An Immunohistochemical Analysis of Cell Cycle Markers in Oral Mucosal Dysplastic Lesions Treated by Laser Therapy. A Pilot Study

Jennifer Lo · Judith McNaughtan · Vidya Rani ·
Dragica Maric · Andrew Smith · Michael McCullough ·
Arun Chandu

Received: 15 February 2011 / Accepted: 20 March 2011 / Published online: 19 April 2011
© Association of Oral and Maxillofacial Surgeons of India 2011

Abstract

Introduction Over the past 15 years, dysplastic oral mucosal lesions have been treated by laser ablation with variable success. A recent study have shown that the type of laser utilized may be important for patient outcome, however, it may also be changes at a cellular level that could be an important factor in determining recurrence outcome. The aims of this study were to assess cellular markers related to oral dysplastic lesions treated by two different laser types.

Methods and Materials Twenty patients with a histopathological diagnosis of dysplasia treated with laser ablation between the years 1992 and 2003 were assessed. Tissue blocks of the original diagnostic biopsy specimens were stained with specific cell cycle markers (Cyclin-D1 and Ki67) via immunohistochemistry and presence of the marker were analysed by virtual microscopy. Patients were assessed according to grade of dysplasia [(mild vs.

moderate vs. severe) and the type of laser used (Potassium Titanyl Phosphate (KTP) vs. Carbon Dioxide (CO₂)].

Results No significant difference in Cyclin-D1 and Ki67 levels were found between the two groups with different grades of dysplasia, however, decreased Cyclin-D1 was found in those patients treated with KTP laser ($P = 0.028$).

Conclusions The findings of the present study may indicate cell cycle makers such as Cyclin-D1, may be responsible for the behaviour of dysplastic lesions treated with laser therapy, rather than the type of laser itself, which was reported in previous studies.

Keywords Oral · Dysplasia · Laser · Leukoplakia · Oral mucosa · Cyclin · Ki67

Introduction

Oral cancer is the 6th most common cancer worldwide [1]. It has a variable incidence and mortality across different countries due to differing risk profiles and availability or access to health services [2, 3]. These tumours are often preceded by dysplastic lesions that are characterised histologically by staging from mild to severe dysplasia [4], which may be considered a continuum of tissue disorganization suggesting a progressively increased risk of malignant change [5]. The aetiology of these lesions includes smoking, alcohol consumption, and betel quid chewing [6]. The risk of neoplastic transformation varies from 0.3 to 25% depending on location, clinical features, degree of dysplasia and aetiological factors [7].

The use of molecular markers, such as Cyclin and Ki67, have been previously investigated as markers to aid in the early diagnosis of oral dysplastic lesions [5]. An increase in epithelial proliferation in increasingly dysplastic tissue

J. Lo · J. McNaughtan · V. Rani · D. Maric · M. McCullough ·
A. Chandu
Melbourne Dental School, The University of Melbourne,
720 Swanston Street, Carlton, Melbourne, VIC 3010, Australia

A. Smith
School of Dentistry, The University of Western Australia,
Crawley, WA 6009, Australia

A. Chandu
Oral and Maxillofacial Surgery, The Royal Melbourne Hospital,
Grattan St, Parkville, Melbourne, VIC 3052, Australia

A. Chandu (✉)
Level 1, 665 Mt Alexander Rd, Moonee Ponds, Melbourne,
VIC 3039, Australia
e-mail: chandua@unimelb.edu.au

with alterations in regulation of cell division, cell cycle progression, DNA synthesis and repair has been found in previous studies [5]. Movement and timing of a cell through normal cell proliferation is controlled by Cyclins [5]. Cyclin-D1 has not only been shown to be the rate-limiting controller of G1-phase progression, but also has been characterized as an oncogene with its expression shown to increase with increasing severity of dysplasia [8].

Ki67 a nuclear antigen is a molecular marker expressed by proliferating cells in late G₁, S, G₂ and M phases [9, 10], but is not present in resting cells (G₀) [11]. Ki67 has been identified in both benign and malignant cells when actively replicating and is more highly expressed in more biologically aggressive lesions [9]. Cells begin to express Ki67 during the S phase with increasing levels during G₂ with maximum levels during mitosis [5]. In previous studies, the percentage of cells that stain positively has been used as a measure of proliferation and as a prognostic factor [12]. Increased Ki67 expression has been shown with increasing dysplasia and has been suggested as a possible marker of early premalignant changes [5].

Lodi and Porter [13] in a comprehensive review of treatment for potentially malignant mucosal lesions of the oral mucosal concluded that although some treatments have been shown to result in clinical resolution, other studies showed no benefit over placebo and that long term follow up was still advised. It would appear that recurrence and malignant transformation of oral dysplastic lesions might be independent of treatment regime.

A number of surgical and medical treatments have been proposed for oral dysplastic mucosal lesions. Surgical methods include excision by scalpel, laser, electrocautery/cryotherapy and photodynamic therapy. Medical treatments include vitamin A (retinoids), antioxidants, bleomycin [14], cyclo-oxygenase [4], and a mouthwash containing attenuated adenovirus [15]. Excision of pre-cancerous lesions has yet to be proven to prevent future malignant changes. Recurrence of dysplastic lesions has been hypothesized by Schwartz et al. [16], in that new epithelium migrates from the periphery to cover the wound after excision or ablation originate from potentially altered mucosa. The continued use of carcinogens, such as tobacco and alcohol may also result in lesion recurrence.

The use of lasers in resecting or ablating dysplastic lesions has been found to be an effective method in the treatment of dysplastic lesions with recurrence rates reported from 7.7 to 66% [17]. The benefits of laser treatment over conventional surgery include reduced inflammation, postoperative swelling, pain and infection [5]. However recurrence and malignant transformation is thought to depend on location, clinical features, degree of dysplasia and aetiological features such as alcohol and smoking [7]. The type of laser used may also have an

impact on the outcomes of dysplastic lesions. A recent study comparing the outcomes of dysplastic lesions treated with either Potassium Titanyl Phosphate (KTP) or Carbon Dioxide (CO₂) laser has shown that those lesions treated with KTP laser had a significantly decreased recurrence rate than those treated with CO₂ laser [17]. Different types of lasers have been purported to result in better disease control when compared to other treatment modalities [5], however, it may be other tissue factors, such as cell cycle markers, rather than the laser type used which may influence treatment outcome. Thus, the aim of the present study was to investigate cell cycle markers Ki67 and Cyclin-D1 immunohistochemically on the initial biopsy specimen of patients who have subsequently undergone laser ablation of oral mucosal dysplastic lesions.

Methods and Materials

Patients with a diagnosis of dysplasia at the Royal Dental Hospital of Melbourne between 1992 and 2003 whose outcomes have been previously described after treatment with laser ablation [17] were included for this pilot study. Patients were recruited based on availability of the initial biopsy specimen to allow immunohistochemistry analysis. Therefore, this sub-group was randomly and blindly selected based on the availability of tissue samples for analysis. Data collected included the patient's age, sex, clinical appearance of lesions, site of lesions and histological diagnosis based on incisional biopsies were included. Histological diagnosis was based on these biopsies as the lesions then underwent laser ablation without further histological analysis. Haematoxylin and Eosin (H & E) stained slides were gathered and classified into mild, moderate or severe dysplasia. Tissue blocks for each patient was obtained then sectioned and immunohistochemically stained with specific cell cycle markers (Cyclin-D1, Ki67) and analysed using Positive Pixel Count Algorithm in an Aperio Scanscope (Aperio technologies, USA). Ethical approval was obtained from the Human Ethics Committee at The University of Melbourne.

Immunohistochemical Analysis

The cell markers investigated were Cyclin-D1 and Ki67, both of which are nuclear stains. Sections of formalin-fixed, paraffin-embedded tissue were cut and subjected to de-paraffinization through a series of hydrations. An antigen-retrieval procedure was performed by placing the sections in Target Retrieval Solution (S3308; Dako, Calif, USA) inside a plastic pressure cooker, which was positioned in a microwave oven. The specimens were treated over 2 cycles for 15 min each. All sections were washed with a citrate-buffered saline solution and incubated with

the primary antibody. The following primary antibodies were used: anti-Cyclin-D1 at a 1:100 concentration (Dako, Calif, USA) and anti-Ki67 at a 1:150 concentration (Dako, Calif, USA). Sections were incubated with a secondary antibody (E0353 and E0354, Dako) for 30 min. A StreptABC Complex/HRP reagent (DAB K3468; Dako, Calif, USA) was applied, and the known control section for each antibody was stained in each run to confirm the presence of appropriate immunostain activity. Negative controls were included to assess background stain. The mean value of the negative controls was considered as the background stain. The background stain value was subtracted from the number of strong positive pixels, then divided by the total number of pixels (positive and negative) analysed. Thus, the ratio of strong positive pixels was used as a measure of tissue positivity.

Statistical Analysis

Mann–Whitney *U* Tests (Statview 5, SAS, Cary NC, USA) was used to compare differences in the levels of Cyclin-D1 and Ki67 for the different laser types including those patients treated with a CO₂ laser (Sharplan 743, Laser Industries, Tel Aviv, Israel) and those treated with the KTP laser (Laserscope Surgical Laser System, model 813, Aura Laserscope, San José, CA). Kruskal–Wallis tests were used to compare Cyclin-D1 and Ki 67 levels in the different levels of dysplasia. A *P*-value of less than 0.05 was considered statistically significant.

Results

A total of 20 patients were included in this study, 8 males and 12 females with an overall mean age of 64 years (range 40–71 years) (Table 1). Biopsy sites included the floor of mouth, buccal mucosa, tongue, soft palate, maxilla and mandible. Dysplasia was seen in all excised lesions and classified into mild, moderate and severe dysplasia (Table 1).

In regards to Cyclin-D1 (Fig. 1), dysplastic lesions in the KTP laser group had significantly less staining with

Cyclin-D1 than lesions treated with CO₂ laser (Table 2), however, when analysing the levels of Cyclin-D1 according to the level of dysplasia, no significant difference was observed between mild, moderate or severe dysplasia (Table 3). Looking at Ki67 (Fig. 2), no significant difference in staining was detected between different laser groups or different levels of dysplasia (Tables 2, 3). These findings are exemplified by boxplot histograms for Cyclin-D1 (Fig. 3a) showing significantly different median points between initial biopsy tissue from patients treated with CO₂ and KTP but similar median points for Ki67 (Fig. 3b).

Discussion

Laser therapy is one of the documented methods to treat premalignant lesions of the oral cavity [17, 18]. Previous reports have found CO₂ lasers to be efficacious in the treatment of oral dysplastic lesions with a low recurrence rate of 7.3% [18] while KTP lasers have been found to decrease the risk of recurrence of oral leukoplakia [17]. Some of the data used in the present study has been used previously to assess both CO₂ and KTP lasers [17, 18], and in these studies, cell cycle markers were not assessed. Thompson et al. [5], is one of the few studies that reports the use of cell cycle markers in patients undergoing laser therapy.

In regards to Cyclin-D1, increased amounts of Cyclin-D1 have been found with increasing grades of dysplasia [14]. Cyclin-D1 was also amplified in 7 out of 10 cases when progressing from dysplastic lesions to carcinoma [14], which also has been found to correlate to an increase in staining and intensity of staining [19]. It has been found that increased Cyclin-D1 levels were identified in poorly differentiated SCC than well differentiated SCC, however, an increase Cyclin-D1 mRNA was found to only exist between normal and dysplastic lesions, but not between dysplastic lesions and SCC [19]. This identified that staining for Cyclin-D1 was possibly a more effective method for identifying dysplastic lesions.

Few studies have assessed different laser types with reference to cell cycle markers. The present study found

Table 1 Patient demographics

Laser type	Gender	Age	Clinical appearance	Histopathological diagnosis
CO ₂	Male = 4	Mean = 63 years	Leukoplakia = 9	Mild = 5
	Female = 7	Range = 40–71 years	Erythroplakia = 2	Moderate = 4 Severe = 2
KTP	Male = 4	Mean = 64 years	Leukoplakia = 8	Mild = 4
	Female = 5	Range = 54–65 years	Erythroplakia = 1	Moderate = 1 Severe = 4

Fig. 1 Immunohistochemical stain for Cyclin-D1 of tissue previously diagnosed as severe dysplasia (50× magnification)

Fig. 2 Immunohistochemical stain for Ki67 of tissue previously diagnosed as severe dysplasia (50× magnification)

Table 2 Analysis of laser type and immunohistochemical staining

Laser type	Cyclin-D1	Ki-67
CO ₂	Median = 31.86 Standard error = 10.61 Range = 12.32–146.74 N = 11	Median = 13.82 Standard error = 4.97 Range = 4.58–47.39 N = 11
KTP	Median = 14.44 Standard error = 12.17 Range = 4.64–120.95 N = 9	Median = 21.57 Standard error = 9.41 Range = 3.20–80.89 N = 9
P-value	0.028	NS

P-value ≤ 0.05 considered significant

Table 3 Analysis of level of dysplasia and immunohistochemical staining

Level of dysplasia	Cyclin-D1	Ki-67
Mild	Median = 24.03 Standard error = 11.79 Range = 4.80–120.95	Median = 21.57 Standard error = 4.27 Range = 6.61–46.96
Moderate	Median = 32.43 Standard error = 23.27 Range = 17.79–146.74	Median = 13.82 Standard error = 7.94 Range = 4.58–47.39
Severe	Median = 27.53 Standard error = 21.32 Range = 4.64–106.42	Median = 8.63 Standard error = 17.03 Range = 3.20–80.89
P-value	NS	NS

P-value ≤ 0.05 considered significant

decreased levels of Cyclin-D1 in patients treated with KTP lasers. In a previous study, KTP laser was found to have a significantly decreased risk of recurrence in patients treated for oral dysplastic lesions as compared to the CO₂ laser [17]. This finding of decreased Cyclin-D1 may explain the

Fig. 3 Box plot of Cyclin-D1 (a) and Ki67 (b) for both CO₂ (number 1) and KTP (number 2)

clinical finding of decreased risk of recurrence in this previous study [17], however, more research would need to be undertaken to confirm this, particularly in relation to outcome. Interestingly, the present study found no significant difference in Cyclin-D1 levels in different grades of dysplasia. This may be a result of the small numbers of

cases analysed and the wide range/standard error of these cases.

Looking at Ki67, Thomson et al. [5] has previously found an increase in Ki67 in increasing grades of dysplasia in patients who have had oral dysplastic lesions treated with laser excision. Of note, the authors also found the amount of staining was significantly associated with the risk of recurrence. The present study showed no significant difference in the quantity of Ki67 staining between different levels of dysplasia and between the different laser subgroups.

There are some limitations of the present study. It is a retrospective study with a small sample size. Nonetheless, decreased levels of Cyclin-D1 were found in lesions treated with KTP laser which may explain the findings of previous studies. These results will form the foundation for a more extensive study of cell cycle markers in both potentially malignant and malignant lesions of the oral cavity.

Acknowledgments This project was supported by the Australian Dental Research Foundation Undergraduate Vacation Research Grant 2008/2009.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Abbas N, El-Sharkawy S, Abbas E, El-Shaer M (2007) Immunohistochemical study of p53 and angiogenesis in benign and preneoplastic oral lesions and oral squamous cell carcinoma. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 103:385–390
2. Petersen PE (2009) Oral cancer prevention and control—the approach of the world health organisation. *Oral Oncol* 45:454–460
3. Yokoo K, Noma H, Inoue T, Hashimoto S, Shimono M (2004) Cell proliferation and tumour suppressor gene expression in iodine unstained area surrounding oral squamous cell carcinoma. *Int J Oral Maxillofac Surgery* 33:75–83
4. Hamadah O, Thomson PJ (2009) Factors affecting carbon dioxide laser treatment for oral precancer: a patient cohort study. *Lasers Surg Med* 41:17–25
5. Thomson P, Hamadah O, Goodson ML, Cragg N, Booth C (2008) Predicting recurrence after oral precancer treatment: use of cell cycle analysis. *Br J Oral Maxillofac Surg* 46:370–375
6. Saito T, Nakajima T, Mogi K (1999) Immunohistochemical analysis of cell cycle-associated proteins p16, pRb, p53, p27 and ki-67 in oral cancer and precancer with special reference to verrucous carcinomas. *J Oral Pathol Med* 28:226–232
7. Kramer IR, Lucas RB, Pindborg JJ, Sobin LH (1978) Definition of leukoplakia and related lesions: an aid to studies on oral precancer. *Oral Surg Oral Med Oral Pathol* 46:518–539
8. Liu SC, Klein-Szanto AJ (2000) Markers of proliferation in normal and leukoplakia oral epithelia. *Oral Oncol* 36:145–151
9. Lazzaro B, Cleveland D (2000) P-53 and Ki-67 antigen expression in small oral biopsy specimens of salivary gland tumours. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 89:613–617
10. Takeda T, Sugihara K, Hirayama Y et al (2006) Immunohistological evaluation of Ki-67, p63, CK 19 and p53 expression in oral epithelial dysplasias. *J Oral Pathol Med* 35:369–375
11. Chrysomali E, Nikitakis NG, Tosios K, Sauk JJ, Papanicolaou SI (2003) Immunohistochemical evaluation of cell proliferation antigen Ki-67 and apoptosis-related proteins Bcl-2 and caspase-3 in oral granular cell tumour. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 96:566–572
12. Wiesner FG, Magener A, Fasching PA et al (2009) Ki-67 as a prognostic molecular marker in routine clinical use in breast cancer patients. *Breast* 18:135–141
13. Lodi G, Porter S (2008) Management of potentially malignant disorders: evidence and critique. *J Oral Pathol Med* 37:63–69
14. Pitiyage G, Tilakaratne W, Tavassoli M, Warnakulasuriya S (2009) Molecular markers in oral epithelial dysplasia: review. *J Oral Pathol Med* 38:737–752
15. Van der Waal I (2009) Potentially malignant disorders of the oral and oropharyngeal mucosa; terminology, classification and present concepts of management. *Oral Oncol* 45:317–323
16. Schwartz F, Maraki D, Yalcinkaya S et al (2005) Cytologic and DNA cytometric follow-up of oral leukoplakia after CO₂ and Er:YAG-laser assisted ablation: a pilot study. *Lasers Surg Med* 37:29–36
17. Lim B, Smith A, Chandu A (2010) Treatment of oral leukoplakia with carbon dioxide and potassium-titanyl-phosphate lasers: a comparison. *J Oral Maxillofac Surg* 68:597–601
18. Chandu A, Smith A (2005) The use of CO₂ laser in the treatment of oral white patches: outcomes and factors affecting recurrence. *Int J Oral Maxillofac Surg* 34:396–400
19. Mishra R, Das B (2009) Cyclin D1 expression and its possible regulation in chewing tobacco mediated oral squamous cell carcinoma progression. *Arch Oral Biol* 54:917–923