

Clinical and Molecular Determinants of Malignant Transformation in Oral Epithelial Dysplasia

Thesis submitted in accordance with the
requirements of the University of Liverpool for the
degree of Doctor in Medicine by

Michael Wing Sung Ho
BDS MBChB MFDS DOHNS FRCS(OMFS) FEBOMFS

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Declarations

The work contained in this thesis was carried out in the Department of Molecular Clinical Cancer Medicine, University of Liverpool between April 2013 and May 2016. Most of the research ideas were conceived jointly between myself and my supervisors. The majority of sample collection, retrieval of tissue blocks, immunohistochemistry, and all of the sample preparation were my own work. The western blot gels were produced jointly with two BSc students (Mark Ryan and Juhi Gupta) the remaining laboratory procedures were my own work. Where the thesis chapters were published, I was the primary author in all but one of the publications (Chapter 5) and made significant contributions to the production of the drafts and final manuscript.

Where the TNM staging system has been cited in this thesis, these were based upon the 7th edition of AJCC/UICC staging for oral squamous cell carcinoma.

All patient samples and clinical data were subject to the proper ethical and consent considerations, as approved by the local ethical committee (Sefton REC now **North West – Liverpool Central**; reference number: EC 47.01). None of this data forms part of any other thesis.

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Abstract: Clinical and Molecular Determinants of Malignant Transformation in Oral Epithelial Dysplasia

Known risk factors for malignant transformation in oral lesions with malignant potential

include the size and appearance, however the importance of site, grade of dysplasia and exposure to environmental carcinogens remains controversial. Patients with a diagnosis of oral epithelial dysplasia (OED) were recruited to a longitudinal observational cohort study in a tertiary oral dysplasia clinic. Clinical, histopathological and risk factor data were assessed to identify clinical determinants of malignant transformation; in patients where the oral dysplasia underwent malignant transformation the treatment outcomes were evaluated.

Ninety-eight eligible patients were recruited and followed up for the median duration of 48 months. Twenty-six (26.5%) patients underwent malignant transformation, with significant predictors as follows: non-smoking status, lateral tongue subsite, non-homogeneous appearance, high histological grade and size of lesion $\geq 200\text{mm}^2$. Whilst these clinical features have previously been associated with malignant transformation in OED, the highly statistically significant finding of malignant transformation in non-smokers, consistent with several cohorts reported in the recent years. This suggests that these patients, apparently with endogenous OED, perhaps with inherited or acquired predisposition, are considered to be at higher risk (compared to smokers) and should form the focus for further investigation.

All 26 patients in whom dysplasia transformed to oral squamous cell carcinoma were presented to the multidisciplinary team (MDT) with stage 1 disease. Ninety-one percent (21/23) were initially treated by wide local excision and 9% (2/23) required tumour resection followed by reconstruction. Twenty-two percent (5/23) of patients developed second primary OSCC, and further diagnoses of oral dysplasia with an estimate that 76% of patients will undergo one or more event in 5 years. Specialist monitoring of OED by a multidisciplinary

team allows detection of OSCC at an early stage, facilitating largely curative treatment with simple and usually minor surgical intervention. The high incidence of second primary OSCC formation in high risk patients with OED supports an intensive targeted surveillance regimen in this group of patients.

The observed higher rate of malignant transformation in non (or light)-smokers supports the hypothesis of an endogenous aetiology. It is hypothesised that loss of FANCD2 and associated proteins lead to genomic instability and oncogenesis.

Longitudinal archival samples were obtained from 40 individuals with OED from time of diagnosis to the most recent clinical review in 23 non-transforming/stable OED or until excision of the SCC in 17 unstable OED undergoing malignant transformation.

Histopathological reassessment, immunohistochemistry for FANCD2 and Western blotting for phosphorylation/mono-ubiquitination status of ATR, CHK1, FANCD2 and FANCG were undertaken on each tissue sample.

Immunohistochemistry studies found under-expression of FANCD2 was observed in the diagnostic biopsy of OED lesions which later underwent malignant transformation when compared with stable OED. Combining the FANCD2 immunohistochemistry scores with histological grading more accurately predicted malignant transformation ($p=0.005$) than histology alone and correctly predicted malignant transformation in 10/17 initial biopsies. A significantly reduced expression of total FANCD2, pFANCD2, pATR, pCHK-1 and pFANCG were observed in OED which underwent malignant transformation.

There is strong evidence that defects in the DNA damage sensing-signalling-repair cascade are associated with malignant transformation in OED. Loss of post-translational modification in FANCD2 and related proteins, was more predictive of malignant transformation when compared to clinical parameters.

Index of contents

Acknowledgements.....	2
Declarations.....	3
Grants and financial support.....	3
Index of contents	7
List of Tables	10
List of Figures.....	12
Chapter 1 Introduction: Challenges in the management of oral epithelial dysplasia and the role of the Fanconi Anaemia Pathway in oncogenesis.....	16
Introduction	16
Classification of oral epithelial dysplasia	16
Challenges in the management of oral epithelial dysplasia	17
The Fanconi Anaemia Pathway and oncogenesis.....	19
Aims of thesis	24
Objectives of thesis	25
Statement of hypothesis.....	26
Chapter 2 The clinical determinants of malignant transformation in oral epithelial dysplasia.....	27
Introduction	27
Patients and methods	28
Results.....	29
Discussion.....	38
Chapter 3 Outcomes of oral squamous cell carcinoma arising from oral epithelial dysplasia: the rationale for monitoring of oral premalignant lesions in a multidisciplinary clinic.....	44
Introduction	44
Patients and methods	45
Results.....	45
Discussion.....	48

Chapter 4 Surgical management of oral dysplasia	54
Introduction	54
CO2 Laser surgery for oral lesions with malignant potential.....	55
Aims and objectives	65
Results	65
Discussion.....	67
Chapter 5 The Liverpool Multidisciplinary Approach to the Management of Oral Epithelial Dysplasia.....	69
Introduction	69
The challenges associated with the management of oral epithelial dysplasia.....	70
The Diagnosis of Oral Epithelial Dysplasia.....	70
Epidemiological, Clinical & Histological Factors as Predictors of Malignant Transformation in Oral Epithelial Dysplasia.....	71
The Management of Oral Epithelial Dysplasia	72
Surveillance of Patients with Oral Epithelial Dysplasia	75
The Liverpool Multidisciplinary Approach to the Management of Oral Epithelial Dysplasia	76
The establishment of the Multidisciplinary Oral Dysplasia Clinic .	76
Conclusion.....	79
Referral of Patients with suspicious oral lesions.....	80
Assessment of Patients	80
The General Management of Patients with Oral Epithelial Dysplasia.....	81
Severe Dysplasia.....	81
Moderate Dysplasia.....	82
Mild Dysplasia.....	82
Surveillance of Patients with Moderate or Severe Dysplasia.....	83
Surveillance and Follow-up of Patients with Mild Dysplasia.....	83

Chapter 6 Loss of FANCD2 and related proteins may predict malignant transformation in oral epithelial dysplasia	85
Introduction	85
Patients, materials and methods	87
Patients.....	87
FANCD2 Immunohistochemistry	88
Western blotting	89
Data/Statistical Analysis	91
Results	92
Patient outcomes	92
FANCD2 immunohistochemistry	94
FANCD2 and associated protein expression	99
Discussion.....	102
Chapter 7 Conclusion: Proposals for the future management of oral epithelial dysplasia - striving for a personalised approach.....	109
Further work	111
References	114
Appendix.....	126
Publications from thesis.....	126
Peer reviewed publications:	126
Book chapter:	126
Oral presentations/abstracts and posters from doctorate	127
Invited faculty/lectures	127
Oral Peer-Reviewed Abstracts.....	127
i. International meetings:	127
ii. National meetings:.....	128
Poster presentations	128

List of Tables

Table 2. 1	Baseline information of 98 patients at initial presentation	30
Table 2. 2	Two and five-year Kaplan-Meier transformation rates % (Standard Error(SE)) .	32
Table 2. 3	Number of patients who experienced malignant transformation of clinical OED/PVL for selected parameters (n=98 unless specified).	35
Table 2. 4	Final model of independent predictors for the 98 patients using multi-variable Cox regression.	36
Table 2. 5	Kaplan-Meier analyses with regard to smoking status and lesion appearance. 2 and 5-year Kaplan-Meier transformation rates (SE).	36
Table 2. 6	Comparison of treatment intent for OED which have undergone malignant transformation vs. stable OED.....	38
Table 2. 7	Total number of biopsies (and surgical excision), number of clinic attendances and follow-up duration for 98 patients.....	38
Table 3. 1	Summary of demographic and treatment outcomes of 23 patients in whom OED underwent malignant transformation (OSCC) MT - malignant transformation, FOM - floor of mouth, WLE - wide local excision (laser or cold steel), NDFFR - tumour resected with neck dissection and microvascular free tissue transfer reconstruction; D=discharged, UF=under follow-up, LF=lost to follow-up.....	47
Table 3. 2	Data from early OSCC from OED cohort from this study and the Liverpool 1992-2002 OSCC cohort(1): treatment details and disease related outcomes.	49

Table 4. 1	Summary of treatment outcomes of oral premalignant lesions with CO2 laser (* follow-up reported in years).....	66
Table 5. 1	High Risk, Predictive Features for Malignant Transformation in the Liverpool Cohort(97).....	72
Table 5. 2	Recurrence and Malignant Transformation Rates following Surgical Intervention for Oral Leukoplakia	74
Table 6. 1	FANCD2 immunohistochemistry scoring system	89
Table 6. 2	Antibodies used in Western blot.....	91
Table 6. 3	Demographic and clinic-pathological features of patients (n = 40) (* at initial diagnostic biopsy)	93
Table 6. 4	FANCD2 immunohistochemistry scores in first diagnostic biopsy of non-transforming (NT) and transforming (T) OED, together with paired OSCC for the transforming group	97
Table 6. 5	FANCD2 IHC-OED grade combined scores in stable OED (NT), OED which underwent malignant transformation (T) and oral squamous cell carcinoma which had arisen from OED.	98
Table 6. 6	Normalised protein expression in initial diagnostic biopsy of NT OED and T OED lesions	100

List of Figures

Figure 1. 1 Activation of the Fanconi anaemia pathway. DNA damage such as interstrand cross-links can cause the replication fork to stall, creating single-stranded regions coated by RPA. This leads to activation of the checkpoint kinase ATR and its downstream effector kinase CHK1. ATR and CHK1 phosphorylate several components of the Fanconi anaemia (FA) core complex and the FANCI–FANCD2 (ID) complex. Phosphorylation of FANCI (solid red circles) may act as a switch, making the ID complex ready for ubiquitination. Phosphorylation of FANCD2 and Fanconi anaemia core components (broken pink circles) affects the efficiency of, but is not essential for, ID ubiquitination by the FA core complex, together with E1 and UBE2T. Analogously, ubiquitination of FANCD2 (solid orange ovals) is essential for DNA repair, activating the ID complex for chromatin binding; on the other hand, FANCI ubiquitination (broken orange oval) affects the efficiency of, but is not essential for, DNA repair. FANCM also has a direct role in DNA repair, as a branch point translocase for stalled replication forks and four-way junctions(48). *Reproduced with permission from the author and publisher: A major switch for the Fanconi anemia DNA damage–response pathway. Weidong Wang. Nature Structural and Molecular Biology 2008. 15(11) 1128-9.21*

Figure 1. 2 Fanconi pathway safeguards chromosomal stability in the repair of inter-strand crosslinks by facilitation of homologous recombination repair and suppression of non-homologous end joining (NHEJ).22

Figure 2. 1 Kaplan-Meier curves for malignant transformation rates: smoking history.....33

Figure 2. 2 Kaplan-Meier curves for malignant transformation rates: appearance of lesion

Figure 2. 3	Kaplan-Meier curves for malignant transformation rates: grade/severity of dysplasia ('others' excluded)	37
Figure 2. 4	Leukoplakia lateral border of tongue in lifelong non-smoker, histology shows severe dysplasia: high risk of malignant transformation (approx. 40-50%).	41
Figure 2. 5	Floor of mouth lesion in heavy smoker with moderate risk (approx. 10-15%) of malignant transformation.....	42
Figure 3. 1	Left lateral tongue microinvasive SCC: pre-treatment (left) and complete healing following excision with laser (right)	50
Figure 3. 2	Right lateral tongue severe dysplasia (top left) underwent malignant transformation to microinvasive SCC (top right) with complete healing following laser excision (bottom centre).....	51
Figure 3. 3	Time from date of malignant transformation to first occurrence date of any of second event (local recurrent SCC, new primary, SCC, further dysplasia, death) or in the absence of an event to the date lost to follow-up.....	52
Figure 4. 1	Non-homogenous premalignant lesion on the lateral tongue in a non-smoker (high risk lesion).	56
Figure 4. 2	Lateral tongue wound left to heal by secondary intention following laser excision of mucosal layer down to intrinsic muscles of tongue with excellent haemostasis. ..	56
Figure 4. 3	Preoperative appearance of homogenous right buccal mucosal lesion (severe dysplasia). 58	
Figure 4. 4	Three-year follow-up appearance of well healed scar following excision.	58
Figure 4. 5	Erythroleukoplakia anterior floor of mouth in a smoker (high risk lesion).	59

Figure 4. 6 Appearance of lesion following staining with Lugol's Iodine solution: area of pallor indicates mucosal dysplastic change.60

Figure 4. 7 Peripheral excision margin delineated with CO2 laser 5mm from area highlighted by Lugol's Iodine staining (1mm beam spot, 6W super-pulsed continuous setting): precise cut edge with excellent haemostasis allowing clear visibility.61

Figure 4. 8 The submandibular ducts were carefully dissected out and divided to achieve safe excision margins. The proximal cut ends were then secured with 6/0 monofilament sutures to prevent retraction and aid localisation.....62

Figure 4. 9 The submandibular ducts positioned posteriorly through a stab incision in the floor of mouth mucosa with a No. 11 scalpel blade and inset in place with 6/0 monofilament sutures. The wound was left to heal by secondary intention following CO2 laser excision. ...63

Figure 4. 10 Left ventral tongue polypoidal mass ("over granulation") can sometimes occur during healing following laser excision.64

Figure 5. 1 A clinical dilemma: Patient with lesions on tongue, demonstrating both high and low risk predictive factors for malignant transformation.....78

Figure 5. 2 The Management Algorithm for cases of Oral Epithelial Dysplasia used by the Liverpool Multidisciplinary Team.79

Figure 6. 1 Representative example of FANCD2 immunohistochemistry during progression to carcinoma. A: lack of FANCD2 expression in normal (non-dysplastic) epithelium; widespread and moderate staining in the nucleus and cytoplasm of suprabasal (differentiating) layers in non-invasive dysplastic epithelium and B: loss of FANCD2 expression when malignant transformation has occurred. Magnification x400.....95

Figure 6. 2 Representative FANCD2 immunohistochemistry of ‘low risk’ dysplasia from NT OED (left): moderate dysplasia displaying strong nuclear and cytoplasmic expression of FANCD2 across the entire thickness of epithelium with widespread distribution and ‘high risk’ T OED (right): severe dysplasia with weak and irregular expression FANCD2 in the nucleus and cytoplasm of especially in the basal two-thirds of the epithelium with multifocal distribution . Top row : low power magnification (x100); bottom row: high power magnification (x400).....96

Figure 6. 3 Representative example of Western blot expression analysis of DNA damage sensing and repair proteins. NT: first diagnostic biopsy of a non-transforming OED; T: first diagnostic biopsy of a transforming OED; Oe: normal oesophagus; CT: control OSCC from a different cohort; CN: control (morphologically) normal from margin of OSCC resection.101

Chapter 1 Introduction: Challenges in the management of oral epithelial dysplasia and the role of the Fanconi Anaemia Pathway in oncogenesis

Introduction

Oral epithelial dysplasia falls within the group of oral lesions with malignant potential. The diagnosis is achieved after histopathological assessment following biopsies of oral lesions which can vary in appearance: homogenous white patch (leukoplakia), red patch (erythroplakia), non-homogenous lesion (erythroleukoplakia) or an erosive or ulcerated lesion. The management of oral epithelial dysplasia is clinically important as it offers the clinical team the opportunity to detect any progression in the grade of dysplasia or more importantly malignant transformation. Despite advances in the management of oral cancer, the main determinant in survival outcomes of treatment is primarily dictated by the stage of oral cancer(1). In tumours which evolve within a step-wise progression model through oral epithelial dysplasia before malignant transformation, timely intervention combined with close clinical surveillance offers the opportunity to optimise survival and functional outcomes whilst utilising simpler treatment modalities i.e. surgery as single modality often without the need for combined modality (adjuvant radiotherapy or chemoradiotherapy).

Classification of oral epithelial dysplasia

The main classification utilised is the 2017 World Health Organisation (WHO) classification where the severity of dysplasia is graded mild, moderate or severe(2). The binary system(3) of low and high grade dysplasia, which was devised to improve clinical decision making and reduce variation of reporting amongst pathologists, has been included as one of the recognised classification systems for oral epithelial dysplasia in the 2017 WHO classification.

Several other less commonly utilised systems include the classifications suggested by Banoczy and Csiba(4), Lumerman et al.(5), Speight et al.(6), Kuffer and Lombardi (oral intraepithelial neoplasia)(7), and Brothwell et al(8). Malignant transformation of oral leukoplakia has been reported as occurring in 0.13-17.5% (9, 10) of cases, over a period of 6 months to 30 years; this wide range is likely to be due to differences in patient groups, length of follow up and treatment modality between studies. It is an interesting observation that, in population studies, 20.1-42.5% of untreated leukoplakia resolved in 5 years(11). Non-homogenous lesions are more likely to undergo malignant transformation (9, 10) and up to seven-fold increase in malignant transformation risk has been reported (12, 13). Around 90% of erythroplakia showed evidence of severe dysplasia or carcinoma on histology(14). The wide difference in outcome indicates the need for histological diagnosis (as opposed to clinically descriptive terms such as leukoplakia or erythroplakia, which could encompass a wide range of diagnoses).

Challenges in the management of oral epithelial dysplasia

Clinicians face several challenges in the management of oral epithelial dysplasia:

1. The number of patients with oral dysplasia managed within secondary care is relatively small and when managed by multiple clinicians from different specialties e.g. oral and maxillofacial surgery, oral medicine, oral surgery or otolaryngology, indications and thresholds for intervention can vary. The need for involvement of multidisciplinary team members i.e. Oral and Maxillofacial Surgery, Oral Medicine, Oral Pathology, Oral Surgery and Biomedical Scientists has been adopted in teaching institutions, however such a set-up can be perceived as resource intensive and might

not be readily available to many secondary care units receiving primary care referrals for such conditions(15).

2. The clinical appearance of oral epithelial dysplasia is similar to various oral lesions e.g. hyperkeratosis, lichenoid reaction, lichen planus or early oral cancer.
3. The evidence for interventions and treatment remain low level despite the decades of experience and increasing number of publications in the literature(16):
 - a. A considerable number of publications still utilise to the term or diagnosis of leukoplakia (or erythroplakia) as the main representation of oral epithelial dysplasia, making extrapolation of findings difficult and limits the generalisability of the reported findings in contemporary practice within a secondary or tertiary setting(17-19),
 - b. Most of the published literature report case series with limited numbers from single centres(4, 20-22),
 - c. The severity of the grade of dysplasia is does not correlate consistently with the clinical behaviour of the oral dysplasia in terms of malignant transformation and
 - d. Although there are clinical features which could be correlated to a higher risk for malignant transformation, this can vary depending of the population studied and perhaps could be influenced by regional endogenous or environmental factors.
4. Most studies aimed at identifying a molecular or pathological marker of malignant change have failed to undertake correlation with longitudinal clinical outcomes, so their translational value has been diminished leading to the lack of clinical application (23-25). Furthermore, most of the reported studies correlate more with the severity

of the grade of dysplasia(26-28) rather than malignant transformation and this approach is difficult as event rate of malignant transformation is low, and studies are therefore prolonged(20).

5. The progression of oral epithelial dysplasia to malignancy can take several years (up to 10-15 years). This low event rate makes time-defined clinical trials utilising malignant transformation as an end-point less feasible for funding. There is a notable paucity of multi-centred/collaborative protocols, and where malignant transformation can be predicted there remains some uncertainty about recommended treatment options.
6. The duration of follow-up for oral epithelial dysplasia remains a subject which generates controversy(20). In a resource constrained climate, the clinical justifications for review of a benign condition needs to be evidence-based. Similar challenges have been faced by gastroenterologists managing Barrett's oesophagus, where patients with the estimated risk of malignant transformation of 0.5% per annum undergo interval surveillance by endoscopy of the upper gastrointestinal tract(29).

The Fanconi Anaemia Pathway and oncogenesis

Several cancer systems, including head and neck squamous cell carcinomas have been found to have dysregulation of the Fanconi Anaemia (FA) pathway(30) The incidence of head and neck squamous cell carcinoma (HNSCC) in patients with the cancer prone syndrome, Fanconi Anaemia, is up to 1400 times that of the general population (31-33) and occurs earlier in life. The Fanconi Anaemia pathway (FAP) facilitates homologous recombination repair of DNA double-strand breaks and is an integral component of the DNA damage repair mechanism

which maintains genomic stability in healthy individuals (34, 35). Defects in the FA pathway leads to increased cancer susceptibility(30). FA is a rare genetic condition, whereby there is a defect in the DNA repair mechanism due to a mutation in a FA gene(36), resulting in bone-marrow failure, developmental abnormalities and increased cancer susceptibility due to cellular sensitivity to cross-linking agents(35). Higher risks of developing cancers such as acute myeloid leukaemia and squamous cell carcinoma have been observed in at least 20% of FA patients(32, 37). The incidence of oral leukoplakia in FA patients who have not undergone haematopoietic stem cell transplant is reportedly high and it has been suggested that this should be included as part of the phenotype of this group of patients and screening for oral leukoplakia should form part of the regular surveillance strategy due to the high risk of head and neck (oral) cancer(38).

The FA pathway is a DNA repair pathway, which responds to DNA damage including errors during replication or DNA cross-linking agents such as diepoxybutane, mitomycin C and cisplatin(39). Diepoxybutane/mitomycin C stress test based on the increased chromosomal instability seen in FA cells, compared to normal controls, remains the front-line diagnostic test. Cisplatin is a first-line chemotherapeutic agent in primary non-surgical management of head and neck cancer. The status of the FAP has been identified as a potential major predictor of response to treatment with cisplatin (as a radiation sensitizer) when utilised with radiotherapy in the treatment of head and neck cancer(40). The FAP consists of at least 22 proteins and has 3 main components (41) (Figure 1.1): (i) the core complex which consists of proteins FANCA, B, C, E, F, G, L and M, the main role of which is to facilitate ubiquitination of (ii) FANCD2 and FANCI protein dimers which then activate (iii) five downstream effector proteins (FANCI, BRCA1, RAD51, FANCD1/BRCA2) that directly participate in DNA damage repair(30). Within the overall DNA damage response, the FAP interacts with the DNA

damage sensing and signalling proteins, ataxia telangiectasia rad-3 (ATR), ataxia telangiectasia mutated (ATM) and checkpoint 1 (Chk1) kinases (42, 43). Major sites of ATR-mediated phosphorylation are found on FANCA, FANCD2, FANCG and FANCI (44-47). Mutations or disruptions in any of these FANC proteins can lead to chromosome instability and cross-linker hypersensitivity(30) .

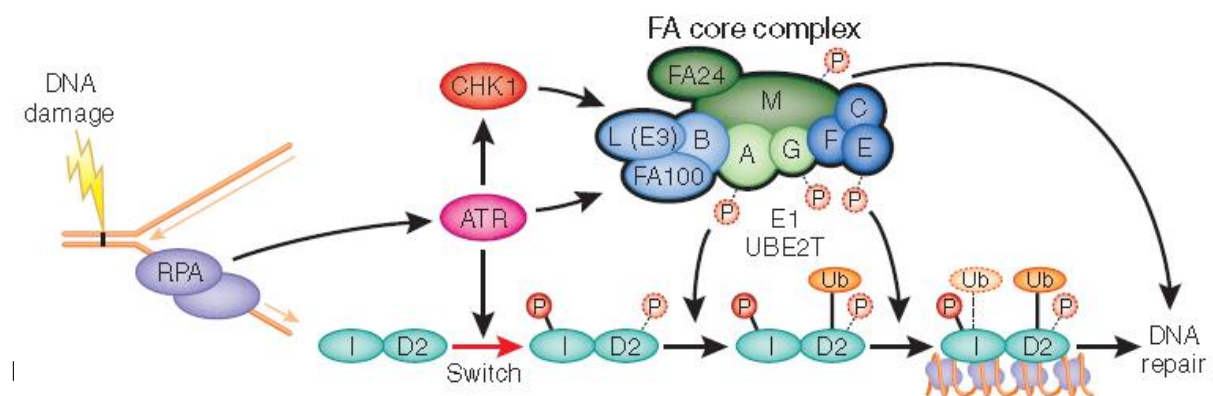


Figure 1. 1 Activation of the Fanconi anaemia pathway. DNA damage such as interstrand cross-links can cause the replication fork to stall, creating single-stranded regions coated by RPA. This leads to activation of the checkpoint kinase ATR and its downstream effector kinase CHK1. ATR and CHK1 phosphorylate several components of the Fanconi anaemia (FA) core complex and the FANCI–FANCD2 (ID) complex. Phosphorylation of FANCI (solid red circles) may act as a switch, making the ID complex ready for ubiquitination. Phosphorylation of FANCD2 and Fanconi anaemia core components (broken pink circles) affects the efficiency of, but is not essential for, ID ubiquitination by the FA core complex, together with E1 and UBE2T. Analogously, ubiquitination of FANCD2 (solid orange ovals) is essential for DNA repair, activating the ID complex for chromatin binding; on the other hand, FANCI ubiquitination (broken orange oval) affects the efficiency of, but is not essential for, DNA repair. FANCM also has a direct role in DNA repair, as a branch point translocase for stalled replication forks and four-way junctions(48). *Reproduced with permission from the author and publisher: A major switch for the Fanconi anemia DNA damage–response pathway. Weidong Wang. Nature Structural and Molecular Biology 2008. 15(11) 1128-9.*

FANCD2 (or FANCD2/FANCI heterodimer) activates the DNA repair pathway once it has been phosphorylated and monoubiquitinated (S331 and K561 respectively) (49). Homologous recombination is the main method of DNA damage repair that the FA signalling pathway selects. However, in FANC deficient cells, attempted repair of lesions occurs through the

error-prone repair process of nonhomologous end joining (NHEJ). This method increases the chances of mutagenesis occurring in FANCD1 deficient cells(37) (Figure 1.2).

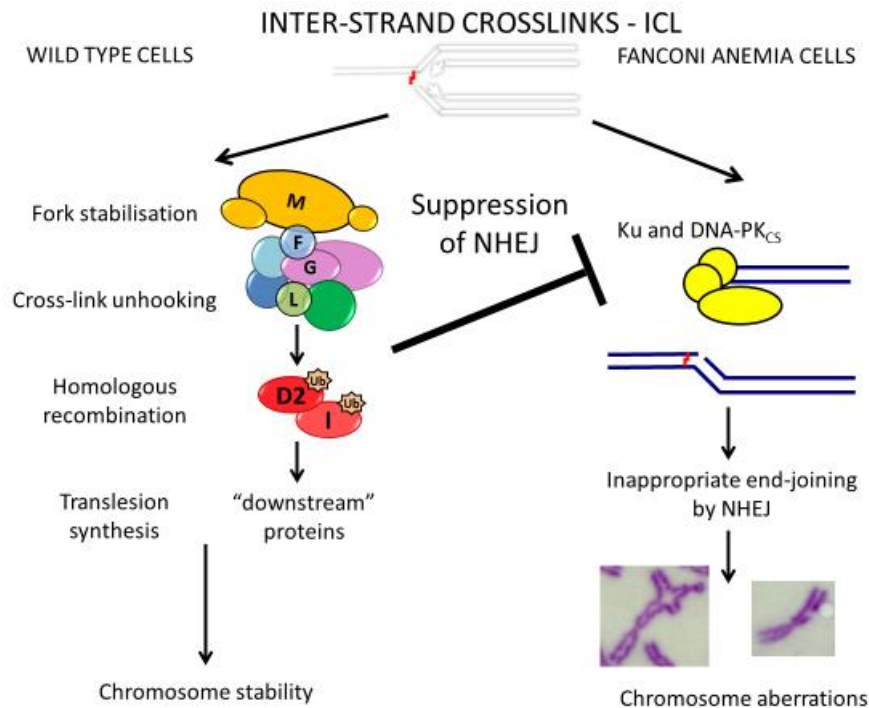


Figure 1.2 Fanconi pathway safeguards chromosomal stability in the repair of inter-strand crosslinks by facilitation of homologous recombination repair and suppression of non-homologous end joining (NHEJ).

The activation of the FANCD1 proteins, which are part of the FA pathway, is brought about by CHK1 (checkpoint kinase) phosphorylation by ATR (Ataxia–telangiectasia and Rad3–related) kinase, which in turn phosphorylates the FANCD1 proteins (Figure 1.1). CHK1 is a checkpoint kinase that regulates FA cells at the G2/Mitosis checkpoint in the cell cycle(30). DNA damage that causes stalled replication fork progression leads to ATR kinase activation of CHK1; CHK1 responds by phosphorylating Cdc25c which halts the transition of the cells from G2 into mitosis. FA tumour cells mainly acquire DNA damage during the S phase of the cell cycle,

when DNA repair through the FAP occurs in non-FA cells(30, 50). ATR phosphorylates CHK1 whereas ATM (ataxia-telangiectasia mutated) phosphorylates CHK2. ATM is part of a DNA repair pathway that runs parallel to the ATR pathway(35). ATM works with ATR to repair double-strand breaks in DNA in NHEJ. ATR phosphorylates several proteins: CHK1, FANCA, FANCI, FANCD2 and FANCG. The phosphorylation of CHK1 by ATR then leads to the activation of FANCE(43, 51, 52). Both CHK1 and CHK2 phosphorylate the FANCD2 protein at different sites, however phosphorylation of one site (S331 – serine residue at position 331) is essential for activation(43). The ATR pathway is of interest because when CHK1 is phosphorylated it in turn phosphorylates FANCD2 leading to ‘activation’ of the protein and the downstream processes in DNA damage repair. Previous studies have shown that the site of phosphorylation of the FANCD2 protein is S331(43). Another functional post translational modification of interest is the phosphorylation site located at S7 in FANCG, as this has been shown to be important in optimal homologous recombination repair(53). Therefore, these post-translational modifications can be used as indicators for the activation of the FA-DNA repair pathway. Such post-translational modifications can be detected by using antibodies against phosphorylated states of the FANC proteins.

In evaluating the activation of the FA pathway, the FANCD2 (or FANCI) protein can be used as an indicator of FA pathway status(49). FANCD2 is both monoubiquitinated and phosphorylated in response to DNA damage. It has been observed that the process leading to early occurrence of oral cancer in FA patients follows a similar pathway as in non-FA cancer patients, which would support a caretaker function for FA genes in the protection against oral carcinogenesis(54). It is therefore hypothesised that failure to activate FANCD2 and/or related proteins would be observed in the OED samples which subsequently underwent

malignant transformation. These phosphorylated sites could potentially be utilised as predictive indicators in oral epithelial dysplasia, in conjunction with clinical and histological assessment to determine whether malignant transformation is likely to occur. This could enable personalisation of patient management, supporting the decision for clinical intervention or observation.

Aims of thesis

- Identify the clinical determinants of malignant transformation in OED for patients managed in the Merseyside Tertiary Oral Dysplasia Multidisciplinary Team (MDT) clinic in Liverpool Dental Hospital,
- Evaluate treatment outcomes of oral squamous cell carcinoma (OSCC) which has arisen from OED in the cohort of patients managed within the Oral Dysplasia MDT clinic in Liverpool Dental Hospital,
- Identify the best surgical technique employed in the management of oral epithelial dysplasia in contemporary management of OED in contemporary clinical practice,
- Establish a rational MDT approach in the management of OED, to personalise management algorithm and ensure effective utilisation of resource with the National Health Service (NHS),
- Investigate the status of the Fanconi Anaemia Pathway in OED and this impacts on the risk of malignant transformation and
- Propose future areas of research and innovation in clinical practice which will enable a personalised approach in the management of OED.

Objectives of thesis

- Identify patients have been prospectively recruited into a molecular biomarker study (Sefton Research and Ethics Committee ethical approval Reference number: EC 47.01) and collate their clinicopathologic data and management outcomes in the Oral Dysplasia MDT clinic through their medical records,
- Identify the clinical risk factors, from clinical outcomes data, for malignant transformation in patients with OED and establish the rate of malignant transformation in this cohort of patients,
- Compare the clinical determinants of malignant transformation in OED in this cohort against the published evidence in the literature to ascertain the clinical relevance and inform future practice,
- Investigate the treatment outcomes (treatment modalities, locoregional recurrence and survival) of patients with OED which have undergone transformation to OSCC and compare their treatment outcomes with patients who have presented with OSCC seen through the fast track suspected cancer pathway (i.e. OSCC which has arisen *de-novo*),
- Review the published literature for published series of surgical management of OED with laser excision and/or ablation, evaluate their treatment outcomes to inform best evidenced clinical practice with regard to their impact on malignant transformation in OED, recurrence of OED, how these techniques impact on tissue archiving initiatives and their medicolegal implications,
- Provide clinical expert guidance in areas of importance in clinical practice i.e. diagnosis, indications for intervention and surveillance protocols, based on the

findings above and decades of cumulative experience within the Oral Dysplasia MDT members, with proposal for a clear strategy in the management of OED,

- Access archival formalin fixed tissue of patients with OED for immunohistochemical expression of FANCD2 protein and extract proteins from these tissue blocks to investigate the status of activation of the FAP-DNA damage repair pathway in these patients,
- Report on the findings of the translational work conducted in a subgroup of patients from the clinical outcome cohort to clarify the status and role of the Fanconi Anaemia Pathway and related proteins in malignant transformation of oral epithelial dysplasia and
- Summarise the findings of this thesis and contextualise them within the current landscape of clinical practice and policies for future development in the NHS, within the framework of recommendations by the Royal College of Surgeons of England.

Statement of hypothesis

It is therefore hypothesized that in OED which has undergone malignant transformation, in the absence of environmental risk factors, an endogenous drive exists which leads to carcinogenesis and this we propose is a defect in the Fanconi Anaemia and its related pathways.

Chapter 2 The clinical determinants of malignant transformation in oral epithelial dysplasia

Introduction

The clinical significance of oral epithelial dysplasia (OED) lies in its association with malignant transformation into oral squamous cell carcinoma (OSCC)(55). OED can present clinically as leukoplakia: homogenous(flat, thin, uniform white) and non-homogenous(white and red/erythroleukoplakia, speckled or nodular, verrucous leukoplakia)(56), or erythroplakia(57). OED can only be diagnosed histologically and in the WHO 2017(2) it is divided into mild, moderate, severe and carcinoma-in-situ, although it represents a spectrum of epithelial change rather than distinct categories. In order to reduce interobserver variability and improve clinical correlation, a binary system(58) has been suggested: low-risk (mild & moderate dysplasia) and high-risk (severe dysplasia and carcinoma-in-situ). The risk of malignant transformation has been reported to be between 6.6-36.4%, systematic review and meta-analysis found the rate to be 12.1%(5, 9, 20, 59, 60). Risk factors associated with malignant transformation include: female gender(10), longer duration of lesion(9, 18), idiopathic leukoplakia(non-smokers) (9, 18, 61), subsite of tongue and/or floor of mouth(61-64), size $\geq 200\text{mm}^2$ (21, 56, 65), non-homogenous appearance(9, 21), higher grade of dysplasia(10, 20, 66), verrucous subtype(5, 9, 67, 68) and the presence of multiple lesions(69). Surgical excision(including laser) has been the preferred modality of treatment in the management of OED(70), despite the lack of randomized controlled trial evidence(16) and recurrence rates of up to 35% have been reported(18). However, lesions that were not excised demonstrated a higher rate of malignant transformation compared with those that were excised(20, 71). Most of the reported literature have presented outcome data on oral leukoplakia/premalignant lesions, with only a few reporting clinical outcomes of OED(5, 59,

61, 62, 66, 71, 72). The purpose of this study was to evaluate the clinical factors that determined the risk of malignant transformation of OED into OSCC in a tertiary Regional Oral Dysplasia Clinic.

Patients and methods

Inclusion criteria

Patients who attended the oral dysplasia clinic with lesions clinically suspicious of OED were prospectively recruited into a clinical and molecular biomarker study since September 1999 and their demographic and pathology details were entered into a computerised database. Ethical approval for this study was given by Sefton REC (now **North West – Liverpool Central**; reference number: EC 47.01). Consent was obtained for retrospective and prospective analysis of tissue samples and clinical records. The principal inclusion criteria were biopsy proven OED or proliferative verrucous leukoplakia (PVL). Any patient with a history of oral squamous cell carcinoma (OSCC), less than 2 years preceding or coincident, with OED was excluded.

Data collection

The following baseline information were recorded: demographic details (age, gender), smoking and alcohol history, site and clinical appearance of lesion (homogenous or non-homogenous), approximate size of lesion (measured as total surface area from clinical photographs, as documented in medical records or measurement of specimen when lesion was surgically excised), number of lesions (single or multiple), histopathological diagnosis of initial biopsy, history of previous OSCC and initial treatment (clinical observation or surgical excision: with definitive diagnosis) . The following longitudinal follow-up data were collected throughout the duration of clinical follow-up: clinical progression of dysplasia grade or malignant transformation (confirmed by biopsy), total number of biopsies/surgical excision

carried out, number of visits to the dysplasia clinic, endpoint clinical behaviour of OED/PVL lesion: remission/ progression of dysplasia severity/ malignant transformation, length and status of follow-up at most recent review: under follow-up, lost to follow-up or discharged. Statistical method: Statistical analysis was carried out using the SPSS (Statistical Package for the Social Sciences) software, IBM Corporation. Kaplan-Meier methods were used to estimate the percentage of patients who underwent malignant transformation of OED/PVL after two and five years, and the log-rank test was used to compare transformation survival curves. Cox regression methods were used to investigate the main independent predictors of survival. Hazard ratios (HR) with 95% confidence interval (95% CI) and p values were reported ($p < 0.05$ was considered statistically significant).

Results

145 patients were identified from the database of consented patients. The medical records of 25 patients were not retrievable, 10 patients did not have a diagnosis OED/PVL and in 12 patients biopsy of the clinical lesion contained OSCC. Therefore 98 eligible patients (with biopsy confirmed OED/PVL) were included for this analysis. The first clinic appointments for these patients were between 11 May 1984 and 16 March 2011.

The baseline information of all patients included in the study is presented in Table 2.1.

		Number of patients (%) n=98
Gender	Male	48(49)
	Female	50(51)
Age (at first appointment)	<50.0	26(26.5)
	50.0-59.9	39(39.8)
	≥60.0	33(33.7)
Site of OED	Floor of mouth (FOM)	40(40.8)
	Buccal mucosa	19(19.4)
	Lateral tongue	15(15.3)
	Others:	24(24.5)
	Soft palate	7
	Mandibular alveolar/gingival	6
	Maxillary alveolar/gingival	2
	Ventral tongue	5
	Dorsum tongue	1
	Hard palate	1
	Tonsillar	1
Size of OED (largest in cases with multiple lesions) *	< 200mm ²	39(40.6)
	≥ 200 mm ²	57(59.4)
Diagnosis (first biopsy)	Mild dysplasia	33(33.7)
	Moderate dysplasia	26(26.5)
	Severe dysplasia	19(19.4)
	Proliferative verrucous leukoplakia	7(7.1)
	Others**	13(13.3)
Number of lesions	Single	74(75.5)
	Multiple	24(24.5)
Clinical appearance	Homogenous white	70(71.4)
	Others:	28(28.6)
	Erythroleukoplakia	19
	Erosive (red patch)	7
	Ulcer	2
Smoking history	Never or <5 pack years	23(23.5)
	5-20 pack years	30(30.6)
	>20 pack years	45(45.9)
Alcohol consumption	Teetotal or <5 units per week	47(48.0)
	5-20 units per week	28(28.6)
	>20 units per week	23(23.5)
Previous OSCC	Yes	5(5.1)
	No	93(94.9)

* not known for 2 patients therefore n=96(100%) for this parameter. The size of OED was derived from a combination of photographic recorded dimensions, clinical notes and pathology records from resected lesions.

**includes chronic hyperplastic candidiasis, lichenoid changes and lichen planus which subsequently when re-biopsied showed features of OED/PVL

Table 2. 1 Baseline information of 98 patients at initial presentation

At the most recent clinical review, the endpoint of OED/PVL lesion were: 26(26.5%) patients had malignant transformation, 6(6.1%) had progressed to a more severe grade of dysplasia and 66(67.4%) were either stable or had resolved spontaneously. Excluding the PVL patients (based on the published diagnostic criteria(67, 73)), 78 patients had a diagnosis of OED from their initial biopsy and within this subgroup, 19 (24.4%) underwent malignant transformation. Overall, the median time from malignant transformation from first clinic appointment was 47.7 months (IQR 16.3-83.9). Three patients underwent malignant transformation from PVL with median transformation time of 33.5 months (IQR 24-145).

Patient factors were analysed with respect to malignant transformation rate of OED/PVL by univariate Kaplan-Meier analyses to compare the transformation curves and estimate the percentage of patients who had malignant transformation after two and five years (Table 2.2).

		Transformation rate estimate			
		Patients	2yr	5yr	Log rank test
	TOTAL	98	12 (4)	23 (5)	
Gender	Male	50	8 (5)	18 (8)	$\chi^2=0.4$ p=0.50
	Female	48	16 (5)	27 (7)	
Age	<50	26	4 (4)	16 (9)	$\chi^2=2.5$ p=0.29
	50-59	39	14 (7)	20 (8)	
	60+	33	17 (7)	33 (10)	
Site	Floor of Mouth	40	3 (3)	8 (6)	$\chi^2=13.0$ p=0.005
	Buccal	19	6 (6)	24 (13)	
	Lateral Tongue	15	29 (12)	53 (16)	
	Other	24	18 (8)	24 (10)	
Size of lesion*	<200 mm ²	39	7 (5)	13 (7)	$\chi^2=3.9$ p=0.05
	200+ mm ²	57	16 (5)	30 (7)	
Diagnosis (first biopsy)	Mild	33	3 (3)	19 (9)	$\chi^2=7.7$ p=0.05
	Moderate	26	0 (-)	17 (11)	
	Severe	19	38 (12)	38 (12)	
	Other	20	18 (10)	24 (11)	
Number of lesions	Multiple	24	18 (8)	26 (11)	$\chi^2=0.4$ p=0.55
	Single	74	10 (4)	22 (6)	
Appearance	Homogenous white	70	5 (3)	16 (6)	$\chi^2=8.9$ p=0.003
	Other	28	29 (10)	41 (11)	
Smoking	Never or <5 pack years	23	28 (10)	43 (12)	$\chi^2=15.7$ p<0.001
	5-20 pack years	30	7 (5)	29 (11)	
	>20 pack years	45	7 (5)	7 (5)	
Alcohol	Teetotal or <5units/week	47	12 (5)	25 (7)	$\chi^2=0.4$ p=0.81
	5-20 units per week	28	18 (9)	26 (11)	
	>20 units per week	23	5 (5)	17 (12)	

*NK for 2

Table 2. 2 Two and five-year Kaplan-Meier transformation rates % (Standard Error(SE))

The strongest univariate predictors were smoking status, appearance and tumour site, followed by severity of dysplasia and size of lesion (Figures 2.1 and 2.2, Table 2.3).

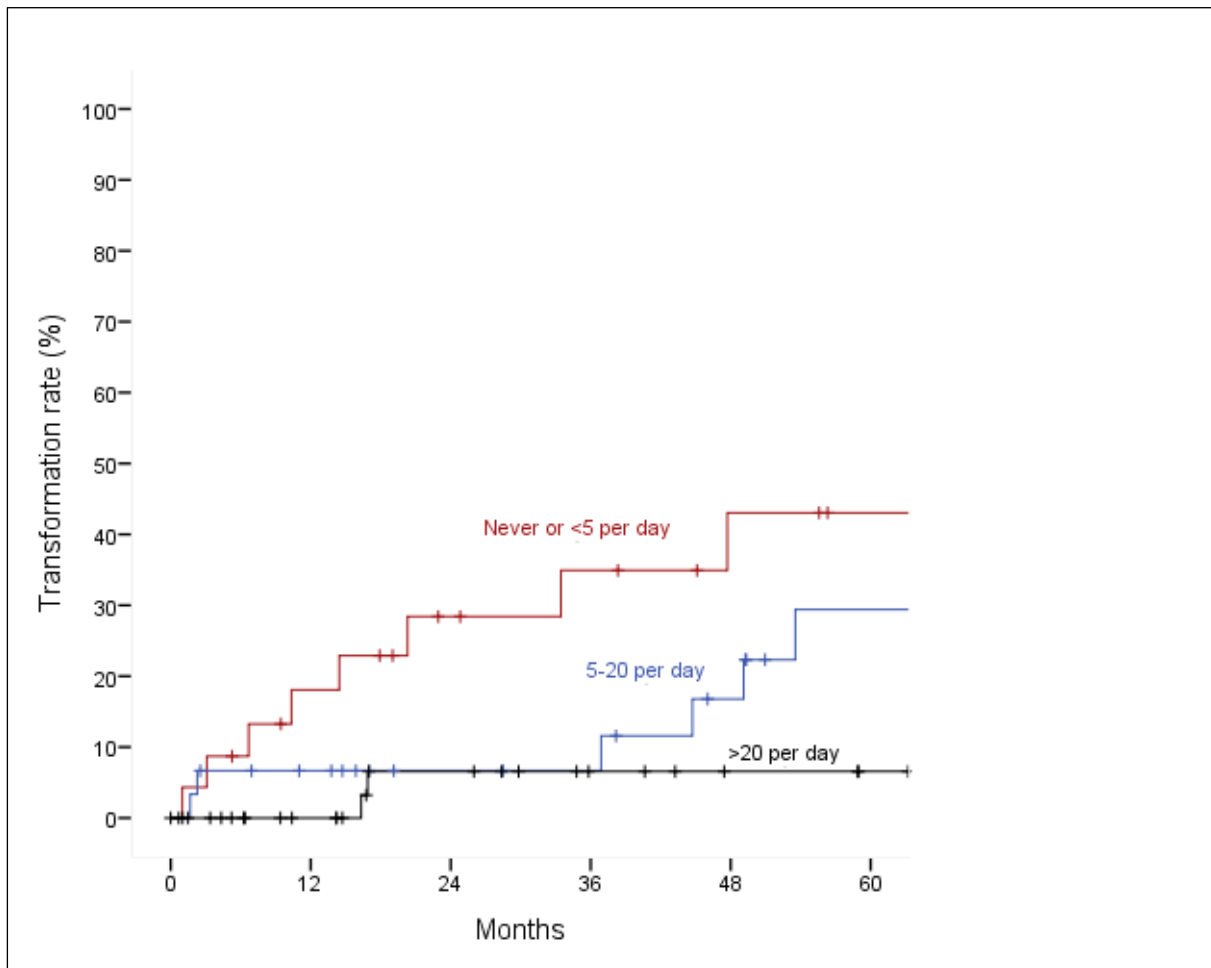


Figure 2.1 Kaplan-Meier curves for malignant transformation rates: smoking history

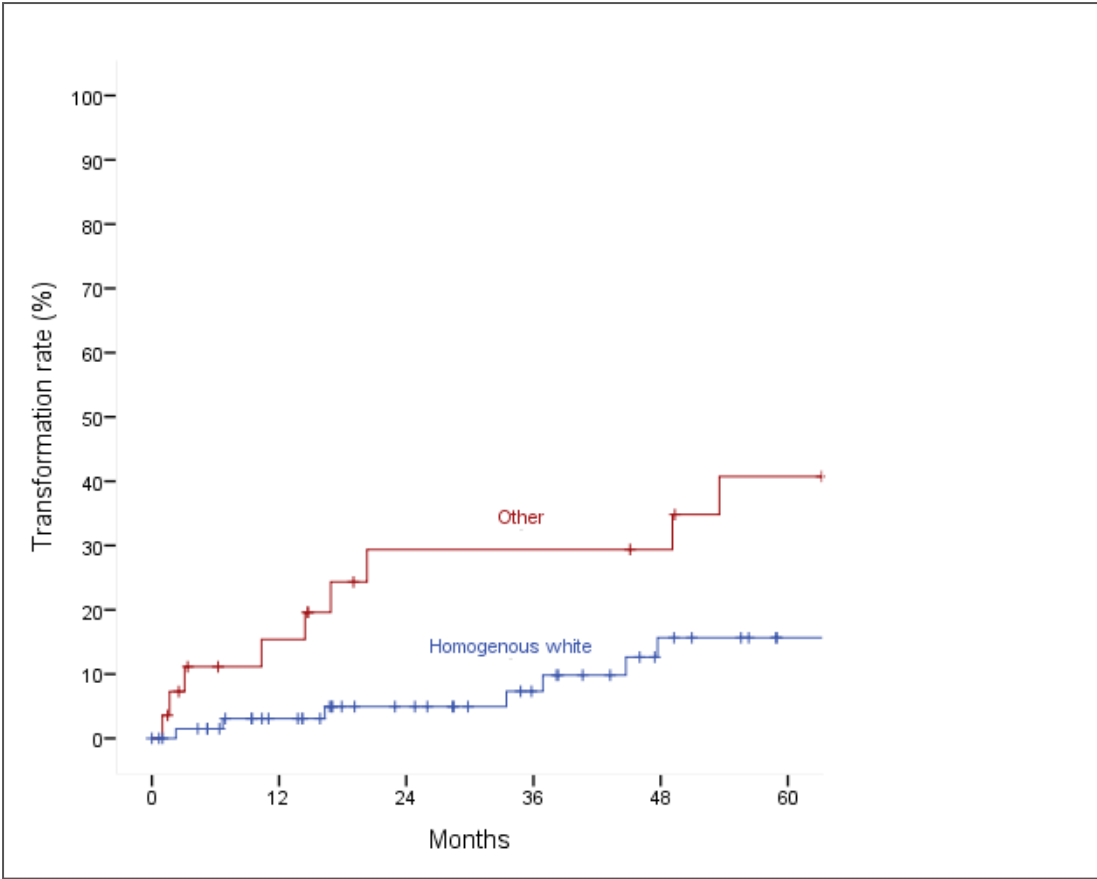


Figure 2. 2 Kaplan-Meier curves for malignant transformation rates: appearance of lesion

	Malignant transformation (%)	Progression of dysplasia (%)	Remission (%)
Smoking history			
Never of <5 pack yrs	12(52.1)	1(4)	10(43.5)
5-20 pack yrs	9(30)	2(6.7)	19(63.3)
>20 pack yrs	5(11.1)	3(6.7)	37(82.2)
Clinical appearance			
Homogenous white	12(17.1)	5(7.1)	53(75.8)
Others	14(50)	1(3.6)	13(46.4)
Site of lesion (n=55)			
FOM	6(15)	2(5)	32(80)
Lateral tongue	9(60)	1(6.7)	5(33.3)
Dysplasia grade (n=78)			
Mild	6(18)	1(3)	26(79)
Moderate	5(19.2)	-	21(80.8)
Severe	8(42.1)	2(10.5)	9(47.4)

Table 2. 3 Number of patients who experienced malignant transformation of clinical OED/PVL for selected parameters (n=98 unless specified).

Patient factors (apart from previous history of OSCC) were used in stepwise multivariable Cox regression modelling. The first predictor into the regression was smoking status (at $p < 0.001$), and then appearance ($p = 0.03$) for its extra independent contribution to the model (extra $\chi^2 = 5.0$) (Table 2.4).

	Hazard ratio (relative risk of malignant transformation)	95% CI of Hazard ratio
Smoking status:		
>20 pack years	Relative to '>20 pack years'	
5-20 pack years	2.3	0.8 - 6.9
Never or <5 pack years	5.2	1.8 -15.0
Appearance:		
Homogenous white	Relative to 'Homogenous white'	
Other	2.4	1.1 – 5.2

Table 2. 4 Final model of independent predictors for the 98 patients using multi-variable Cox regression.

Kaplan-Meier estimates for smoking status stratified by appearance were then determined (Table 2.5).

		Patients	Transformation rate estimate		Log rank test
			2yr	5yr	
Appearance: Homogenous white					
Smoking	Never or <5pack yrs	12	8 (7)	33 (16)	$\chi^2=6.5$ p=0.03
	5-20 pack yrs	19	5 (5)	22 (11)	
	>20 pack yrs	34	4 (4)	4 (4)	
Appearance: Other					
Smoking	Never or <5pack yrs	8	58 (17)	58 (17)	$\chi^2=6.3$ p=0.08
	5-20 pack yrs	10	10 (10)	38 (18)	
	>20 pack yrs	8	20 (18)	20 (18)	

Table 2. 5 Kaplan-Meier analyses with regard to smoking status and lesion appearance. 2 and 5-year Kaplan-Meier transformation rates (SE).

When smoking status was excluded, then only appearance was selected in the regression modelling, with tumour site borderline for independent entry at p=0.08 in addition to appearance. The risk of malignant transformation for the different levels of smoking, adjusted for appearance was significant (Chi-squared 12.7, p=0.002), as was that for the

different levels of appearance, adjusted for smoking status (Chi-squared 5.7, $p=0.02$) (Table 2.5). A chi-squared test comparing the malignant transformation Kaplan-Meier curves of severe dysplasia (high-risk) with mild and moderate dysplasia (low-risk) (Figure 2.3) was significant ($p=0.012$) and Cox regression gave a hazard ratio of 3.2 (95% CI 1.0-7.9; $p=0.01$).

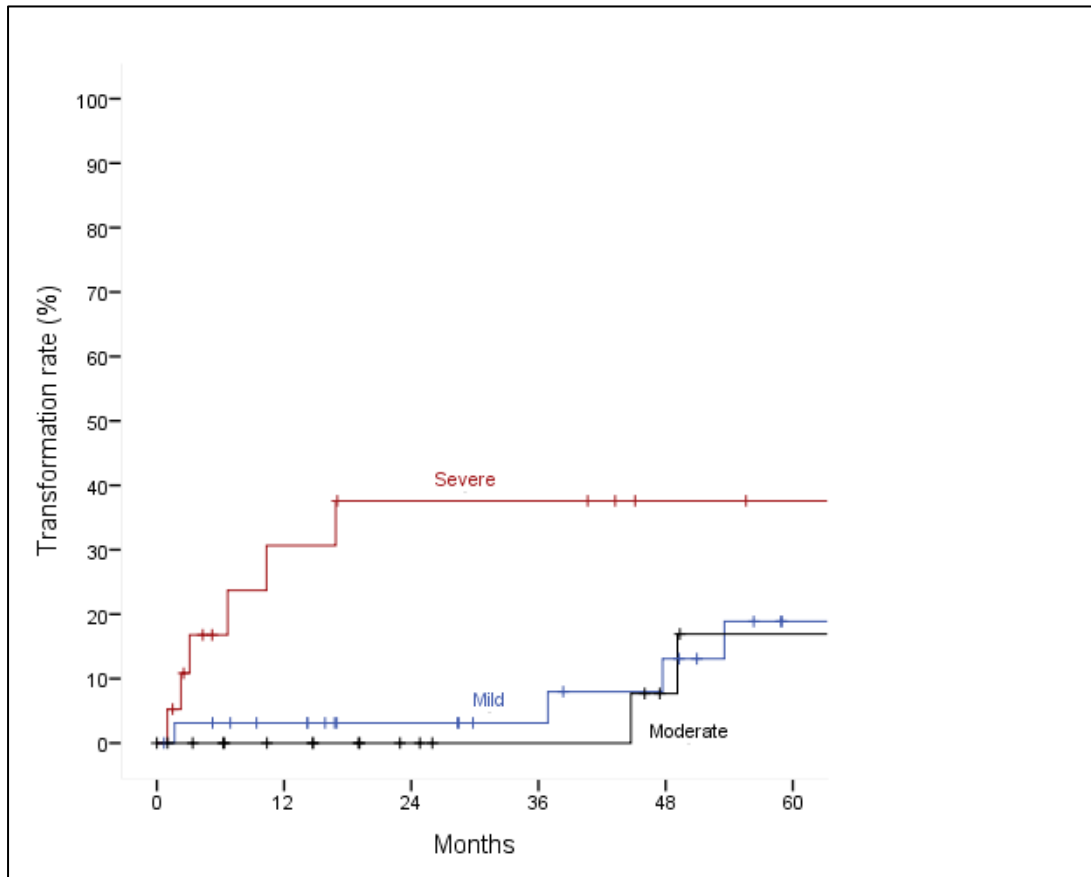


Figure 2.3 Kaplan-Meier curves for malignant transformation rates: grade/severity of dysplasia ('others' excluded)

The clinical intention to treat OED/PVL lesions by the clinician after initial assessment (excise vs review) did not influence malignant transformation rate (Table 2.6).

Data related to the number of incisional biopsies and surgical excisions, number of clinic attendances and follow-up duration is shown in Table 2.7. The follow-up status of patients at

the time of reporting was: 48(49%) under follow-up, 38(38.8%) lost to follow-up and 12(12.2%) discharged to follow-up in the primary/secondary care (low-risk lesions).

Groups n = 98	Treatment intention: surgical excision – n(%)	Treatment intention: clinical surveillance – n(%)	Fisher exact test
Malignant transformers (n=26)	5 (5)	21 (21)	p = 0.534
Stable and progression (n=72)	10 (10)	62 (64)	

Table 2. 6 Comparison of treatment intent for OED which have undergone malignant transformation vs. stable OED.

Group	Total number of biopsies (and surgical excision) Median (IQR)	Number of clinic attendances Median (IQR)	Follow-up period (months) Median (IQR)
Malignant transformers (n=26)	4(3.0-6.25)	15(10.75-29)	80.5(35.2-125.6)
Stable and progression (n=72)	1(1-2)	9(5-18)	41.9(15.0-80.3)
All patients (n=98)	2(1-3)	12(6-19)	49(19-95)

Table 2. 7 Total number of biopsies (and surgical excision), number of clinic attendances and follow-up duration for 98 patients

Discussion

In this study, we discovered a notably high malignant transformation rate of 26.5% amongst patients diagnosed with OED offered prolonged follow-up. Factors such as non-smoking, lateral tongue site and non-homogenous appearance were all associated with a malignant transformation rate in excess of 40%, although the former two were inter-dependent factors. Non-smokers were 5.2 times more likely to undergo malignant transformation compared to

heavy smokers. Malignant transformation occurred after a median of 4 years following diagnosis of dysplasia.

The overall malignant transformation rate in this study was 26.5% compared with 7-27%, for OED, reported in several hospital-based cohorts(5, 59, 66); thus, falling at the higher end of the spectrum. One possible reason for this could be recruitment bias due to the tertiary nature of the Regional Dysplasia Clinic, although this setting is common to most other studies. It is, of course, not a population-based cohort, therefore the characteristics and outcomes of those lesions not referred to the clinic remains unknown. Most publications in the literature report oral leukoplakia, a clinical entity(9, 10, 16, 74) but the inclusion criteria of biopsy confirmed OED in this study will also likely contribute to the high rates seen. The advantages of following a tight inclusion protocol with prolonged follow-up seen in this series are self-evident. PVL was included within the analysed group as clinically the appearance may be similar, and the life-long implications and risks of malignant transformation may be considered broadly comparable(67, 75, 76).

The significant high-risk nature (5.2 x) of lesions in non-smokers is particularly noteworthy and was also reflected in the consistency of trend, with the intermediate group (5-20 pack years) having intermediate (2.3 x) risk more likely to undergo transformation than heavy smokers. When the effect of smoking on gender with relation to malignant transformation was assessed, male non-smokers had a transformation rate of 57% (4/7) when compared with female non-smokers (50%; 8/16). This contrasted with findings of previous authors, who reported OED with more aggressive behaviour only in female non-smokers(9, 10). It has to be clarified that these observations do not imply that smoking is protective of malignant transformation in OED, but in the presence of environmental triggers for the process of carcinogenesis, elimination of these triggers such as tobacco use has been known to result in

resolution of OED especially in the floor of mouth subsite(77). In contrast, the finding of OED in non-smokers (and/or drinkers) would suggest an endogenous aetiology. The disease pattern in 'endogenous' OED has been seen more in the lateral tongue subsite and has been reported in several cohorts to have a higher risk of malignant transformation than those seen in smokers have a higher proportion of OED located in the floor of mouth or palate when compared to non-smokers(61, 62, 78, 79). There is emerging evidence to suggest that it is likely that these underlying "endogenous" factors, in the absence of tobacco smoking, act irrespective of gender(61).

Non-homogenous lesions were 2.4 times more likely to undergo malignant transformation, consistent with several previous reports(9, 10, 21, 65), one of which found this to be the only factor predictive of malignant transformation(59). The non-homogenous appearance of the lesion, amongst other patient factors, has raised clinical suspicion and resulted in the prescription of a more aggressive approach to treatment by surgical excision (Table 2.6).

Although method of treatment did not influence malignant transformation rates, it was felt this would likely be strongly influenced by prescription bias so have not felt further analysis particularly valuable.

In terms of histological grade, high grade OED (42.1% malignant transformation, 8/19) were 3.2 times more likely to undergo malignant transformation compared with low grade lesions. The validity of grading of OED has been questioned due to the possibility of intra and inter-observer variability(59) and therefore a binary system has been suggested to reduce this effect and enhance clinical relevance(58). In our study, the pathology specimens were reported by two oral pathologists (Julia Woolgar and Asterios Triantafyllou), giving consistency in grading.

The lateral tongue and floor of mouth have both previously been associated with an increased risk of malignant transformation(5, 18). Recent evidence suggests that the characteristics of OED in smokers vs. non-smokers differ especially in relation to the site of OED as shown in this cohort and their respective transformation rates, higher in non-smokers and lateral tongue(61). Whilst the site of OED had significant impact on the risk of malignant transformation, the findings reported, and emerging evidence supports the influence of smoking status on OED behaviour in relation to its location. In our study, lesions on the lateral tongue had the highest propensity for malignant transformation (60%; 9/15) whilst the commonest site with OED/PVL was the FOM, making up 40.8% (40/98) of lesions, with malignant transformation seen in only 6 (15% of FOM lesion) patients. This unexpectedly large difference highlights the need for close supervision in lateral tongue lesions (Figures 2.4 and 2.5).



Figure 2. 4 Leukoplakia lateral border of tongue in lifelong non-smoker, histology shows severe dysplasia: high risk of malignant transformation (approx. 40-50%).



Figure 2. 5 Floor of mouth lesion in heavy smoker with moderate risk (approx. 10-15%) of malignant transformation.

The size of the lesion, although not independently predictive of malignant transformation, was statistically significant when the transformation rates were compared, and larger lesions ($\geq 200\text{mm}^2$) appear to have a higher propensity for malignant transformation supported by findings of earlier reports(21, 65).

The median time to malignant transformation of 47.7 months in our cohort supports the need for long-term clinical follow-up of OED/PVL, with the longest case taking 256.5 months for malignant transformation, not dissimilar to results of a recent meta-analysis(20). . In the indication for intervention, there could potentially have been some prescription and selection bias, and this could vary between clinicians. The sample size, while comparable to reported cohorts in the literature, is still relatively small and the difficulties encountered with statistical power in such situations apply here as well. Furthermore, when logistic regression was

applied the box size becomes even smaller, thus data has to be interpreted within this context.

The clinical determinants of malignant transformation reported here will guide the advice given and interventions prescribed to patients. The observations reported also highlight the need to understand the differing risk, and perhaps differing mechanism of carcinogenic route, between smoking-associated and non-smoking associated OED. It is also notable that the prolonged follow-up and multiple interventions in a multi-disciplinary clinic described above carry significant financial costs. In the face of apparently high risk lesions these costs appear to be justified. The outcomes of intervention in patient who underwent malignant transformation would inform the viability of the approach adopted. Safe de-escalation of care for those with lower risk, for example, to review in primary care may also be possible. Stratification of patients will likely depend on clinical and histological features, but additionally with the development of predictive biomarkers reflecting the known genetic(80, 81) and epigenetic(82, 83) determinants of malignant progression.

Chapter 3 Outcomes of oral squamous cell carcinoma arising from oral epithelial dysplasia: the rationale for monitoring of oral premalignant lesions in a multidisciplinary clinic

Introduction

Prompt intervention in the management of early stage oral squamous cell carcinoma (OSCC) remains the most reliable means of achieving the best possible survival and functional outcomes(1, 84-86). Therefore, the infrastructure of service provision should facilitate the process of early detection and diagnosis of OSCC followed by a seamless care pathway, where there is minimal delay to definitive surgery. Recent reviews(87-90) have suggested that opportunistic screening in the high-risk group of patients within the primary health care setting by general dental practitioners may be feasible and cost-effective and there is emerging evidence that a population based approach, targeting high-risk groups can improve early detection rates of oral cancer(91). Extrapolation of these suggestions would support targeted screening of patients with potentially malignant oral lesions (mostly oral epithelial dysplasia (OED)) within a secondary or tertiary multidisciplinary clinic (with representatives from oral and maxillofacial surgery, oral medicine and oral pathology). The multidisciplinary team approach improves patient care by concentration of expertise, which would be enhanced by experience in the management of complex cases, leading to improved patient care. This environment also creates an excellent training opportunity and facilitates patient recruitment into clinical studies which could inform future practice and identify areas for further research(83). It has been alluded to the fact that this group of patients reported in Chapter 2 justified long term follow-up to manage a presumably increased risk of second primary tumours and further OED. The aim of this chapter is to describe the clinical

management and outcomes of OSCC arising from monitored OED in a dedicated multidisciplinary clinic.

Patients and methods

Inclusion criteria

The inclusion and exclusion criteria of the OED cohort has been described in the previous chapter. Briefly, this included histologically diagnosed OED in 91 patients who were recruited prospectively into a clinical and molecular biomarker study since September 1999 (ethical approval by Sefton REC; Reference number: EC 47.01), with first clinic appointment dates between May 1984-March 2011 (median follow up of 48 months (IQR 18-96)). The data presented here includes all those patients within the cohort who underwent malignant transformation.

Data collection

The following data fields were extracted from the main database: demographic details (age, gender), smoking and alcohol history, site and initial grade of OED, cTNM on presentation to the Head and Neck Multidisciplinary Team, extent and modality of surgical intervention, time from first appointment in the Liverpool Regional OED Clinic to malignant transformation, and local recurrence of excised OSCC (defined as recurrence within same subsite within 2 years). Additional features noted include the development of metachronous OED in a separate oral cavity subsite, follow-up duration and clinical status at the most recent update of database.

Results

Twenty-three (25%) patients underwent malignant transformation over the period of this study. The median age at transformation was 57.5 years (IQR 50.1-68.8), median time to malignant transformation from first clinic appointment was 40.3 months (IQR 12.1-67.2). The

median duration of follow-up following cancer diagnosis was 24 months (IQR 11-58). Four patients had surgical excision of clinically unsuspected OSCC following planned interval biopsies. In the remaining 19, malignant transformation was detected following biopsy triggered by clinical suspicion (i.e. unplanned biopsies). All transforming patients were presented to the Head and Neck Multidisciplinary Team with Stage 1 OSCC (cT1N0M0). Twenty-one patients underwent wide local excision of their OSCC (including one localised maxillary alveolectomy and one mandibular rim resection, for marginal clearance); and 2 required tumour resection, selective neck dissection and reconstruction with microvascular free tissue transfer (fasciocutaneous radial forearm and vascularised iliac crest). All 23 patients were confirmed as pT1N0M0 following surgical excision. One required adjuvant radiotherapy for close deep margin. Three (13%) patients developed local recurrence, one (4%) contralateral regional recurrence and one (4%) distant metastasis (pulmonary). Five patients (22%) developed new primary OSCC and another five had histological diagnosis of (22%) further OED on subsequent biopsy. All local recurrences and second primary tumours were surgically excised and the patient who had regional recurrence underwent extended selective neck dissection and adjuvant radiotherapy. The disease specific survival was 100% and overall survival 96% (22/23) when clinical outcome data was updated at the endpoint of study. The demographics and treatment outcomes of these patients have been summarised in Table 3.1.

Table 3. 1 Summary of demographic and treatment outcomes of 23 patients in whom OED underwent malignant transformation (OSCC) MT - malignant transformation, FOM - floor of mouth, WLE - wide local excision (laser or cold steel), NDFFR - tumour resected with neck dissection and microvascular free tissue transfer reconstruction; D=discharged, UF=under follow-up, LF=lost to follow-up

ID	Age	Sex	Smoking	Alcohol units/week	Site	Index Diagnosis (grade of dysplasia)	Time to malignant trans from 1st clinic appt(mths)	Surgery	Local recurrent SCC	Further new SCC	Further dysplasia	No of further excision	Total follow-up in OED clinic (mths)	Clinical status
1	85.2	M	Never	Teetotaller	Lat tongue	severe	6.67	WLE	Y	N	N	1	94.8	UF
22	49.2	F	<5 pack years	5-20 units	Lat tongue	severe	65.22	WLE	Y	N	N	1	111.6	UF
21	57.5	M	5-20 pack years	<5	Lat tongue	moderate	83.65	WLE	N	Y	N	1	147.6	UF
17	69.3	F	Never	Teetotaller	Lat tongue	severe	14.78	WLE	N	Y	Y	1	36	UF
8	46.1	F	Never	<5	Lat tongue	mild	36.80	WLE	N	N	Y	1	58.8	UF
23	37.5	M	5-20 pack years	21-40	Lat tongue	mild	53.55	WLE	N	N	Y	0	115.2	UF
4	44.7	F	5-20 pack years	<5	Lat tongue	severe	78.59	WLE	N	N	N	0	82.8	UF
6	54.7	F	Never	5-20 units	Lat tongue	severe	13.90	WLE	N	N	N	0	13.2	UF
12	52.0	M	Never	5-20 units	Lat tongue	moderate	4.04	WLE	N	N	N	0	32.4	UF
13	65.1	F	Never	Teetotaller	Lat tongue	moderate	82.96	WLE	N	N	N	0	121.2	LF
7	45.2	F	5-20 pack years	<5	Ventral tongue	mild	40.34	WLE	N	Y	Y	1	24	UF
11	68.8	F	5-20 pack years	Teetotaller	Ventral tongue	moderate	42.64	WLE	N	N	N	0	52.8	UF
15	72.6	F	5-20 pack years	5-20 units	Buccal	mild	35.94	WLE	Y	N	N	1	118.8	LF
5	44.6	F	>20 pack years	Teetotaller	Buccal	moderate	69.19	Localised alveolectomy	N	Y	N	0	256.8	UF
20	58.0	F	5-20 pack years	<5	Buccal	moderate	49.12	WLE	N	Y	Y	2	145.2	UF
3	51.8	M	Never	<5	Buccal	moderate	4.30	NDFFR	N	N	N	0	196.8	UF
2	87.2	F	Never	Teetotaller	Retromolar	severe	4.86	NDFFR	N	N	N	0	25.2	UF
10	68.8	M	5-20 pack years	21-40	FOM	severe	2.10	WLE	N	N	N	0	21.6	UF
9	68.7	F	>20 pack years	21-40	FOM	moderate	63.34	WLE	N	N	N	0	78	UF
14	53.1	M	>20 pack years	5-20 units	FOM	mild	90.61	WLE	N	N	N	0	116.4	LF
16	72.9	M	Never	<5	FOM	moderate	10.38	Rim mandible	N	N	N	0	57.6	UF
18	60.9	M	5-20 pack years	>40	FOM	severe	90.18	WLE	N	N	N	0	145.2	D
19	50.9	F	>20 pack years	5-20 units	Soft palate	severe	16.43	WLE	N	N	N	0	21.6	UF

Discussion

The treatment outcomes of early OSCC which had arisen from OED in this cohort appeared to have excellent regional recurrence and disease specific survival outcomes when compared with previous OSCC cohorts(1, 86, 92, 93); reported as regional recurrence 10-31% and disease specific survival 46-86%. The local recurrence rate was marginally higher than previously reported, 8-12%(1, 86, 92, 93) and the second primary tumour rate was within the higher end of reported figures, 7-32%(1, 94). These excellent outcomes were achieved following relatively minor treatment: surgical intervention consisted of localised treatment to the primary tumour sites in 87% (20/23) of patients (including treatment for local and regional recurrences). It is understood that this cohort is the first report of treatment outcomes of OSCC which had arisen from OED monitored in a dedicated oral dysplasia multidisciplinary team clinic.

In acknowledging the limitations of this data, it has been recognised that an element of bias towards high risk OED cases were included within the research cohort. The overall number of OSCC arising from OED within this cohort is modest. It was assumed in the analysis that all the early stage OSCC would have progressed to larger tumours if left untreated, and this is perhaps a matter for debate as occasional tumours may regress spontaneously.

In order to conclusively prove the benefit of specialist surveillance by a multidisciplinary team, comparison with a control group of, for example, OSCC arising from OED but not subject to surveillance, would be required, however this data is not available. Anecdotally, there have been cases of T2, T3 and T4 OSCC at presentation to the MDT despite previous histological diagnoses of OED *not* subject to formal surveillance, however there is no reliable mean of capturing this data. Patient satisfaction in this cohort was not formally measured,

however, there was a low non-attendance rate. Those patients who underwent malignant transformation and were counselled by members of the multidisciplinary team received their treatment through a seamless care pathway with minimal delay.

In order to give context to the outcomes of this group of 23 patients, data from 489 OSCC patients treated by primary surgery 1992-2002(1) is summarised and contrasted in Table 3.2 (patients from the same geographical catchment area).

	Early OSCC from OED 1984-2012 (23 patients) % (n)	Liverpool OSCC cohort 1992-2002 (489 patients) %(n)
Treatment details:		
Surgery to primary site only	91(21)	18(89)
Neck dissection	9(2)	82(400)
Free flap reconstruction	9(2)	76(373)
Primary surgery & adjuvant radiotherapy	4(1)	40(194)
Disease related outcomes:		
Local recurrence	14(3)	10(50)
Regional*/loco-regional** recurrence	4*(1)	21**(103)
New primary OSCC	22(5)	7(35)
Freedom from loco-regional recurrence	83(19)	76(373) ^a
Disease specific survival	100(23)	74(362) ^a

a. Data based on Kaplan Meier curves estimate at 5 years

Table 3. 2 Data from early OSCC from OED cohort from this study and the Liverpool 1992-2002 OSCC cohort(1): treatment details and disease related outcomes.

Although direct comparison between this cohort and other OSCC cohorts would not be entirely reliable (as it is all but certain that all the previously reported cohorts would have contained patients with precursor OED), the contrasting outcomes offers some perspective. The relatively minor nature of surgical intervention and excellent outcomes in the management of OED derived OSCC (Figures 3.1 & 3.2) are apparent.

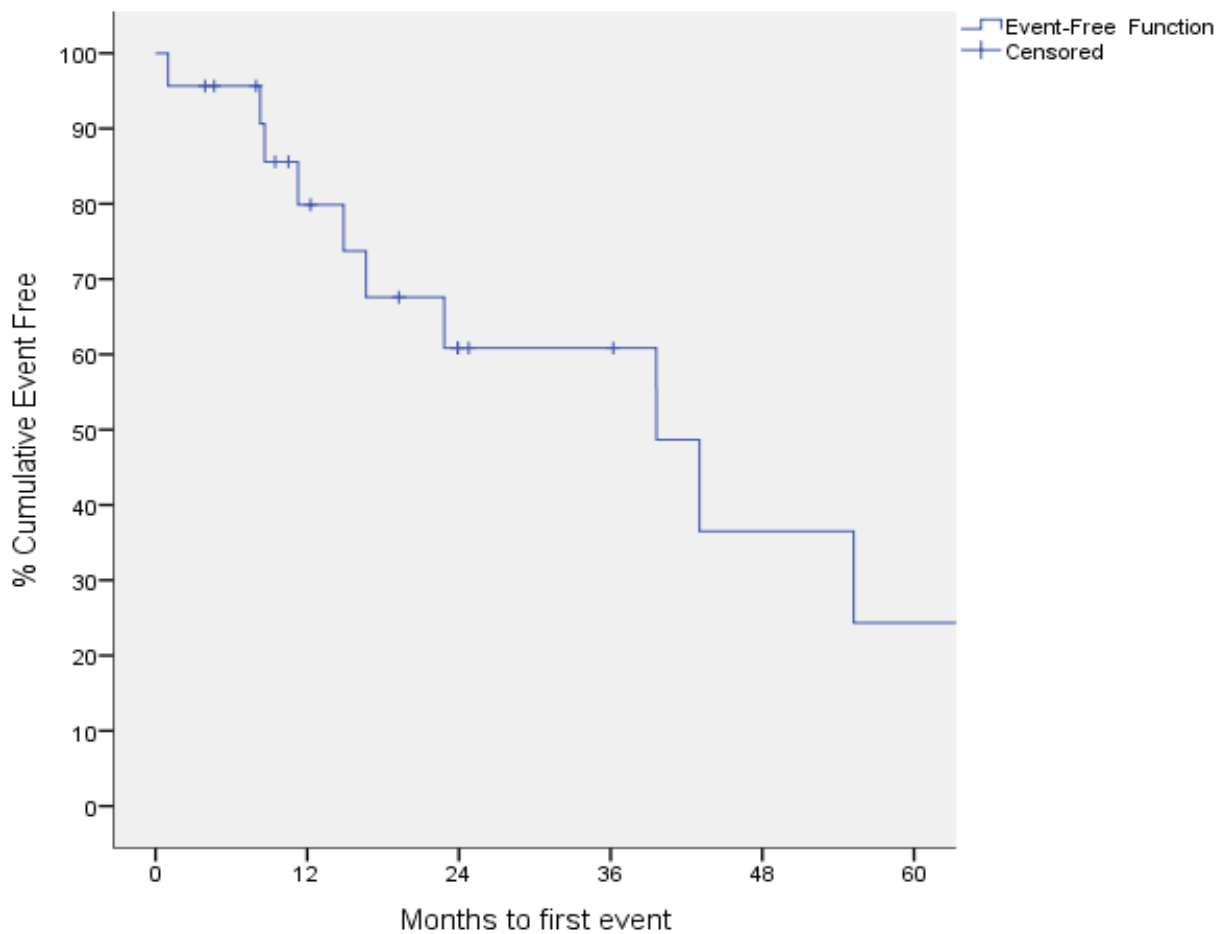


Figure 3. 1 Left lateral tongue microinvasive SCC: pre-treatment (left) and complete healing following excision with laser (right)



Figure 3. 2 Right lateral tongue severe dysplasia (top left) underwent malignant transformation to microinvasive SCC (top right) with complete healing following laser excision (bottom centre)

This would indicate rather less patient morbidity, fewer serious complications and a greatly reduced burden of treatment to patients and health service overall(95, 96). The higher local recurrence and second primary tumour rate reported is likely to reflect the high-risk nature of patients followed up in the Regional Oral Dysplasia clinic, in whom the oral mucosa is unstable (field change) (Figure 3.3).



Kaplan-Meier estimates of 12-month event free survival (with standard error): 80% (SE 9%)
Kaplan-Meier estimates of 24-month event free survival (with standard error): 61% (SE 12%)
Kaplan-Meier estimates of 60-month event free survival (with standard error): 24% (SE 14%)

Figure 3. 3 Time from date of malignant transformation to first occurrence date of any of second event (local recurrent SCC, new primary, SCC, further dysplasia, death) or in the absence of an event to the date lost to follow-up.

As previously suggested(97), it is postulated that there are possible endogenous factors within some of the patient population which could account for this behaviour, in addition to the most common environmental factors such as tobacco and alcohol exposure. The additional cost of long-term regular follow-up of OED in a multidisciplinary setting needs to be weighed against the costs of surveillance and might make a health economic approach worthwhile. The data presented demonstrates a pivotal role for multidisciplinary input with

representation from the Head and Neck Multidisciplinary team, in this case facilitated by oral and maxillofacial surgery in the oral dysplasia clinic, acting as intermediary between surveillance in the oral dysplasia clinic and the MDT.

The outcomes reported in this chapter appear to favour long term clinical monitoring of OED within a multidisciplinary clinic. The advantages of long-term review for OED patients are clear, necessitated by the median interval to malignant transformation >3 years and high local recurrence and second primary tumour rates (76% at 5 years). Better predictive models for transformation would aid inclusion to a surveillance programme and tailoring of that programme to the patients' individual risks. As in other areas of personalised medicine, these models will likely include histopathology, clinical and demographic characteristics as well as molecular biomarkers.

Chapter 4 Surgical management of oral dysplasia

Introduction

Surgical excision has been the preferred modality of treatment in the management of OED(70), despite the lack of evidence from randomised controlled trials(16). Even with a policy of surgical excision, recurrence rates of up to 35% have been reported(18), however, it has been suggested that monitored lesions demonstrate a higher rate of malignant transformation compared with those that are excised(20, 71). Although molecular biomarkers such as DNA aneuploidy(98), loss of heterozygosity(99) or DNA methylation(83) of key tumour suppressor genes are actively researched and demonstrate correlation to risk of malignant transformation, these have yet to translate to mainstream clinical practice. The decision to offer surveillance or surgical excision is therefore made on clinical grounds using the factors described(97), and histological grading.

Surveillance of OED results in a number of newly diagnosed OSCC. Therefore, the infrastructure of service provision should facilitate the process of early detection and diagnosis of OSCC followed by a seamless care pathway, where there is minimal delay to definitive surgery. Prompt intervention in the management of early stage OSCC remains the most reliable means of achieving the best possible survival and functional outcomes(1, 84-86). It is helpful to manage such lesions in dedicated tertiary multidisciplinary clinic (with representatives from oral and maxillofacial surgery, oral medicine and oral pathology). There has been some local evidence to suggest that such arrangements allow cases that (inevitably) transform to frank malignancy to be managed rapidly and with excellent results(100). These cases can usually be treated with single modality and with superior recurrence and survival outcomes compared to OSCC arising from other referral pathways(1, 100) (Table 3.2).

CO2 Laser surgery for oral lesions with malignant potential

CO2 laser is the most commonly used laser in the surgical management oral cavity soft tissue excision or ablation(101-109). Alternative laser systems include NdYAG or diode laser(71). It has been proven to be safe and effective as its coherent beam is almost completely absorbed by intracellular water. When used as a precise cutting instrument in the oral cavity, 1mm diameter focus beam at 5-7W super-pulsed continuous setting, it also achieves haemostasis by photo thermocoagulation allowing clear visibility to the surgical site and sealing off nerve endings resulting in less pain post-surgery. It can also be used to achieve ablation, 3mm diameter defocused beam at 4-6W super-pulsed continuous setting. A micro-manipulator can be used for improved accuracy in posterior lesions but in practice most oral cavity laser surgery is carried out by a free handpiece. Utility of an operating microscope could enhance tissue preservation in the absence of a malignant diagnosis, however the set-up and procedure could be more time consuming.

Peripheral excision margins of 3-5mm depending on the size and severity of dysplasia grade(22), of the lesion would seem appropriate with depth of excision down to the connective tissue layer (adipose/muscular tissue depending on site) (Figures 4.1 and 4.2).



Figure 4. 1 Non-homogenous premalignant lesion on the lateral tongue in a non-smoker (high risk lesion).



Figure 4. 2 Lateral tongue wound left to heal by secondary intention following laser excision of mucosal layer down to intrinsic muscles of tongue with excellent haemostasis.

It should be borne in mind that a significant proportion of laser resections for dysplasia harbour OSCC on the final histological report(101). Demarcation of the oral dysplastic lesion and its margins is sometimes enhanced with the use of toluidine blue(110) or Lugol's Iodine solution(111), although the objective benefit of either has yet to be demonstrated in high quality studies, their role in identification of high risk lesions, selecting biopsy sites and helping to ensure clear margins is potentially promising (112-114). Laser surgery offers the reported advantage of; haemorrhage control, improved visibility and shortening of operative time, along with decreased post-operative pain and swelling, minimal scarring and good post-operative tissue mobility(115). Laser excision is usually preferred over ablation as it allows definitive diagnosis through histopathological tissue examination, which aids clinical management, bio banking, research and documentation for clinical governance or medico-legal purposes.

Healing is primarily by secondary intention with complete re-epithelialisation after 4-6 weeks(116) (Figures 4.3 and 4.4).



Figure 4. 3 Preoperative appearance of homogenous right buccal mucosal lesion (severe dysplasia).



Figure 4. 4 Three-year follow-up appearance of well healed scar following excision.

The buccal mucosa has the tendency for more adverse scarring and fibrosis which can cause trismus. Therefore, for more extensive buccal lesions (>2cm diameter), it is advocated that reconstruction should be considered with one of the following options: the buccal fat pad, split thickness skin graft 'immobilised' by a bolster dressing or free tissue transfer.

In the anterior floor of mouth, which is a very common site for OED, management the submandibular duct(s) need to be carefully considered. In the event that the submandibular duct becomes stenosed or obstructed after excision, the resultant obstructive sialadenopathy causes patient discomfort(117) and, critically, may mask or simulate cervical metastasis. The submandibular duct(s) should be identified by careful dissection during laser excision and posteriorly repositioned (Figures 4.5-4.9).



Figure 4. 5 Erythroleukoplakia anterior floor of mouth in a smoker (high risk lesion).



Figure 4. 6 Appearance of lesion following staining with Lugol's Iodine solution: area of pallor indicates mucosal dysplastic change.



Figure 4. 7 Peripheral excision margin delineated with CO2 laser 5mm from area highlighted by Lugol's Iodine staining (1mm beam spot, 6W super-pulsed continuous setting): precise cut edge with excellent haemostasis allowing clear visibility.

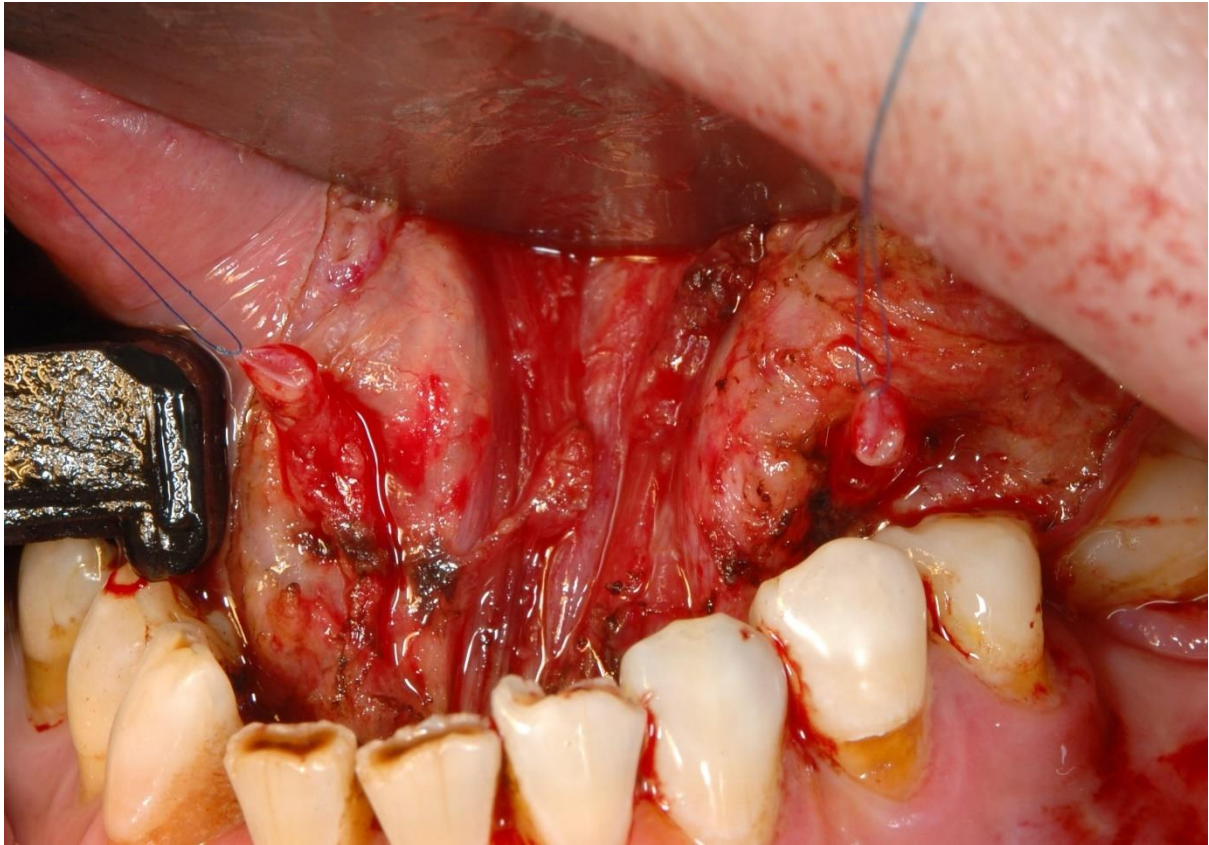


Figure 4. 8 The submandibular ducts were carefully dissected out and divided to achieve safe excision margins. The proximal cut ends were then secured with 6/0 monofilament sutures to prevent retraction and aid localisation.

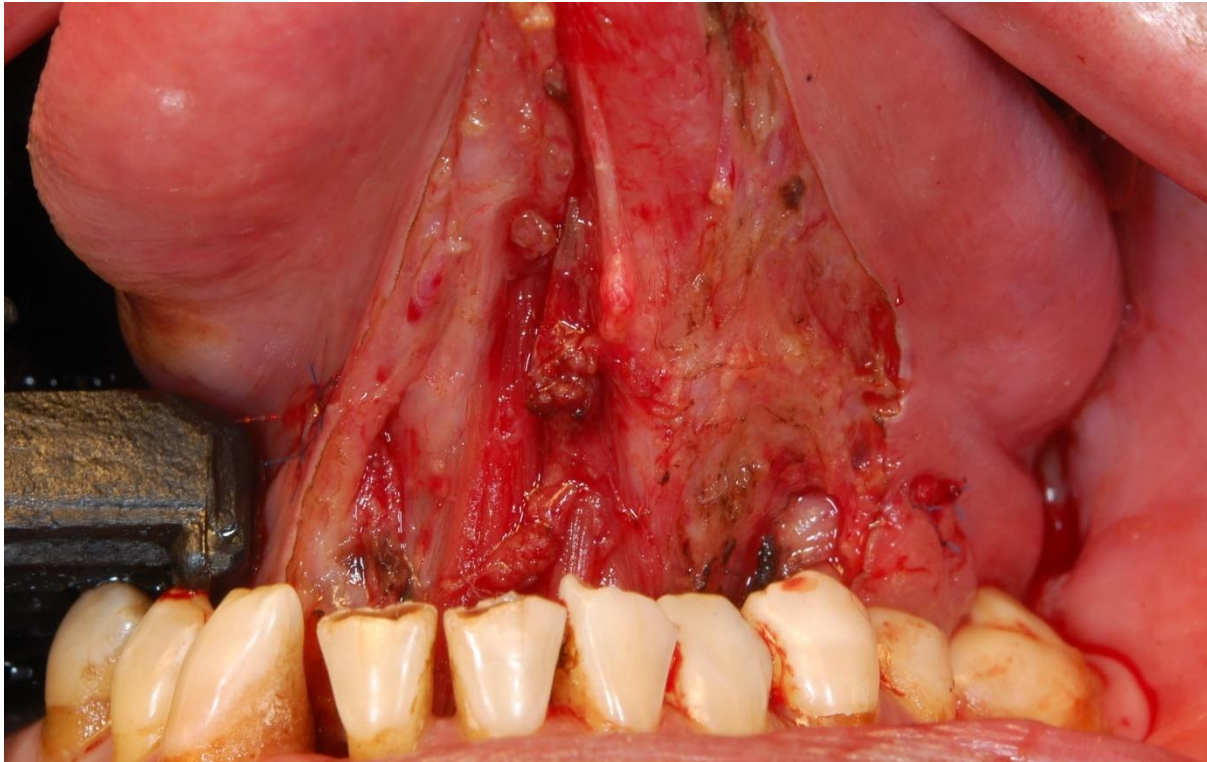


Figure 4. 9 The submandibular ducts positioned posteriorly through a stab incision in the floor of mouth mucosa with a No. 11 scalpel blade and inset in place with 6/0 monofilament sutures. The wound was left to heal by secondary intention following CO2 laser excision.

This is a technically demanding procedure but it effective in preservation of asymptomatic submandibular salivary gland function.

The excision of small (or anterior) oral premalignant lesions can usually be done as a day surgery procedure under local or general anaesthesia. Where possible nerve blocks should be utilised in preference to local infiltration as the latter could mask bleeding blood vessels due to vasoconstriction by epinephrine. Excision of larger (or posterior) lesions is usually more successful using general anaesthesia to allow optimal control of the surgical site and for patient comfort, and it may be necessary for patients to stay for a period of observation due to increased risk of bleeding and need to resume adequate oral alimentation.

Complications are occasionally seen after laser excision of OED. If infection occurs in the granulating lesion, there may be bleeding which although usually minor may on occasion

require readmission and operative haemostasis. Following excision of larger lesions, there is frequently a period of post-operative pain which can last for up to 6 weeks. Furthermore, some lesions, particularly in the tongue can be subject to “over-granulation” resulting in an exophytic polypoid mass at the site of laser excision (Figure 4.10). Although these will often settle with conservative management, there is sometimes a diagnostic uncertainty regarding presence of malignancy in such lesions so re-excision is occasionally mandated.



Figure 4. 10 Left ventral tongue polypoidal mass ("over granulation") can sometimes occur during healing following laser excision.

Aims and objectives

The literature was reviewed for publications which have reported on the utility of interventional laser surgery in the management of oral lesions with malignant potential (OLMP). Treatment outcomes such as recurrence of OLMP and malignant transformation were the main endpoints assessed. These outcomes were compared between patients who were treated by laser excision, laser ablation and cohorts treated by a combination of laser excision and/or ablation in the management of oral dysplasia. The main objective of this review was to ascertain if there was any difference in recurrence and malignant transformation rates between the different techniques of laser surgery in the management of OLMP.

Results

The studies identified from the literature with clear reported outcomes of recurrence following laser treatment of OLMP (which included oral dysplasia, leukoplakia, erythroplakia and proliferative verrucous leukoplakia) and malignant transformation rates in their respective cohorts have been summarised in Table 4.1. The average recurrence rates of OLMP for the three groups of laser interventional surgery (based on pooled data from Table 4.1) were: laser ablation 52.5%, laser excision 11.6% and laser excision and/or ablation 20%. The reported rates of malignant transformation were 0-13%. The finding of an incidental oral squamous cell carcinoma in excised OLMP was seen in up to 12% of patients (118).

Surgical technique	Author (year)	Number of patients (number of lesions)	Mean follow-up (range) – months	Recurrence rate (%)	Transformation rate (%)
Laser ablation	Flynn(103) (1988)	14	29 (12-41)	8/14 (57)	1/14 (7)
	Chandu(105) (2005)	39 (69)	42 (2-102)	33/69 (48)	5/39 (13)
Laser excision	Chiesa(101) (1986)	54	24	2/54 (2)	0
	Chu(102) (1988)	28	65 (36-114)	5/28(18)	3 (11)
	Roodenburg(119) (1991)	70 (103)	64 (6-144)	10/103 (10)	0
	Van der Hem(109) (2005)	200 (282)	52 (1-219)	28/282 (10)	3/200 (1)
	Yang(116) (2011)	114	41 (21-109)	20/114 (18)	13/114 (11)
Laser excision and/or ablation	Frame(120) (1984)	(44)	10 (4-24)	6/44 (14)	0
	Thomson(108) (2002)	55	18 (1-44)	12/55 (22)	4 (7)
	Jerjes(106) (2012)	77 (123)	77	15/77 (20)	8/77 (10)
	Del Corso(121) (2015)	77	60 (± 32)	22 (29)	3 (4)
	Thomson(118) (2017)	590 (773)	7.3 (0-19) *	87/590 (15)	28/590 (5)

Table 4. 1 Summary of treatment outcomes of oral premalignant lesions with CO2 laser (* follow-up reported in years)

Discussion

The main finding from the review of the literature would suggest that in terms of recurrence of OLMP laser excision appeared to have a lower rate of recurrence when compared to laser ablation. The combined techniques of laser excision and/or ablation resulted in recurrence rates better than laser ablation alone but inferior to laser excision. One possible explanation for this reported difference could be related to the fact that laser excision requires removal of more tissue depth when compared to laser ablation hence allowing more complete excision of OLMP. Furthermore, the technique of laser ablation relies on the systematic coverage of the area occupied by the OLMP with a defocused laser and treatment outcomes would be more sensitive to variability between operators and/or ablation techniques utilised. Perhaps of more clinical importance is the finding of an incidental oral cancer in excised OLMP (118). Laser excision has in these cases contributed to the early detection of oral cancer which might not have been possible in ablative techniques. Therefore, it could be argued that from a patient safety and medicolegal perspective, laser excision would be the preferred approach over laser ablation. The lower recurrence rates in CO₂ laser excision when compared to NdYAG laser ablation has been observed in a recent case series, and the malignant transformation rate in the reported cohort was higher in the laser ablation group of patients(121). Due to the heterogeneity of pathology in the reported studies (Table 4.1), statistical comparison between these groups for recurrence and malignant transformation rates of a specific histological diagnosis e.g. oral dysplasia, would be difficult and ideally require access to original data for a metaanalysis. This was not explored or reported in a recently published systematic review on the treatment of oral leukoplakia with CO₂ laser(122). Recurrence rates of up to 100% were reported when lesions were excised with a

clearance margin of less than 2mm; this figure fell to 10% when lesions were excised with a margin of greater than or equal to 3mm(22). As alluded to earlier, there is potentially a role for utility of visual adjuncts in the surgical management of oral dysplasia (or OLMP) as the pathology is confined to the epithelial layer and when compared to cancer resections, the concern with deep marginal clearance becomes less of an issue. A multi-centre, randomised controlled trial has completed recruitment, including 419 patients with oral or oropharyngeal cancer, with the aim of assessing the effectiveness of Lugol's iodine in helping to achieve clear mucosal margins(123) . Patients will be followed up for a minimum of 2 years to assess the impact of the presence of dysplasia at margins on recurrence, the publication of outcomes from this trial is awaited. Based on the evidence reviewed, it would therefore suggest that laser excision of oral dysplasia/OLMP would be preferred over ablative procedures and potential exists for utility of a cost-effective visual adjunct which would be easy to use and is widely available.

Chapter 5 The Liverpool Multidisciplinary Approach to the Management of Oral Epithelial Dysplasia

Introduction

Oral epithelial dysplasia (OED) together with lichenoid lesions and other conditions such as submucous fibrosis, are potentially malignant oral mucosal diseases (PMD). The significance of OED lies in its association with malignant transformation into oral squamous cell carcinoma (OSCC)(55, 97). OED presents histologically as a spectrum of epithelial changes, rather than distinct categories. The WHO (2005 and 2017) system grades it as mild, moderate, severe and carcinoma-in-situ(2, 124). There are other classifications for scoring OED, however it is recognized that one limitation of using histological criteria is the subjectivity of the WHO grading system.(124-126)

The major challenge facing the clinician, when a patient is diagnosed as having OED, is deciding on their optimal management strategy. The two pivotal questions, for which there are currently no clear-cut answers are: what is the likelihood of an oral lesion with OED undergoing malignant transformation to OSCC, and what intervention(s) can mitigate against this? A supplemental question relates to the follow-up of the patient – what is an appropriate surveillance programme and how best can this be delivered?

The first part of this chapter discusses the challenges associated with managing patients with OED, including initial diagnosis, the significance of epidemiological, clinical and histological factors, management strategies and their efficacy in patients with OED. It is widely recognised that there is currently a lack of high-quality evidence, from randomised control trials, regarding malignant transformation of OED to formulate evidence-based guidelines.

However, clinicians have a duty of care to patients and their relatives/carers to offer advice and management, based on the best available evidence.

The second part of this chapter outlines a multidisciplinary approach to the management of patients with OED, in the form of an algorithm. The algorithm is based on best available evidence, albeit inconclusive, and the experience of a multidisciplinary, tertiary clinic based in the Oral Medicine Unit at Liverpool University Dental Hospital; established to manage high-risk OED.

The challenges associated with the management of oral epithelial dysplasia

The Diagnosis of Oral Epithelial Dysplasia

Despite several studies into alternative methods(3, 112, 127, 128), routine histopathological examination remains the gold standard for diagnosis of OED. It is acknowledged that there would be some variability between pathologists in their interpretation of the architectural and cellular changes(127). The use of less invasive, diagnostic measures as adjuncts or alternatives to histopathology, such as the use of Lugol's iodine, toluidine blue, bio-optical imaging and cytological specimens have been researched(112, 123, 128-131), but require validation. To date, none of these methods can reliably replace histopathology. Concerns have been raised about the reliability of histopathological examination of incisional biopsy specimens, especially in larger lesions as the sample may not be representative of the lesion, and these have been comprehensively reviewed elsewhere in the literature(126). A negative

biopsy result may not completely exclude carcinoma or its potential, up to 10% of excised oral leukoplakia may contain previously undetected malignant or dysplastic tissue(101). Apparently normal mucosa, contralateral to mucosa with confirmed OSCC or OED has also been found to harbour dysplastic tissue: in a study of 26 patients with unilateral OSCC or OED, 9 (35%) of 'mirror-image' biopsies found evidence of dysplasia or microinvasive carcinoma(132).

Epidemiological, Clinical & Histological Factors as Predictors of Malignant Transformation in Oral Epithelial Dysplasia

The malignant transformation rate of OED has been reported as occurring in 6.6 – 36.4% of cases (5, 9, 20, 59, 60, 97, 133, 134) a systematic review and meta-analysis indicated a rate of 12.1%(20). An average 'time to transformation' has been reported of 0.5 – 17 years (9, 60, 97, 100). Mild dysplasia has been reported as showing less than 5% malignant transformation, with moderate and severe dysplasia having rates of 3-15% and 7-50% respectively(18, 133).

The findings from Chapter 2 showed 22% of patients undergoing malignant transformation within five years, with significant predictive factors being: non-smoking status, site, non-homogenous appearance and size (>200mm²) of lesion (Table 5.1).

Size >200mm ²
Non-homogenous lesion
Lateral tongue lesion
Non-smoking status

Table 5. 1 High Risk, Predictive Features for Malignant Transformation in the Liverpool Cohort(97)

Gender, age, number of lesions and alcohol history did not predict for malignant transformation (97). The role of predictive biomarkers for malignant transformation in OED has yet to be established in clinical practice. Several studies have identified predictive molecular biomarkers that may yet translate into clinical tools (62, 83, 135-137).

The Management of Oral Epithelial Dysplasia

No consensus exists on the appropriate management of dysplastic lesions of the oral mucosa. Surgical excision is frequently carried out and there is some evidence to suggest that this reduces the risk of malignant transformation when compared with active surveillance(71, 138). A systematic review of OED, involving 992 patients, showed that lesions that were not excised showed significantly higher transformation rates than those that were excised(20). Smoking more than 20 cigarettes per day and consuming more than 100g of alcohol a day increases the risk of developing OED; this risk declines, following smoking cessation, back to that of the general population after 10-15 years(139). Smoking cessation can result in the disappearance of a substantial number of oral leukoplakia (138-140). Paradoxically, oral

epithelial dysplasia has been found to have less malignant potential when associated with a smoking habit(9, 10).

Despite a lack of randomised control trial evidence with regards to OED and smoking cessation/alcohol reduction, these habits are risk factors for the development of oral cancer and therefore smoking cessation advice and alcohol reduction counseling is advisable in the management of patients with OED(141). It has been suggested that, for lesions thought to be at low risk of progression, e.g. mild/moderate dysplasia in a low-risk site, active monitoring may be the preferred management plan(140). Active monitoring includes clinical examination, photographic records (15) and re-biopsy as guided by clinical change. Most clinicians would excise lesions showing evidence of severe dysplasia or carcinoma-in-situ as they would be considered at high risk of malignant transformation. A summary of recurrence and malignant transformation rates of OED treated by scalpel excision can be found in Table 5.2.

Study cohort	Type of Treatment	Recurrence (%)	Malignant transformation rate – treated cases (%)	Comments
Pandey 2001(142)	Surgical Excision	10.1	0	12 months follow-up
Holmstrup 2006(21)	Scalpel Excision	13.5	12.4	20% of malignant transformers were non-homogenous lesions compared with 3% homogenous
Kuribayashi 2012 (22)	Scalpel Excision (with Lugol's Iodine)	15.1	1.9	Gingivae most common site for recurrence
Thomas 2012 (143)	Scalpel Excision	4.2	4.1	4.1% of lesions were on the tongue

Table 5. 2 Recurrence and Malignant Transformation Rates following Surgical Intervention for Oral Leukoplakia

Non-surgical treatment would offer obvious advantages to patients and photodynamic therapy was reported to achieve a good response in one study (144); however, it is not a widely available modality and most studies have small sample sizes and insufficient follow up(145). Chemoprevention (vitamin A, beta-carotene and 13-cis-retinoic acid) has been used in the management of OED but studies report early/high incidence of recurrence(146-148). Topical bleomycin has been shown to reduce the size of OED and reduce the severity histopathological grade compared to placebo (149). The latest Cochrane review regarding management of oral leukoplakia concluded that; there is insufficient high-quality evidence to support the use of non-surgical interventions for treatment of oral epithelial dysplasia(16). A recent randomised control trial of freeze-dried black raspberry gel has shown promising

short-term results for reduction in lesion size, grade of dysplasia and loss of heterozygosity events in OED lesions (26).

In the UK, the British Association of Head and Neck Oncologists (BAHNO) Head and Neck Cancer Multidisciplinary Management Guidelines 2011 (150), advise on the targeted use of biopsy and histopathological assessment, along with advice on reduction of environmental carcinogens (tobacco use and alcohol), followed by surgical excision of the lesion where the size of the lesion and subsequent function allows (this was updated in 2016 without any change). The 2004 Fourth World Workshop on Oral Medicine have reviewed the management of oral epithelial dysplasia(55) and concluded that there is a lack of randomised studies assessing the effectiveness of surgical intervention in preventing malignant transformation. However, surgical excision (laser or scalpel) remains the most appropriate/accessible treatment option for many patients.

Surveillance of Patients with Oral Epithelial Dysplasia

No consensus exists for exact duration of follow-up or follow-up intervals (15). Lifelong follow-up at intervals of no more frequent than every 6 months has been recommended(140). Recent evidence suggests that long term surveillance, up to 15 years, might be required(151). The BAHNO Management Guidelines have recommended long-term surveillance of OED (150). The majority of clinicians re-biopsy in the event of clinical deterioration of the lesion (152), however, a programme of scheduled re-biopsy may be justified in light of findings that clinically unsuspected OSCC have been detected as a result of scheduled re-biopsy (100).

The clinical setting used for surveillance may be significant for patient outcome: patients managed within the multidisciplinary oral dysplasia clinic, whose lesions underwent

malignant transformation, presented with significantly earlier stage cancers than those who presented via other routes(100). This permitted more limited surgical intervention (wide local excision) and only occasional requirement for neck dissection, flap reconstruction and adjuvant radiotherapy, when compared to higher stage tumours presenting via other clinical pathways.

It is however recognised that there is a significant economic cost attached to running these tertiary clinics, which may not be feasible to set up in some regions. It may be reasonable to monitor lower risk lesions out with the multidisciplinary dysplasia clinic, but active surveillance would be required in either primary or secondary care.

Whether, or not, it is acceptable in respect of risk management, to discharge patients considered in a 'low risk' cohort to primary care for review of their OED remains controversial, particularly in view of the ongoing uncertainties regarding the late risk of malignant transformation. This approach would require close liaison between primary and secondary care and, ideally, the establishment of managed clinical networks.

The Liverpool Multidisciplinary Approach to the Management of Oral Epithelial Dysplasia

The establishment of the Multidisciplinary Oral Dysplasia Clinic

The rationale for establishing the Liverpool Regional Oral Dysplasia Clinic was that, by concentrating on the combined expertise of senior clinicians from Oral & Maxillofacial Surgery, Oral Medicine and Oral Pathology, patient care would be enhanced, particularly in complex and high-risk cases. This clinical environment would also create undergraduate and

postgraduate teaching and training opportunities and allow for the recruitment of patients into clinical studies which could inform future practice(83, 97, 100) and, ultimately, evidence-based guidelines.

When this clinic was first established referral criteria were agreed, as limited resources necessitated careful selection of cases. Initially, patients with histopathology indicating either moderate or severe dysplasia were seen. These had a range of clinical presentations, i.e. leukoplakia (including proliferative verrucous leukoplakia), erythroplakia, erythroleukoplakia and speckled lesions. Patients were subsequently identified who had been attending Oral Medicine or Oral and Maxillofacial Surgery clinics and had a histopathological diagnosis of mild dysplasia, despite a clinically suspicious oral lesion, such as speckled leukoplakia. Targeted re-biopsy and specialist oral pathology review are indicated in these cases, following assessment on the multidisciplinary dysplasia clinic.

It was also agreed that patients who had previously undergone ablative head and neck surgery would be followed up by the joint head and neck oncology team, rather than the dysplasia clinic, as they require follow up with several specialties including clinical oncology, speech therapy, dieticians, dentists and clinical nurse specialists.

After twenty years of experience in both the multidisciplinary OED clinic and a routine Oral Medicine clinic, our approach to the management of OED has evolved and is now applied to patients referred from primary or secondary care. Not all will be transferred to the multidisciplinary clinic, but the same approach to management is applied.

Figure 5.1 illustrates an example of a management dilemma, with large lesions at the limits of what can be resected with good function outcome. Geographic biopsies of the homogenous leukoplakia in a 70-year old man show mild dysplasia, however the patient is a lifelong non-smoker and the predominant site is lateral tongue. The patient has a similar but smaller

lesion on the contralateral tongue (and buccal mucosa). Thus, with a balance of high and low-risk features, the decision on whether to intervene surgically or offer surveillance is finely balanced.



Figure 5.1 A clinical dilemma: Patient with lesions on tongue, demonstrating both high and low risk predictive factors for malignant transformation.

The management algorithm of patients referred with OED to the Liverpool Oral Dysplasia Multidisciplinary Team, has been summarized in Figure 5.2.

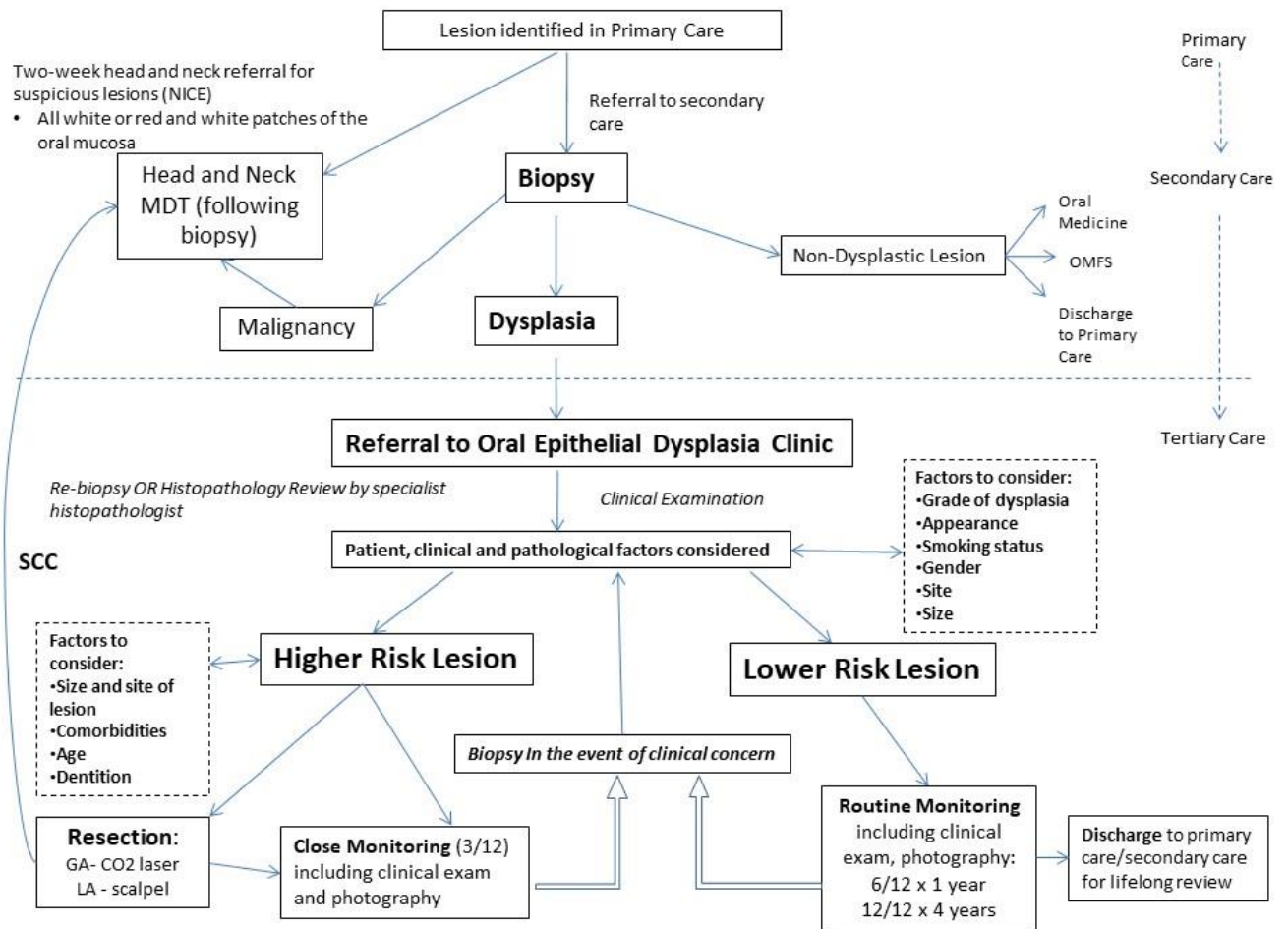


Figure 5.2 The Management Algorithm for cases of Oral Epithelial Dysplasia used by the Liverpool Multidisciplinary Team.

Conclusion

The management of patients with OED remains problematic and poses significant challenges to the clinician. Risk stratification for malignant transformation currently relies on the clinical and histological features.

It is acknowledged that the principles underlying the Liverpool Multidisciplinary approach to OED are not new or unique. However, this has formed the basis of the management of OED in a tertiary supra-regional practice and led to excellent survival outcomes(100). Whilst the

establishment of a multidisciplinary specialist clinic would be the ideal to strive towards and should be encouraged, the management of OED, in a considerable proportion of cases, is still based in secondary care, either in Oral Medicine or, Oral and Maxillofacial Surgery. The principles that underlie the Liverpool Approach could be transferable to other clinical settings, supported by fast-track referral to a Head & Neck cancer team as needed, thus allowing timely management of patients undergoing malignant transformation.

Referral of Patients with suspicious oral lesions

Most patients are referred from primary dental care and have potentially malignant oral mucosal lesions identified from opportunistic screening. Referrals from primary medical care are often prompted by the patient's self-examination. Some referrals are via the 2 Week Head & Neck Suspected Cancer Pathway; others are triaged from referral letters as needing Urgent (within 2 weeks) or Priority (within 6 weeks) appointments.

Assessment of Patients

A comprehensive history including medical, smoking (and/or betel quid use), alcohol, social and dental history should be documented. After extra-oral examination, the oral mucosa is carefully visualised with attention paid to the floor of mouth, lateral borders of the tongue and oropharynx, as these areas can be more difficult to examine. Fiberoptic nasendoscopy is arranged for any patients where visualisation of the affected mucosa is difficult. All mucosal lesions are photographed, with requisite labelling of patient details, and the appearance of lesions, as well as results of palpation. The importance of taking an adequate and

representative tissue sample cannot be over-emphasised and if necessary, the biopsy site is marked on a print-out of the clinical photograph. In complex and non-homogenous lesions, the biopsy site is chosen by a senior, experienced clinician; this is not devolved to a trainee without close supervision. Biopsies close to or overlapping opening of salivary ducts are undertaken by an Oral and Maxillofacial Surgery consultant.

Larger lesions (>200 mm²) usually require multiple geographic biopsies and for these, and posterior-placed lesions, a general anesthetic (or intravenous sedation) is considered and discussed with the patient.

The General Management of Patients with Oral Epithelial Dysplasia

Patients are counselled about smoking cessation and offered appropriate access to support. Their alcohol intake is also addressed. The biopsy result(s), indicating the level of dysplasia as well as the clinical appearance of the lesion(s), influences the management approach and these factors, together with any possible surgical interventions, are discussed with the patient and family or carer, as appropriate. Patient factors such as co-morbidities and frailty are considered, but paramount are the patient's wishes and their informed consent to any procedures. The following approach is adopted in Liverpool in respect of histopathological findings and clinical presentation.

Severe Dysplasia

Patients are offered surgical excision with the aim of achieving clear margins. For extensive lesions, essentially deemed unresectable, excision is not compatible with function, therefore

active surveillance with re-biopsy is undertaken. This policy is sometimes needed even for high risk lesions. The application of toluidine blue or Lugol's iodine is considered to aid visibility of dysplasia during surgical excision/ablation. In higher risk cases, reconstruction with skin graft, local flaps or microvascular free tissue transfer is considered; these patients are referred to Head and Neck MDT for imaging and management decisions.

Moderate Dysplasia

Excision is considered for some patients, providing the lesion is not too extensive to allow acceptable post-operative function. Patients with high risk lesions (see Table 5.1) are recommended to have surgical intervention. For those patients without high risk factors, a period of active surveillance, with re-biopsy in the event of clinical change, may be considered. A decision concerning patients with co-morbidities or who are not willing to have any surgical / laser intervention can be similarly delayed and management options re-visited.

Mild Dysplasia

Patients with homogenous oral mucosal lesions and a histopathological diagnosis of mild dysplasia are offered surveillance, together with smoking cessation and alcohol-related advice, as appropriate. If the oral lesion has high risk features (Table 5.1) then re-biopsy is undertaken, or a decision made to proceed directly to excision. The latter would usually be preceded by a detailed discussion with the patient and their clear expressed informed consent.

Surveillance of Patients with Moderate or Severe Dysplasia

Patients with severe or moderate dysplasia are followed-up in the multi-disciplinary clinic on a long-term basis, regardless of whether there has been surgical or laser treatment. They undergo a full assessment at each review, and this includes up-to-date clinical photographs. A decision is made concerning the need for follow-up biopsy (or biopsies), this is primarily prompted by clinical concerns about the change in appearance of the lesion (or lesions) and involves a team approach. If the patient reports (rare) discomfort from a previously asymptomatic lesion under surveillance, this is regarded as a red flag (warning symptom) unless there are obvious causative factors, such as trauma from a loose denture or teeth. The follow-up interval is usually one-month post-surgery/laser excision and then 3, 6 or 12 monthly depending on the clinical assessment, histology and previous history.

Surveillance and Follow-up of Patients with Mild Dysplasia

Most patients with mild dysplasia are reviewed in the routine Oral Medicine or Oral and Maxillofacial Surgery clinic, i.e. secondary care, for up to 5 years. If their lesion undergoes significant changes, or following a repeat biopsy indicating a higher grade of dysplasia, they are referred to the multidisciplinary OED clinic. Some patients with mild dysplasia and high-risk clinical features will also be offered long term review in the routine Oral Medicine clinic. A decision concerning referral back to primary or secondary care for ongoing surveillance is made based on several factors, including past dental history and attendances, patient preference and liaison with the patient's general dental practitioner. If the patient and dentist are involved in this decision and provided with all copies of relevant histopathology and updated photographs, this can be a mutually beneficial option for follow-up. It is

essential that both the patient's general dental and medical practitioners are kept informed of the patient's progress from their first hospital visit. Safeguards for this arrangement include copying the discharge letter to the patient so they are aware of the need to attend their general dental practitioner for follow-up and providing the general dental practitioner with the appropriate contact details so re-referral can be promptly arranged if concerns arise with the oral mucosal lesion.

Chapter 6 Loss of FANCD2 and related proteins may predict malignant transformation in oral epithelial dysplasia

Introduction

In the management of oral epithelial dysplasia (OED), histopathological grading and clinical determinants of malignant transformation to oral squamous cell carcinoma (OSCC) have been the primary influence in the treatment approach adopted (20, 55, 97), despite the numerous studies identifying putative molecular and other predictors of malignant change (28, 135, 153). Most studies aimed at identifying a molecular or pathological marker of malignant change have failed to undertake correlation with longitudinal clinical outcomes, so their translational value has been diminished leading to the lack of clinical application (23-25). Furthermore, they have mostly not been formally validated in independent series - this approach is difficult as the event rate of malignant transformation is low, and studies are therefore prolonged (3). There is a notable paucity of multi-centre/collaborative protocols, and, where malignant transformation can be predicted, there remains some uncertainty about recommended treatment options. The clinical outcomes of a cohort of patients managed in the Liverpool Multidisciplinary Oral Dysplasia Clinic, identified non-smoking status and the non-homogenous appearance of OED as the strongest independent predictors of malignant transformation (HR 5.9 and 2.3 respectively) (97). The estimated malignant transformation rate in this study was 22% over 5 years. The more aggressive behaviour of lesions observed in non-smoking patients (or light smokers) with OED supports an endogenous aetiology: while this might seem counter-intuitive, this trend has been seen in other models of carcinogenesis (154, 155).

The incidence of head and neck squamous cell carcinoma (HNSCC) in patients with the cancer prone syndrome, Fanconi Anaemia, is 1400 times greater than that of the general population

(31, 32) and occurs earlier in life. The Fanconi Anaemia pathway (FAP) removes interstrand crosslinks (ICL) lesions and facilitates homologous recombination repair of DNA double-strand breaks and is an integral component of the DNA damage repair mechanism which maintains genomic stability in healthy individuals (34, 35, 52). The FAP consists of 22 proteins (FANCC – FANCW) and has 3 main components (41) (Figure 1.1): (i) The FANCM-MHF1-MHF2 complex senses ICLs and localises to the DNA, acting as a recruitment site for the core complex through FANCM-FANCF interactions. The main role of the core complex is to facilitate ubiquitination of (ii) FANCD2 and FANCI protein dimers. The core complex mediates FANCD2/FANCI monoubiquitination, at residues K561 and K532 through FANCL E3 ubiquitin ligase activity, which then activates (iii) downstream effector proteins which include nucleases, translesion polymerases, homologous recombination proteins, and de-ubiquitinases to complete the DNA repair process and to attenuate FAP signalling. The detection of prognostic markers of DNA damage such as single-stranded DNA and replication stress, leads to the ataxia telangiectasia rad-3 (ATR) and checkpoint 1 (CHK1) kinases mediated cell cycle arrest in G2 or S phase, and activation of key components of the FA pathway via phosphorylation at several functional residues, including; FANCA at S1449, FANCM at S1045, FANCD2 at S717, S222, S331 and T691, FANCG at S387, S383 and S7, and FANCE at S374 and T346. (44-47).

If left unrepaired, ICL lesions progress through the cell cycle, resulting in the stalling of replication forks and the eventual formation of double-strand breaks, resulting in genomic instability. (156).

We hypothesise that in transforming OED (particularly in non-smokers), an aberration in the DNA damage sensing, signalling or repair pathways leads to accumulation of DNA mutations and malignancy. These aberrations in the tumour suppressor mechanism leads to

phenotypical and genotypical changes which occur in the early phase of carcinogenesis. In OED, the potential exists for identification of these aberrations in these lesions with potential for malignant transformation. The objective of this study was to investigate the status of FANCD2 and related proteins (ATR, Chk1 and FANCG) in the DNA damage repair pathway of OED in patients who presented to the Liverpool Multidisciplinary Oral Dysplasia clinic, specifically to elucidate if this information could be obtained from the initial diagnostic biopsy as a predictive tool which might have been utilised to influence clinical management, and to correlate these findings to clinicopathologic characteristics.

Patients, materials and methods

Patients

Forty patients with OED, were identified from the clinical cohort in Chapter 2 and were included in this study after giving informed consent. All specimens for this study additionally met the additional criteria that the diagnostic FFPE block had not been previously utilised for translational research. The tissue size on the diagnostic biopsy FFPE block allowed for four 0.6mm core to be extracted for RNA extraction/Western blotting (23 non-transforming OED – NT and 17 malignant transforming OED – T). Four micrometre sections of the initial diagnostic incisional biopsy FFPE from each patient were stained with haematoxylin and eosin (H&E) and reported blinded and independently by two oral and maxillofacial pathologists to confirm the presence of OED (Initial attempts to construct a tissue micro-array was unsuccessful as the thickness of the incisional biopsy FFPE specimen was variable and inadequate to allow reproducibility of sections which enabled consistent and meaningful

comparison(23)). In this study, the WHO 2017 dysplasia classifications(2) were then grouped as follows: Group 0: severe dysplasia and Group 1: mild or moderate dysplasia

FANCD2 Immunohistochemistry

FFPE sections from each clinical timepoint, including the initial diagnostic biopsy, for these 40 patients were stained with anti-FANCD2 antibody (F117: sc-20022, Santa Cruz diluted 1/100) and Biogenex Supersensitive Polymer HRP detection kit (Launch Diagnostics QD430-XAKE) as previously described(155). The expression of FANCD2 was scored blinded by two independent observers (Asterios Triantafyllou and Michael Ho), one being an oral and maxillofacial pathologist. The stained sections were identified by their pathology identification number alone, thus the observers were blinded to the clinical details and outcome of OED i.e. either stable OED or OED which underwent malignant transformation. Consequently, staining was classified by localisation, extent and intensity using a descriptive binary scoring system (Table 6.1). Any discrepancy in scoring was reviewed jointly and a mutually agreed score determined. Control tissue from normal and OED areas of tissue adjacent to OSCC from a different cohort of anonymised patients (n=3) were stained using the same method.

Feature		Binary score	
		0	1
Nuclear Staining	Extent	Absent-Focal	Multifocal-Widespread
	Intensity	Absent-Weak	Moderate-Strong
Cytoplasmic Staining	Extent	Absent-Focal	Multifocal-Widespread
	Intensity	Absent-Weak	Moderate-Strong

Table 6. 1 FANCD2 immunohistochemistry scoring system

Western blotting

Areas with the highest grade of dysplasia in the incisional biopsies were marked on the H&E stained sections and 0.6mm cores were obtained from the corresponding FFPE blocks (minimum of 2 cores from each block). Cores of the same diameter were obtained from anonymised controls as follows: normal tissue from oesophagus (n=5), areas of OSCC from a different cohort (n=3) and tissue with normal histological architecture located adjacent to OSCC (n=3).

The protein extraction protocol utilised was modified (based on local expertise from supervisor J. B. Wilson) from one previously published (157): FFPE cores were placed in Eppendorf safe-lock tubes (Eppendorf, Hamburg, Germany) and deparaffinised by incubation at room temperature in xylene for 10min. After each incubation, the tissue was pelleted at 12000×g for 3 min, and incubation/centrifugation steps were repeated two more times. The deparaffinised tissue pellets were then rehydrated with a graded series of ethanol (100% for 5 minutes, 90% for 5 minutes, 70% for 5 minutes and a further 5 minutes in 70%) and centrifuge at 1300 RPM for 30 secs to remove excess ethanol. Following this, 150µl of

Laemmli sample buffer (Sigma-Aldrich) were added. All samples were subjected to high-temperature extraction at 100°C for 20min, and then cooled in ice for 5 minutes. Extracts were centrifuged for 30min at 13000 RPM and the supernatant collected and stored at -20°C until needed.

The expression of β -actin, ATR, pATR (s428), CHK1, p-CHK1 (s317), FANCD2 (non-and mono-ubiquitinated isoforms), pFANCD2 (s331), FANCG and pFANCG (s7) were assessed in a blinded fashion (to clinicopathologic details and outcome: stable OED or OED which underwent malignant transformation) by western blotting using anti- mouse or anti-rabbit fluorescently labelled 680/800 secondary antibodies (1:10 000 dilution) (Invitrogen, Paisley UK) (Table 6.2) (43), extracted protein was separated on SDS- polyacrylamide (PAGE) gels: samples were prepared for loading, by the addition of 20 μ L of protein extract to 40 μ L of Laemmli loading buffer and immediately heated at 100 °C for 5 minutes, prior to loading. The samples were loaded on to a 15% SDS-PAGE gel which were ran overnight at constant 50 V. The protein was then transferred to a nitrocellulose membrane - 4 hours at constant 350 mA (158). This was blocked in LI-COR buffer for 1 hour at room temperature prior to the addition of specific antibodies at the specified dilutions.

Antibody against	Raised in	Company	Catalogue number	Dilution	Citations
β -actin (C4)	Mouse	Santa Cruz	sc-47778	1:5,000	(159)
ATR	Rabbit	Atlas	HPA028264	1:1,000	(160)
pATR	Mouse	Gift of Dr G. M. Kupfer		1:1,000	(43, 51)
CHK1	Mouse	Santa Cruz	sc-56291	1:1,000	(161)
pCHK1 (S317)	Rabbit	Calbiochem	DR1025	1:1,000	(161)
FANCD2*	Mouse	Santa Cruz	sc-28194	1:500	(46)
pFANCD2* (S331*)	Mouse	Gift of Dr G. M. Kupfer		1:1,000	(43, 51)
FANCG*	Rabbit	Santa Cruz	sc-28219	1:1,000	(46)
pFANCG* (S7*)	Mouse	Gift of Dr G. M. Kupfer		1:1,000	(51, 162)

**selected as markers of activation of the Fanconi Anaemia pathway availability and reliability from previously published data(43, 53)*

Table 6. 2 Antibodies used in Western blot

An Odyssey Infrared Imaging System was used to visualise band intensity (Li-Cor Biosciences, Cambridge, UK). Densitometry values of band intensities were measured and calculated using ImageJ®. For each protein, these values were normalised against the oesophageal normal tissue scores (positive FANCD2 expression (<https://www.proteinatlas.org/ENSG00000144554-FANCD2/tissue>), anonymised normal tissue readily available), then ranked and divided into tertiles representing low expression, moderate expression and high expression. The normal oesophageal control values always lay within the central tertile.

Data/Statistical Analysis

Mann Whitney and Fisher exact tests were utilised to compare FANCD2

immunohistochemistry scores between samples from NT and T OED lesions. The Fisher-

Freeman-Halton exact test was used as a measure of significant difference and comparison between the densitometry ratios of NT and T OED samples. Statistical analyses were conducted, using the statistical software (IBM SPSS Statistics for Windows, Version 22.0). A p value of less than 0.05 was considered significant.

Results

Patient outcomes

The demographic and clinicopathologic details of the forty patients included in this study are summarised in Table 6.3.

As previously noted in Chapter 2, the malignant transforming (T) group had a higher proportion of non-smokers ($p = 0.05$), lateral tongue lesions ($p = 0.01$) and non-homogenous OED ($p = 0.001$) when compared with the non-transforming (NT) OED group. The median follow-up period was 5.3 years (range 0 - 21 years) with a median of 3.4 years (range 0 - 14 years) for the NT group and 6.9 (range 1 – 21 years) for the T group. The median time to malignant transformation was 3.4 years (range 0 – 7.6 years). The dysplasia binary score at first diagnostic biopsy was not significantly different between the groups, with 5/17 (29%) transformers classified as high risk compared with 3/23 (13%) non-transformers ($P=0.189$). These 3 patients were progression-free for 18, 52 and 107 months, respectively, following diagnosis. The median time to malignant transformation for T OED in patients with the higher risk binary score at diagnosis was 14.8 months when compared with T OED with a low risk binary score, 44.7 months ($p = 0.1$).

		Non-transformers (n =23)	Transformers (n=17)	P value
Age	Median (range)	55.9 (27.9 – 78.4)	57.5 (37.5-85.2)	0.56
Gender n (%)	Male Female	13 (57) 10 (43)	6 (35) 11 (65)	0.22
Site n (%)	Lateral tongue Floor mouth Buccal Alveolar mucosa	4 (17) 11 (49) 4 (17) 4 (17)	10 (58) 3 (18) 4 (24) -	0.01
Appearance n (%)	Homogenous white Red-white Red	20 (87) 2 (9) 1 (4)	7 (41) 10 (59) -	0.001
Lesion size n (%)	< 200mm ² ≥ 200mm ²	5 (22) 18 (78)	3 (18) 14 (82)	1
Smoking n (%)	Never 5-20 pack years >20 pack years	5 (22) 7 (30) 11 (48)	7 (41) 8 (47) 2 (12)	0.05
Alcohol n (%)	Teetotal Current drinker	6 (26) 17 (74)	5 (29) 12 (71)	1
Grade of dysplasia* n (%)	Mild/moderate Severe/Cis	20 (87) 3 (13)	12 (71) 5 (29)	0.19
Definitive treatment	Surveillance Excision	17 (74) 6 (26)	0 17 (100)	0.0001

Table 6. 3 Demographic and clinic-pathological features of patients (n = 40) (* at initial diagnostic biopsy)

FANCD2 immunohistochemistry

Nuclear and cytoplasmic staining for FANCD2 was absent in morphologically normal oral epithelium (Figure 6.1), however, FANCD2 staining increased in intensity in non-invasive dysplastic epithelium and was then lost again in micro-invasive OSCC (Figure 6.1).

A decrease in the intensity of both nuclear and cytoplasmic staining were observed in the first diagnostic biopsy of OED lesions that were destined to undergo malignant transformation when compared with non-transforming OED, although the distribution of staining was not statistically different (the quantitative, rather than qualitative, element of FANCD2 staining was predictive of malignant transformation) (Figure 6.2; Table 6.3).

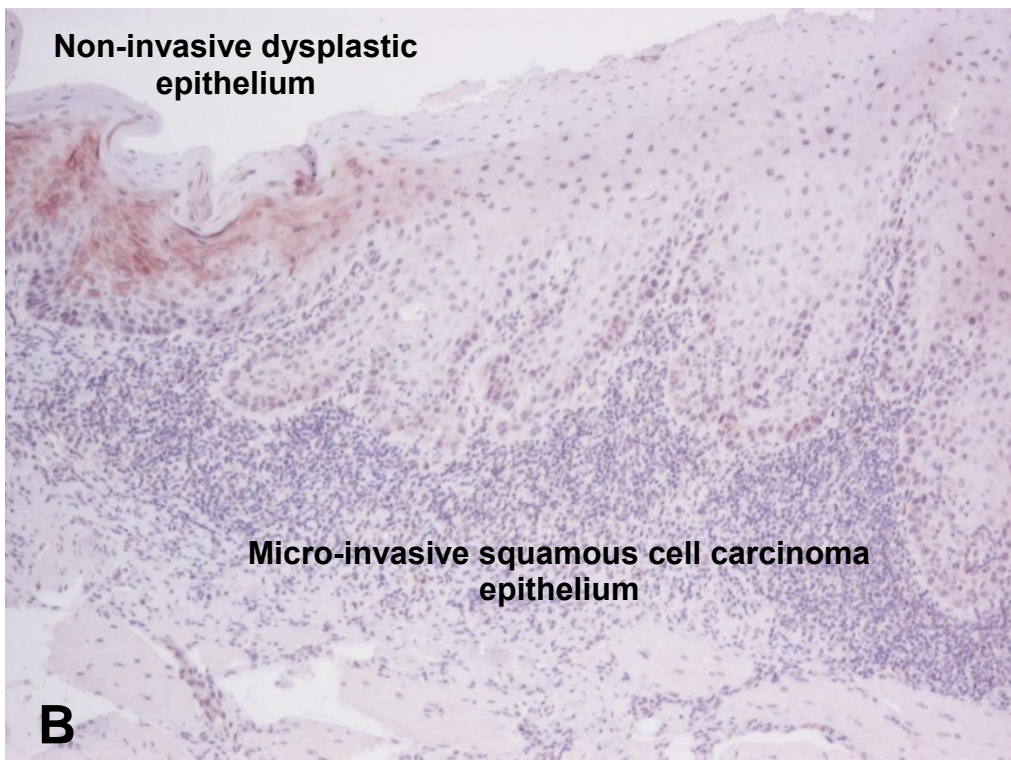
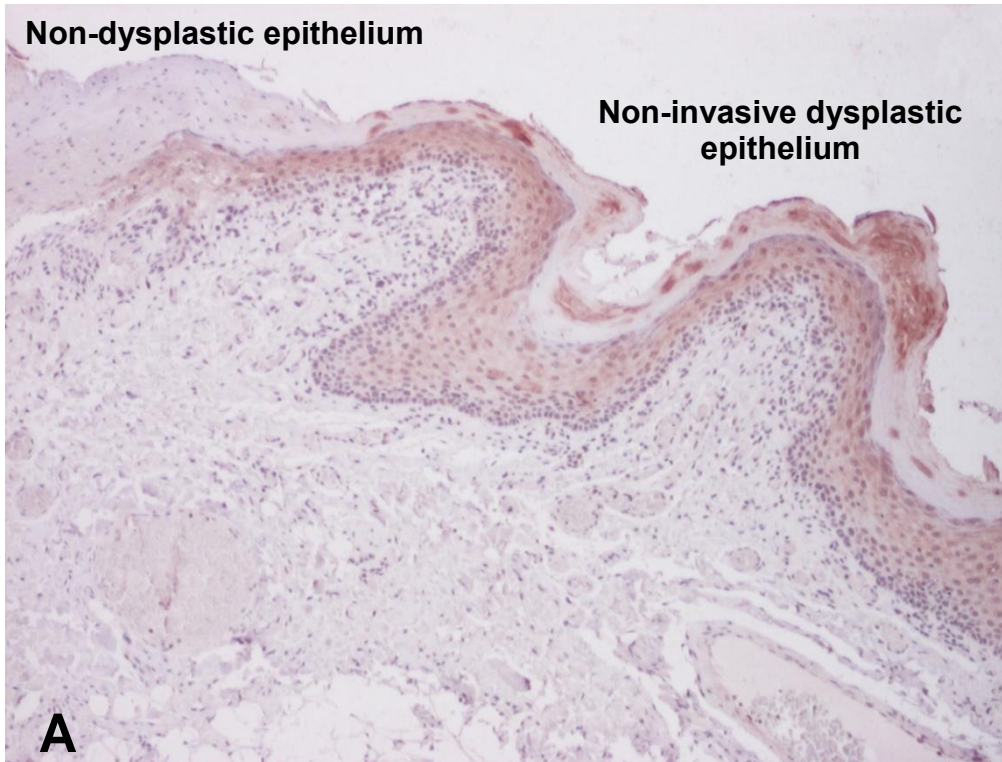


Figure 6. 1 Representative example of FANCD2 immunohistochemistry during progression to carcinoma. A: lack of FANCD2 expression in normal (non-dysplastic) epithelium; widespread and moderate staining in the nucleus and cytoplasm of suprabasal (differentiating) layers in non-invasive dysplastic epithelium and B: loss of FANCD2 expression when malignant transformation has occurred. Magnification x400

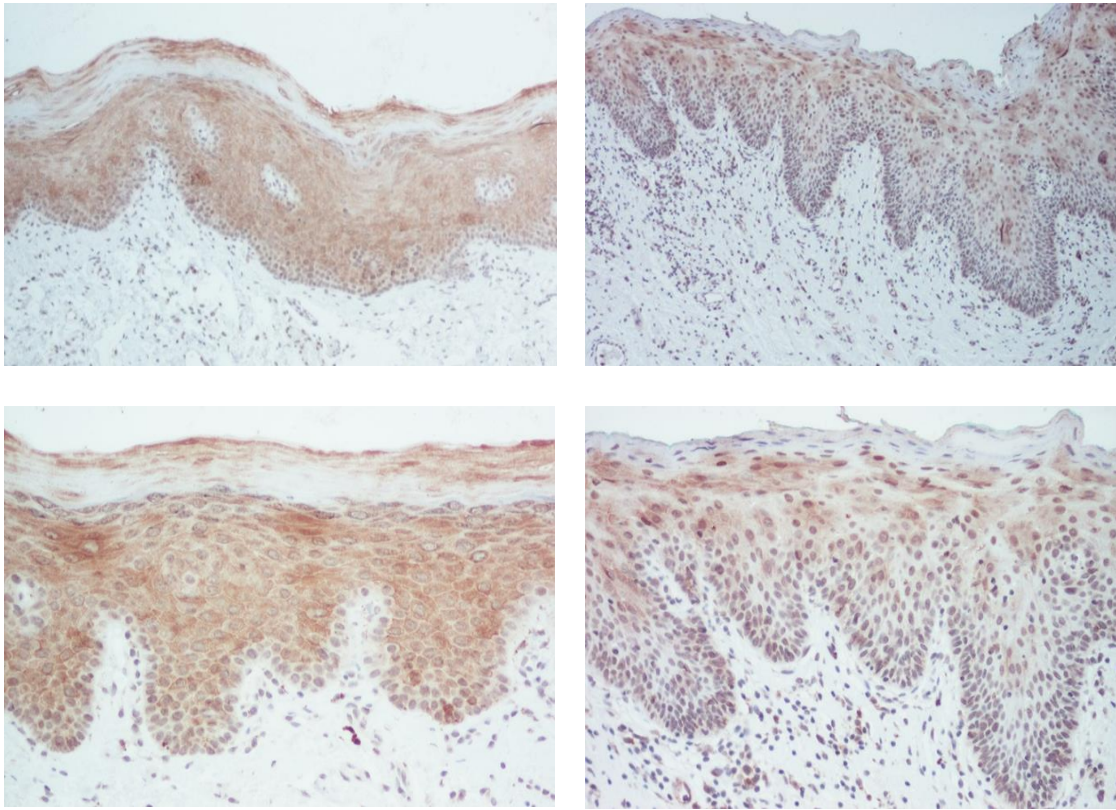


Figure 6. 2 Representative FANCD2 immunohistochemistry of 'low risk' dysplasia from NT OED (left): moderate dysplasia displaying strong nuclear and cytoplasmic expression of FANCD2 across the entire thickness of epithelium with widespread distribution and 'high risk' T OED (right): severe dysplasia with weak and irregular expression FANCD2 in the nucleus and cytoplasm of especially in the basal two-thirds of the epithelium with multifocal distribution . Top row : low power magnification (x100); bottom row: high power magnification (x400).

Therefore, only intensity scores were incorporated into a final composite FANCD2-OED score as below. In OSCC sections from transformed OED lesions, both intensity and distribution of FANCD2 staining were reduced compared with the first diagnostic biopsy from the same patient (Table 6.4).

Site of staining	Pattern of staining	Biopsy		
		NT - first diagnostic n (%)	T – first diagnostic n (%)	T – OSCC diagnostic n (%)*
Nuclear	Distribution			
	Absent/Focal	6 (26)	4 (24)	9 (60)
	Multifocal/Widespread	17 (74)	13 (76)	6 (40)
	Intensity**			
	Absent/Weak	13 (57)	16 (94)	14 (93)
	Moderate/Strong	10 (43)	1 (6)	1 (7)
Cytoplasmic	Distribution			
	Absent/Focal	1 (4)	1 (6)	4 (27)
	Multifocal/Widespread	22 (96)	16 (94)	11 (73)
	Intensity***			
	Absent/Weak	5 (22)	8 (47)	10 (67)
	Moderate/Strong	18 (78)	9 (53)	5 (33)

*Specimens for two patients were not retrievable from archival records

** : NT v T (first diagnostic biopsy) P<0.008 (Mann-Whitney Test)

***: NT v T (first diagnostic biopsy) P=0.089 (Mann-Whitney Test)

Table 6. 4 FANCD2 immunohistochemistry scores in first diagnostic biopsy of non-transforming (NT) and transforming (T) OED, together with paired OSCC for the transforming group

Combining the FANCD2 nuclear and cytoplasmic intensity scores with the histological grading of OED (mild/moderate dysplasia assigned score of 1 and severe dysplasia/carcinoma-in-situ/SCC assigned score 0) produced a score that more accurately predicted transformation (p=0.005) (Table 6.5), with a cut off score of 1 or less being significantly associated with a higher risk of malignant transformation in OED (p=0.001). This FANCD2-OED Risk Score in all the retrieved OSCC archival tissue from OED which underwent malignant transformation was ≤ 1 (n = 15; 2 archival OSCC specimen not retrievable).

FANC D2–OED Risk Score	NT OED n = 23		T OED n = 17		P value (Mann Whitney test)	*P Value (Fisher's exact test)
3	4	21*	2	7*	0.005	0.001
2	17		5			
1	2	2*	8	10*		
0	0		2			
FANC D2–OED Risk Score			OSCC n = 15			
1			7	15		
0			8			

Illustration of how FANCD2-OED Risk Score was derived:

e.g. Severe dysplasia (0) with absent/weak nuclear staining (0) and moderate/strong cytoplasmic stain (1) = 0 + 0 + 1 = 1

Table 6. 5 FANCD2 IHC-OED grade combined scores in stable OED (NT), OED which underwent malignant transformation (T) and oral squamous cell carcinoma which had arisen from OED.

Of the 2 NT OED lesions with low (≤ 1) FANCD2-OED Risk scores, one patient presented with severe dysplasia which was excised shortly after presentation and has now been progression free for 52 months and one presented with mild dysplasia that has been progression-free for 19 months. Eighty-three percent (10/12) of patients with FANCD2-OED Risk score ≤ 1 in the OED cohort reported went on to develop OSCC compared with 25% (7/28) in patients with a score of ≥ 2 . The diagnostic biopsies for the 7 transforming lesions with high (≥ 2) FANCD2-OED Risk scores were obtained a median of 5.8 years (range 0.3-7.0 years) prior to transformation, while the biopsies from the 10 transforming lesions with low (≤ 1) scores were taken a median of 2.4 years (range 0.2-5.3 years) prior to transformation. In all 15 of the available oral squamous cell carcinomas which had arisen from OED, the expression of FANCD2, and the concomitant FANCD2-OED score, was low (Table 6.5). The FANCD2-OED scores were not significantly different when the following parameters were assessed: age, gender, site of lesions, appearance of lesion, smoking or alcohol history and time to

transformation, but as expected, were associated with grade of dysplasia ($P < 0.0005$). In total, 1/3 'false positive' and 5/12 'false negative' dysplasia grade scores were correctly predicted by the FANCD2-OED score.

FANCD2 and associated protein expression

No significant differences in the normalised expression of β -actin, ATR, CHK1 and FANCG were observed between T and NT OED lesions (Table 6.6; Figure 6.3). As the biopsy specimens in OED were much smaller in comparison with those obtained in established OSCC, the Western blots were performed on a single occasion due to the limited quantity of available protein.

However, significantly reduced expression of FANCD2 (non-ubiquitinated and mono-ubiquitinated forms), pFANCD2, pATR, pCHK-1 and pFANCG were observed in OED prior to malignant transformation with the FANCD2, pFANCD2 and pFANCG observations mirrored in the control tumour tissue. These parameters did not correlate with any clinicopathological features such as site or smoking history. The median ratio of mono-ubiquitinated: non-ubiquitinated forms of FANCD2 (an indication of activation of the FA pathway) were 0.9 and 0 for NT and T OED, respectively ($p < 0.001$).

Normalised densitometry score ratio*		Low expression n (%)	Moderate expression n (%)	High expression n (%)	P value (Fisher-Freeman-Halton exact test)
ATR	T	9	3	5	0.24
	NT	7	8	8	
pATR	T	14	3	0	< 0.0005
	NT	1	9	13	
Chk1	T	5	7	5	> 0.29
	NT	10	5	8	
pChk1	T	13	3	1	< 0.0005
	NT	3	8	12	
FANCD2 (S & L [#])	T	10	2	5	< 0.025
	NT	10	8	5	
pFANCD2-s331	T	16	1	0	< 0.0005
	NT	1	12	10	
FANCG	T	4	6	7	0.61
	NT	10	4	9	
pFANCG-s7	T	16	1	0	< 0.0005
	NT	1	10	12	

* See materials and methods

Sum of 'small' and 'large' FANCD2 isoforms. - L corresponds to the monoubiquitylated (activated) isoform p: phosphorylated proteins; s331 & s7: location of phosphorylation

Table 6. 6 Normalised protein expression in initial diagnostic biopsy of NT OED and T OED lesions

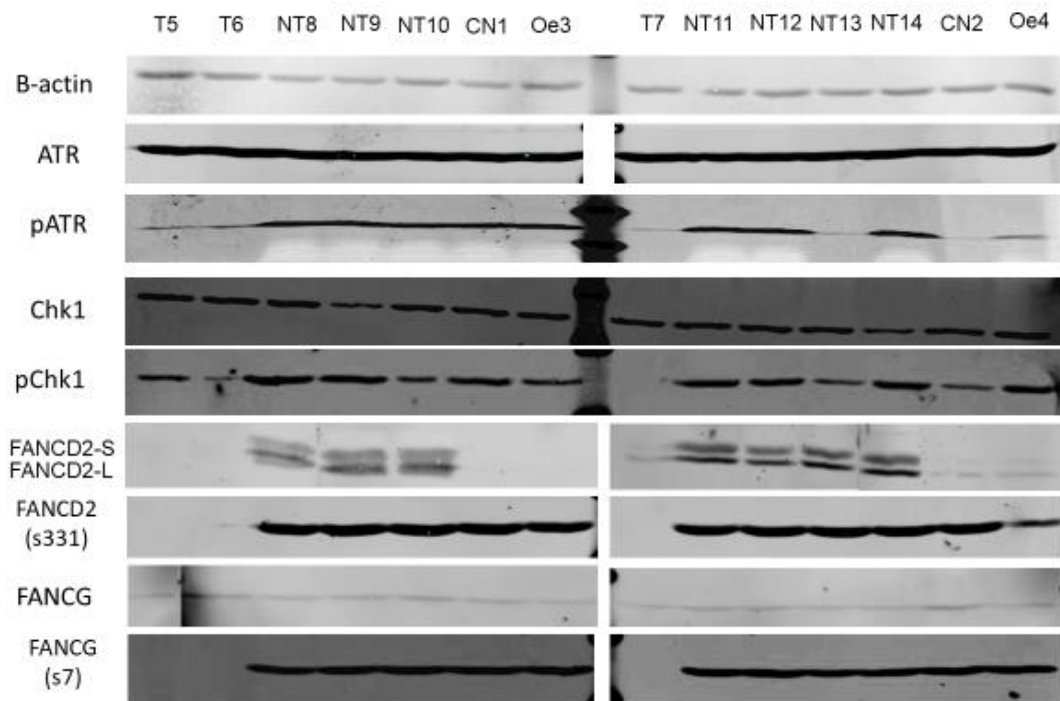
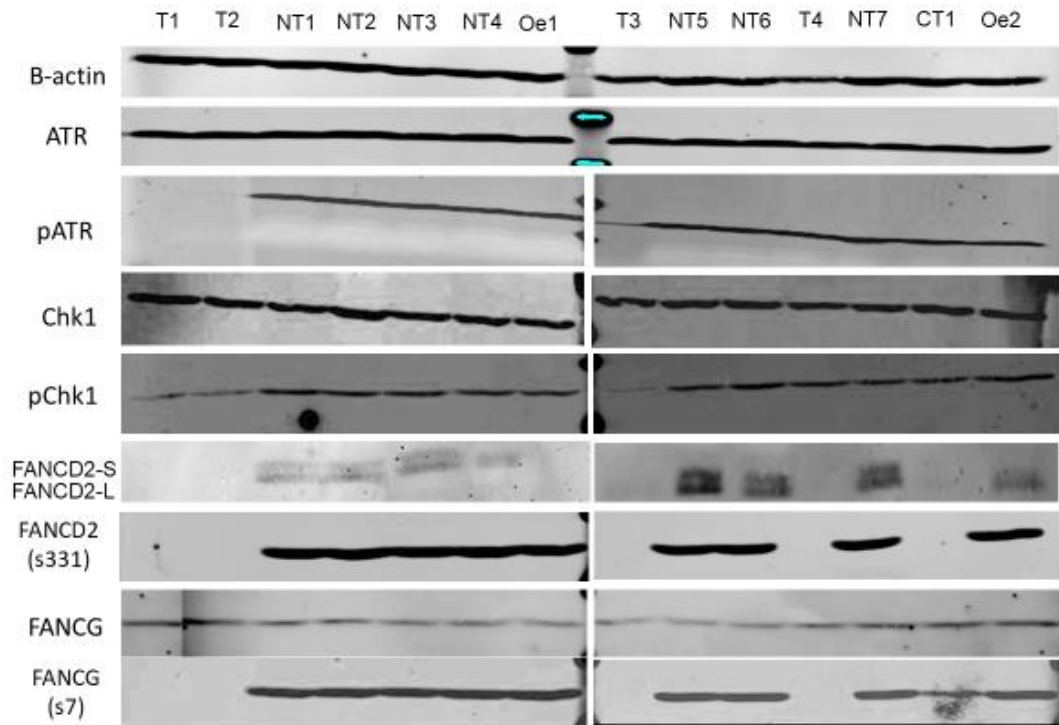


Figure 6.3 Representative example of Western blot expression analysis of DNA damage sensing and repair proteins. NT: first diagnostic biopsy of a non-transforming OED; T: first diagnostic biopsy of a transforming OED; Oe: normal oesophagus; CT: control OSCC from a different cohort; CN: control (morphologically) normal from margin of OSCC resection.

Discussion

The results and observations of this study have evidenced support to the notion that loss of FANCD2 and associated protein (in the DNA damage repair pathway) are associated with malignant transformation in OED. The lower, statistically significant, expression of post-translationally modified proteins in T OED when compared to NT OED is indicative of inactivation of the pathways and was a more accurate predictor of malignant transformation than clinical parameters such as smoking history and site. Furthermore, immunohistochemical staining for FANCD2 coupled with a binary dysplasia score could correctly predict malignant transformation in 10/17 initial biopsy samples obtained prior to transformation, with a 'false positive' rate of 2/23 non-transforming OED biopsies. This performed better than dysplasia grading alone, either as a binary score or the current WHO classification. The significantly different rates of malignant transformation in between patients with low FANCD2 (83%) expression and higher FANCD2 (25%) expression further validate its potential utility in the clinical setting in determination of the risk of carcinogenesis. The data of Rudland et al (155) indicates that cytoplasmic as well as nuclear staining may be prognostic, but their complicated system for scoring extent and intensity of staining was not appropriate for our samples which were generally small in nature. There could be scope for future validation studies to utilise resection specimens if necessary, however there is generally some degree of heterogeneity in tissue architecture/grade of dysplasia and interpretation of these findings require further thought and definition of the scoring criteria utilised. Both CHK1 and CHK2 phosphorylate the FANCD2 protein at different sites, but only phosphorylation of the s331 residue activates the protein complex. Upstream,

the ATR pathway is of interest because when CHK1 is phosphorylated, it in turn activates FANCD2 by phosphorylation.

The loss of FANCD2 expression has to be viewed within the context of the presence of OED or OSCC, hence the importance of including the grade of OED (or diagnosis of OSCC) into the FANCD2-OED Risk Score (Table 6.5). In several anonymised normal oesophageal control samples (Figure 6.3) the expression of FANCD2 was not observed similar to the normal oral epithelium (obtained from the margins of resected OSCC/OED specimen) (Figure 6.1A). This suggests that in the absence of OED or OSCC, the lack of FANCD2 expression would indicate that the Fanconi Anaemia Pathway has not been activated. The difference in these normal controls with transforming OED is that quite consistently the post-translational modifications e.g. pATR, pChk1, pFANCD2(s331) and pFANCG(s7) have all been preserved in the control specimen. These warrants further investigation in order to explain the mechanism of the Fanconi Anaemia Pathway in normal and diseased tissue within the context of the process of carcinogenesis.

In this analysis, moderate dysplasia was classified together with mild dysplasia as 'low risk' while severe dysplasia was classified as 'high risk'. Other researchers have classified moderate dysplasia as 'high risk', but recent discussion in the literature suggests that both suggested binary classifications are simplistic (125, 163). Alternative suggestions for binary classification of oral dysplastic lesions rely heavily on pathological interpretation, which could be prone to intra and inter-observer reliability problems (164, 165). It is acknowledged, therefore, that the proposed histopathology/IHC classification in its current format will have skewed the moderately dysplastic lesions in this study towards higher FANCD2-OED scores and is thus prone to false negatives, although it still performed better than histopathology alone. Its strength may be in identifying lesions which will NOT transform, but development

into a routine, clinical test requires a more robust definition of reduced immunostaining utilising data from a larger number of transforming and non-transforming lesions. This study is best categorised as proof-of-concept as the design and the cohort are not adequate for a robust prognostic biomarker study.

When the NT and T groups were compared, there were significant differences in site, smoking aetiology and appearance between the two cohorts. These are such strong predictors of transformation in our modest cohort that it was impossible to adequately match the two groups. The validity of diagnosis of the grade of OED from an incisional biopsy could potentially be questioned as the issue of heterogeneity of OED especially in a large lesion is a valid consideration. In the Liverpool Oral Dysplasia MDT clinic, the biopsy of OED lesions is carried out by senior surgical members of the team where the area of most clinical concern is sampled. Experience in our practice would suggest that concordance of initial biopsy and definitive histopathology diagnoses were very high and had not adversely impacted on patient outcomes. This was demonstrated in the reported cohort of patients where patients who have their OED excised did not have their grade of OED upgraded but the more often scenario was that the area with the most severe grade of OED has been completely excised by the incisional biopsy. Therefore, the findings observed for FANCD2 immunohistochemistry and Western blotting were valid from the statistical and clinical perspectives.

Six of the twenty-three NT lesions (that we would have expected to transform) were totally excised – therefore some of the differences observed between NT and T groups might potentially relate to the method of treatment rather than the inherent cancer risk of the sample (Table 6.3) This could be a reflection that there is such strong prescription bias on

grade and/or clinical features of lesions that it would be impossible to adequately match the cohorts.

The median follow-up period was less in the NT vs the T group [3.4 years (range 0 - 14 years) vs 6.9 (range 1 – 21 years)], and this may partially explain the observation of NT OED lesions with low (≤ 1) FANCD2-OED scores, one of whom had only been followed for 19 months, and T OED lesions with high (≥ 2) FANCD2-OED scores, 6 of which were obtained more than 3 years prior to transformation. These data may give some indication as to the sensitivity of this technique for prediction of malignant transformation prior to the event. For example, of the 6 T OED patients in whom we had non-neoplastic tissue from an intermediate timepoint, we observed that the FANCD2-OED scores decreased in 2 patients (from ≥ 2 to ≤ 1 in both cases) and remained static in 4 patients (3/4 of whom scored ≤ 1 at the initial biopsy), with all the OSCC from these patients scoring 0 (n=5) or 1 (n=1).

It has been reported that phosphorylated proteins are more labile, and that epitope degradation can occur within 30 minutes of ischaemia in formaldehyde (166) leading to loss of certain post-translational modifications (PTMs), specifically phosphorylation. Utilising the protein extraction protocol described in this study, the expression of PTMs has been shown to be consistently down-regulated across 4 different phosphorylation sites (ATR (s428), CHK1 (s317), FANCD2 (s331) and FANCG (s7)) in biopsy tissue destined to transform (T-OED) compared with that which was not (NT-OED). This finding is significant given the lack of availability of fresh tissue collections from cohorts of dysplasia patients and suggests that PTM biomarkers of transformation may be developed for FFPE tissue. This could potentially be used to influence treatment decisions in clinical practice or when utilised within the context of a clinical trial in the management of OED to stratify treatment/intervention arms.

The hypothesis that malignant change in OED involves alteration of the FA pathway is supported by both our immunohistochemistry and western blotting data. These reinforce the previously described differences observed in the appearance and site of OED in these two groups of patients(97) but are not simply a reflection of these differences as no associations were observed between site or appearance of lesion and PTM of these proteins. Samples from T OED lesions showed significant reduction in the phosphorylation of ATR, CHK1, FANCD2 and FANCG in comparison to non-transforming samples, indicating a lack of ATR-CHK1 activation following DNA damage and/or replicative stress. It may be argued that these observations are due to a lack of stimuli in the transforming group as they have a preponderance of non-smokers, but analysis of our data does not support this as no significant difference in PTM expression was observed between smokers and non-smokers. The ability of CHK1 to phosphorylate several functionally important sites for optimal function and activation of the FA pathway appears to be compromised in these patients which, it is proposed, will lead to the impairment of the functionality of the FA core complex and lead to a reduction in subsequent HRR activity (43, 46, 51, 167). In contrast, non-transforming samples showed high levels of FANCD2 s331 and FANCG s7 phosphorylation, indicating that these sites were successfully phosphorylated by activated CHK1, and could function effectively in DNA repair, thus reducing the burden of DNA damage in these cells and reducing the risk of malignant transformation.

FANCD2 monoubiquitylation, which is thought to be promoted by ATR-CHK1 mediated FANCD2 phosphorylation (45, 161), is a critical step in FA pathway activation(51, 168) and evidence suggests that a reduction in FANCD2 monoubiquitylation has a greater influence on genomic instability than down regulation of FANCD2 expression (169). In our study, it was

observed that transforming samples have lower levels of FANCD2 monoubiquitylation (FANCD2 L expression) compared to non-transformers, and interestingly, they consistently displayed lower levels of total FANCD2 expression. These findings agree with our concurrent immunohistochemistry data where we observed a lack of FANCD2 protein expression in T-OED compared with NT-OED lesions.

The direct evaluation of the DNA sensing-signalling-damage repair cascade in OED has not been previously reported, although there is recent evidence that individuals with reduced, systemic, double strand break repair capacity are more prone develop to head and neck cancer (38). It has been suggested that activation of DNA damage response might be protective in the early stages of oral carcinogenesis, but progressive deregulation over time could eventually result in the failure to suppress malignant transformation(170). The results of the current study indicate that additional evaluation of these pathways is worthwhile to understand their capability to predict malignant transformation in OED at the initial diagnostic biopsy, especially as time to transformation may be as long as 7 years (20, 97). Loss of heterozygosity (LOH) status at putative tumour suppressor gene loci (3p14, 9p21, 9p22 and 17p13) is currently the most reliable predictor in malignant transformation in OED (79, 171, 172) and there is evidence to suggest that LOH is secondary to homologous recombination deficiency/DNA damage repair deficiencies at 15 cancer sites, including head and neck squamous cell carcinoma (173), indicating a possible link with the current data.

In the process of validating the FANCD2-OED Risk score, a multi-centred setting with larger sample size would be desired, to control for as many variables as possible matched for smoking, clinical appearance, age and management (surveillance vs excision). This would with

the numbers needed to treat analysis inform the sensitivity and specificity requirements of the potential for the FANCD2-OED Risk score to serve as a potential biomarker for malignant transformation in OED(174). This should explore possibilities of refining the processes presented in this study, to streamline the diagnostic pathway so that clinical application would be feasible and cost effective. It remains to be seen if the FANCD2-OED Risk score could be more accurate in the prediction of malignant transformation when compared to loss of heterozygosity(175), or perhaps they could be utilised jointly. This could inform the development of future novel treatment strategies in the management of OED.

Chapter 7 Conclusion: Proposals for the future management of oral epithelial dysplasia - striving for a personalised approach

There has been more clarity over the last decade in the high-risk features of OED based on clinical presentation. These include lateral tongue subsite and/or floor of mouth(62-64, 97), female gender(10), longer duration of lesion(9, 18), idiopathic leukoplakia(non-smokers) (9, 18, 97), , size $\geq 200\text{mm}$ (21, 56, 65, 97), non-homogenous appearance(9, 21, 97), higher grade of dysplasia(10, 20, 66, 97), verrucous subtype(5, 9, 67, 68) and the presence of multiple lesions(69). Intervention to date consists of primary surgical excision or conservative clinical surveillance, as systemic or topical therapies have not been adopted widely or shown to be effective(16, 149, 176, 177). Management of patients with oral epithelial dysplasia should incorporate be primary/secondary prevention and patient education around the role of tobacco and/or alcohol in carcinogenesis(141, 178). Laser surgical excision has been the more widely adopted practice in general as it offers intraoperative advantages in terms of visibility and fine precision, when compared to cold steel. It also offers better local control when compared with laser ablation and allows for histopathological evaluation of the entire resected lesion which in some occasions may harbour subclinical occult early squamous cell carcinoma and in larger lesions, there could be heterogeneity in the severity of dysplasia grade. The latter factors could impact on patient prognosis and regularity at which patients are followed up. There is emerging evidence that the risk of malignant transformation could be lower in patients who have their OED excised compared to those in which the lesions are managed conservatively(20, 71). The preceding chapters have demonstrated that in the management of OED management of high-risk patients/lesions within a MDT setting can be justified by the treatment outcomes within the context of early detection of malignant

transformation, simpler treatment modalities and excellent survival outcomes(100). Expert guidance derived from local outcomes(76, 97, 100) provides the basis for a clinical management and surveillance protocol(179). These clinical determinants are of immense value in guiding the selection of management strategies, however remains mainly retrospective and have been based on outcomes in single centres. The availability of a molecular indicator for underlying deficiencies in DNA damage repair through the FA pathway as demonstrated through the proof of concept/pilot study in this cohort based upon the initial biopsy of OED following presentation offers the opportunity to combine the clinical and molecular determinants of malignant transformation on an individual basis. Due to the small sample size in the reported series within this thesis, the proposal for a multi-centre study which allows for matching/controlling of clinical factors such as smoking history, gender, site, size and appearance of lesion would enable more accurate interpretation of findings and allow for the them to be applied to the wider population. Further options for progress lie in the potential for less invasive methods of sampling the oral mucosal lesions e.g. oral scrapes or brush biopsies instead of an incisional biopsy, or surrogate sampling such as utilisation of salivary samples (containing oral epithelial cells). This has been piloted in a selected group of patients and there was very close correlation of FANCD2 and related protein expression when compared with the blots obtained from proteins extracted from cores of archival FFPE (Gupta et. al – unpublished data).

The practice of Oral and Maxillofacial Surgery is undergoing structural change within the UK and it would be foreseeable that in the coming decade, further centralisation of services will occur especially where multidisciplinary, subspecialty and/or translational services are involved(180). There would be potential for the relevant specialty associations i.e. British Association of Oral and Maxillofacial Surgeons, British Society of Oral Medicine and The

Pathological Society of Great Britain and Ireland, in collaboration with NHS Improvement, to formalise a registry for oral epithelial dysplasia in order that national level data can be collated to form sufficient data to progress understanding of the condition especially in relation to malignant transformation as this would be in line with the NHS 10-year plan to improve early diagnosis of oral cancer and utilise large datasets (clinical and molecular) to formulate artificial intelligence supported algorithms in formulation of personalised management strategies. There is potential for such a registry to inform the duration and interval of follow-up. Utilised in combination with molecular prognosticators, it could help risk stratification of patients so that high risk patients can be reviewed in tertiary multidisciplinary clinics and lower risk patients could be managed in 'spoke' secondary care centres or in the primary care within the context of a managed clinical network.

Several chemoprevention trials have unfortunately not demonstrated significant success in the management of oral lesions with malignant potential(181-183). The clinical outcomes reported within this thesis(97) and the reported reduction in the risk of smoking-related head and neck cancer in a cohort of USA war veterans(184) has in part led to the conception of the SAVER (Sodium Valproate for Epigenetic Re-programming in Management of High Risk Oral Epithelial Dysplasia) trial: a randomised, double blind, placebo controlled clinical trial with embedded mechanistic and feasibility studies, in the UK and Ireland.

Further work

The next phase in advancement in the understanding and management oral dysplasia requires more collaborative efforts on a national/multi-national level, to capitalise on the information technology available in capturing large clinical datasets which could then be

correlated to translational elements where the support exists. A multidisciplinary and multiprofessional initiative will be required to ensure meaningful engagement of clinicians involved in the diagnosis, management and follow-up of OED within the primary, secondary and tertiary care sectors. The healthcare infrastructure currently in place requires important but minor enhancements which would uniquely position the UK National Health Service to develop a prevention and early detection collaborative network similar to that which exists in British Columbia(62). This should aid early detection of oral squamous cell carcinoma which arise from oral epithelial dysplasia. Less invasive methods for the diagnosis and clinical surveillance of OED needs to be developed to reduce the morbidity which might arise from the need for multiple (interval) incisional biopsies. Techniques to improve the diagnostic accuracy of less invasive sampling such as oral scrapes or brush cytology will need to be developed. The role of surrogate sampling from saliva samples and how this can be utilised to allow early diagnosis of OED/OSCC and in the follow-up of these patients remain areas of potential research which has yet to be fulfilled.

Outcome of existing chemoprevention studies should emerge within the next decade to inform clinicians with regard to the viability of pharmaceutical primary prevention for oral cancer and/or regression of oral epithelial dysplasia. Mechanistic studies would have been embedded within these trials to investigate and validate the known molecular determinants of malignant transformation in oral dysplasia(79, 83, 185) against patient and clinicopathological characteristics and management outcomes. In the surgical management of OED, utility of new technologies such as visual/optical aids (114, 186) and intelligent scalpel/laser technologies utilising desorption electrospray ionisation mass spectrometry(DESI-MS) (187, 188) has the potential to improve the completeness of excision of OED. This allows mass spectrometry testing of aspirated tissue plumes intraoperatively,

potentially allowing precision surgical excision with preservation of vital normal structures in the oral cavity to preserve and optimise residual function.

The proposed combined approach in future research and innovation in the management of OED adopts a holistic approach to ensure that the delivery of information-based management algorithm is combined with translational initiatives which would feedback into the process of patient care, and refinement of treatment methods with new emerging technologies to reduce morbidity, enhance patient outcomes and quality of life.

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Appendix

Publications from thesis

Peer reviewed publications:

1. Field EA, McCarthy CE, Ho MW, Rajlawat BP, Holt D, Rogers SN, Triantafyllou A, Field JK, Shaw RJ. Response to Oral epithelial dysplasia in oral submucous fibrosis: A challenge. *Oral Oncol.* 2016 Mar;54: e20
2. Field EA, McCarthy CE, Ho MW, Rajlawat BP, Holt D, Rogers SN, Triantafyllou A, Field JK, Shaw RJ. The management of oral epithelial dysplasia: The Liverpool algorithm. *Oral Oncol.* 2015 Oct;51(10):883-7
3. Ho MW. Patients with both oral leukoplakia and oral submucous fibrosis had a higher rate of malignant transformation compared to patients with oral leukoplakia or oral submucous fibrosis alone. *J Evid Based Dent Pract.* 2013 Dec; 13(4):183-4. PubMed PMID: 24237747.
4. Ho MW, Field EA, Field JK, Risk JM, Rajlawat BP, Rogers SN, Steele JC, Triantafyllou A, Woolgar JA, Lowe D, Shaw RJ. Outcomes of oral squamous cell carcinoma arising from oral epithelial dysplasia: rationale for monitoring premalignant oral lesions in a multidisciplinary clinic. *Br J Oral Maxillofac Surg.* 2013 Oct; 51(7):594-9. PubMed PMID: 23601832.
5. Ho MW, Risk JM, Woolgar JA, Field EA, Field JK, Steele JC, Rajlawat BP, Triantafyllou A, Rogers SN, Lowe D, Shaw RJ. The clinical determinants of malignant transformation in oral epithelial dysplasia. *Oral Oncol.* 2012 Oct; 48(10):969-76. PubMed PMID: 22579265.

Book chapter:

- Book chapter - Transoral Laser Microsurgery of Benign and Malignant Lesions of the Upper Aerodigestive Tract: CO2 laser in the management of lesions with malignant potential in the Oral Cavity (Thieme) MW Ho and RJ Shaw 2015.

Oral presentations/abstracts and posters from doctorate

Invited faculty/lectures

- Course organiser/director and faculty: Yorkshire Oral Lesions with Malignant Potential Study Day (multidisciplinary faculty and multi-specialty audience), 23 June 2017
 - Oral dysplasia: update and review of evidence in the literature
 - Oral dysplasia: translational science, evidence in the literature and local experience
 - Oral dysplasia: role of the multidisciplinary clinic and proposal for minimum dataset
- Current evidence for management of oral epithelial dysplasia: Yorkshire OMFS Specialty Registrar Regional Study Day, May 2016
- The role of double strand DNA break repair regulation in malignant transformation of Oral Dysplasia
 - 6th International Meeting for Regional and Targeted Therapies for Cancer, Zhongshan Hospital, Shanghai, China November 2014

Oral Peer-Reviewed Abstracts

i. International meetings:

- Evaluation of the DNA damage repair pathway through oral epithelial dysplasia scrapes: its role in the determination of malignant transformation (Cancer Biology) Michael Ho, Mark Ryan, Juhi Gupta, Asterios Triantafyllou, Janet Risk, Richard Shaw, James Wilson
 - o American Head and Neck Society: 9th International Conference for Head and Neck Cancer, Seattle – July 2016
- The clinical determinants of malignant transformation in oral epithelial dysplasia. Ho MW, Risk JM, Woolgar JA, Shaw RJ et al.
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ii. National meetings:

- Management of Proliferative Verrucous Leukoplakia: justification for a conservative approach – E Okoturo et. al (University of Liverpool)
 - o BAOMS, Birmingham June 2017

- Evaluation of the DNA damage repair pathway through oral epithelial dysplasia scrapes: its role in the determination of malignant transformation. M. Ho · J. Gupta · J. Risk · A. Triantafyllou · R. Shaw · J. Wilson
 - o BAOMS, Liverpool July 2015

- The role of ATR-Fanconi Anaemia pathway in malignant transformation of oral epithelial dysplasia. Ho MW, Triantafyllou A, Risk JM, Shaw RJ and Wilson JB.
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- The clinical determinants of malignant transformation in oral epithelial dysplasia. Ho MW, Risk JM, Woolgar JA, Rogers SN, Shaw RJ et al.
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Poster presentations

- Evaluation of outcomes of proliferative verrucous leukoplakia in Leeds Dental Institute. T Austin, T Wei, P Chengot and MW Ho
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- Transoral laser resection of oral squamous cell carcinoma and oral premalignant lesions – complications, morbidity and outcomes (2003-2014). OP Krishnan, C Wan, J Johnson, TA Boye, DA Mitchell, DN Sutton and MW Ho
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- The role of ATR-Fanconi Anaemia pathway in malignant transformation of oral epithelial dysplasia. Ho MW, Triantafyllou A, Risk JM, Shaw RJ and Wilson JB.
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- The clinical determinants of malignant transformation in oral epithelial dysplasia. Ho MW, Risk JM, Woolgar JA, Rogers SN, Shaw RJ et al.
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- o BAHNO Annual Meeting, London, April 2012
- o British Society of Oral Medicine ASM, Liverpool, May 2012