IJC
International Journal of Cancer

S100A7 overexpression is a predictive marker for high risk of malignant transformation in oral dysplasia

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Early detection of oral lesions (OLs) at high risk of cancer development is of utmost importance for intervention. There is an urgent unmet clinical need for biomarkers that allow identification of high-risk OLs. Recently, we identified and verified a panel of five candidate protein biomarkers namely S100A7, prothymosin alpha, 14-3-3ζ, 14-3-3σ and heterogeneous nuclear ribonucleoprotein K using proteomics to distinguish OLs with dysplasia and oral cancers from normal oral tissues. The objective of our study was to evaluate the potential of these candidate protein biomarkers for identification of oral dysplastic lesions at high risk of cancer development. Using immunohistochemistry, we analyzed expressions of these five candidate protein biomarkers in 110 patients with biopsy-proven oral dysplasia and known clinical outcome and determined their correlations with p16 expression and HPV 16/18 status. Kaplan–Meier survival analysis showed reduced oral cancer-free survival (OCFS) of 68.6 months ($p = 0.007$) in patients showing cytoplasmic S100A7 overexpression when compared to patients with weak or no S100A7 immunostaining in cytoplasm (mean OCFS = 122.8 months). Multivariate Cox regression analysis revealed cytoplasmic S100A7 overexpression as the most significant candidate marker associated with cancer development in dysplastic lesions ($p = 0.041$, hazard ratio = 2.36). In conclusion, our study suggested the potential of S100A7 overexpression in identifying OLs with dysplasia at high risk of cancer development.

The development of oral squamous cell carcinoma (OSCC) is a multistep process, wherein frank malignancy is often preceded by oral lesions (OLs).[1] Histological assessment of a biopsy with evidence of dysplasia is considered as the gold standard for determining the risk of malignant transformation.[2–5] Increasing grade of dysplasia (mild/moderate/severe) has been associated with a high rate of malignant transformation; however, the progression rates vary from 6 to 36%. This

Key words: S100A7, hnRNPK, PTMA, 14-3-3ζ, 14-3-3σ, oral cancer, biomarker, immunohistochemistry, dysplasia, progression, transformation

Additional Supporting Information may be found in the online version of this article.

Conflicts of interest: Nothing to report

Author's contributions: RR and IL conceptualized the study. RR, KWMS, IL, IW, TJC, CM, JK and AM contributed to the study design and to the manuscript. JK, IK, GS, AM and JA conducted the experimental work. IK and JA performed the chart reviews for clinical data, follow-up and data collection and established the clinical database. IL, IW, CM and TJC provided the clinical samples, clinical parameters and the follow-up data. IL and CM performed the histopathology reporting of all the patients tissues analyzed. JK and AM did the statistical analysis and had access to the raw data. JK, AM, IL and RR interpreted the data. The manuscript was drafted by JK and AM, edited by RR and submitted for comments to all the authors of this study. The investigators incorporated their suggestions and all authors approved the final version of the manuscript. PGW provided the infrastructure for this study.

Grant sponsors: Canadian Institutes of Health Research (CIHR) and International Science and Technology Partnerships (ISTP) Canada–Department of Biotechnology (DBT), India

DOI: 10.1002/ijc.28473

History: Received 1 Dec 2012; Accepted 21 Aug 2013; Online 1 Oct 2013

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What's new?

Identification of oral lesions with dysplasia at high risk of malignant transformation remains a major clinical challenge, and is of utmost importance for identifying patients who would benefit from early intervention. Currently, there are no biomarkers that are being routinely used in clinics to predict high-risk lesions. Here, the authors evaluated the potential of five candidate protein biomarkers and correlated their expression with p16 and HPV 16/18 to identify oral lesions with dysplasia at high risk of cancer development. S100A7 overexpression demonstrated the potential to serve as a useful marker for estimating the risk of oral dysplasia progressing to cancer.

wide variation is attributed to differences in diagnosis, patient characteristics, influence of demographic traits, human papillomavirus (HPV) infection, tobacco habits, genetics, treatment and follow-up time intervals.[2–5] Moreover, dysplasia grading is subjective, not often associated with malignant transformation; some dysplastic lesions may remain static or even regress, whereas the nondysplastic lesions may occasionally become malignant. The standard treatments for OLs include surveillance, chemoprevention and surgical resection.[4,6,7] Recurrences are observed in 10–20% cases and cancer development in 3–9% of areas of excised lesions.[6,7] Patients with recurrent dysplasia may require additional surgery, which further compromises their quality of life.

Accurate assessment of oral dysplasia and identification of lesions at high risk of malignant transformation remains a major clinical challenge and is of immense importance for identifying patients in whom early intervention will lead to more effective disease management.[6,7] The key to early detection and effective management of the disease lies in better understanding of the molecular mechanisms implicated in malignant transformation of OLs with dysplasia. Advances in molecular technologies have provided tools for developing markers to identify high-risk OLs.[8] Many genes and signaling pathways have been associated with oral dysplasia. These include alterations in genes/pathways that regulate genomic stability, cell cycle, apoptosis, cytoskeleton and angiogenesis. Higher Ki67/Mcm2 ratio; overexpression of cyclin D1, p53, COX-1/COX-2, E-cadherin, beta-catenin, APC and vimentin; galectins, cyclin D1 amplification, loss of p16 and increased telomerase activity have been reported in oral dysplasia.[9,10] Loss of heterozygosity (LOH) in chromosomal loci at 3p, 9p21, 17p13, 13q11, 13q21 and 14q31 has been reported in squamous hyperplasia and dysplasias.[11,12] In addition, HPV has also been implicated in OLs as an infecting agent. Compared to normal oral mucosa, HPV is detected frequently in oral dysplastic lesions and carcinoma with a higher prevalence of HPV 16 or 18 genotypes.[13,14]

Recently, our group identified differentially expressed proteins in oral dysplasia and cancer in comparison with normal oral mucosa using quantitative proteomics with isobaric tags for absolute and relative quantitation (iTRAQ) and multidimensional liquid chromatography–mass spectrometry (LC-MS/MS).[15,16] Of these, we selected and verified expression of five candidate protein biomarkers including S100A7, prothymosin alpha (PTMA), 14-3-3ζ, 14-3-3σ and heterogeneous nuclear ribonucleoprotein K (hnRNP K), in an independent cohort of oral dysplasia and OSCCs.[17–20] Notably, our studies revealed that overexpression of these proteins correlated significantly with poor prognosis of oral cancer patients.[17–20] Moreover, these proteins have been shown to play important roles in inflammation, transcription, cell cycle regulation, proliferation and survival in several epithelial malignancies including head and neck, breast, lung, bladder, skin, colorectal, esophageal and gastric cancers.[21–27] On the basis of their roles in regulating several cellular processes and their clinical significance in OSCCs, we hypothesized that overexpression of these proteins may be associated with malignant transformation of oral dysplasia.

In our study, we aimed to investigate the clinical significance of this panel of five candidate protein biomarkers and correlate with p16 expression and HPV 16/18 status in an independent set of oral dysplasias with known clinical outcome, and evaluate their potential to determine the risk of cancer development among these patients. Our findings underscored the potential of S100A7 overexpression to serve as a biomarker for identifying dysplastic lesions at high risk of cancer development.

Material and Methods

Study population characteristics and criteria

Our study was approved by the research ethics board of Mount Sinai Hospital, Toronto, Canada, before commencement. The patients' charts with clinicopathological diagnosis of dysplasia from 2000 to 2010 were retrospectively reviewed to obtain the clinical information and follow-up data in the Department of Pathology, Mount Sinai Hospital. Information regarding gender, age, site of lesions at the time of the initial diagnosis of dysplasia and smoking history was documented in the clinical database.

Inclusion criteria. Patients with OLs having histopathological evidence of dysplasia and a known clinical outcome were included in the study.

Exclusion criteria. Patients with OLs with dysplasia but with no available follow-up data or patients diagnosed with OLs with dysplasia concomitant with OSCC at the first visit were excluded from the study.

Based on these criteria, an independent set of 110 patients with dysplasia were selected in this cohort for further analysis. There was no overlap of dysplasia cases used in this study with the samples used in the discovery set published previously.[16] In case patients with OLs had multiple biopsies, the

first biopsy section with histological evidence of dysplasia was used for immunostaining.

Management protocol. All patients with OLs had an initial biopsy. The patients with histopathological evidence of mild dysplasia were monitored at 6 monthly intervals. A repeat biopsy was performed if the lesion changed in appearance. The patients with moderate or higher grades of dysplasia had excision of the lesions wherever feasible clinically. In case excision was not feasible, the patient was continually monitored with repeat biopsies for clinically suspicious areas for cancer development.

Histopathology

The histopathologic diagnosis of all cases was re-examined and confirmed according to the World Health Organization (WHO) criteria by the oral pathologists (IL and CM) at Mount Sinai Hospital. The dysplastic areas were selected from hematoxylin and eosin (H&E)-stained section of each tissue. Dysplastic lesions were classified into mild, moderate or severe dysplasia grades based on WHO standard criteria.[2] These cases included mild ($n = 58$), moderate ($n = 39$) and severe ($n = 13$) dysplasia. Of 110 tissue blocks reviewed for inclusion in our study, 86 dysplasia cases were used for construction of tissue microarrays (TMAs), whereas 24 cases used for immunohistochemistry were whole tissue sections.

Construction of TMAs

The TMA blocks were constructed by relocating small cylindrical tissue cores (two cores per tissue block representing the dysplasia sections) from individual donor blocks and placing them in a recipient block with defined array coordinates. Arrays were constructed from formalin-fixed paraffin-embedded (FFPE) tissues by the removal of 0.6-mm-diameter tissue cores from donor blocks. A total of two morphologically representative areas of interest from each donor block were identified under the microscope by the pathologists (IL and CM) using a stained H&E section as a guide. Using a precise spacing pattern on manual TMA instrument, 150–200 cores could be transferred to the recipient paraffin block in a grid-like fashion, retaining a link to the original block and its pathology. Consecutive 4-μm sections were cut from the recipient block and used for immunohistochemical staining for p16, five candidate protein biomarkers and HPV 16/18 status using chromogenic *in situ* hybridization (cISH) as described below.

Immunhistochemistry of candidate markers in OLs using TMA

TMA slides were immunostained using Vectastain Elite ABC kit (PK-6100) rapid protocol as described by the manufacturers (Vectastain Laboratories, Burlingame CA). Antigen retrieval was performed using microwave in Tris-EDTA buffer, pH 9.0, containing 0.05% Tween-20 for 15 min at 450 W followed by 5 min at 900 W. Slides were immunostained with the respective mouse monoclonal antibodies: anti-p16 (sc-1661, Santa Cruz Biotechnology, Dallas TX) at 1:100 dilution; anti-S100A7 (sc-52948, Santa Cruz Biotechnology, Dallas TX) at 1:500 dilution; anti-PTMA (LS-B2322, Lifespan Biosciences, Seattle WA) at 1:3,500 dilution; anti-hnRNPK (ab23644, Abcam, Cambridge MA) at 1:5,000 dilution; anti-14-3-3σ (ab14116-50, Abcam, Cambridge MA) at 1:2,500 dilution and 14-3-3ζ (IMG-6664A, Imgenex, San Diego CA) at 1:100 dilution. Tissue sections of liver were used as positive control in the TMA slides. The sections were evaluated by light microscopic examination. Images were captured using the Visiopharm Integrator System (Horsholm, Denmark).

Evaluation of immunohistochemical staining

Immunopositive staining was evaluated in five areas of the tissue sections as described earlier.[24–28] Sections were scored as positive if epithelial cells showed immunopositivity in the cytoplasm and/or nucleus when observed by the evaluators (JK and IK under the supervision of IL and CM) who were blinded to the clinical outcome. These sections were scored as follows: 0, <10% cells; 1, 11–30% cells; 2, 31–50% cells; 3, 51–70% cells and 4, >70% cells showed immunoreactivity. Sections were also scored semiquantitatively on the basis of intensity as follows: 0, none; 1, mild; 2, moderate and 3, intense. Finally, a total score (ranging from 0 to 7) was obtained by adding the scores of percentage positivity and intensity for each of the tissue sections. The immunohistochemical data were subjected to statistical analysis as described below. The scoring by two observers was discrepant in about 2% cases and a consensus on the final result was reached by re-evaluation of these slides and discussion. An inter-rater reliability analysis using the *K*-statistic was performed to determine consistency among evaluators. The inter-rater reliability for the evaluators was found to be $K = 0.921$ ($p < 0.001$, 95% CI = 0.83–1.01).

cISH for HPV16/18 detection

HPV 16/18 status was determined in formalin-fixed oral dysplasia tissue sections in TMA using cISH protocol, routinely being used in our hospital for patient care. Briefly, FFPE oral dysplasia tissue sections in TMA were deparaffinized in xylene. Sections were treated by digestion with proteinase K, followed by hybridization with biotinylated DNA probes for high-risk (HR)-HPV genotypes 16/18 or human embryonic DNA used as a control for determining genomic DNA integrity as described earlier.[28] Cervical cancer tissue section was used as a positive control for detection of HPV 16/18 infection. For determining HPV 16/18 status, diffuse nuclear staining was considered as indicative of episomal HPV, whereas point-form nuclear staining was characteristic of integrated HPV16/18 DNA.[28]

Follow-up data and statistical analysis

Statistical analysis was performed using the software packages SPSS version 20.0 for Windows (SPSS, Chicago, IL) and R-statistical software version 2.12.2 (R Foundation, http://www.r-project.org, Vienna, Austria). In our study, malignant transformation *versus* no transformation of oral dysplastic

Table 1. Analysis of clinical parameters with transformation potential of oral dysplasia patients

Characteristics		Transformed *N* (%)	Untransformed *N* (%)	*p*-Value	OR (95% CI)
Dysplasia (110 cases)		39 (35.4)	71 (64.5)		
Age (years)	≤59	15 (38.5)	37 (52.1)		
	>59	24 (61.5)	34 (47.9)	0.170	1.741 (0.7–3.85)
Gender	Female	21 (53.8)	30 (42.2)		
	Male	18 (46.2)	41 (57.8)	0.243	0.627 (0.3–1.4)
Site	Tongue	26 (66.7)	53 (74.6)		
	Others[1]	13 (33.3)	18 (25.4)	0.373	0.679 (0.3–1.6)
Histopathological grade	Mild	12 (30.8)	46 (64.8)		
	Moderate	18 (46.2)	21 (29.6)	**0.008**[3]	3.286 (1.3–8.1)
	Severe	9 (23.1)	4 (5.6)	**0.001**[4]	8.625 (2.3–32.8)
Smoking history[2]	Yes	15 (51.7)	31 (52.5)		
	No	14 (48.3)	28 (47.5)	0.942	0.968 (0.4–2.4)

[1]Others included buccal mucosa ($n = 18$), floor of mouth ($n = 12$) and lip ($n = 1$).
[2]Smoking history available for 88 cases only.
[3]*p*-Value obtained from Chi-square analysis for mild*vs*. moderate dysplasia.
[4]*p*-Value obtained from Chi-square analysis for mild*vs*. severe dysplasia.
Abbreviations: OR: odds ratio; 95%CI: 95% confidence interval.

lesions was considered to be the clinical outcome of the patients. A descriptive analysis was performed on clinical and pathological factors. Based on our earlier studies, a predefined cutoff value of each candidate protein biomarker was chosen for defining positivity (low/high score). The χ^2 test and Fischer's exact test were used to assess the associations among categorical variables. In addition, significance of our null hypothesis was also verified using Mann–Whitney test.

Follow-up period of dysplasia patients for oral cancer-free survival (OCFS) was defined as the interval from the time when patient underwent first biopsy to malignant transformation (*i.e*., events) or no transformation at last consultation (for censored observations). Dysplasia patients were monitored for a maximum period of 150 months (mean 43 months and median 36.5 months). Notably, malignant transformation of oral dysplasia was observed in 39 of 110 (35.4%) patients. However, 71 patients (64.5%) did not show any histological evidence of malignant transformation until the end of the follow-up period. Life tables were created to determine the median OCFS among patients with mild, moderate or severe dysplasia. OCFS was determined using time-to-event analysis, Kaplan–Meier method and log-rank test. Cox proportional hazards models were used for evaluation of clinicopathological factors including age, gender, degree of dysplasia, smoking habits, p16 immunostaining and overexpression of candidate markers in predicting risk of cancer development. Hazard ratios (HRs) with 95% confidence interval (95% CI) and significant *p*-values were reported. All tests were two-sided, and *p*-values < 0.05 were considered statistically significant. The systematic and rigorous assessment of positive and negative predictive values (PPV and NPV, respectively) for prognostic markers was carried out as described earlier.[17–20]

Results

Patient characteristics

Of the 110 patients with dysplasia included in our study, follow-up data were available for up to a maximum of 150 months with the mean follow-up period of 43 months. Of these, 39 patients (35.4%) developed invasive squamous cell carcinoma of oral cavity with the mean time for malignant transformation of 27.9 months (range, 2–118 months). The baseline clinicopathological characteristics of patients with dysplastic lesions including age, site of lesion, histopathological grade and smoking history are presented in Table 1. The average age at diagnosis was 59 years (range, 30–88 years). On the basis of histopathological characteristics, these lesions were subclassified as mild (58 cases, 52.7%), moderate (39 cases, 35.4%) and severe dysplasia (13 cases, 11.8%). No significant differences were observed in age, gender, site of lesion and smoking history between patients with dysplasia that showed malignant transformation (*i.e*., developed oral cancer) in comparison to the untransformed dysplasia (Table 1). Notably, 12 of 58 (20.7%) cases with mild dysplasia, 18 of 39 (46.1%) cases with moderate dysplasia ($p = 0.008$, odds ratio, OR = 3.286, 95% CI = 1.3–8.1, Table 1) and nine of 13 (69.2%) with severe dysplasia developed malignancy ($p = 0.001$, OR = 8.625, 95% CI = 2.3–32.8, Table 1).

Analysis of candidate marker overexpression and clinicopathological parameters

Our immunohistochemical analysis revealed that 79.1% (87 of 110) dysplastic lesions show increased expression of S100A7 protein in either cytoplasm and/or nucleus of epithelial cells (Figs. 1*i*, *a–c* and Table 2). The intensity of S100A7

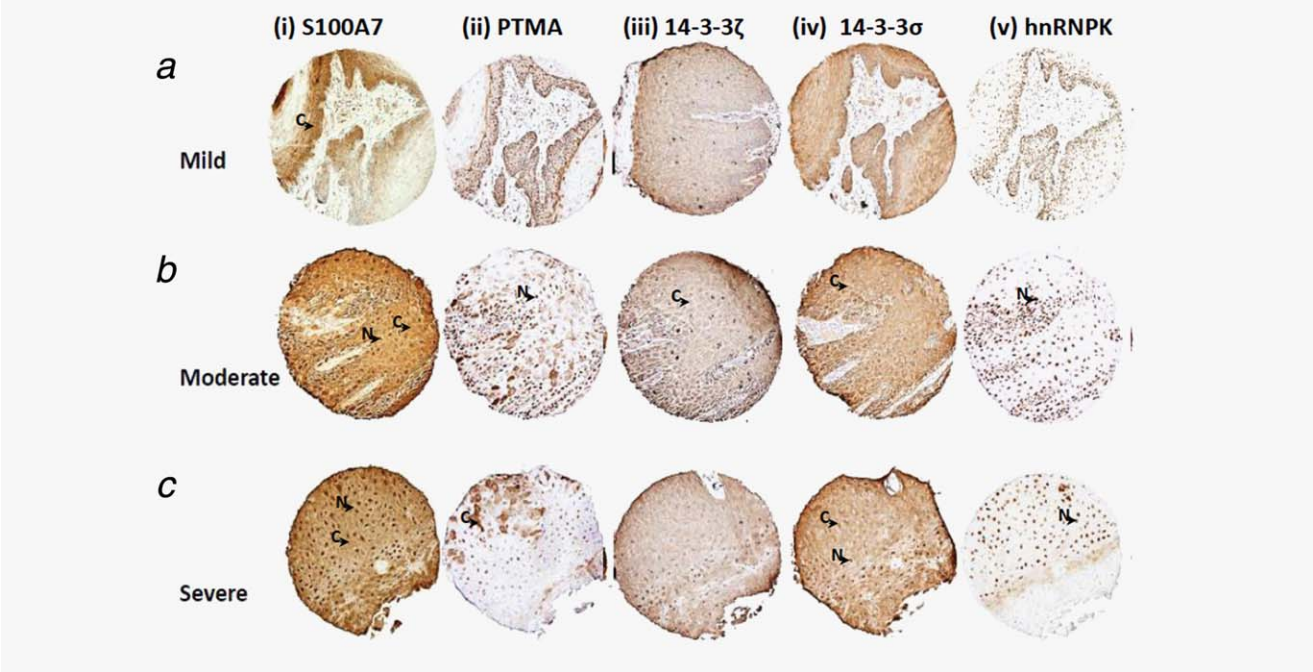

Figure 1. Immunohistochemical analysis of five candidate markers in oral lesions with dysplasia. Immunohistochemistry was carried out in tissue sections from oral lesions with dysplasia using specific antibodies for S100A7, prothymosin alpha (PTMA), 14-3-3ζ, 14-3-3σ and heterogeneous nuclear ribonucleoprotein K (hnRNP K) as described in Material and Methods section. Panel shows cytoplasmic and/or nuclear immunostaining of (*i*) S100A7, (*ii*) PTMA, (*iii*) 14-3-3ζ and (*iv*) 14-3-3σ in (*a*) mild, (*b*) moderate and (*c*) severe dysplasia. Panel (*v*) shows nuclear hnRNP K in (*a*) mild, (*b*) moderate and (*c*) severe dysplasia; no detectable cytoplasmic expression of hnRNPK was observed in dysplasia sections used in this study. Arrows show cytoplasmic (C) or nuclear (N) staining in cells (original magnification, ×200).

Table 2. Correlation of candidate protein markers expression with transformation in oral dysplasia patients

Characteristics		Transformed *N* (%)	Untransformed *N* (%)	*p*-Value	OR	95% CI
Dysplasia (n = 110)		39	71	–	–	–
p16 expression	Nuclear positive	32 (82.1)	53 (74.6)	0.375	1.553	0.5–4.1
HPV 16/18+		0	0	–	–	–
S100A7$^+$	Overexpression (cytoplasm/nuclear)	36 (92.3)	51 (71.8)	**0.014**	4.706	1.3–17.1
	Cytoplasm	32 (82.1)	38 (53.5)	**0.003**	3.970	1.5–10.2
	Nuclear	35 (89.7)	49 (69.0)	**0.018**	3.929	1.2–12.4
PTMA$^+$	Overexpression (cytoplasm/nuclear)	37 (94.9)	67 (94.4)	0.911	1.104	0.2–6.3
	Cytoplasm	25 (64.1)	36 (50.7)	0.176	1.736	0.8–3.8
	Nuclear	36 (92.3)	63 (88.7)	0.743	1.524	0.4–6.1
14-3-3ζ$^+$	Overexpression (cytoplasm/nuclear)	37 (94.9)	69 (97.2)	0.536	0.536	0.1–5.3
	Cytoplasm	31 (79.5)	62 (87.3)	0.277	0.563	0.2–1.6
	Nuclear	21 (53.8)	38 (53.5)	0.974	1.013	0.4–2.2
14-3-3σ$^+$	Overexpression (cytoplasm/nuclear)	27 (69.2)	62 (87.3)	**0.040**	0.327	0.1–0.8
	Cytoplasm	25 (64.1)	57 (80.3)	0.062	0.439	0.2–1.1
	Nuclear	18 (46.2)	46 (64.8)	0.071	0.466	0.2–1.1
hnRNP K$^+$	Nuclear[1]	39 (100)	70 (98.6)	1.000	0.986	0.9–1.1

[1]No cytoplasmic staining was observed for p16 and hnRNP K in tissue sections used in this study.
Abbreviations: OR: odds ratio; 95%CI: 95% confidence interval.

expression in dysplasia sections ranged from weak to strongly positive among different grades of dysplasia (Figs. 1*i*, *a–c*). Thirty-four of 58 (58.6%) mild dysplasia, 26 of 39 (66.7%) moderate dysplasia and ten of 13 (76.9%) severe dysplasias showed cytoplasmic S100A7 overexpression (Supporting Information Table S1). Notably, 32 of these 70 dysplasia cases (45.7%) showing S100A7 overexpression in cytoplasm transformed to cancer ($p = 0.003$, OR = 3.97, 95% CI = 1.5–10.2, Table 2). Nuclear S100A7 expression was observed in 40 of 58 cases of mild dysplasia (68.9%), 33 of 39 (84.6%) moderate dysplasia and 11 of 13 (84.6%) severe dysplasia (Supporting Information Table S1). Thirty-five of these 84 (41.6%) dysplasia cases showing nuclear S100A7 developed malignancy ($p = 0.018$, OR = 3.929, 95% CI = 1.2–12.4, Table 2). However, no significant correlation was observed between S100A7 overexpression (cytoplasm/nucleus) and degree of dysplasia ($p > 0.05$, Supporting Information Table S1).

Immunhistochemistry (IHC) analysis showed PTMA expression in either cytoplasm (25 of 39 cases, 64.1%) or nuclei (36 of 39 cases, 92.3%) in epithelial cells of dysplasia that progressed to cancer (Figs. 1*ii*, *a–c* and Table 2). Similarly, increased expression of both the 14-3-3ζ and 14-3-3σ isoforms was observed in cytoplasm and/or nuclei of epithelial cells in mild, moderate and severe dysplasia (Figs. 1*iii*, *a–c* and *iv*, *a–c*, Table 2). Among dysplasia cases that transformed to cancer, 79.5% (31 of 39 cases) showed cytoplasmic expression of 14-3-3ζ, whereas 21 cases (53.8%) showed its expression in nuclei of epithelial cells (Table 2). For 14-3-3σ, 64.1% cases showed its expression in cytoplasm, whereas 46.2% cases showed nuclear expression in addition to cytoplasmic staining (Table 2). Nuclear hnRNP K was observed in all dysplasia patients, but no detectable expression was observed in cytoplasm (Figs. 1*v*, *a–c* and Table 2). No significant difference was observed for expression of cytoplasmic or nuclear PTMA, 14-3-3ζ, 14-3-3σ and hnRNP K in dysplasia that transformed to cancer when compared to those that did not progress to cancer (Table 2). Mann–Whitney test also showed significant association of S100A7 overexpression in cytoplasm ($p = 0.002$) and nucleus ($p = 0.008$) in dysplasia cells among patients with OLs who progressed to cancer in comparison to patients who did not progress to cancer, thereby giving an independent evaluation of association of protein markers with dysplasia.

Evaluation of p16 expression and HPV 16/18 status

Expression of p16 was evaluated in all oral dysplasia cases analyzed in our study. Nuclear p16 was observed in 85 of 110 cases (77.3%), whereas 25 cases (22.7%) showed low or no detectable expression of nuclear p16 in oral dysplasia cells (Supporting Information Fig. S1). Thirty-two of 39 (82.1%) dysplasia cases that transformed to cancer showed nuclear p16 expression (Table 2). Nuclear p16 expression was observed in 49 of 58 (84.4%) mild, 28 of 39 (71.7%) moderate and eight of 13 (61.3%) severe dysplasia (Supporting Information Table S1). However, no significant correlation was observed between nuclear p16 and any of the five candidate protein biomarkers showing overexpression in oral dysplasia cases analyzed in our study (Supporting Information Table S2).

Our study revealed no detectable levels of HPV 16/18 in all the dysplasia cases analyzed, irrespective of their p16 status (positive/negative) (Supporting Information Fig. S1B, i, Table 2). Oral dysplasia tissue section used as a negative control showed no detectable levels of HPV 16/18 (Supporting Information Fig. S1B, ii). Cervical cancer tissue sections used as positive control showed strong positivity for HPV 16/18 (Supporting Information Fig. S1B, iii). TMA tissue sections used as control to determine genomic DNA integrity showed strong positive staining (Supporting Information Fig. S1B, iv). No significant correlation was observed between nuclear p16 and HPV16/18 in oral dysplasia cases. Together, our data clearly suggest lack of association of p16 or HPV 16/18 with S100A7 expression and transformation of oral dysplasia.

Table 3. Evaluation of clinical parameters and S100A7 overexpression for determining OCFS in dysplasia patients

	Kaplan–Meier Survival analysis, un-adjusted *p*-value	Multivariate Cox regression analysis, adjusted *p*-value	Hazard ratio (HR)	95% CI
Age	0.411	0.771	–	–
Gender	0.391	0.612	–	–
Site	0.740	0.284	–	–
Smoking history	0.755	0.124	–	–
Dysplasia grade				
Mild*	–	–	–	–
Moderate	**0.004**	**0.013**	**2.54**	**1.6–10.8**
Severe	**<0.001**	**<0.001**	**5.42**	**2.6–23.2**
p16 expression	0.995	0.892	–	–
S100A7 overexpression				
Cytoplasm$^+$	**0.007**	**0.041**	**2.36**	**0.9–8.4**
Nuclear$^+$	**0.041**	0.570	–	–

Evaluation of S100A7 overexpression as a marker for OCFS

Among the five candidate protein biomarkers analyzed in our study, S100A7 overexpression in cytoplasm or nuclei showed significant association with malignant transformation of dysplastic lesions (Table 2). Hence, we further determined the potential of S100A7 overexpression in identifying patients having OLs with dysplasia at high risk of cancer development using Kaplan–Meier survival analysis to determine the probability of OCFS for these dysplasia patients. In univariate analysis, we analyzed the association between S100A7 overexpression in cytoplasm or nuclei of dysplasia, loss of p16 expression and other potential risk factors including dysplasia grade (mild, moderate and severe), smoking history,

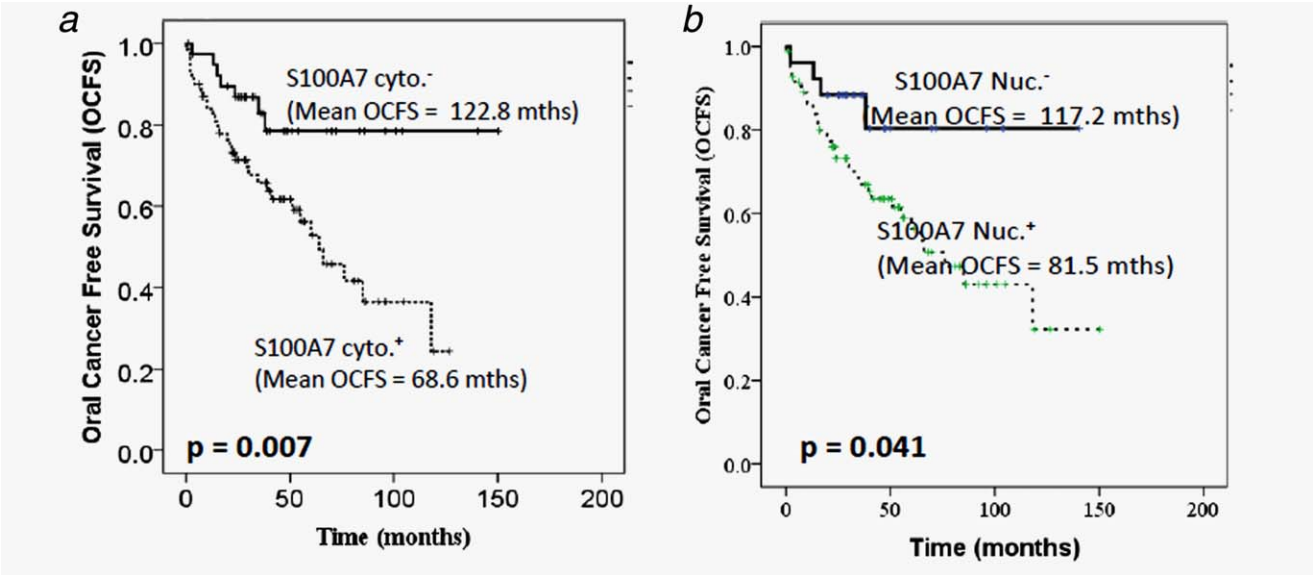

Figure 2. Kaplan–Meier survival analysis for evaluation of oral cancer-free survival (OCFS). Kaplan–Meier survival analysis was performed to determine association of S100A7 overexpression (cytoplasm/nucleus) with prognosis of dysplasia patients. Panel shows Kaplan–Meier survival curves for (*a*) cytoplasmic S100A7 expression showing reduced OCFS ($p = 0.007$) and (*b*) nuclear S100A7 expression ($p = 0.041$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

age, gender and site of lesion with OCFS (Table 3). Notably, dysplasia patients showing cytoplasmic S100A7 had significantly reduced OCFS (mean OCFS = 68.6 months, $p = 0.007$) when compared to patients with weak or no S100A7 immunostaining in cytoplasm (mean OCFS = 122.8 months, Fig. 2*a* and Table 3). Similarly, dysplasia patients showing nuclear S100A7 had reduced OCFS (mean OCFS = 81.5 months, $p = 0.041$) in comparison with patients with weak or no S100A7 immunostaining in nucleus (mean OCFS = 117.2 months, Fig. 2*b* and Table 3). Among clinical parameters, degree of dysplasia (moderate or severe) showed a significant correlation with OCFS ($p < 0.05$, Table 3). Patients with moderate dysplasia demonstrated low mean OCFS of 58.59 months ($p = 0.004$), whereas in severe dysplasia cases, patients showed mean OCFS of 38.9 months ($p < 0.001$, Supporting Information Fig. S2) when compared to mild dysplasia (mean OCFS = 116.25 months, Supporting Information Fig. S2). None of the other clinical parameters including age, gender, site of lesion or smoking history showed any significant correlation with OCFS ($p > 0.05$, Table 3).

Our life table and Kaplan–Meier analysis clearly showed the low mean OCFS in moderate and severe dysplasia patients showing S100A7 overexpression in cytoplasm or nuclei of dysplasia cells. Notably, 13 of 18 (72.2%) moderate dysplasia and nine of nine (100%) severe dysplasia patients who developed oral carcinoma showed cytoplasmic S100A7 overexpression, whereas 17 of 18 (94.4%) moderate and eight of nine (88.8%) severe dysplasia had nuclear overexpression of S100A7. However, no significant correlation was observed for differences in OCFS in moderate/severe dysplasia patients showing S100A7 overexpression in cytoplasm or nuclei ($p > 0.05$) as revealed by Kaplan–Meier analysis (Supporting Information Figs. 3a–3c).

Analysis of risk factors for transformation of dysplasia into cancer was performed using the Cox proportional hazards model (Table 3). Importantly, cytoplasmic S100A7 overexpression ($p = 0.041$, HR = 2.36) and degree of dysplasia (moderate dysplasia, $p = 0.013$ and severe dysplasia, $p < 0.001$) emerged as an independent factors for identifying high-risk dysplasia (Table 3). This clearly demonstrated the significance of cytoplasmic S100A7 overexpression in predicting malignant transformation of dysplasia.

Based on our data, the additional prognostic value that S100A7 overexpression in cytoplasm provided for predic ting (PPV) or excluding (NPV) malignant transformation in oral dysplasia patients was measured by the ratios: $PPV_{transformation}$/dysplasia (118 months|S100A 7 $cyto^+$)/ $PPV_{transformation}$/dysplasia (118 months) = 75.6/60.0; $NPV_{transformation}$/dysplasia (118 months|S100A7 $cyto^+$)/$NPV_{transformation}$/dysplasia (118 months) = 78.5/40.0 (Figs. 3*a* and 3*b*). Increase in PPV and NPV for S100A7 in comparison to dysplasia grade underscores the potential of S100A7 as a marker for predicting malignant transformation in dysplastic lesions.

Discussion

Early prediction for malignant potential of oral epithelial dysplasia is crucial for clinical management of patients with the disease. In our study, we verified the expression of five candidate protein biomarkers, namely, S100A7, PTMA, 14-3-3ζ, 14-3-3σ and hnRNP K, in oral dysplasia and correlated with p16 expression as well as HPV 16/18 status. Notably, majority of the dysplasia (mild/moderate/severe) that progressed

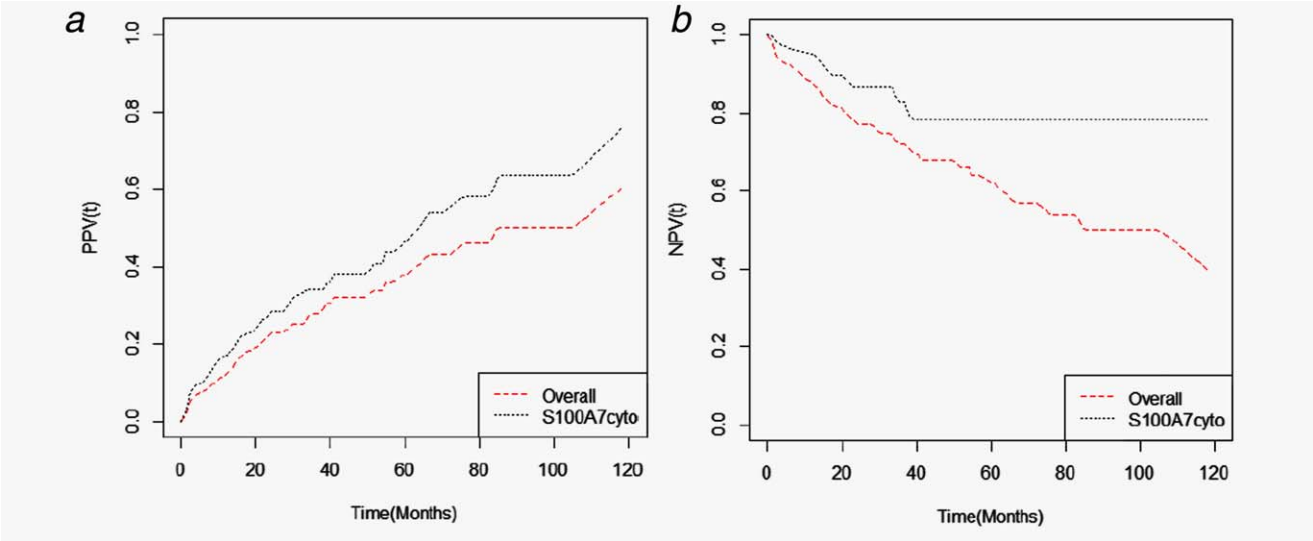

Figure 3. (*a*) Positive predictive values [PPV(t)] for time to malignant transformation for 39 oral dysplasia patients with S100A7 expression and for all 110 dysplasia patients with survival data (overall); (*b*) negative predictive values [NPV(t)] for time to malignant transformation for 39 patients with S100A7 overexpression, and for all 110 patients (overall). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

to malignancy showed S100A7 overexpression (cytoplasm/nuclear), emphasizing the potential of S100A7 overexpression for stratifying dysplasia patients at higher risk of cancer development. Of the five candidate protein biomarkers analyzed, S100A7 overexpression in cytoplasm emerged as the most significant risk factor for cancer development in patients having OLs with dysplasia with PPV (75.6%) and NPV (78.5%), regardless of age, gender, site of lesion, smoking habits and grade of dysplasia. Unlike S100A7, nuclear p16 expression showed no significant difference in the expression among dysplasia patients who transformed to cancer in comparison to those who did not transform to cancer. Moreover, HPV 16/18 was not detected in any of the dysplasia cases analyzed in our study using cISH, irrespective of their p16 status or transformation to malignancy. There are controversial reports regarding the use of p16 expression as a surrogate marker for HR-HPV infection or as a marker for progression in dysplasia.[29–33] Moreover, involvement of both p16 and HR-HPV in development of oral epithelial dysplasia and their roles in transformation to malignancy have not been shown unequivocally as demonstrated in squamous cell carcinomas of pharynx.[34–36] In support of our findings, several studies reported discordance between p16 expression and HPV 16/18 in oral dysplasia and OSCCs.[14,29,30]

It is noteworthy that increased levels of S100A7 transcripts have also been reported in oral dysplasia in comparison with normal oral mucosa using microarray analysis.[37] Recently, Winter *et al.*[38] using 15 leukoplakia biopsies showed S100A7 overexpression and DOC1 downregulation by reverse-transcriptase polymerase chain reaction and proposed that the combined investigation of both genes may be a marker for estimating the risk of cancer development in intraoral lesions. The major limitation of the study was the limited sample size and no correlation with clinical outcome in a longitudinal study. Further, majority of studies examining markers for OLs are cross-sectional analyses and do not provide data on these lesions over time, thus rendering them unsuitable for identifying patients with dysplasia at high risk of transformation. Longitudinal long-term follow-up studies of OLs are required to determine the robust signature of markers for predicting fate of dysplastic lesions. Our study has an advantage of long-term follow-up analysis for dysplasia patients unlike those reporting biomarkers for OLs without any follow-up data. To the best of our knowledge, this is the first report demonstrating the association of S100A7 overexpression in cytoplasm with increased risk for malignant transformation of oral epithelial dysplasia.

In our independent studies, we recently reported S100A7 overexpression as an important risk factor associated with reduced disease-free survival of OSCC patients.[19] Moreover, S100A7 overexpression also showed a significant correlation with well-differentiated OSCCs, suggesting its role in differentiation in addition to proliferation and invasion.[19] In support of these observations, using orthotopic mouse models of oral cancer, Zhou *et al.*[39] demonstrated that S100A7 overexpression resulted in degradation of β-catenin by the noncanonical pathway, independent of GSK3β, and promoted tumor differentiation in oral cancer cells. Altered expressions of other members of S100 family of proteins have also been associated with diagnosis and/or prognosis of OSCC patients.[40–42] S100A7 forms both homodimers and heterodimers with other members of the family interacting with c-*jun* activation domain-binding protein 1 (Jab1), Ran-binding protein M (RanBPM), epidermal fatty acid-binding protein (EFABP) and transglutaminase.[43–46] Interactions with RanBPM have been shown to promote migration of renal cell

carcinoma, suggesting a role for S100A7 in enhancing the invasive potential of cancer cells.[45]

Recently, S100 family of proteins including S100A7 has also been reported to play important roles in inflammation and carcinogenesis.[23,47] Nasser *et al.*[23] reported enhanced proliferation and production of proinflammatory molecules such as cytokines and chemokines (IL-1a, IL-11, CSF2, CXCL1 and CXCL8) in S100A7 overexpressing MDA-MB-231 breast cancer cells in comparison to vector controls. Inflammation-related markers such as oncostatin M (OSM) and interleukin-6 (IL-6) have been suggested to regulate the expression and activity of S100A7 in breast cancer by regulating PI3K, STAT3 and Erk signaling.[48] S100A7 overexpression has also been associated with poor patient outcome in ER-negative invasive breast cancer patients.[49] In view of the involvement of IL-6 and PI3K signaling in oral cancer demonstrated by our laboratory and others, we speculate that these mechanisms may extend to development of oral cancer as well. However, the unequivocal experimental evidence for the role of S100A7 in malignant transformation of oral dysplastic cells remains to be obtained.

In conclusion, our findings revealed the clinical significance of S100A7 overexpression in oral dysplastic lesions. S100A7 overexpression alone or in combination with dysplasia grade might have the potential to serve as a useful marker for estimating the risk of oral dysplastic lesions progressing to malignancy. We are cognizant of the fact our study is limited by number of cases that progressed to cancer; nevertheless, our findings support the utility of S100A7 as a potential biomarker in clinical practice in future as an IHC tool for oral biopsies to stratify risk of malignancy, similar to the use of p16 status in cervical intraepithelial disease, currently being used to improve grading of dysplasia, and in some studies as a predictive marker for progression.[50]

Acknowledgements

R.R. gratefully acknowledges the financial support from Canadian Institutes of Health Research for Chair in Advanced Cancer Diagnostics. Alex and Simona Shnaider Chair in Thyroid Cancer is gratefully acknowledged by P.G.W. The authors are thankful to Dr. David Swanson, Mount Sinai Services, Mount Sinai Hospital, who provided them support for carrying out cISH for determining the HPV 16/18 status in this study.

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