Implications on pathogenesis and risk of oral lichen planus neoplastic transformation: an ex-vivo retrospective immunohistochemical study

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Running title

OLP and risk of neoplastic transformation

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Conflict of interest statement

All the authors certify that they haven’t any conflict of interests relevant to the study to declare.

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Summary

Aims: to evaluate OPN, MCM7, Ki-67, p53, Bel-2 and 53BP1 presence, together with the abnormal adaptive CD4 and CD8 T-cell response markers expression in a series of oral lichen planus (OLP) affected patients and assess their combined contribution for a more objective disease classification.

Methods and results: In this ex-vivo retrospective analysis, biopsy specimens from 28 adults with a clinical diagnosis of OLP at different progression degree (16 reticular, 2 plaque-like, 1 erosive and 9 mixed type) were collected. Sections were immunohistochemically investigated for the proinflammatory cytokine osteopontin (OPN), alpha-beta CD4 and CD8 positive T cells, DNA replication licensing factor (MCM7), proliferating cell marker (Ki-67), apoptotic and tumor antigen (p53), apoptosis modulator (Bcl-2) and cellular response regulator to double-strand breaks tumor suppressor p53-binding protein 1 expression.

Statistical analysis revealed that 53BP1 is highly represented among the OLP study patients (p<0.05). Moreover, on the basis of the quantification results of the highly expressed parameters, two illness categories with different severity were evidenced. The classification hypothesis was confirmed by i) OLP lesion persistence, ii) the development of oral severe lesions in the patients belonging to high grade activity OLP group (HGA-OLPs) and iii) the ascertainment of the same evidence both in the oral squamous cell tumor controls (OSCC) and in HGA-OLP cases.

Conclusion: This study completes the scenario with respect to early detection, thanks to a more precise histological analysis, for rationalizing the clinical and histological findings toward a sharable international disease scoring system.

Keywords

Oral lichen planus, oral squamous cell carcinomas, chronic inflammation, DNA damage response, neoplastic transformation and classification.
INTRODUCTION

OLP is a common T-cell mediated chronic and recurrent inflammatory disease with an essential role in cell-mediated autoimmunity, characterized by multiple bilateral mucosal lesions with keratotic and/or atrophic-erosive, rarely bullous, findings (Nico et al., 2011; Alrashdan et al., 2016). Predominant patterns are mainly seen, with a predilection for female gender, as white reticulated and lace-like lesions of the cheek, gum, lip, tongue and gingiva, associated to cutaneous clinical manifestations in about 15% of the affected patients. Several etiological hypotheses, none of them fully satisfactory up to now, for its exogenous (infective, from drugs, chemical agents or mechanical damage) and endogenous (oral and gut microbiota influence, autoimmunity, hormonal status and genetic predisposition) pathogenesis have been formulated (Mignogna et al., 2004; Roopashree et al., 2010; Kurago, 2016; He et al., 2017; Baek and Choi, 2018).

Histologic findings are characterized by a subepithelial dermal band-like infiltration, aggressive against the basal layer, made up by mononuclear T cells attracted by cytokines released from damaged keratinocytes (Zhou et al., 2008).

A role of OLP as precancerous condition has been identified by World Health Organization (WHO), since its neoplastic progression towards oral squamous cell carcinoma (OSCC) has been observed in up to 12.5% of cases, although with discrepancies among the series reported (Gonzalez-Moles et al., 2008a; Liu et al., 2010; Wang and van der Waal, 2015); nevertheless source, cause and risk rate of malignant transformation are still unknown.

Amongst the molecules investigated for their putative role in OLP neoplastic transformation, OPN, a proinflammatory glycoproteic cytokine synthesized by immune cells may deserve attention. It is involved in cell-adhesion/migration through its interaction with CD44 receptors and activation of the EGF pathway, and in immune cell recruitment promotion (Cantor and Shinohara, 2009; Liu et al., 2011; Santarelli et al., 2015). Although OPN has a supposed role in the neoplastic transformation of precancerous disorders such as leukoplakia and oral submucous fibrosis (OSMF) (Chang et al., 2008; Zhou et al., 2008; Subramani et al., 2015), only few studies have reported about
its hypothetical involvement in OLP pathogenesis and neoplastic transformation (Devoll et al., 1997; Kunii et al., 2009; Valcz et al., 2010; Routray et al., 2013). Among the key factors produced in response to OPN, immune CD4$^+$ and CD8$^+$ T cells are essentially recruited, activated and maintained during the typical chronic inflammatory response generated during the mucosa hypersensitivity reaction where the clinical symptoms are evident (Morimoto et al., 2011). Furthermore, OLP may predispose the mucosal tissues toward an oxidative stress, which in turn causes DNA damage (Madhulika et al., 2015).

Double-strand breaks (DSBs) are normally recognized by peculiar sensors, able to activate the DNA damage response (DDR) that leads to immune system activation and DNA repair process (Gorgoulis et al., 2018). However, if the immune cascade activation is excessive, as happens in autoimmune diseases, self and foreign DNA are no more discriminated and chronic inflammation and oxidative stress in turn favor DNA damage and immune disease progression. In such a context, the epigenetic p53-binding protein 1 (53BP1), formed in G1 and lost in early S phases, can indirectly measure cell DNA damage levels (Park et al., 2010; Bi et al., 2015; Dillenburg et al., 2015; Nakad and Schumacher, 2016; Fernandez-Vidal et al., 2017), creating an interplay between the DDR and the immune signaling, thus orchestrating the response to genomic aberrations and surveillance against tumors.

In concert, the mini-chromosome maintenance complex MCM7, a helicase of about 90 KDa typical of the late G1/early S phases, finely orchestrates the initiation of eukaryotic DNA replication and S phase entry, thus acting as a prognostic marker for cancer associated diseases (Pramod et al., 2014) such as OSCC (Tampa et al., 2018), in which it is well recognized that alterations in apoptotic pathways likely seem to occur. In this regard changes in Bcl-2 and Ki-67 expression, respectively anti-apoptotic modulator of the intrinsic pathway responsible for the inflammatory state maintenance (Pigatti et al., 2015), and proliferative activity marker, might add further details in the predisposition of OLP to cancer progression.
On this basis, in the present study, made up of a series of biopsies from 28 patients with clinical and histological diagnosis of OLP, we evaluated, by immunohistochemical tools, the intraepithelial and stromal distribution of OPN in correlation with the expression of MCM7, Ki-67, p53, Bcl-2 and 53BP1. On the basis of the quantification results of the highly expressed parameters we then split OLP lesions into low grade activity (LGA) and HGA cases. To understand if this OLP classification hypothesis was realistic, we therefore included in the study 10 OSCC affected control patients, and we compared the findings obtained with the data of the OLP case series.

MATERIALS AND METHODS

PATIENTS AND TISSUE SPECIMENS

Twenty eight patients (15 females and 13 males) with a clinical and histological diagnosis of OLP, of which 16 classified as reticular, 2 plaque-like, 1 erosive and 9 of mixed type (reticular and others at the same time), were collected between 2002 and 2014 at the Dentistry Unit of the School of Medicine (UPO, Novara, Italy), and selected from the Pathology Unit files for inclusion in this retrospective study. All histological slides were revised by an expert pathologist (G.V.). Clinical information and main characteristics of the patients included in the study are shown in Table 1a. Age ranged from 27 to 87 years (mean 54) and the site of the lesions was more frequently the mucosa of the cheek (24 cases), while in the other 4 cases the lesions were respectively in gum, lip, retromolar trigone and tongue mucosa. No patient underwent topical or systemic corticosteroid treatment two months before biopsy. The observation period of the OLP patients examined in this ex-vivo retrospective study was between 5 and 11 years.

Ten OSCCs, obtained from 1 female and 9 males with an age range from 50 to 79 years (mean 64.7), were used as control tumor cases, whose information and main characteristics are reported in Table 1b; while ten archival healthy oral mucosa samples, collected from the discarded margins of surgical procedures, were used as normal controls.
All subjects gave informed consent to participate to the study conducted according to the Declaration of Helsinki.

ETHICAL APPROVAL

An approval for the feasibility of this study and publication of individuals’ data, carried out in accordance with the declaration of Helsinki as revised in 2013, was granted by the local Hospital Research Ethics Committee.

IMMUNOHISTOCHEMICAL ANALYSIS

Five µm thick serial sections of each case were mounted on superfrost ultraplus glass slides (Menzel, Thermo Scientific, Monza, Italy), deparaffined and rehydrated.

For CD4 (clone 4B12), CD8 (clone C8/144 B), Ki-67 (MIB-1), p53 (clone DO-7) and Bcl-2 (clone 124; all monoclonal antibodies were from DAKO and distributed by Agilent Technologies Italia S.p.A., Milan, Italy) antigens, stainings were performed with a Ventana benchmark XT Automated Platform (Ventana Medical Systems, Tucson, AZ, USA).

For OPN, antigen retrieval was carried out in a 10 mM pH 6 citrate buffer unmasking solution in a microwave oven at 600 Watt for 10’. After that, slides were left to cool for 20’ at room temperature (RT). Endogenous peroxidase was quenched in 3% hydrogen peroxide in PBS 1x. After blocking in 10% NHS, 1% BSA in PBS 1h at RT, a mouse monoclonal anti-OPN antibody (clone MAb53, Abcam, distributed by Prodotti Gianni, Milan, Italy) was diluted 1:200 in 5% NHS and incubated ON at 4°C.

A mouse monoclonal antibody to mini-chromosome maintenance protein 7 (MCM7, clone 47DC141), obtained from Neomarkers (distributed by Thermo Fisher Scientific), was 1:300 diluted; the slides were incubated ON at 4 °C.

For 53BP1 retrieval, sections were incubated in 1mM EDTA pH 8.5 for 25’ at 95°C. After blocking in modified phosphate-buffered gelatin (PBG 1X), composed of 2% BSA and 1% gelatin (from cold
water fish skin, Sigma Aldrich, Milan, Italy) for 1h at RT, a rabbit polyclonal anti-53BP1 (working dilution 1:1000 in 1% BSA, Abcam) antibody was used, followed by a biotinylated secondary antibody and a streptavidin peroxidase reagent.

All sections were developed at RT with 3-3’ diaminobenzidine (DAB) substrate (Vector Laboratories, distributed by DBA Italia Srl, Segrate, Milan, Italy), counterstained in Mayer’s hematoxylin (BioOptica, Milan, Italy), dehydrated and mounted with a xylene-based mounting medium (VectaMount permanent mounting medium, H-5000, Vector, distributed by DBA Italia Srl). Negative controls were also included by omitting the primary antibodies (data not shown).

CRITERIA FOR INTERPRETATION OF IMMUNOHISTOCHEMICAL STAININGS

Images were acquired by the digital microscope Pannoramic MIDI II and analyzed using the Pannoramic Viewer software (3DHistech Ltd, Budapest, Hungary, distributed by Diapath, S.p.A., Italy). Two observers (B.A. and G.V.) independently scored the stainings.

Regarding markers expression, staining intensity was scored into 2 groups.

Expression of both CD4 and CD8 antigens within the epithelial mucosa was semiquantitatively evaluated, by counting positive cells in at least 5 random high-magnification representative fields (x200 magnification); score 0 corresponded to staining absence or less than 3 positive cells/field, score 1 to presence of more than 3 positive cells/field.

CD4 and CD8 positivity of the band-like infiltration in the papillary stroma was evaluated as follows: 0 when immunonegative or less than 30% positive cells/field were seen, and 1 when positivity was in more than 30% cells.

When OPN epithelial expression was absent or limited to the basal epithelial stratum (weak), a score 0 was assigned, while in case of full-thickness staining (moderate-strong), score was 1.

Stromal positivity was evaluated with the same method.

MCM7 expression was scored by evaluating the percentage of G1/S epithelial cells with positive nuclear staining: a score 0 was assigned when no or weak (1+=≤10%, only basal stratum) positivity
was seen, score 1 when moderate (2+=10 to ≤25%, basal and parabasal cell positivity) or strong (3+= >25%, positivity of almost all epithelium) expression was observed.

Ki-67 expression was scored by evaluating the percentage of positive epithelial nuclei: a score 0 was assigned when no or weak (1+=≤10%, only few cells) positivity was seen, 1 when moderate (2+=10 to ≤25%, basal and parabasal cell positivity) or strong (3+= >25%, positivity of almost all epithelium) expression was observed.

Similarly, p53 expression was scored by evaluating the percentage of positive epithelial nuclei: a score 0 was assigned when weak (1+=≤10%, limited to basal and suprabasal layers) positivity was seen and 1 when moderate (2+=10 to ≤25%, basal and parabasal cell positivity) or strong (3+= >25%, positivity of almost all epithelium) expression was observed.

Bcl-2 expression was evaluated both in the epithelium and in the inflammatory stromal counterpart: a score 0 was assigned when no or weak (1+=≤10%) positivity was observed and 1 when moderate (2+=10 to ≤25%) or strong (3+= >25%) positivity was observed.

Cases with G1/S epithelial 53BP1 positive nuclei were identified with a value of 1.

STATISTICAL ANALYSIS

Continuous variables are presented as means and range (min-max) and categorical variables as numbers and percentages. A Fisher’s exact test was applied to determine correlations between biological markers and clinical findings of each patient and associations among biological markers.

A p value of less than 0.05 was considered statistically significant.

RESULTS

The quantitative data and the immunostaining patterns are shown in Tables 1a, 1b, 2a, 2b, and Figure 1, respectively.

HISTOLOGIC FINDINGS - We analyzed some histologic findings to determine the differences from OLP case to case and in relation to the clinical presentation: in most of them (22/28), the
band-like lymphocytic infiltration thickness was in the range 0.16-1.2 mm with 11/28 patients showing a reticular pattern, while squamous epithelium was quite subtle (0.1-0.5 mm) in 17 patients. In 12 out of 17 the clinical pattern was reticular and hyperkeratosis was seen; moreover, in only 6 cases (without predilection for a clinical pattern) epithelium showed mild dysplastic findings. Regarding tumor control lesions, whose localization sites were mainly the same as the OLPs, 3 out of 10 were severe dysplasia, while the remaining 7 were carcinomas. In all cases the inflammatory infiltrate (I.I) thickness was higher than 0.16 mm, while the epithelium thickness (E.T.) was between 0.51 and 9.5 mm in carcinoma lesions only.

IMMUNOHISTOCHEMICAL FINDINGS - CD4 and CD8 immunoreactivity was mainly observed in the band-like lymphocytic infiltration (Figure 1 B, B’ and C, C’) and, to a lesser extent, it was displayed by lymphocytes infiltrating inside the epithelium of OLPs; this finding was more evident, and statistically significant, in male gender (p=0.01 and 0.03 respectively; Table 3). Simultaneous expression of CD4 and CD8 inside the epithelium was statistically significant (p=0.01; Table 3). As expected, CD4 and CD8 markers were highly represented (Table 2b) both in the epithelium and the stroma of OSCC controls as shown in Figure 1 B’’ and C’’.

Cytoplasmic positivity for OPN was displayed both in the squamous epithelium (as diffuse staining) and in the stromal infiltrating lymphocytes in a similar number of cases in OLP specimens (16/28 and 18/28, respectively; Figure 1 D, D’), with an almost statistically significant correlation between their expression within the two compartments (p=0.0054; Table 3). Amongst the positive cases OPN was more represented, even without statistical significance, when infiltration band was in the range 0.16-1.2 mm; no staining was ever seen in healthy mucosa (data not shown). OPN epithelial expression highly correlated to CD4 intraepithelial expression (p=0.05; Table 3). Worth of note, 9 and 8 out of 10 OSCCs showed OPN in the epithelium and stroma respectively (Table 2b and Figure 1 D’’).

MCM-7 expression in squamous epithelial cells of OLP cases was only poorly present in the nuclei of cases with less intense lymphocytic infiltration, while it was detectable in the basal and
intermediate G1/S positive keratinocytes of the highly infiltrated mucosa (Figure 1 E, E’) with a significant correlation with CD4 intraepithelial positivity (p=0.04; Table 3). MCM-7 expression was highly correlated with OPN positivity, both in the epithelium and in the stroma (p=0.002 and 0.001 respectively; Table 3). MCM7 was also highly expressed and always present in OSCC tumor samples (Table 2b and Figure 1 E’’). Similarly, the ubiquitary proliferation marker Ki-67 highly correlated with MCM7 in OLPs (p=0.021; Table 3 and Figure 1 F, F’) and with OPN positivity, both in the epithelium and in the stroma (p=0.006 and 0.047 respectively; Table 3). This marker was also present in all control cases (Table 2b and Figure 1 F’’).

p53 expression was limited to the basal/suprabasal layers in only 17.86% of OLP cases, while in the remaining cases it was observed in the basal and parabasal strata (Table 2a and Figure 1 G, G’). Its expression highly correlated with CD4 in the stroma (p=0.0149; Table 3).

In fourteen out of 28 (50%) and 13/28 (46.43%) OLPs, p53 and MCM7 or Ki-67 were moderate-highly expressed respectively, while of the 25 OLP cases 53BP1 positive, 21 (84%) had a p53 score of 1. Moreover 15/16 (93.75%) OLPs with an epithelial OPN score of 1 also showed a moderate-strong p53 expression and finally 16/18 (88.89%) with a stromal OPN score of 1 had also a moderate-strong p53. As shown in Table 2b and Figure 1 G’’, p53 was always present in tumor controls.

Bel-2 was expressed at high levels in the inflammatory cells of 15/28 OLPs, in high correlation with CD4 stromal expression (p=0.011; Table 3) and in all OSCCs, while a weak positivity was noticed in epithelial cells in OSCC samples only, as depicted in Tables 2a and b and Figure 1 H- H’’.

Nine of the 15 OLPs moderately or strongly positive for stromal Bel-2 (32.14%) were positive for both epithelial OPN and MCM7, 10 (35.71%) for Ki-67, 12 (42.86%) for 53BP1 and stromal OPN and 13 (46.43%) for p53.

Finally, the regulator of the cellular response to DSBs 53BP1 was confined inside few peculiar epithelial nuclei foci (Figure 1 I, I’, J, J’) in 89% of the cases (Table 2a), demonstrating that DNA damage levels involve most OLPs. Of the 25 53BP1 positive cases, 12 (46.42%) and 11 (39.29%)
were also positive for Ki-67 and MCM7 markers respectively. However, no correlations were evident between this marker and clinical findings. 53BP1 was displayed in all tumor samples (Table 2b and Figure 1 I”, J”), while it was rarely observed in normal mucosa (data not shown). All the data obtained with the Fisher’s exact test are available upon request.

**DISCUSSION**

OLP is an autoimmune premalignant disorder for which a univocal and overall accepted classification criterion has not been identified yet. In fact clinical and histopathological findings are often discordant; for this reason several patients with high neoplastic progression risk, which with the currently available knowledge are up to 12.5%, probably could not be correctly diagnosed. Moreover, since a clear explanation on the origin of several OSCC cases has not been found and that some OLP lesions are not well recognized yet, this percentage could be higher with respect to the currently identified one (Kinouchi et al., 2018).

On these bases, the main aim of this study was to retrospectively IHC analyze and correlate some molecules, already independently known for their potential association with OLP (Santarelli et al., 2015; Sagari et al., 2016), and try to identify OLP cases at greater progression risk by assessing their combined contribution and comparing these findings with those obtained in OSCC controls.

As a matter of fact, despite the abundant literature on OLP and its etiology, the mechanisms at the basis of this immune-mediated disease are largely unknown. Till now most researchers believe that this disorder may be triggered by diverse external and internal stimuli that orchestrate, with a circular feedback, the abnormal accumulation of CD4+ and CD8+ infiltrating T lymphocytes in characteristic sub-epithelial bands, which induces epithelial damage and promotes the clinical onset of the disease (Chabas et al., 2001; Ohshima et al., 2002).

Since the link between chronic inflammation and malignant transformation of OLP seems to be fully related to a huge release of cytokines (Redder et al., 2014), it is fundamental to identify the molecules involved in the transition from the active inflammation to dysplastic changes.
In this context, OPN antigen has been evidenced as a key factor both in the development of the immune response and in the tissue destruction/repair processes promoted during inflammation (Mignogna et al., 2004). Since it is expressed not only by several immune cells, but also by epithelial, endothelial and fibroblast cells of the inflamed tissues (Devoll et al., 1997; Zhou et al., 2008), it is obvious to deduce that its specific biological activities are necessarily regulated by a plethora of receptors able to recognize signaling motifs themselves conditioned by OPN post-translational modifications. Moreover, OPN maintains the epithelial barrier and participates in inflammation (Zhou et al., 2008), thus making it difficult to understand its role not only in physiologic processes, but also in mucosal inflammatory conditions.

In our ex-vivo OLP samples series, OPN was highly expressed in both the epithelium and the stroma in more than 50% of the cases, in correlation with the presence of intraepithelial CD4 and CD8 lymphocytes. This may be due to a preferential involvement of OPN and its isoforms in recruiting and polarizing alpha-beta CD4 (“helper”) and CD8 (“cytotoxic”) T lymphocytes, as well as macrophages, inside the squamous epithelium. Furthermore, it was absent in normal mucosa, as previously reported (Zhou et al., 2008), while it was present in almost all OSCC control samples. On the other hand, OPN expression also correlated to MCM7, a protein expressed in G1/S phase that finely orchestrates S phase entry and DNA replication (Redder et al., 2014; Hadzi-Mihailovic et al., 2012), and to Ki-67 antigen; this evidence supports an OPN role in activating epithelial proliferation.

DSBs are generally repaired by a coordinated DDR that leads to immune system activation and DNA lesion removal. In autoimmune disorders such as OLP, this activation is excessive, leading to the typical chronic inflammation and oxidative stress that favors DNA damage and the immune disease through a circular loop mechanism (Vidyalakshmi et al., 2016). In such a context, 53BP1 can indirectly measure cell DNA damage levels (Squarzanti et al., 2018), creating an interplay between the DDR and the immune signaling, thus orchestrating the response to genomic aberrations.
and surveillance against tumors. In both our OLP cases and OSCC controls, 53BP1 resulted highly expressed, suggesting and confirming the activation of the DNA damage response.

To reinforce once more these data, we also checked for the expression of the survival protein Bcl-2, known for its role in inhibiting apoptosis and tumor promotion. Bcl-2 mainly affected the subepithelial portion in more than half of the analyzed OLP lesions (of which two thirds were also highly positive for the proliferation markers) and of all the OSCCs, thus supporting its ability, as suggested by Pigatti et al., 2015, to create a microenvironment prone to the persistence of the inflammatory state.

As above underlined, OLP creates a cytokine-based milieu that increases the proliferation index and, in a percentage of cases, determines the conditions for neoplastic transformation (Subramani et al., 2015).

Based on the quantification results of the highly expressed parameters, we split the whole OLP series into two groups not harboring any statistical difference concerning clinical presentation or disease history. Fifteen of the 28 patients selected for this study were classified as LGA, since they presented highly expressed only 7 or less of the examined parameters, while those with more than 7 were categorized as HGA-OLPs. In this regard all OSCC controls showed a high expression in more than 8 parameters, a condition also observed in the 84.7% of the HGA group lesions.

Twelve out of 13 HGA-OLPs exhibited both 53BP1 and cycling cells markers, i) suggesting that the altered DDR led to further genomic injury amplification, ii) confirming that their expression grows as the degree of dysplasia increases as described by Takeda et al., 2006 and iii) reinforcing their contribution to the disruption of the DDR pathway.

Finally, all the HGA-OLPs showed an association between basal and suprabasal p53 levels and Ki-67, MCM7 and 53BP1, as also published by Gonzalez-Moles et al., 2008b and Valente et al., 2001, emphasizing p53 ability to preferentially activate the cell cycle for DNA repair and its role as a specific marker for lesions that are at higher risk of malignant transformation (Cruz et al., 2002; Varun et al., 2014). This could appear contradictory since Ki-67 and MCM7 evidence proliferation,
while p53 marks quiescence or apoptosis; one possible explanation, as also pointed out by other authors, could be that these proliferating and quiescent cells are different populations within the same OLP lesion (Gonzalez-Moles et al., 2006).

A definite common report of the observation period (5-11 years) of the patients enrolled in the study was not available because some of them did not attend the scheduled dental checks, probably because they referred to other medical centers or the lesions regressed.

Although the OLP cases low number does not allow a statistical conclusion, it is symptomatic that among the patients we included in the HGA group, 5 subjects had dysplasia and one developed severe oral lesions together with an aggressive form of Parkinson's disease. Since no literature data exist on the malignant progression mean time of these lesions types, the remaining patients, identified as belonging to the HGA group and without apparent signs of tumor progression, but with lesions that still persist, could more likely progress over time.

In conclusion, this study completes the scenario with respect to early detection, thanks to a more precise histological analysis, for identifying LGA and HGA categories of this potentially evolutive autoimmune disorder, for the improvement of the therapeutic approach efficacy and management, by rationalizing the clinical and morphological findings toward a sharable international disease scoring system (Wang and van der Waal, 2015).

Acknowledgments

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DF Squarzanti performed most of the IHC experiments, prepared the figures and all the tables; R Sorrentino performed some IHC experiments and image acquisition; T Cena did the statistical analysis; M Migliario collected patients’ clinical data; A Chiocchetti and L Rimondini contributed
essential reagents; B Azzimonti designed the research study, interpreted the data and wrote the paper; G Valente interpreted the data and wrote part of the discussion.

B Azzimonti and DF Squarzanti thank Fondazione Cariplo (prot. 2013-0954) that partially supported the research. The funder had no role in study design, data collection and interpretation or in the decision to submit the work for publication.

List of abbreviations

B-cell lymphoma protein 2 (Bcl-2), bovin serum albumin (BSA), 3-3’ diaminobenzidine (DAB), DNA damage response (DDR), double-strand breaks (DSBs), epidermal growth factor (EGF), epithelium thickness (E.T.), high grade activity (HGA), inflammatory infiltrate (I.I), low grade activity (LGA), mini-chromosome maintenance complex (MCM7), normal horse serum (NHS), oral lichen planus (OLP), oral squamous cell carcinoma (OSCC), oral submucous fibrosis (OSMF), osteopontin (OPN), over-night (ON), p53-binding protein 1 (53BP1), phosphate-buffered gelatin (PBG), room temperature (RT), World Health Organization (WHO).

References


Tables and figure legends

Table 1. Patients’, OLP (a) and OSCC (b) lesions main characteristics. Each category variable is expressed as frequencies (%).

Table 2. OLP (a) and OSCC (b) markers expression. Data are represented as frequencies (%).

Table 3. Associations among the biological markers evaluated in OLP biopsies. Statistically significant values (p<0.05), calculated with Fisher’s exact test, are in bold.

Figure 1. Immunostainings of LGA-, HGA-OLPs and OSCC control cases. Hematoxylin and eosin staining of the i) two representative OLP categories with a low (A), high (A’) expression protein degree pattern and of the ii) OSCC control cases (A’’). Histological stainings for CD4 (B-B’’), CD8 (C-C’’), OPN (D-D’’), MCM7 (E-E’’), Ki-67 (F-F’’), p53 (G-G’’), Bcl-2 (H-H’’), and 53BP1 (I-J’’) with DAB substrate; counterstaining with hematoxylin. Bar scales in A-I’’ 20 x fields= 200 μm; J-J’’ 40x fields= 20 μm.
Table 1. Patients’, OLP (a) and OSCC (b) lesions main characteristics

(a)

<table>
<thead>
<tr>
<th>N (%)</th>
<th>Age (y): mean min-max</th>
<th>Site N (%)</th>
<th>Inflammatory infiltrate (I.I.) thickness (mm) N (%)</th>
<th>Epithelium thickness (E.T.) (mm) N (%)</th>
<th>Other oral lesions N (%)</th>
<th>Cutaneous lesions N (%)</th>
<th>Hyperkeratosis N (%)</th>
<th>Mild dysplasia N (%)</th>
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<tr>
<td>Patients</td>
<td>28 (100) 54</td>
<td>Cheek</td>
<td>24 (85.71)</td>
<td>1 (3.57) 1 (3.57) 6 (21.43)</td>
<td>0.05 ≤ I.I. ≤ 0.15 0.16 &lt; I.I. ≤ 1.2 0.1-0.5</td>
<td>22 (78.57)</td>
<td>11 (39.29)</td>
<td>17 (60.71)</td>
</tr>
<tr>
<td>Gender</td>
<td>Females</td>
<td>15 (53.57)</td>
<td>58 (27.87)</td>
<td>13 (46.43)</td>
<td>0 (0) 0 (0) 3 (10.715)</td>
<td>0.16 &lt; I.I. ≤ 1.2</td>
<td>12 (42.86)</td>
<td>9 (32.14)</td>
</tr>
<tr>
<td>Males</td>
<td>13 (46.43)</td>
<td>49</td>
<td>11 (39.28)</td>
<td>0 (0) 1 (3.75)</td>
<td>0.1-0.5</td>
<td>0.51-1.5</td>
<td>Hard/soft palate, buccal mucosa, lip, alveolar process</td>
<td>Forearm, shoulder</td>
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</table>

(b)

<table>
<thead>
<tr>
<th>N (%)</th>
<th>Age (y): mean min-max</th>
<th>Site N (%)</th>
<th>I.I. thickness (mm) N (%)</th>
<th>E.T. (mm) N (%)</th>
<th>Severe dysplasia</th>
<th>Carcinoma</th>
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<tr>
<td>Patients</td>
<td>10 (100) 64.7</td>
<td>Palate</td>
<td>2 (20)</td>
<td>1 (10) 6 (60)</td>
<td>0.16 &lt; I.I. ≤ 1.9</td>
<td>0.1-0.5</td>
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<td>Gender</td>
<td>Females</td>
<td>1 (10)</td>
<td>57</td>
<td>0 (0) 0 (0) 1 (16.7)</td>
<td>0 (0) 1 (10)</td>
<td>0 (0) 1 (10)</td>
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<tr>
<td>Males</td>
<td>9 (90)</td>
<td>65.6</td>
<td>2 (20)</td>
<td>1 (10) 5 (43.3)</td>
<td>9 (90)</td>
<td>3 (30)</td>
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Table 2a. OLP markers expression

<table>
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<tr>
<th>Marker expression, N (%)</th>
<th>Immunoreactivity intensity</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Absent-weak (0)</td>
<td>Moderate-strong (1)</td>
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</tr>
<tr>
<td>CD4 epithelium</td>
<td>23 (82.14)</td>
<td>5 (17.86)</td>
<td></td>
</tr>
<tr>
<td>CD4 stroma</td>
<td>8 (28.57)</td>
<td>20 (71.4)</td>
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</tr>
<tr>
<td>CD8 epithelium</td>
<td>24 (85.71)</td>
<td>4 (14.29)</td>
<td></td>
</tr>
<tr>
<td>CD8 stroma</td>
<td>9 (32.14)</td>
<td>19 (67.86)</td>
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<tr>
<td>OPN epithelium</td>
<td>12 (42.86)</td>
<td>16 (57.14)</td>
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<tr>
<td>OPN stroma</td>
<td>10 (35.71)</td>
<td>18 (64.29)</td>
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</tr>
<tr>
<td>MCM7</td>
<td>13 (46.43)</td>
<td>15 (53.57)</td>
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</tr>
<tr>
<td>Ki-67</td>
<td>14 (50)</td>
<td>14 (50)</td>
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</tr>
<tr>
<td>p53</td>
<td>5 (17.86)</td>
<td>23 (82.14)</td>
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<tr>
<td>Bcl-2 epithelium</td>
<td>28 (100)</td>
<td>0 (0)</td>
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<tr>
<td>Bcl-2 stroma</td>
<td>13 (46.43)</td>
<td>15 (53.57)</td>
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</tr>
<tr>
<td>53BP1</td>
<td>3 (10.71)</td>
<td>25 (89.29)</td>
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Table 2b. OSCC markers expression

<table>
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<th>Marker expression, N (%)</th>
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<td>8 (80)</td>
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<tr>
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<td>CD8 epithelium</td>
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<td>p53</td>
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Table 3. Associations among the biological markers evaluated in OLP biopsies.

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<th>Biological markers</th>
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<th>OPN stroma</th>
<th>CD4 epi</th>
<th>CD4 stroma</th>
<th>CD8 epi</th>
<th>CD8 stroma</th>
<th>53BP1</th>
<th>MCM7</th>
<th>Ki-67</th>
<th>p53</th>
<th>Bcl-2 epi</th>
<th>Bcl-2 stroma</th>
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