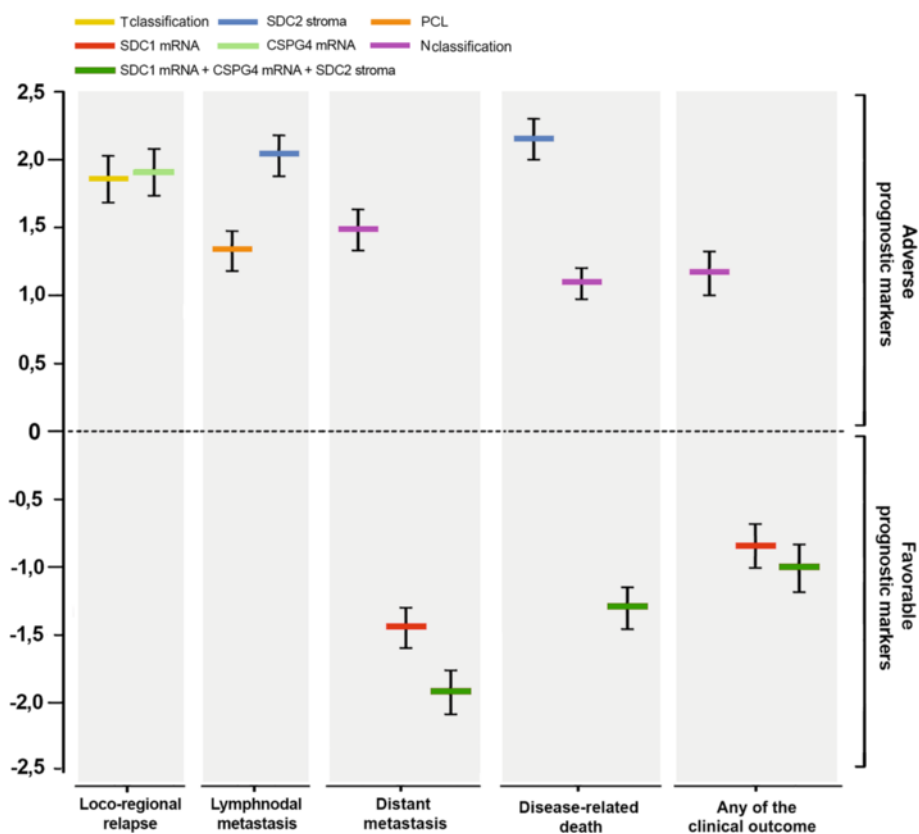


Figure 5 Cox proportional hazard analysis. Plot overview of Cox proportional hazard estimated regression coefficients of the resulting independent prognostic factors for loco-regional recurrence, lymphnodal metastases, distant metastases, disease-related death and the occurrence of any of the clinical outcomes.

intralesional stroma than in the neoplastic cells and, hence, the PG showed an expression pattern that was complementary to SDC1. Notably, SDC2 was also observed to be strongly enriched in neovascular structures, where it appeared to be associated with both endothelial cells and pericytes. Our present mapping study is the first to reveal a *de novo* expression of SDC3 and SDC4 in oral cavity HNSCC and the accumulation of SDC4 in areas of cell-cell contact [22,24,25] within such lesions. Intracellular abundance of SDCs in HNSCC cells may reflect the incapacity of the cells to complete the post-translational processing of these PGs and/or their transport and intercalation into the cell membrane, or an accentuated internalization and intracellular recycling process.

Several GPCs were also found to be misexpressed in oral cavity HNSCC lesions, albeit with frequencies that were generally lower than those seen for SDCs. GPC1, known to be highly expressed in pancreatic and breast carcinomas [32,33], was found to be the prevalent GPC of these lesions, alongside with GPC3, which has independently been reported to be up-regulated in several other tumour types and has a recognized value as prognostic

factor and putative therapeutic target in hepatocellular carcinomas [34,35,37]. In this context, it is, however, worth noting that GPC3 has also been proposed to act as a potential tumour suppressor in certain neoplasia, showing a putative transformation-dependent silencing of the glypican [30,71-73].

When we applied univariate and multivariate meta-analytical methods to correlate the observed PG expression patterns with clinically relevant disease outcomes we unveiled striking associations. Appearance of NG2/CSPG4, a PG with a precedent prognostic impact in numerous solid tumours [74-82], was found to tightly correlate with loco-regional tumour recurrence and, hence, was disclosed to be the first ever to be described molecular relapse predictor in oral cavity HNSCC. Enhanced expression of GPC1 in the stromal compartment of these lesions also closely correlated with tumour recurrence and paralleled the more predictable prognostic implication of tumour staging. Beside its prognostic role in pancreatic cancer, there is currently no other indication that altered expression of GPC1 may influence the course of any tumour type. Another crucial finding of this study was the close association between SDC2 up-regulation in

the intralesional stromal compartment and the overall survival of the patients carrying such SDC2-rich primary lesions. Even in this case, the present study provides the first evidence for such a prognostic relationship in any cancer type and, similarly to the potential of NG2/CSPG4, emphasizes that SDC2 may serve as a putative target for prevention and/or treatment of relapsing oral cavity HNSCC.

Conclusion

The present study provides the first evidence that altered expression of cell surface-bound PGs is strongly linked to the formation and progression of oral cavity HNSCC. Elective modulation of PG expression in primary oral cavity HNSCC lesions correlates, in a predictive manner, with several clinical outcomes and may therefore serve as an adjunct in the molecular diagnosis of these tumours. More specifically, enhanced expression of SDC2 in the tumour stroma significantly correlates with overall survival and is indicative of lymphonodal metastasis, whereas aberrant increased of SDC1 transcription is indicative of the presence of distant metastases. Strikingly, up-regulation of NG2/CSPG4 is tightly linked to loco-regional recurrence of the tumour, underscoring the potential of this biomarker to forcefully predict the clinical course of oral cavity HNSCC patients.

Additional files

Additional file 1: Table S1. Patient demographic and clinic-pathological features.

Additional file 2: Figure S1. Overview of the distribution of clinical outcomes manifested by oral cavity HNSCC patients during the entire follow-up period subdivided in 6-months time intervals.

Additional file 3. Supplemental and detailed Materials and methods.

Additional file 4: Table S2. Listing of the individual relative PG expression data.

Additional file 5: Figure S2. Representative immunostaining on healthy control tissue sections: GPC1 (a), GPC3 (b), GPC4 (c), GPC6 (d), NG2/CSPG4 (e), SDC1 (f), SDC2 (g), SDC3 (h) and SDC4 (i).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conception and design: AF, SR, NB and RP. Development of methodology: EMS, TP, ES, CM, RC, MPF and RP. Acquisition of data: AF, RS, NB, MG, DL, MTM, EL and AAS. Analysis and interpretation of data: AF, RS, NB, MG, LM, EL, MPF and RP. Writing, review and/or revision of the manuscript: AF, SR, NB and RP. Administrative, technical, or material support: all co-author. Study supervision: TP, ES, MPF and RP. All authors read and approved the final version of the manuscript.

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